Charles University
Faculty of Sciences

Distribution and functions of γ-tubulin and its complexes

Mgr. Vadym Sulimenko

PhD Thesis

Supervisor: Pavel Dráber, PhD

Department of Biology of Cytoskeleton,
Institute of Molecular Genetics,
Academy of Sciences of the Czech Republic

Prague 2006

I would like to thank everybody who participated somehow in the preparation of this PhD thesis. I am very thankful to my supervisor Dr. Pavel Dráber who managed and led my work. Thanks to all my colleagues from the Department of Biology of Cytoskeleton who helped me with the experiments and discussed the results. My special thanks to all my friends involved in this project, particularly to Dr. Alexander Gakh and Mgr. Natalia Kokurina for their help with English correction. Finally, I am very thankful to my family, especially to my wife Tetyana, for unlimited patience and trust. This work would never be done without kind help of all these people. Thank you all!

CONTENTS

ABBREVIATIONS

ATP	Adenosine triphosphate
BMMC	Bone marrow mast cell
Cdk	Cyclin-dependent kinase
CLIP	Cytoplasmic linker protein
FcεRI	High affinity IgE receptor
γ-TuSC	γ-Tubulin small complex
γ-TuRC	γ-Tubulin ring complex
GCP	γ-Tubulin complex protein
GDP	Guanosine diphosphate
GST	Glutathione-S-transferase
GTP	Guanosine triphosphate
MAP(s)	Microtubule-associated protein(s)
MB	Marginal band
MIP	Microtubule interacting proteins
MTOC(s)	Microtubule organising center(s)
NP-40	Nonidet P-40
PCM	Pericentriolar material
Plk	Polo-like serine/threonine kinase
RA	Retinoic acid
SDS	Sodium dodecyl sulfate
SH	Src homology domain
siRNA	Small interfering RNA
+TIPs	Plus-end tracking proteins
TTL	Tubulin tyrosine ligase

I. INTRODUCTION

I.1. MICROTUBULES

The cytoskeleton is a complex of fibrillar structures in eukaryotic cells, which plays a role in many essential functions, such as spatial organization, growth, division, movement and adaptation to different circumstances. The cytoskeleton is composed of three basic structures: *microtubules* – hollow cylinders with an outer diameter of 25-28 nm, *microfilaments* – two-stranded helical polymers with a diameter of 6-8 nm and *intermediate filaments* – rope-like fibers with a diameter of 10 nm. All three types of filaments are assembled from protein subunits. The basic building components of microtubules are α- and β-*tubulin* subunits. Microfilaments are formed by α-, β- or γ-actin molecules and intermediate filaments, depending on the cell type, consist of different types of structural subunits. Moreover, the cytoskeleton network includes a large amount of associated and interacting proteins that modulate properties of the cytoskeleton.

Although the word cytoskeleton suggests a rigid a structure, it is, on the contrary, a dynamic system. Except for a few types of intermediate filaments, the cytoskeletal fibrillar structures are extremely dynamic. Regulations of filament nucleation, polymerization and depolymerization, play an important role in the formation of transient cytoskeletal structures. The dynamic behavior of cytoskeletal filaments allows eukaryotic cells to build an enormous range of structures with different properties from relative low amounts of basic components. As this thesis focuses on microtubules, they will be described in more detail in further chapters.

I.1.1. Microtubule functions

The microtubular network is one of the prominent components of the cytoskeleton. Microtubules are essential in the following cellular functions: maintenance of cellular shape and polarity, formation of mitotic and meiotic apparatus, organization within the cytoplasm, intracellular transport, participation in intracellular signal transduction and formation of specific structures such as cilia and flagella. Microtubules are usually dynamic, but they can be stabilized in response to the cell's requirements. During mitosis microtubules are necessary for the translocation of each pair of chromosomes to the mitotic midplate and to the spindle poles. Microtubules also participate in cytokinesis, ensuring the distribution of organelles

between daughter cells. In interphase microtubules form a network that originates from microtubule organizing centers (MTOCs). In concert with actin and intermediate filaments microtubules establish and maintain the overall internal architecture of the cytoplasm. Disruption of the microtubular network, using specific microtubular drugs, results in changes to the cell's shape and the redistribution of cytoplasmic components. A direct role of cytoplasmic microtubules in the intracellular translocation of membranous components, was first demonstrated in neurons. Video enhanced microscopy showed that oriented superstable microtubule arrays in axons serve as the tracks along which vesicles and organelles are translocated from the cell center, to the periphery, by molecular motors (Allen et al., 1985; Vale et al., 1985). Microtubules are also important for the polarization of epithelial cells (Meads & Schroer, 1995). Except for well-known functions, the microtubular network also plays an essential role in intracellular signal and regulatory pathways. Many fundamental signaling molecules, such as trimeric and small G-proteins and different kinases and phosphatases, can bind to microtubules that provide a large surface for their interactions. Signaling proteins can also be transported along microtubules by molecular motors (Gunderson & Cook, 1999).

Highly specific and stable microtubular structures are formed in some protozoa. In fact, up to seventeen distinct microtubular structures, in a single cell, have been described in ciliates (reviewed in Libusová & Dráber, 2006). Stable microtubules are also characteristic for basal bodies, cilia and flagella.

I.1.2. Microtubule structure and dynamics

Microtubules, assembled from heterodimers of α- and β-tubulin and from a collection of microtubule associated proteins (MAPs), are hollow cylindrical structures of various lengths (Dustin, 1984). The cylinder of a microtubule is built out of 13 protofilaments – long linear strings of αβ-tubulin dimers connected in a head-to-tail manner (Tilney et al., 1973). The single protofilament, in which the average diameter is about 5 nm, associates with neighbor protofilaments by lateral connections, mainly between the same types of tubulin subunits, i.e. α/α and β/β interactions. Tubulin subunits of neighbor protofilaments are shifted about 1 nm in the direction of the microtubular axis, forming a left-hand helix. This helix is not continual, but each microtubule has a seam where α-tubulins interact with β-tubulins (Nogales et al., 1999; Li et al., 2002). Each protofilament in a microtubule is assembled from subunits pointing in the same direction. Due to this orientation, each microtubule has a distinct

structural polarity where α-tubulin is exposed at one end of the microtubule, called the minus (-) end and β-tubulin at the opposite side, called the plus (+) end (Amos & Hirose, 1997). Plus and minus ends differ in assembling and disassembling characteristics. Tubulin subunits are added to the fast growing (+) end of the microtubule and are preferentially lost from the (-) end.

In order for microtubule polymerization to occur energy from the hydrolysis of GTP, bound to β-tubulin, is used. Tubulin dimers are added with GTP (GTP cap), on the (+) end of the microtubules. After polymerization GTP on β-tubulin is hydrolyzed to GDP. Individual microtubules containing a GTP cap favor growth. When the GTP cap is lost, fast depolymerization (shrinking) proceeds. In some cases, fast depolymerization is stopped and the microtubule starts to grow (rescue). This phenomenon of repeated slow growing, fast shrinking and rescue of microtubules, is named "dynamic instability of microtubules" (Mitchison & Kirshner, 1984). There is also another phenomenon connected with the presence of microtubule polarity called "treadmilling". It was shown, that *in vitro,* due to different rates of the association and dissociation of dimers at the (+) and (-) ends, a microtubule can maintain a constant length. However, single subunits flux through the polymer. Regulation of microtubule length is a complex process. Ca^{2+} inhibits assembly of microtubules from dimers, while Mg^{2+} stimulates polymerization (Solomon et al., 1977). Microtubule dynamics is a temperature-dependent process – polymerization is usually promoted at 37 °C, while depolymerization takes place at 4 °C. Polymerization proceeds only when tubulin dimers are present at a critical concentration. Repeated cycles of temperature-dependent polymerization and depolymerization are often used for the isolation of microtubules.

The mechanisms allowing the formation of microtubules under subcritical concentrations of tubulin dimers are not as well known. Microtubule dynamics depends on the presence of microtubule-associated proteins (MAPs) and microtubule-interacting proteins (MIPs). *In vivo* microtubules are anchored by their (-) ends. In MTOCs these ends are capped by stabilizing proteins (Keating & Borisy, 1999). MTOCs are extremely important for rigorous organization of the microtubular network. Their function is to initiate (nucleation) microtubule polymerization and anchor their (-) ends. MTOCs have a different morphology, but a strong functional homology, in different cell types. The most known MTOCs are centrosomes and basal bodies in animal cells (Doxsey et al., 2005) and spindle pole bodies in yeast (Jaspersen & Winey, 2004). Acentriolar organizing centers exist in higher plants (Vaughn & Harper,

1998). Different MTOCs have very similar basic components – they contain structural and nucleating proteins, molecular motors and regulating and signaling proteins (Šmejkalová & Dráber, 2003). One of the most important proteins associated with MTOCs is the third member of the tubulin family – γ-tubulin. This will be described more thoroughly in chapter I.2.

I.1.3. αβ-Tubulin heterodimers

Both α- and β-tubulins have a molecular weight of about 50 kDa. Due to the relatively low dissociation constant of the tubulin heterodimer $K_d = 10^{-6}$, there is only a small amount of tubulin monomers present in a water solution (Detrich & Williams, 1978). Limited proteolysis of tubulin subunits with various proteases, revealed that they are composed of three structural domains: N-terminal, intermediate and C-terminal domain (Mandelkow et al., 1985). N-terminal domains are highly conserved and are buried in the microtubule wall. C-terminal domains are more variable and are exposed on the surface of the microtubule (Dráber et al., 1989). External parts of the C-terminal structural domains are acidic and bind various associated proteins. Electron crystallography has revealed the three-dimensional structure of the αβ-tubulin heterodimer (Lowe et al., 2001), as well as its position in the microtubule wall (Nogales et al., 1999). Each tubulin subunit has a binding site for one molecule of GTP on the N-terminal domain. GTP bound to an exchangeable "E-site" (Weisenberg et al., 1976) of β-tubulin can be hydrolyzed to GDP. In contrast, GTP bound to a nonexchangeable "N-site" on α-tubulin is physically trapped at the dimer interface and therefore is not hydrolyzed. Both E- and N-sites bind specifically to Mg^{2+} ions and these divalent cations are involved in GTP binding to tubulin and control the stability and structure of the αβ-tubulin dimer (Correia et al., 1988; Menendez et al., 1998). A variety of drugs bind to tubulin and affect its polymerization (Hamel et al., 1996). Microtubule depolymerizing drugs (colchicine, nocodazole, vinblastine, vincristine, podophyllotoxin) bind preferentially to soluble dimers in contrast to microtubule stabilizing drugs (taxoids, epothilones and discodermolide), which have higher affinity for the polymerized tubulin. All of these drugs dramatically modify microtubule dynamics (Caplow & Zeeberg, 1981; Panda et al., 1995). Microtubular drugs are especially harmful for dividing cells, which need a functional mitotic spindle. Both microtubule-stabilizing and destabilizing agents are widely used for the treatment of cancer (reviewed in Pellegrini & Budman, 2005; Zhou & Giannakakou, 2005). A development of

new-generation anti-cancer drugs, based on conjugation of microtubule-destroying agents and monoclonal antibodies, was recently reported (Lambert et al., 2005).

Genes for α- and β-tubulins are very conservative and the sequence identity between phylogenetically different organisms is very high. For example, there is a 74% identity between human α-tubulin and the α-tubulin of the yeast *Candida* (Daly et al., 1997) and an 82% identity between human β-tubulin and the β-tubulin of the protozoa *Leishmania* (Fong & Lee, 1988). Sequence identity between α- and β-tubulins is approximately 35-40% (Cleveland & Sullivan, 1985). With exception of the single β-tubulin gene in the budding yeast *Saccharomyces* (Neff et al., 1983), both the α- and β-tubulins are encoded by multiple gene families. At least 6 genes for α-tubulin and 6 genes of β-tubulin were described (Cleveland et al., 1987; Luduena et al., 1998). Using high-resolution isoelectric focusation, it was shown that both α- and β-tubulins are present in multiple charge variants, or isoforms (Wolff et al., 1982). The number of isoforms in some cases can be much higher than the number of expressed tubulin genes. At least 11 isoforms of α-tubulin and 14 isoforms of β-tubulin, were described in brain tissue (Linhartová et al., 1992) and a large number of isoforms were also observed in plant cells (Smertenko et al., 1997).

I.1.4. Posttranslational modifications of α- and β-tubulins

The heterogeneity of tubulin subunits is based not only on the presence of multiple genes, but also on extensive posttranslational modifications. Tubulins are substrates for acetylation, tyrosination/detyrosination, nitrotyrosination, polyglutamylation, polyglycylation, palmitoylation and phosphorylation. Most of these modifications, except for acetylation, are located on C-terminal domains of tubulin subunits, which are exposed on the microtubule surface (Nogales et al., 1999).

Acetylation

Acetylation on the ε-amino group of a conserved lysine residue at position 40 of the α-tubulin was first described in *Chlamydomonas* (L'Hernault & Rosenbaum, 1985). Acetylation is often present on less dynamic, stable microtubules. It was shown that acetylation is not essential for cell survival. The complete elimination of tubulin acetylation, by direct mutagenesis of the lysine-40 residue to arginine in *Tetrahymena,* had no visible effect on the phenotype (Gaertig et al., 1995). The disruption of the HDAC6 gene encoding histone deacetylase 6, responsible for the deacetylation of tubulin, led to the extensive acetylation of

tubulin. Highly increased levels of acetylated α-tubulin did not significantly affect proliferation and differentiation of embryonic stem cells (Zhang et al., 2003).

Tyrosination/detyrosination

The mRNA for most α-tubulins encode for carboxy-terminal tyrosines. This tyrosine is thereafter removed by a tubulin tyrosine carboxypeptidase (TTCP). Therefore, primary modification of α-tubulin is through detyrosination (Argarana et al., 1978). The detyrosinated α-tubulin has a carboxy-terminal glutamic acid. Modified α-tubulin is often referred to as Glu-tubulin. The tRNA-independent incorporation of terminal tyrosine into tubulin was first observed in brain homogenates (Barra et al., 1973; Argarana et al., 1977). The addition of terminal tyrosine is an ATP-dependent reaction carried out by tubulin tyrosine ligase (TTL). Detyrosination itself does not stabilize microtubules in cells (Webster et al., 1990), but it can be used as a marker in microtubule dynamics studies. Stable microtubules are characterized by the presence of Glu-tubulin. Only TTL can attach tyrosine to Glu-tubulin.

When terminal glutamic acid is removed, "Δ2-tubulin" is formed (Paturle-Lafanechere et al., 1991). Δ2-Tubulin is not a substrate for TTL and therefore escapes the tyrosination-detyrosination cycle. It is also interesting to note, that Δ2-tubulin is more inclined to undergo other modifications such as polyglutamylation (Paturle-Lafanechere et al., 1991) or polyglycylation (Banerjee et al., 2002).

Nitrotyrosination

TTL can also catalyze the incorporation of 3-nitrotyrosine, generated by the reaction of nitric oxide with tyrosine, to α-tubulin. It has been suggested that nitrotyrosination is an irreversible process and that the accumulation of nitrotyrosine leads to microtubule dysfunction and cellular injury in epithelial lung carcinoma cells (Eiserich et al., 1999). In contrast, Bisig et al. (2002) has shown that nitrotyrosination of α-tubulin is reversible and has no detrimental effect on dividing cells. Nitration of the C-terminal domain of α-tubulin during neuronal differentiation of PC12 cells (Cappelletti et al., 2003), as well as the identification of nitrated α-tubulin in the neuronal cells of *Sepia* (Palumbo et al., 2002), indicate that the nitration of tubulin could play a role in the regulation of microtubules during neuronal morphogenesis.

Polyglutamylation

Polyglutamylation is the attachment of a polyglutamate side chain of variable length to the glutamate residues in the carboxy-terminal part of both tubulin subunits. It was described for

the first time in α-tubulin from the mammalian brain (Edde et all., 1990). Polyglutamylation occurs in the protist *Giardia,* which is considered to be the most primitive of eukaryotes (Weber et al., 1997). In protists polyglutamylation is a predominant modification of stable microtubules, such as flagellar axonemes, basal bodies and the submembranous network (Bre et al., 1994). In mammalian cells polyglutamylated tubulins were found in centrioles, mitotic spindles and midbodies, as well as in the axons of neurons (Bobinnec et al., 1998). Polyglutamylation is a cell cycle-dependent process. The activity peak of polyglutamylase is in G2, but the level of glutamylated tubulin is at its maximum during mitosis (Larcher et al., 1996). It was suggested that polyglutamylation of the outer centriole walls could play a role in the anchoring mechanism of the centriolar matrix (Bornens et al., 2002).

Polyglycylation

Polyglycylation is the attachment of a polyglycine side chain to the carboxyl groups of conserved glutamate residues presented in the C-terminal regions of α- and β-tubulins. This prominent modification of axonemal microtubules was first observed in tubulins isolated from the cilia of *Paramecium* (Redeker et al., 1994). Up to 34 glycine residues can be present in one side chain and many neighboring glutamate residues can be polyglycylated. Such extensive modification is characteristic for axonemal tubulins, whereas the cytoplasmic pool of tubulin usually has only a few glycine residues (Bré et al., 1998). It seems that the polyglycylation of β-tubulin is extremely important for the axonemal organization and cytokinesis of cells.

Palmitoylation

The ability of α-tubulin to incorporate radioactively labeled [^{3}H] palmitate has been shown in the budding yeast *Saccharomyces,* as well as in cell-free studies (Caron et al., 1997; Ozols & Caron, 1997). Palmitate is predominantly bound at the cysteine 377 residue of α-tubulin. Mutation of the corresponding residue to serine in the budding yeast, reduced the *in vivo* palmitoylation level by 60% (Caron et al., 2001). In PC12 cells, [^{3}H]-palmitic acid was incorporated into tubulin in a time- and cell-density-dependent manner and most of the radioactive marker was located in the plasma membrane-enriched fraction. Detergent-extracted tubulin from the plasma membrane was more palmitoylated than tubulin obtained from the cytoplasm. It seems that palmitoylated tubulin can be directly incorporated into the plasma membrane, but its functions are still unclear (Zambito & Wolff, 2001).

Phosphorylation

Phosphorylation of serine residues (Ser442/444) within the carboxy-terminal domain of β-tubulin was first reported in the brain (Eipper et al., 1974). Later, it was demonstrated that both α- and β-tubulin subunits were phosphorylated on serine and threonine residues by polo-like kinase, both *in vivo* and *in vitro* (Feng et al., 1999). Tubulin phosphorylation probably plays an important role in the process of neuronal differentiation, as neuron-specific βIII-tubulin was phosphorylated during the differentiation of mouse neuronal cells (Gard & Kirschner, 1985). Tubulin dimers are also phosphorylated at tyrosine, by Src kinase, in the nerve growth cone membranes isolated from fetal rat brain (Matten et al., 1999). Although Src-family kinases have tubulin as a direct substrate, activation of the other tyrosine kinases, such as Fes, also affected tubulin dynamics (Laurent et al., 2004). Essential roles of the Src-family kinase Fyn in microtubule reorganization during the second meiotic division was reported in rat eggs, where Fyn kinase was associated with microtubules and phosphorylated α-tubulin (Talmor-Cohen et al., 2004). Phosphorylation of tyrosine residues on α-tubulin was described in stimulated T lymphocytes (Ley et al., 1994). Syk-dependent phosphorylation on tyrosine residue of α-tubulin was also reported in activated B-lymphocytes (Faruki et al., 2000). Recently it was shown that the phosphorylation of Ser-173 of β-tubulin by Cdk1 kinase, impaired GTP binding and thus modulated microtubule assembly during mitosis (Fourest-Lieuvin et al., 2005).

I.1.5. Other members of tubulin superfamily

α-, β- and γ-Tubulins belong to a large tubulin superfamily. The other members of this family are delta (δ), epsilon (ε), zeta (ζ) and eta (η) tubulin. The Gene database GenBank (http://www.ncbi.nlm.nih.gov) contains sequences of the other proteins that belong to the tubulin superfamily – iota (ι), theta (θ) and kappa (κ) tubulins, in which genes were found in the genome of *Paramecium* (Dutcher et al., 2003). Localization of these proteins and their functions are still unknown.

δ-Tubulin is responsible for organization of C-tubules in basal bodies (Dutcher & Trabuco, 1998) and for the anchoring of basal bodies (Fromherz et al., 2004). It was also found in the centrosomes of mammals, as well as in the nuclei of animal cells (Dutcher et al., 2001). The pool of δ-tubulin was observed in a soluble form in the cytoplasm (Smrzka et al., 2000). ε-Tubulin was primarily localized in the pericentriolar matrix of human cells (Chang & Stearns,

2000) and is essential for the duplication of centrioles in S-phase of the cell cycle (Chang et al., 2003). Functions of ζ-tubulin are still unknown. It was localized in the basal bodies of *Trypanosoma* and *Leishmania* (Vaughan et al., 2000), as well as in the centrosomes of vertebrates (McKean, 2001). The gene originally named SM19, found in *Paramecium* (Ruiz et al., 2000), was later named η-tubulin. The mutations affecting SM19 led to a progressive reduction of basal bodies, decreasing of cell length and changes in cell shape. SM19 mutants showed relocation of γ-tubulin from the centrosomal region. It was suggested that η-tubulin might tether γ-tubulin complexes to the nucleation site in basal bodies (Ruiz et al., 2000). Further experiments have shown an interaction between η- and β-tubulin molecules (Ruiz et al., 2004). η-Tubulin was also found in the centrosomes of *Xenopus* (Dutcher et al., 2003).

I.1.6. Microtubule-associated and microtubule-interacting proteins

Microtubules are associated with numerous proteins that modulate microtubule dynamics, bind microtubules to other proteins or use microtubules for intracellular transport. Except for MTOCs and proteins connecting the (-) end of microtubules, there are three major groups of proteins associated with microtubules (Mandelkow & Mandelkow, 1995). Microtubule-associated proteins (MAPs) strongly interact with microtubules and therefore it is easy to enrich them by repeated temperature-dependent cycles of polymerization-depolymerization (Wiche et al., 1998). This group includes MAP1A, MAP1B, MAP2, tau (τ)-proteins, MAP4, 205 kDa MAP and STOPs (stable tubulin only polypeptides). These proteins play a structural role – they bind to the surface of the microtubule, bridge several tubulin subunits and possibly abolish the repulsive negative charge on the microtubule surface. They promote tubulin polymerization and stabilize microtubules. Structural MAPs are phosphorylated and are regulated by the usual intracellular signal transductors – kinases and phosphatases.

The polarized organization of microtubules is used by mechanochemical ATPases (kinesins, dyneins) for transport of various cellular components (reviewed in Hirokawa & Takemura, 2004; Vallee et al., 2004). Microtubule motor proteins use the energy from ATP hydrolysis for their movement along microtubules. Microtubule motors play essential roles during mitosis – they cross-bridge and slide microtubules, transport specific mitotic cargoes along spindle microtubules and regulate microtubule assembly dynamics (Sharp et al., 2000). Some members of the large kinesin superfamily, such as XKCM1 and XKIF2, destabilize microtubules and thus participate in their depolymerization (Walczak et al., 1996; Desai et al., 1999; Hirokawa & Takemura, 2004).

Microtubule interacting proteins (MIPs) are proteins with a lower affinity to microtubules and do not copolymerize with microtubules during repeated cycles of polymerization and depolymerization. Some of MIPs are located on (+) ends of microtubules. "Plus-end tracking proteins" (+TIPs) (Schuyler & Pellman, 2001) contain, for example, end-binding proteins EB1, EB2 and EB3, cytoplasmic linker proteins CLIP115 and CLIP170, adenomatous polyposis coli (APC) protein (Galjart & Perez, 2003) and CLIP-associated proteins (CLASPs) (Mimori-Kiyosue et al., 2005). +TIPs stabilize the growing ends of microtubules and participate in chromosome segregation during mitosis (Galjart et al., 2005). Some +TIPs are regulated by other proteins that stabilize microtubules at specific cellular sites in response to signaling cues. Except for +TIPs, there are also other proteins that interact with microtubules. For example, MA-01 antigen participates in the interaction between microtubules and intermediate vimentin filaments (Dráberová & Dráber, 1993) and the protein Op18/stathmin, which affects microtubule dynamics by sequestering tubulin dimers and promoting GTP hydrolysis at the E-site (Brannstrom et al., 2003). Katanin (the Japanese word "katana" meaning sword) uses the energy of ATP hydrolysis to sever microtubules from MTOCs, which enables fast depolymerization of filaments that are not anchored (Quarmby et al., 2000). Many proteins of signal pathways are specifically connected with microtubules and can change the dynamics of the cytoskeleton (Gundersen & Cook, 1999). For example, microtubule-binding protein Costal-2 was originally identified as a participant in the *hedgehog* signal pathway (Ohlmayer & Kalderon, 1998). Microtubules can also be used as a scaffold for many viruses. The HIV-Tat protein binds to microtubules and can impair the mitotic process (Battaglia et al., 2005). The Ebola virus protein VP40 associates with microtubules and enhances their polymerization ratio (Ruthel et al., 2005). Heald & Nogales (2002) offered the general scheme of important proteins interacting with tubulins and affecting microtubule dynamics.

I.2. GAMMA-TUBULIN

I.2.1. Discovery of γ-tubulin

γ-Tubulin, with a molecular weight of about 48 kDa, was first discovered in the filamentous fungus *Aspergillus* (Oakley & Oakley, 1989) as a product of the *mipA* gene during genetic screening for proteins interacting with β-tubulin. Later, it was shown that disruption of the γ-

tubulin gene led to a depletion of microtubules and growth arrest (Oakley et al., 1990). Immunolocalization of γ-tubulin revealed that it was enriched in both the centrosomes and mitotic spindle pole bodies (Stearns et al., 1991, Horio et al., 1991). On the basis of these observations, it was proposed that γ-tubulin is an essential component for microtubule nucleation. Using the γ-tubulin cDNA sequence from *Aspergillus* for screening of the cDNA from the fruit fly *Drosophila*, a corresponding γ-tubulin sequence was cloned. The obtained sequence was subsequently used for the cloning of human γ-tubulin (Zheng et al., 1991). Using low stringency hybridization, it was also possible to clone the γ-tubulin gene of *Schizosaccharomyces* (Horio et al., 1991) and a portion of the mouse γ-tubulin gene (Joshi et al. 1992). γ-Tubulin is a ubiquitous protein, which is present in all eucaryotes. The importance of γ-tubulin for microtubule nucleation was confirmed by a microinjection of the anti-γ-tubulin antibody into living mouse cells (Joshi et al., 1992). The antibody disrupted the nucleation of microtubules from the centrosome in interphase cells, as well as the formation of the mitotic spindle. A γ-tubulin-related protein with a molecular weight of about 58 kDa was detected in acentriolar plant cells of *Allium, Glycine and Nicotiana*, using antibodies raised against a synthetic peptide corresponding to a conserved region of known γ-tubulins (Liu et al., 1993).

I.2.2. γ-Tubulin distribution

Initially, γ-tubulin was considered to be a typical protein of MTOC. It is located in the spindle pole bodies of *Aspergillus* (Oakley & Oakley, 1989), in the centrosomes of interphase cells of different metazoan species and on the poles of mitotic spindles during mitosis (Zheng et al., 1991; Stearns et al., 1991; Joshi et al., 1992). Later, it was found in the midbodies during cytokinesis (Julian et al., 1993, Nováková et al., 1996), as well as along microtubules of the mitotic spindle (Lajoie-Mazenc et al., 1994; Nováková et al., 1996). γ-Tubulin was also detected in the core of the mammalian centriole (Fuller et al., 1995). It is also a component of basal bodies of retinal photoreceptors and multiciliated epithelial cells (Muresan et al., 1993), basal bodies of green alga *Chlamydomonas* (Dibbayawan et al., 1995) and basal bodies of ciliated (Liang et al., 1996) and flagellated protozoa (Scott et al., 1997, Nohýnková et al., 2000). The distribution of γ-tubulin in MTOC is dependent on the presence of centrioles, as can be interferred from its detection in acentriolar centrosomes of fertilized oocytes during early mouse development (Gueth-Hallonet et al., 1993) as well as in centrosomes of the

acentriolar cell line of *Drosophila* (Debec et al., 1995). A unique distribution of γ-tubulin occurs in the acentriolar cells of higher plants, where punctuate labeling of γ-tubulin was associated with all microtubular arrays (Liu et al., 1993). The presence of γ-tubulin in premitotic nuclei and its association with the kinetochore/centromeric region, suggests its role in the spindle organization of higher plants (Binarová et al., 1998; Binarová et al., 2000). In the human parasite *Entamoeba*, γ-tubulin was found in the nucleus, but not in cytoplasm (Vayssié et al., 2004). The γ-Tubulin of *Entamoeba* forms the intra-nuclear MTOC responsible for the organization of microtubular arrays (Gomez-Conde et al., 2000). Collectively, these findings documented the fact that γ-tubulin is an essential component of active MTOCs, irrespective of their structural diversity.

MTOCs are the most obvious place of the localization of γ-tubulin, where it can be easily detected, due to its high concentration. However, in many cell types, a large amount of γ-tubulin is present in the soluble form, which is sometimes visible as diffuse staining of the cytoplasm.

I.2.3. γ-Tubulin genes and structure

γ-Tubulin is a minor member of the tubulin superfamily. According to quantitative immunoblot analysis, γ-tubulin is present in mammalian cells at a low copy number of about 10^4 molecules per cell, which is less than 1% of the level of either α- or β-tubulin (Stearns et al., 1991). Quantification of γ-tubulin mRNA in *Aspergillus*, revealed that it represented only 5% of β-tubulin mRNA (Oakley & Oakley, 1989). While the α- and β-tubulins are encoded by multiple genes, only a limited number of γ-tubulin genes were found in the majority of organisms studied thus far. For example, there are only two functional genes in *Arabidopsis* (Liu et al., 1994), *Drosophila* (Wilson et al., 1997) and humans (Wise et al., 2000). Human γ-tubulin genes encode proteins with an amino acid identity greater than 98%. The two *Drosophila* γ-tubulins are more divergent. Their amino acid identity is 82% and they differ in expression patterns during gametogenesis and development (Wilson et al., 1997). They also exhibit distinct biochemical properties (Raynaud-Messina et al., 2001). Despite this fact, they are physiologically interchangeable. This is consistent with reported functional replacement of the *Schizosaccharomyces* γ-tubulin by human TUBG1 γ-tubulin (Horio & Oakley, 1994).

Recent analysis of two mouse γ-tubulin genes has shown, that although identity of these genes is very high (97%), their distribution in tissues is different. TUBG1 is expressed

ubiquitously, whereas TUBG2 is primarily detected in the brain (Yuba-Kubo et al., 2005). The generation of TUBG1- and TUBG2-deficient mice revealed functional differences between these genes. *Tubg1*$^{-/-}$ mutants stopped their development at the morula/blastocyst stages due to mitotic arrest. Expression of TUBG2 in blastocysts could not rescue the TUBG1 deficiency. On the other hand, TUBG2-deficient *(Tubg2*$^{-/-}$*)* mice were born, developed and intercrossed normally. Although histochemical screening identified no abnormalities in Tubg2$^{-/-}$ mice, these mutants exhibited behavioral defects and changes in neuronal activities. Only one γ-tubulin gene was found in *Trypanosome* (Scott et al., 1997), *Chlamydomonas* (Silflow et al., 1999), as well as in myxomycete *Physarum* (Lajoie-Mazenc et al., 1996). In the latter case, the gene encodes two γ-tubulins differing in relative molecular weight, sedimentation properties and the ability to bind to the MTOCs. However, it is still unknown whether these two forms arise from alternative splicing of mRNA, or from posttranslational modifications. The advantage of retaining two genes that encode very similar γ-tubulins is still unclear. Expression of these genes could be differentially regulated and this regulation could play a role in ensuring an effective level of γ-tubulin in particular tissues and/or in particular stages of development. Alternatively, γ-tubulin is such key molecule for the cell, that there is a selective advantage in functional redundancy. If one γ-tubulin gene is damaged, the other one might substitute the production of functional γ-tubulin (Wise et al., 2000).

γ-Tubulins are phylogenetically conserved proteins. Comparison of amino acid sequences from extremely divergent organisms has shown at least 66% amino acid identity (Oakley, 1999). γ-Tubulin sequences of human and *Xenopus* have an amino acid identity of 98% (Oakley et al., 1994). There are two exceptions to the conservation of γ-tubulin. The baker's yeast *Saccharomyces* γ-tubulin (the product of the Tub4 gene) is highly divergent. It shares approximately 35% amino acid identity with 16 conventional γ-tubulins (Sobel & Snyder, 1995). The other divergent γ-tubulin with a 40-45% amino acid identity is the nematode *Caenorhabditis* γ-tubulin (Bobbinec et al., 2000).

γ-Tubulins are similar to α- and β-tubulins. They share approximately 28-35% amino acid identity and similarity will increase up to 60% when conserved substitutions of amino acids are considered (Oakley et al., 1994). γ-Tubulin does not have an acidic C-terminal end, which is very important in the αβ-tubulin heterodimer, for binding of the MAPs. γ-Tubulin has similar secondary and tertiary structures to α- or β-tubulin, which implies that highly conserved regions among tubulins might be responsible for the binding of GTP (Burns et al.,

1991). A structural model of possible interactions between αβ-tubulin heterodimers and γ-tubulin was derived from electron crystallography of tubulin dimers and the sequence of γ-tubulin (Inclan & Nogales, 2000). γ-Tubulin is likely to contact the (-) end of α-tubulin and may also associate laterally with tubulin dimers. γ-Tubulin might also be capable of self-assembly, forming either dimers or protofilament-like oligomers. Using the SPOT peptide technique, lateral association of γ-tubulin with a αβ-dimer has been predicated (Llanos et al., 1999). Recently, a 2.7 Å crystal structure of γ-tubulin bound to a non-hydrolysable analog of GTP was described (Aldaz et al., 2005). Binding of GTP and its hydrolysis might change the γ-tubulin conformation and its affinity for α-tubulin. It was suggested that the GTP hydrolysis on γ-tubulin provides a mechanism for the observed release of microtubules from the centrosome, or tuning of the nucleating activity of γ-tubulin complexes (Aldaz et al., 2005).

I.2.4. Soluble complexes of γ-tubulin

Soluble γ-tubulin forms complexes with other proteins. The presence of γ-tubulin in soluble complexes, which was not associated with centrosomal fractions, was first detected in embryonal extract from *Drosophila* (Raff et al., 1993). γ-Tubulin is associated with two previously identified centrosomal MAPs – DMAP60 and DMAP190. Large γ-tubulin complexes were first identified in *Xenopus* oocytes (Zheng et al., 1995). They were characterized as large (~ 2.2 MDa) γ-tubulin ring complexes (γ-TuRC) containing except of γ-tubulin other proteins. Such complexes were also found in *Drosophila* embryos (Moritz et al., 1995) and in different mammalian cells (Meads & Schroer, 1995; Moudjou et al., 1996; Detraves et al., 1997). γ-TuRC has the appearance of an open ring, with approximately the same diameter as a microtubule (~ 25 nm). The purified γ-TuRCs nucleates microtubules *in vitro* (Zheng et al., 1995).

In addition to γ-TuRCs, γ-tubulin was also found in smaller complexes (around 280 kDa) called γ-tubulin small complexes (γ-TuSC) (Moritz et al., 1998). Small complexes isolated from *Drosophila* embryos contained excerpts of γ-tubulin as well as the proteins Dgrip84 and Dgrip91 (Oegama et al., 1999). γ-TuSC is formed by two molecules of γ-tubulin and one molecule of Dgrip84 and Dgrip91. γ-TuSC is a structural subunit of γ-TuRC, which contains additional proteins. γ-TuRCs are salt-sensitive. In the presence of 1 M NaCl or KCl they disintegrate into a few γ-TuSCs and scaffolding proteins (Moritz et al., 1997). The γ-Tubulin

of the yeast *Saccharomyces* exists only in the form of γ-TuSC that contains two molecules of γ-tubulin and one molecule of Spc97p and Spc98p (Knop et al., 1997; Knop & Schiebel, 1997). These proteins are homologues to the human proteins hGCP2 and hGCP3/HsSpc98, (Murphy et al., 1998; Tassin et al., 1998), the *Xenopus* protein Xgrip109 (Martin et al., 1998), as well as the *Drosophila* proteins Dgrip84 and Dgrip91. These proteins are now known to be core proteins of γ-TuSC and are named GCP2 and GCP3.

Electron microscopic tomography revealed that associated proteins, not present in γ-TuSC, form the cap of the ring structure (Moritz et al., 2000). The large γ-TuRCs contain excerpts of γ-tubulin/GCP2/GCP3 subunits, GCP4 (Fava et al., 1999) and GCP5 and GCP6 proteins (Murphy et al., 2001). All GCP proteins are related, but they are not redundant in their functions. It was suggested that γ-tubulin/GCP2/GCP3 subunits are involved in interactions with αβ-tubulin dimers, while GCP4, GCP5 and GCP6 enable the interaction of γ-TuRC with the centrosome, or its equivalent in other organisms (Murphy et al., 2001). Pericentriolar material is especially rich for γ-TuRCs. Hundreds of γ-tubulin rings were found in *Drosophila* pericentriolar material (Moritz et al., 1995). This correlates with the ability of centrosomes to nucleate microtubules.

Apart from γ-TuRCs and γ-TuSCs, soluble γ-tubulin was detected in the TCP-1 chaperonin complex, which is responsible for the folding of tubulins (Melki et al., 1993). Large complexes which contain γ-TuRCs, pericentrin and some other proteins, were found in extracts of *Xenopus* eggs (Dictenberg et al., 1998). Using different methods of fractionation of cell extracts, γ-tubulin was also detected in other complexes, except for γ-TuRCs and γ-TuSCs. Such complexes were found in *Drosophila* (Oegema et al., 1999), *Xenopus* (Tassin et al., 1997; Martin et al., 1998), different mammalian cells (Meads & Schroer, 1995; Murphy et al., 1998; Feng et al., 1999; Dráberová et al., 1999) and sheep brain (Detraves et al., 1997). Complexes of various sizes were also reported in plants (Binarová et al., 2000; Stoppin-Mellet et al., 2000). Multiprotein complexes containing α-, β-, γ-tubulin, the heat shock protein HSP70 and elongation factor 1α were also described (Marchesi & Nhu Ngo, 1993). Moreover, the association of γ-tubulin with Src family tyrosine kinase (Dráberová et al., 1999), Wee1 kinase (Stumpp et al., 2005) and Plk serine/threonine kinase (Feng et al., 1999) was described. In yeast, there is a family of genes called GIMs (genes involved in microtubule biogenesis), whose proteins bind to yeast γ-tubulin Tub4p (Geissler et al., 1998). GIMs proteins form multiprotein complexes, which promote the formation of functional Tub4p and

α-tubulin. Collectively, these data demonstrate that γ-tubulin can be present in various protein complexes.

I.2.5. Role of γ-tubulin in microtubule nucleation

γ-Tubulin is the key component for the nucleation of microtubules. Disruption of the γ-tubulin genes in *Aspergillus* caused blockage of nuclear division, supported by the absence of mitotic spindles and a dramatic reduction in the number of cytoplasmic microtubules (Oakley et al., 1990). In *Schizosaccharomyces*, a defect in γ-tubulin caused failure in nuclear division. A subnormal level of γ-tubulin led to the assembly of defective mitotic spindles, which lacked some of the microtubules necessary for the normal completion of mitosis (Horio et al., 1991). γ-Tubulin is also involved in the formation of microtubular assemblies in interphase mammalian cells. Microinjection of antibodies against γ-tubulin into human HeLa cells, mouse fibroblast 3T3 and human pancreatic epithelial cells, did not markedly affect the microtubule arrays. However, when microtubules were disassembled, either by cold or nocodazole, injected antibodies prevented the reassembly of microtubules after the cells were warmed or the nocodazol was removed. Injection of anti-γ-tubulin antibodies into mammalian cells reduced number of mitotic spindle microtubules and normal division was damaged. Finally, these antibodies blocked the assembly of microtubules from purified brain tubulin, on centrosomes using detergent-extracted models (Joshi et al., 1992). *Xenopus* egg extract, depleted of γ-tubulin, lost its ability to form microtubule asters from centrosomes (Felix et al., 1994). The application of small interfering RNA (siRNA), which was highly efficient in silencing the γ-tubulin of the mRNA of *Entamoeba,* resulted in the loss of organized microtubular arrays, which correlated with a significant reduction of γ-tubulin protein (Vayssié, 2004). On the other hand, an overexpression of γ-tubulin in mammalian cells caused γ-tubulin accumulation in the cytoplasm, where it formed multiple microtubule nucleation sites (Shu & Joshi, 1995). Expression of the human γ-tubulin gene in *Schizosaccharomyces*, with defective endogenous γ-tubulin, led to a replacement of γ-tubulin functions. Human γ-tubulin was localized in the yeast spindle pole body (Horio & Oakley, 1994). This experiment demonstrated the functional conservation of γ-tubulins in human and yeast, even though the homology between *Schizosaccharomyces* and human genes is only 71%.

I.2.6. Models of microtubule nucleation

To explain how the γ-TuRC nucleates microtubules, two models were proposed. They are known as the "template" and "protofilament" microtubule nucleating models. The template model predicts that γ-TuRC corresponds to a 3-start helix of the microtubule, with γ-tubulin subunits 5 nm apart and connected by lateral bonds. Thirteen γ-tubulin subunits form the complete circuit in which each γ-tubulin binds longitudinally to an α-tubulin subunit, forming 13 protofilaments in each microtubule. γ-TuRC thus forms the cap on the (-) end of microtubules (Zheng et al., 1995). The template model is depicted in Fig. 1.

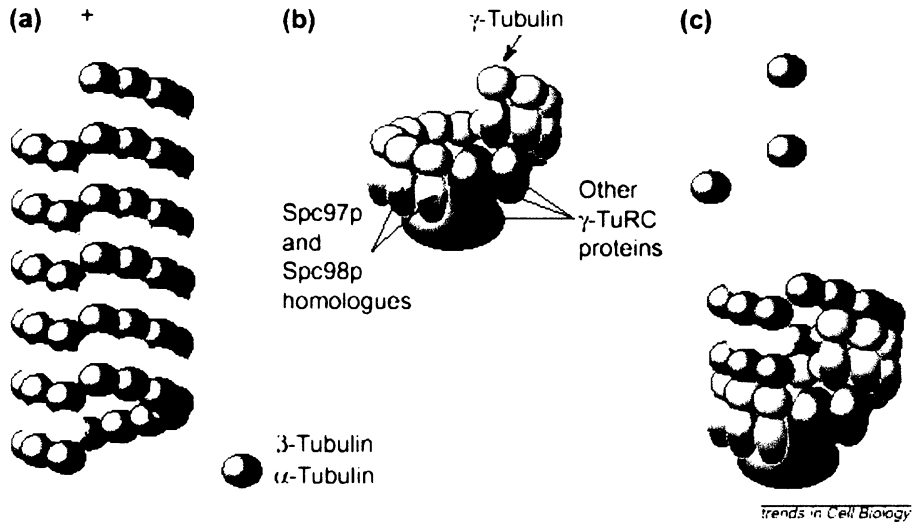


Fig. 1. Template model of microtubule nucleation by γ-tubulin (from Zheng et al., 1995).

The second, protofilament model, predicts that γ-TuRC corresponds to γ-tubulins with their subunits 4 nm apart and connected by longitudinal bonds. Sixteen subunits form the complete circuit, but only a few γ-tubulins bind laterally to an αβ-tubulin dimer to initiate a protofilament nucleus (Erickson & Stoffler, 1996). It is still unclear which model is more correct to explain the molecular mechanism of nucleation. Comparison of both nucleation models is shown in Fig. 2. Electron microscopic tomography of the γ-TuRC has shown a dome-like cap, where outer circular framework is formed by γ-tubulin subunits, whereas the dome is formed by associated proteins (Moritz et al., 2000). Similar cap-like structures on

microtubule (-) ends have been shown by immunoelectron microscopy (Keating & Borisy, 2000; Wiese & Zheng, 2000). This data seems to confirm the template model. However, other studies indicate that for the nucleation of microtubules, lateral bonds between γ- and β-tubulin are much more important than weaker longitudinal bonds between γ-tubulin and the protofilaments (Leguy et al., 2000). There are reports that γ-TuSCs (Oegema et al., 1999) and even single molecules of γ-tubulin (Leguy et al., 2000), can nucleate microtubules *in vitro*. This supports a modified protofilament model (Erickson, 2000).

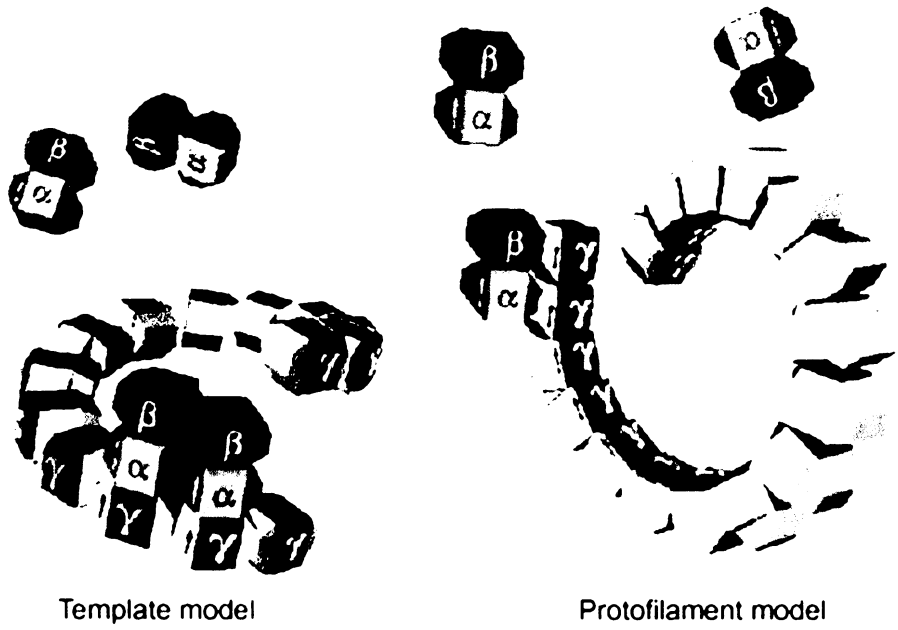


Fig. 2 Proposed structures of the γ-TuRCs as predicted by the template and protofilament models (from Erickson, 2000)

One of the best known places for microtubule nucleation and organization is the centrosome. It contains a pair of centrioles surrounded by an electron-dense pericentriolar material (PCM), which is sometimes called the centrosome matrix. This matrix is a complicated tangle of fibers and granular material with non-specifically associated proteins (Kellog et al., 1994). PCM is rich in γ-tubulin, which is in the form of γ-TuRCs. These γ-TuRCs are organized into a lattice, with the help of pericentrin (Dictenberg et al., 1998). Although pericentrin itself is not a component of γ-TuRC, it seems that it can organize complexes containing 1 molecule of pericentrin, 2 γ-TuRCs and other proteins. These

complexes can be organized into supercomplexes containing 2 molecules of pericentrin and 4 γ-TuRCs. Authors have offered a hypothesis, that pericentrin may facilitate the assembly of γ-tubulin complexes into the centrosome lattice. Previously, it was shown that purified γ-TuRCs were unable to assemble onto salt-stripped centrosomes (Moritz et al., 1998). The model of the centrosome lattice assembly is shown in Fig. 3.

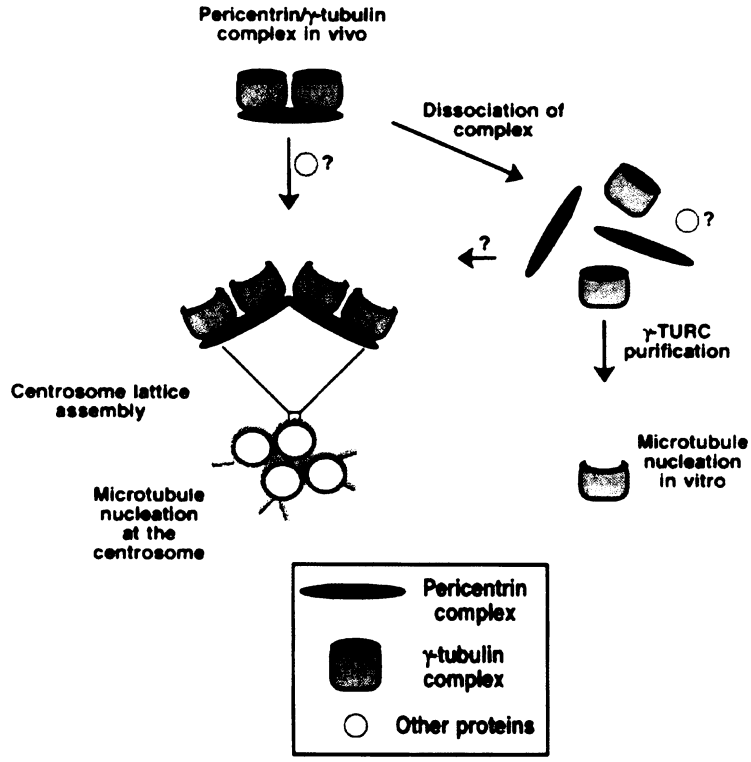


Fig. 3. A model for centrosome lattice assembly (from Dictenberg et al., 1998)

An interesting mechanism of microtubule nucleation was offered for higher plants, which lacks classical MTOCs (Murata et al., 2005). It was shown that both in living cells and in the cell-free system, microtubules were nucleated as branches on existing cortical microtubules. The branch points contained γ-tubulin, which is highly abundant in the cytoplasm of plant cells. Microtubule nucleation in the cell-free system was prevented when γ-tubulin was depleted with a specific antibody.

Although the role of γ-tubulin in microtubule nucleation is indisputable, there are a few interesting observations showing that microtubules can be also nucleated by other

mechanisms. It was shown that the overexpressed centrosomal protein RanBMP caused ectopic microtubule nucleation and led to reorganization of the microtubular network (Nakamura et al., 1998). Antibodies against RanBMP inhibited microtubule aster formation and the same effect had nonhydrolyzable GTP analog bound to Ran. Ran (Ras-like small GTPase), which is well-known as signal transductor, is involved in the regulation of microtubular nucleation. Microtubules can also be nucleated and self-organized in the vicinity of chromosomes (Vernos & Karsenti, 1995). Artificial chromatin-coated beads could also form bipolar microtubule spindles in the presence of microtubule motor proteins (Heald et al., 1996).

I.2.7. γ-Tubulin and kinases

Kinases play an important role in the regulation of microtubule rearrangement. Although phosphorylation of αβ-tubulin dimers is a minor posttranslational modification, it is one of the main modifications of MAPs, which plays an essential role in changing the microtubular network. Kinases also phosphorylate γ-tubulin and/or its associated proteins in γ-tubulin complexes. It was suggested that the Polo-like serine/threonine kinase (Plk) can phosphorylate not only αβ-tubulin dimers, but also γ-tubulin (Feng et al., 1999). Plk plays an essential role in the centrosome maturation in *Drosophila.* The inability to recruit the centrosomal protein CP190, seen in *polo*-defective embryos, leads to a failure to recruit γ-TuRC to the centrosome and therefore disturbs the nucleation of microtubules (Glover et al., 2005). A failure of the centrosomes to recruit γ-tubulin has also been observed in HeLa cells, following a microinjection of antibodies against Plk (Lane & Nigg, 1996), as well as after the depletion of the Plk by RNAi (Bettencourt-Dias et al., 2004). One of the possible targets of Plk is the ninein-like protein Nlp, which is important for proper centrosome maturation and spindle assembly (Casenghi et al., 2003).

Tyrosine kinases are also involved in the regulation of γ-tubulin functions. Microtubule organization in the budding yeast *Saccharomyces* is affected by phosphorylation of the Tyr445 residue of γ-tubulin (Vogel et al., 2001). The level of γ-tubulin phosphorylation is dependent on the phase of the yeast cell cycle. The acidic, phosphorylated form of γ-tubulin was found predominantly in the G1 phase, was dramatically reduced in S phase and was absent in the M phase of cell cycle. It seems that phosphorylation of yeast γ-tubulin during the cell cycle, controls a number of microtubules and their organization. The Tyr445 residue of

the DSYL motif of Tub4p is conserved within the carboxy terminal residues of many γ-tubulins, suggesting that γ-tubulin might be phosphorylated in different organisms. Recently, the interaction of tyrosine kinase Wee1 with γ-TuRC was described in *Drosophila* (Stumpff et al., 2005). γ-Tubulin phosphorylation was dependent on the presence of Wee1 in embryo extracts. Absence of Wee1 kinase in mutant embryos, had a dramatic effect on microtubule organization, including the formation of ectopic foci of microtubules and multipolar spindles. Centrosomes were displaced from the embryo cortex. Wee1 kinase is known as a regulator of cyclin-dependent kinase 1 (Cdk1). The treatment of plant cells from *Vicia* with Cdk specific inhibitors, led to abnormal spindle formation (Binarová et al., 1998). An association of γ-tubulin, with the protein tyrosine kinase $p53/56^{lyn}$ in rat basophilic leukemia cells, was also described (Dráberová et al., 1999). It is known that the microtubule cytoskeleton participates in the process of the activation of mast cells, which culminates in the release of allergy mediators. Molecular mechanisms of interactions between signal transduction molecules and microtubules are still poorly understood. The presence of γ-tubulin, in complex with Lyn kinase and tyrosine-phosphorylated proteins, suggests that γ-tubulin might be involved in the early stages of mast cell activation. Collectively, these data suggest the importance of kinases during the regulation of microtubule nucleation

I.2.8. Other functions of γ-tubulin

Except for well-known microtubule nucleation, γ-tubulin may have some additional functions. γ-Tubulin can cap the (-) ends of preformed microtubules, which modulates the dynamics of the (-) end and prevents the depolymerization of capped microtubules (Wiese & Zheng, 2000). Accumulated data support the idea that γ-tubulin could participate in microtubule stabilization. γ-Tubulin was found on stable kinetochore microtubules that are resistant to treatment with anti-microtubule drugs in *Arabidopsis* (Liu et al., 1995), as well as on kinetochore/centromeric regions of plant chromosomes (Binarová et al., 1998). The γ-Tubulin in acentriolar higher plant cells might be directly or indirectly involved in the modulation and/or stabilization of kinetochore-microtubule interactions. In animal cells, γ-tubulin was detected in the cold-stable fraction of microtubules isolated from the brain (Détraves et al., 1997). γ-Tubulin was present in protein complexes of heterogeneous sizes and different composition. These complexes were able to bind to taxol-stabilized microtubules. Experiments with γ-tubulin mutations in *Schizosaccharomyces* indicated that γ-

tubulin could play an important role, even on assembled microtubules (Paluh et al., 2000). The involvement of γ-tubulin in the coordination of post-metaphase events, segregation of chromosomes and cytokinesis, was implicated from experiments with the mutagenesis of human γ-tubulin and the phenotype analysis of each mutant allele in *Schizosaccharomyces* (Hendrickson et al., 2001). The overlapping roles of γ-tubulin and the kinesin-like protein KLPA, in the establishment of spindle bipolarity, were described by (Prigozhina et al., 2001). γ-Tubulin strongly interacts with BRCA1 protein, which is a suppressor of tumorigenesis in the breast and ovary. It was suggested that an attenuated interaction between BRCA1 and γ-tubulin might induce an increase in aneuploid cell population and thus contribute to tumorigenesis (Hsu & White, 1998; Hsu et al., 2001). Finally, the analysis of the γ-tubulin knockout gene in mice has shown that γ-tubulin might play an important role in the development of the neuronal system during ontogenesis. *Tubg2* deficient mice appeared to exhibit peculiar behavioral deficits, abnormalities in circadian rhythm and reactions to painful stimulations (Yuba-Kubo et al., 2005).

II. AIMS OF THE STUDY

The long-term goal of the Laboratory of Biology of Cytoskeleton is to explain in molecular terms the role which individual microtubule components play in functional diversity of microtubules. The research of the laboratory is also focused on regulatory mechanisms of microtubule nucleation. To this end we have prepared the panel of antibodies against different microtubule proteins including α-, β- and γ-tubulins. The characterization of γ-tubulin in various cells has shown that γ-tubulin is highly conserved protein (Nováková et al., 1996; Nohýnková et al., 2000) that can interact with protein tyrosine kinases (Dráberová, 1999). To increase our knowledge about γ-tubulin functions in different cell types we have analyzed properties of γ-tubulin and mechanisms of microtubule formation in more detail.

Partial aims of this PhD thesis were the following:

1. Determination of γ-tubulin distribution in cells with highly specialized microtubule structures.

2. Biochemical characterization of γ-tubulin, its soluble forms and its possible post-translational modification.

3. Analysis of γ-tubulin complexes and elucidation of mechanisms that could be involved in regulation of γ-tubulin functions.

III. COMMENTS ON PRESENTED PUBLICATIONS

III.1. Distribution of γ-tubulin

III.1.1.

Linhartová I., Novotná B., **Sulimenko V.**, Dráberová E., Dráber P.: *Gamma-tubulin in chicken erythrocytes: changes in localization during cell differentiation and characterization of cytoplasmic complexes.* **Developmental Dynamics, 223**: 229-240, 2002.

This work deals with the distribution of γ-tubulin in developing chicken erythrocytes and the possible role of γ-tubulin in the formation and stability of microtubule marginal bands (MB). The formation of the MB represents the most striking event during the differentiation of avian spherical erythroblasts to ellipsoidal erythrocytes, which is accompanied by rearrangements of the cytoskeleton. One of the key questions of MB biogenesis is whether the nucleation of MB microtubules arises from MTOCs or from microtubule-nucleating activity located at the plasma membrane.

Quantitative immunoblotting revealed changes in the amount of γ-tubulin in differentiating erythrocytes. Immunofluorescence microscopy showed that γ-tubulin in erythroid cells of 5-day-old embryos was located in MTOCs, spindles and on MB, whereas it was not detectable in immature erythrocytes. To investigate whether γ-tubulin is involved in the nucleation of MB in erythroid cells, microtubules were depolymerized by cold treatment, followed by rewarming. In cold-treated cells γ-tubulin was detected in the form of large clusters around the nucleus, similar to tubulin dimers. After rewarming the clusters disappeared and γ-tubulin was located in MTOCs and on MB. In cold-treated cells no distinct nucleating centers were present at the cell periphery. Mature erythrocytes were not stained with anti-γ-tubulin antibodies, but contained a relatively high amount of soluble γ-tubulin. We therefore examined whether γ-tubulin was present in the form of soluble complexes in the cells. Gel filtration chromatography revealed that γ-tubulin was distributed in complexes of various sizes and those large complexes were sensitive to salt treatment. Immunoprecipitation experiments showed that soluble complexes of γ-tubulin contained αβ-tubulin dimers.

Two-dimensional electrophoretic analysis of γ-tubulin unveiled multiple charge variants. The number of γ-tubulin increased during the course of differentiation and at least six isoelectric variants of γ-tubulin, in mature erythrocytes, were detected. As two genes for γ-tubulin are present in vertebrates, multiple charge variants suggest the presence of posttranslational modifications.

The presented data demonstrated for the first time, that γ-tubulin is a substrate for developmentally regulated posttranslational modifications and that the binding properties of γ-tubulin or its complexes change during differentiation events.

III.1.2.

Libusová L., Sulimenko T., **Sulimenko V.,** Hozák P., Dráber P. *γ-Tubulin in Leishmania: cell cycle dependent changes in subcellular localization and heterogeneity of its isoforms.* **Experimental Cell Research, 295**: 375-386, 2004.

The main goal of this work was to determine subcellular localization of γ-tubulin in *Leishmania tropica* during the cell cycle. *Leishmania* belongs to flagellated protozoa with elaborated microtubule structures. γ-Tubulin was detected using a panel of six antibodies against different epitopes on N- and C-terminal domains of the γ-tubulin molecule. Multiple γ-tubulin isoforms were discernible, but in contrast to mammalian and plant cells γ-tubulin was found only in insoluble cytoskeletal fractions. Immunofluorescence microscopy revealed the presence of γ-tubulin in basal bodies, the posterior pole of the cell and in the flagellum. Furthemore, the antibodies showed punctuate staining in the subpelicullar microtubules. In dividing cells γ-tubulin was accumulated in the posterior poles, where it co-distributed with 210 kDa microtubule-interacting protein (Libusová et al., 2001) and vimentin-like 57-kDa protein. The role of γ-tubulin at the posterior pole is unclear, but it could be involved in anchoring or stabilizing of the subpellicular corset of microtubules.

The unexpected finding of γ-tubulin localization along the flagellum was verified by immunoblotting, immunofluorescence and immunogold electron microscopy on isolated flagella. Immunogold electron microscopy revealed that γ-tubulin is specifically associated with the so called paraflagellar rod (PFR) that runs adjacent to the axonemal microtubules. The function of γ-tubulin in flagella is uncertain, but it might be associated with the scaffold of proteins that become attached with PFR components during intraflagellar transport.

The complex cell-cycle dependent pattern of γ-tubulin localization and its properties indicate that γ-tubulin in *Leishmania* might have other functions besides microtubule nucleation.

III.2. γ-Tubulin and its complexes

III.2.1.

Sulimenko V., Sulimenko T., Poznanovic S., Nechiporuk-Zloy V., Bőhm K.J., Macůrek L., Unger E., Dráber P.: *Assotiation of brain γ-tubulin with αβ-tubulin dimers.* **Biochemical Journal, 365**: 889-895, 2002.

This paper is focused on the important question in the γ-tubulin field - whether soluble γ-tubulin exists only as a component of γ-TuRCs and γ-TuSCs, or if there are other complexes containing γ-tubulin. Therefore, we performed biochemical characterization of soluble brain γ-tubulin.

After gradient centrifugation of high-speed extract, γ-tubulin was distributed continually in a large zone that reflected its presence in complexes of various sizes. At least some of the complexes were distinct from γ-TuRCs and γ-TuSCs. αβ-Tubulin dimers had a similar pattern of distribution. Interestingly, during the course of preparation of microtubule proteins γ-tubulin co-distributed with αβ-tubulin dimers. It was possible to co-precipitate γ-tubulin by anti-α-tubulin antibody. Also αβ-tubulin dimers were detected in complexes precipitated by anti-γ-tubulin-antibodies. The same co-precipitation results were observed with small- and high-molecular-mass fractions obtained by gradient centrifugation. These experiments indicated that soluble γ-tubulin can form complexes with αβ-tubulin dimers.

The separation of microtubule proteins under non-denaturating conditions revealed the ability of γ-tubulin to form itself and/or with help of other proteins oligomers which were distinct from similar oligomers formed by αβ-tubulin dimers. In contrast to αβ-tubulin oligomers, those of γ-tubulin did not contain monomer subunits. Using SDS of different grades, we were able to separate on one-dimensional electrophoresis two forms of γ-tubulin from brain extract and microtubule proteins. The γ-tubulin doublet was observed in both low- and hig-molecular mass fractions after gradient centrifugation and in immunoprecipitated

material. There is still an open question, if the observed γ-tubulin isoforms reflect posttranslational modifications of γ-tubulin or the presence of two γ-tubulins coded by different genes. In any case, two-dimensional electrophoretic analysis revealed multiple isoelectric variants of γ-tubulin, both in pig brain extract and in microtubule proteins. Therefore, brain γ-tubulin is posttranslationally modified.

In conclusion, we have demonstrated for the first time, that there are two γ-tubulin isoforms in the brain, persisted in different sized complexes. We have found that γ-tubulin is able to form oligomers. The association of γ-tubulin with αβ-tubulin heterodimers, irrespective of the size of the complexes, indicates the presence of other γ-tubulin forms apart from γ-TuRC and γ-TuSCs.

III.2.2.

Dryková D., Cenklová V., **Sulimenko V.,** Volc J., Dráber P., Binarová P.: *Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes.* **Plant Cell, 15**: 465-480, 2003.

The main goal of this work was to investigate the distribution, properties and functions of γ-tubulin forms in acentriolar plant cells that do not contain classical MTOCs and that have different mechanisms of microtubule organization. Gel filtration chromatography of cell extracts from *Arabidopsis* revealed that γ-tubulin was present in protein complexes of various sizes, similar to those of animal cells. In contrast to large γ-tubulin complexes of animals, plant complexes were more stable in the presence of high concentrations of salt. Plant γ-tubulin was associated with αβ-tubulin dimers in immunoprecipitated experiments, cosedimented with microtubules polymerized *in vitro* and localized along their whole length. γ-Tubulin was detected by immunofluorescence microscopy on the poles of acentrosomal spindles that are associated with the plasma membrane or the membranes of polar vacuoles. Double-label staining showed an association of γ-tubulin with the kinetochore microtubules and with Golgi membranes. To analyze the interaction of plant γ-tubulin with membranes cell extracts were separated by differential centrifugation into cytosolic and membrane fractions. It was shown that γ-tubulin and αβ-tubulin dimers are associated with plant membranes in high-speed microsomal fractions. Blue-native electrophoresis of detergent-solubilized microsomes showed that γ-tubulin complexes were larger than 1 MD. αβ-Tubulin dimers

were also found in large complexes associated with membranes. This is the first description of γ-tubulin association with the membranes of plant cells. The ability of large complexes to nucleate microtubules was revealed by *in vitro* nucleation experiments, where brain tubulin was added to the fractions obtained from gel filtration. Nucleation and microtubule polymerization were observed neither in fractions containing γ-tubulin in the form of small complexes, nor in fractions depleted of γ-tubulin by differential centrifugation.

In conclusion, our results indicate that the association of γ-tubulin complexes with dynamic membranes might ensure the flexibility of non-centrosomal microtubule nucleation.

III.2.3.

Kukharskyy V., **Sulimenko V.,** Macůrek L., Sulimenko T., Dráberová E, Dráber P. *Complexes of γ-tubulin with non-receptor protein tyrosine kinases Src and Fyn in differentiating P19 embryonal carcinoma cells.* **Experimental Cell Research, 298**: 218-228, 2004.

In this work, we have investigated the interactions of γ-tubulin with protein tyrosine kinases of the Src family during the neuronal differentiation of P19 embryonal carcinoma cells. It was shown previously that these kinases are regulators of neuronal differentiation and that tubulin dimers are substrates for Src family kinases.

Using quantitative immunoblotting, we have demonstrated an increased expression of Src and Fyn kinases during the neuronal differentiation of P19 cells stimulated by retinoic acid (RA). This increased expression corresponded with the increased level of tyrosine-phosphorylated proteins. While the expression of γ-tubulin was unchanged, the expression of neuron-specific β-tubulin class III increased. The amount of acetylated α-tubulin (a marker of stable microtubules) also increased during the course of differentiation. Two-dimensional electrophoresis revealed the presence of multiple isoforms of γ-tubulin and treatment with alkaline phosphatase decreased the number of charged variants. This data documented, for the first time, that γ-tubulin is phosphorylated in mammalian cells. Immunoprecipitation experiments showed the interaction of γ-tubulin with Fyn and Src kinases, as well as with other unknown proteins phosphorylated on tyrosine. In cells stimulated by RA the level of phosphorylated proteins co-precipitated with γ-tubulin was higher than in resting cells. Using the Src family selective tyrosine kinase inhibitor PP2 confirmed that the formation of γ-tubulin complexes with phosphorylated proteins was dependent on the activity of Src family

kinases. *In vitro* kinase assays revealed directly, that several kinase substrates are in complexes immunoprecipitated by anti-γ-tubulin antibodies. A lower level of phosphorylation was clearly detected when an *in vitro* kinase assay was done in the presence of PP2.

To investigate possible mechanisms of the association of γ-tubulin with active kinases, *in vitro* binding experiments with GST fusion proteins containing SH2 and SH3 domains of Src and Fyn kinases were performed. These domains are important in the assembly of protein complexes containing the Src family kinases. γ-Tubulin bound to GST-Fyn-SH2 and GST-Src-SH2 fusion proteins, but not to GST-SH3 or GST alone. Phenyl phosphate, an analogue of phosphotyrosine, inhibited the association of γ-tubulin with SH2 domains. These data indicated that γ-tubulin can directly or indirectly bind to the regulatory domain of kinases.

The combined data show, that in the course of neuronal differentiation, the Src family protein tyrosine kinases Src and Fyn form complexes with γ-tubulin by interactions with their SH2 domains. γ-Tubulin can be phosphorylated *in vivo* and the activity of Src family kinases might regulate the interaction of γ-tubulin with tubulin dimers or other proteins during neurogenesis.

III.2.4.

Sulimenko V., Dráberová E, Sulimenko T., Macůrek L., Richterová V., Dráber Pe., Dráber Pa. *Regulation of microtubule formation in activated mast cells by complexes of γ-tubulin with Fyn and Syk kinases.* **Journal of Immunology, 176**: 7243-7253, 2006.

In this work, we continued the analysis of γ-tubulin complexes with protein tyrosine kinases. It is well established, that after the aggregation of high affinity IgE receptors (FcεRI) on the surface of granulated mast cells the Src family kinases are activated and the granules containing inflammatory mediators move to the cell periphery. It was suggested that microtubules may play a pivotal role in this process, but the molecular mechanisms that control microtubule rearrangement during this process are poorly understood. It was shown previously, that γ-tubulin forms complexes with tyrosine phosphorylated proteins and the Src family kinase Lyn, in rat basophilic leukemia cells (Dráberová et al., 1999). To estimate whether Lyn kinase is indispensable for the formation of such complexes in mast cells, we analyzed interactions of γ-tubulin with kinases and their substrates in resting and activated wild type and Lyn-deficient bone marrow mast cells (BMMCs).

The activation of BMMCs induced by FcεRI aggregation or treatment with pervanadate led to rapid polymerization of microtubules. This polymerization was not dependent on the presence of Lyn kinase, as determined by experiments with BMMCs $Lyn^{-/-}$. γ-Tubulin was found in both soluble and insoluble fractions after NP-40 extraction, which corresponded with the distribution of Fyn kinase. Several lines of evidence indicated that γ-tubulin extracted from mast cells with non-ionic detergent NP-40, formed complexes with signal transduction molecules that could modulate microtubule arrays. First, tyrosine-phosphorylated proteins were found to be associated with immunoprecipitated γ-tubulin in resting cells and the amount of these proteins increased after activation. Second, *in vitro* kinase assays revealed that γ-tubulin formed complexes containing kinases and their substrates. Increased levels of ^{32}P labeled proteins were observed when kinase assays were performed with lysates from cells exposed to pervanadate or cells activated by FcεRI aggregation. Third, the activity of γ-tubulin associated kinases was inhibited by the pre-treatment of cells with the Src-family specific (PP2) and Syk-specific (piceatannol) inhibitors. This suggests that the Src and Syk family kinases have an important role in the formation of γ-tubulin signaling complexes. Fourth, the association of Fyn kinase with γ-tubulin complexes was confirmed by immunoprecipitations experiments and the same holds true for Syk kinase. Finally, kinases in γ-tubulin complexes were capable of utilizing the tubulin dimer as a substrate and its phosphorylation was inhibited by both PP2 and piceatannol. These data suggested that the Syk and Src family kinases were involved in the initial stages of microtubule formation. The interaction of γ-tubulin with Fyn kinase was corroborated by pull-down experiments, in which the γ-tubulin complex bound to the recombinant SH2 domain of Fyn kinase and this interaction was of the phosphotyrosine type. Inhibition with phenylphosphate and synthetic peptides from potential binding sites on the γ-tubulin molecule failed to confirm a direct interaction of γ-tubulin with these domains. Thus, the association of γ-tubulin with the SH2 domain is most likely mediated via adaptor-like tyrosine-phosphorylated protein(s).

In conclusion, we have shown that activation of BMMCs leads to rapid reorganization of microtubules. γ-Tubulin forms complexes with Fyn and Syk kinases and other signal transduction molecules in the process of cell activation. We propose that Fyn and Syk kinases are involved in the regulation of binding properties of γ-tubulin and/or its associated proteins, and thus modulate microtubule formation in activated mast cells.

IV. CONCLUSIONS

Major results of this PhD thesis can be summarized as follows:

A complex distribution pattern of γ-tubulin was found in cells with highly specialized microtubule structures. γ-Tubulin was unexpectedly located on the marginal band of microtubules in differentiating avian erythrocytes. In protozoa *Leishmania* γ-tubulin was associated with the posterior pole of the cell and with the paraflagellar rod in the flagella. Moreover γ-tubulin was found in membrane microsomal fraction in plants and in detergent-resistant fractions in mammalian cells. These findings indicate that γ-tubulin might have in different cell types other functions besides microtubule nucleation.

We have demonstrated that γ-tubulin is able to form oligomers and to form complexes with αβ-tubulin dimers. γ-Tubulin is present in cytoplasmic complexes of various size and its binding properties change during differentiation events. Our data indicate that γ-tubulin is present in other molecular complexes apart from earlier described large (γ-TuRCs) and small (γ-TuSCs) complexes. We have demonstrated for the first time that in various cell types γ-tubulin is posttranslationally modified and that in mammalian cells it can be phosphorylated. We suggest that posttranslational modifications of γ-tubulin might regulate interactions of γ-tubulin with αβ-tubulin heterodimers or other associated proteins.

γ-Tubulin is associated with protein tyrosine kinases involved in signal transduction events. γ-Tubulin complexes with the active Src family kinases and other tyrosine-phosphorylated proteins were found in embryonal carcinoma cells as well as in mast cells. Activation of mast cells led to rapid polymerization of microtubules that was dependent on the activity of the Src family kinases. γ-Tubulin interacts with protein tyrosine kinases of the Src family through their regulatory SH2 domains and this interaction is phosphotyrosine type. Our data indicate that the association of γ-tubulin with SH2 domain is probably mediated via adaptor-like tyrosine-phosphorylated protein(s). We propose that Src family kinases are involved in the regulation of binding properties of γ-tubulin and/or microtubule nucleation.

V. REFERENCES

1. Aldaz H., Rice L.M., Stearns T., Agard D.A. (2005) Insights into microtubule nucleation from the crystal structure of human γ-tubulin. *Nature*, 435: 523-527

2. Allen R.D., Wiess D.G., Hayden J.H., Brown D.T., Fujiwake H., Simpson M. (1985) Glideng movement of and bidirectional transport along single native microtubules from squid axoplasm: evidence for an active role of microtubules in cytoplasmic transport. *J. Cell Biol.*, 100: 1736-1752

3. Amos L.A., Hirose K. (1997) The structure of microtubule-motor complexes. *Curr. Opin. Cell Biol.*, 9: 4-11

4. Argarana C.E., Arce C.A., Barra H.S., Caputto R. (1977) In vivo incorporation of [^{14}C]-tyrosine into C-terminal position of the α subunit of tubulin. *Arch. Biochem. Biophys.*, 180: 264-268

5. Argarana C.E., Barra H.S., Caputto R. (1978) Release of [^{14}C]-tyrosine from tubulinyl-[^{14}C]-tyrosine by brain extract. Separation of a carboxypeptidase from tubulin-tyrosine ligase. *Mol. Cell. Biochem.*, 19: 17-21

6. Banerjee A. (2002) Coordination of posttranslational modifications of bovine brain α-tubulin. Polyglycilation of Δ2-tubulin. *J. Biol. Chem.*, 277: 46140-46144

7. Barra H.S., Rodriguez J.A., Arce C.A., Caputto R. (1973) A soluble preparation from rat brain that incorporates into its own proteins [^{14}C]-arginine by a ribonuclease-sensitive system and [^{14}C]-tyrosine by a ribonuclease-insensitive system. *J. Neurochem.*, 20: 97-108

8. Battaglia P.A., Ponti D., Naim V., Venanzi S., Psaila R., Gigliani F. (2005) The HIV-Tat protein induces chromosome number aberrations by affecting mitosis. *Cell Motil. Cytoskel.*, 61: 129-136

9. Bettencourt-Dias M., Giet R., Sinka R., Mazumdar A., Lock W.G., Balloux F., Zafiropoulos P.J., Yamaguchi S., Winter S., Carthew R.W., Cooper M., Jones D., Frenz L., Glover D.M. (2004) Genome-wide survey of protein kinases required for cell cycle progression. *Nature*, 432: 980-987

10. Binarová P., Hause B., Dolezel J., Dráber P. (1998) Association of γ-tubulin with kinetochore/centromeric region of plant chromosomes. *Plant J.*, 14: 751-757

11. Binarová P., Cenklová V., Hause B., Kubátová E., Lysák M., Dolezel J., Bogre L., Dráber P. (2000) Nuclear gamma-tubulin during acentriolar plant mitosis. *Plant Cell*, 12: 433-442

12. Bisig C.G., Purro S.A., Contin M.A., Barra H.S., Arce C.A. (2002) Incorporation of 3-nitrotyrosine into the C-terminus of alpha-tubulin is reversible and not detrimental to dividing cells. *Eur. J. Biochem.*, 269: 5037-5045

13. Bobinnec Y., Moudjou M., Fouquet J.P., Desbruyeres E., Edde B., Bornens M. (1998) Glutamylation of centriole and cytoplasmic tubulin in proliferating non-neuronal cells. *Cell Motil. Cytoskeleton*, 39: 223-232

14. Bobinnec Y., Khodjakov A., Mir L.M., Rieder C.L., Edde B., Bornens M. (1998) Centriole disassembly in vivo and its effect on centrosome structure and function in vertebrate cells. *J. Cell Biol.*, 143: 1575-1589

15. Bobbinec Y., Fukuda M., Nishida E. (2000) Identification and characterization of *Caenorhabditis elegans* γ-tubulin in dividing cells and differentiated tissues. *J. Cell Sci.*, 113: 3747-3759

16. Bornens M. (2002) Centrosome composition and microtubule anchoring mechanisms. *Curr. Opin. Cell Biol.*, 14: 25-34

17. Brannstrom K., Segerman B., Gullberg M. (2003) Molecular dissection of GTP exchange and hydrolysis within the ternary complex of tubulin heterodimers and Op18/stathmin family members. *J. Biol. Chem.*, 278: 16651-16657

18. Bre M.H., de Nechaud B., Wolff A., Fleury A. (1994) Glutamylated tubulin probed in ciliates with the monoclonal antibody GT335. *Cell Motil. Cytoskeleton*, 27: 337-349

19. Bre M.H., Redeker V., Vinh J., Rossier J., Levilliers N. (1998) Tubulin polyglycylation: differential posttranslational modification of dynamic cytoplasmic and stable axonemal microtubules in *Paramecium. Mol. Biol. Cell*, 9: 2655-2665

20. Burns R.G. (1991) α-, β- and γ-tubulins: Sequence comparisons and structural constraints. *Cell Motil. Cytoskel.*, 20: 181-189.

21. Caplow M., Zeeberg B. (1982) Dynamic properties of microtubules at steady state in the presence of taxol. *Eur. J. Biochem.*, 127: 319-324

22. Cappelletti G., Maggioni M.G., Tadeschi G., Maci R. (2003) Protein tyrosine nitration is triggered by nerve growth factor during neuronal differentiation of PC12 cells. *Exp. Cell Res.*, 288: 9-20

23. Caron J.M. (1997) Posttranslational modifications of tubulin by palmitoylation: I. In vivo and cell-free studies. *Mol. Biol. Cell*, 8: 621-636

24. Caron J.M., Vega L.R., Fleming J., Bishop R., Solomon F. (2001) Single site α–tubulin mutation affects astral microtubules and nuclear positioning during anaphase in *Saccharomyces cerevisiae*: possible role for palmitoylation of α-tubulin. *Mol. Biol. Cell*, 12: 2672-2687

25. Casenghi M., Meraldi P., Weinhart U., Duncan P.I., Korner R., Nig E.A. (2003) Polo-like kinase 1 regulates Nlp, a centrosome protein involved in microtubule nucleation. *Dev. Cell*, 5: 113-125

26. Chang P., Stearns T. (2000) Delta-tubulin and epsilon-tubulin: two new human centrosomal tubulins reveal new aspects of centrosome structure and function. *Nat. Cell Biol.*, 2: 30-35

27. Chang P., Giddings T.H.Jr., Winey M., Stearns T. (2003) Epsilon-tubulin is required for centriole duplication and microtubule organization. *Nat. Cell Biol.*, 5: 71-76

28. Cleveland D.W., Sullivan K.F. (1985) Molecular biology and genetics of tubulin. *Annu. Rev. Biochem.*, 54: 331-365

29. Cleveland D.W. (1987) The multitubulin hypothesis revisited: what have we learned? *J. Cell Biol.*, 104: 381-383

30. Correia J.J., Beth A.H., Williams R.C.Jr. (1988) Tubulin exchanges divalent cations at both guanine nucleotide-binding sites. *J. Biol. Chem.*, 263: 10681-10686

31. Daly S., Yacoub A., Dundon W., Mastromei G., Islam K., Lorenzetti R. (1997) Isolation and characterization of a gene encoding α-tubulin from *Candida albicans*. *Gene*, 187: 151-158

32. Debec A., Detraves C., Montmory C., Geraurd G., Wright M. (1995) Polar organization of gamma-tubulin in acentriolar mitotic spindles of *Drosophila melanogaster* cells. *J. Cell Sci.*, 108: 2645-2653

33. Desai A., Verma S., Mitchison T,.J., Walczak C.E. (1999) Kin I kinesins are microtubule-destabilizing enzymes. *Cell*, 96: 69-78

34. Détraves C., Mazarguil H., Lojoie-Mazenc I., Julian M., Raynaud-Messina B., Wright M. (1997) Protein complexes containing gamma-tubulin are present in mammalian brain microtubule protein preparations. *Cell Motil. Cytoskel.*, 36: 179-189

35. Detrich H.W.3rd, Williams R.C. (1978) Reversible dissociation of the αβ dimer of tubulin from bovine brain. *Biochemistry*, 17: 3900-3907

36. Dictenberg J., Zimmerman W., Sparks C.A., Young A., Vidair Ch., Zheng Y., Carrington W., Fay F.S., Doxsey J. (1998) Pericentrin and γ-tubulin form a protein complex and are organized into novel lattice at the centrosome. *J. Cell Biol.*, 141: 163-174

37. Dibbayawan T.P., Harper J.D.I., Elliott J.E., Gunninig B.E.S., Marc J. (1995) A γ-tubulin that associates specifically with centrioles in HeLa cells and the basal body complex in *Chlamydomonas*. *Cell Biol. Internat.*, 19: 559-567

38. Doxsey S., McCollum D., Theurkauf W (2005) Centrosomes in cellular regulation. *Annu. Rev. Cell Dev. Biol.*, 21: 411-434

39. Dráber P., Dráberová E., Linhartová I., Viklický V. (1989) Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies. *J. Cell Sci.*, 92: 519-528

40. Dráberová E., Dráber P. (1993) A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with icrotubules. *J. Cell Sci.*, 106: 1263-1273

41. Dráberová L., Dráberová E., Surviladze Z., Dráber Pe., Dráber Pa. (1999) Protein tyrosine kinase $p53/56^{lyn}$ forms complexes with gamma-tubulin in rat basophilic leukemia cells. *Int. Immunol.*, 11: 1829-1839

42. Dustin P. (1984) Microtubules. Springer, Berlin

43. Dutcher S.K., Trabuco E.C. (1998) The UNI3 gene is required for assembly of basal bodies of *Chlamydomonas* and encodes delta-tubulin, a new member of the tubulin superfamily. *Mol. Biol. Cell,* 9: 1293-1308

44. Dutcher S.K. (2001) The tubulin fraternity: alpha to eta. *Curr. Opin. Cell Biol.*, 13: 49-54

45. Dutcher S.K. (2003) Long-lost relatives reappear: identification of new members of the tubulin superfamily. *Curr. Opin. Microbiol.*, 6: 634-640

46. Edde B., Rossier J., Le Caer J.P., Desbruyeres E., Gros F., Denoulet P.(1990) Posttranslational glutamylation of α-tubulin. *Science*, 247: 83-85

47. Eipper B.A. (1974) Properties of rat brain tubulin. *J. Biol. Chem.*, 249: 1407-1416

48. Eiserich J.P., Estevez A.G., Bamberg T.V., Ye Y.Z., Chumley P.H., Beckman J.S., Freeman B.A. (1999) Microtubule dysfunction by posttranslational nitrotyrosination of a-tubulin: a nitric oxide-dependent mechanism of cellular injury. *Proc. Natl. Acad. Sci. USA*, 96: 6365-6370

49. Erickson H.P., Stoffler D. (1996) Protofilament and rings, two conformations of the tubulin family conserved from bacterial FtsZ to α/β and γ tubulin. *J. Cell Biol.*, 135: 5-8

50. Erickson H.P. (2000) γ-Tubulin nucleation: template or protofilament? *Nature Cell Biol.*, 2: E93-E96

51. Faruki Sh., Geahlen R.L., Asai D.J. (2000) Syk-dependent phosphorylation of microtubules in activated B-lymphocytes. *J. Cell Sci.*, 113: 2557-2565

52. Fava F., Raynaud-Messina B., Leung-Tack J., Mazzolini L., Li M., Guillemot J.C., Cachot D., Tollon Y., Ferrara P., Wright M. (1999) Human 76p: a new member of the gamma tubulin associated protein family. *J. Cell Biol.*, 147: 857-868

53. Felix G.W., Antony C., Wright M., Maro B. (1994) Centrosome assembly in vitro: role of γ-tubulin recruitment in *Xenopus* sperm aster formation. *J.Cell.Biol.*, 124: 19-31

54. Feng Y., Hodge D.R., Palmieri G., Chase D.L., Longo D.L., Ferris D.K. (1999) Association of polo-like kinase with α-, β- and γ-tubulins in a stable complex. *Biochem. J.*, 339: 435-442

55. Fong D., Lee B. (1988) Beta tubulin gene of the parasitic protozoan *Leishmania mexicana. Mol. Biochem. Parasitol.*, 31: 97-106

56. Fourest-Lieuvin A., Peris L., Gache V., Garcia-Saez I., Juillan-Binard C., Lantez V., Job D. (2006) Microtubule regulation in mitosis: tubulin phosphorylation by the cyclin-dependent kinase Cdk1. *Mol. Biol. Cell*, 17: 1041-1050

57. Fromherz S., Giddings T.H., Gomez-Ospina N., Dutcher S.K. (2004) Mutations in alpha-tubulin promote basal body maturation and flagellar assembly in the absence of delta-tubulin. *J. Cell Sci.*, 117: 303-314

58. Fuller S.D., Gowen B.E., Reinsch S., Sawyer A., Buendia B., Wepf R., Karsenti E. (1995) The core of the mammalian centriole contains gamma-tubulin. *Curr. Biol.*, 5: 1384-1393

59. Gaertig J., Cruz M.A., Bowen J., Gu L., Pennock D.G., Gorovsky M.A. (1995) Acetylation of lysine 40 in a-tubulin is not essential in *Tetrahymena thermophila. J.Cell Biol.*, 129: 1301-1310

60. Galjart N., Perez F. (2003) A plus-end raft to control microtubule dynamics and function. *Curr. Opin. Cell Biol.*, 15: 48-53

61. Galjart N. (2005) CLIPs and CLASPs and cellular dynamics (2005) *Nature Rev. / Mol. Cell Biol.*, 6: 487-498

62. Gard D.L., Kirschner M.W. (1985) A polymer-dependent increase in phosphorylation of b-tubulin accompanies differentiation of a mouse neuroblastome cell line. *J. Cell Biol.*, 100: 764-774

63. Geissler S., Siegers K., Schiebel E. (1998) A novel protein complex promoting formation of functional α- and γ-tubulin. *EMBO J.*, 17: 952-966

64. Glover D.M. (2005) Polo kinase and progression through M phase in *Drosophila*: a perspective from the spindle poles. *Oncogene*, 24: 230-237

65. Gomez-Conde E., Lopez-Robles M.C., Hernandez-Rivas R., Hernandez-Jauregui P., Vargas-Mejia M. (2000) Structural organization of gamma-tubulin in the microtubule organizing center (MTOC) during the nuclear division of *Entamoeba hystolitica* trophozoites. *Arch. Med. Res.*, 31: S205-S206

66. Gueth-Hallonet C., Antony C., Aghion J., Santa-Maria A., Lajoie-Mazenc I., Wright M., Maro B. (1993) γ-Tubulin is present in acentriolar MTOCs during early mouse development. *J. Cell Sci.*, 105: 157-166

67. Gundersen G.G., Cook T.A. (1999) Microtubules and signal transduction. *Curr. Opin. Cell Biol.*, 11: 81-94

68. Hamel E. (1996) Antimitotic natural products and their interactions with tubulin. *Med. Res. Rev.*, 16: 207-231

69. Heald R., Tournebize C., Blank T., Sandantzopoulos R., Becker P., Hyman A.R., Karsenti E. (1996) Self-organization of microtubules into bipolar spindles around arteficial chromosomes in *Xenopus* egg extracts. *Nature*, 385: 420-425

70. Heald R & Nogales E. (2002) Microtubule dynamics. *J. Cell Sci.*, 115: 3-4

71. Hendrickson T.W., Yao J., Bhadury S., Corbett A.H., Joshi H.C. (2001) Conditional mutations in gamma-tubulin reveal its involvement in chromosome segregation and cytokinesis. *Mol. Biol. Cell*, 12: 2469-2481

72. Hirokawa H., Takemura R. (2004) Kinesin superfamily proteins and their various functions and dynamics. *Exp. Cell Res.*, 301: 50-59

73. Horio T., Uzawa S., Jung M.K., Oakley B.R., Tanaka K., Yanagida M. (1991) The fission yeast γ-tubulin is essential for mitosis and is localized at the microtubule organizing centers. *J.Cell. Sci.*, 99: 693-700

74. Horio T., Oakley B.R. (1994) Human γ-tubulin functions in fission yeast. *J. Cell. Biol.*, 126: 1465-1473.

75. Hsu L.C., White R.L. (1998) BRCA1 is associated with the centrosome during mitosis. *Proc. Nat. Acad. Sci. USA*, 95: 12983-12988

76. Hsu L.C., Doan T.P., White R.L. (2001) Identificationof a gamma-tubulin-binding domain in BRCA1. *Cancer Res.*, 61: 7713-7718

77. Inclan V.M., Nogales E. (2000) Structural models for the self-assembly and microtubule interactions of γ-, δ- and ε- tubulin. *J. Cell Sci.*, 114: 413-422.

78. Jaspersen S.L., Winey M. (2004) The budding yeast spindle pole body: structure, duplication and function. *Annu. Rev. Cell Dev. Biol.*, 20: 1-28

79. Joshi H.C., Palacios M.J. McNamara L., Cleveland D.W. (1992) γ-Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. *Nature*, 356: 80-83

80. Julian M., Tollon Y., Lajoie-Mazenc I., Moisand A., Mazarguil H., Puget A., Wright M. (1993) γ-Tubulin participates in the formation of midbody during cytokinesis in mammalian cells. *J. Cell Sci.*, 105: 145-156

81. Keating T.J., Borisy G.G. (2000) Immunostructural evidence for the template mechanism of microtubule nucleation. *Nature Cell Biol.*, 2: 352-357

82. Kellogg D.R., Moritz M., Alberts B.M (1994) The centrosome and cellular organization. *Annu. Rev. Biochem.*, 63: 639-674

83. Knop M., Pereira G., Geissler S., Grein K., Schiebel E. (1997) The spindle pole body component Spc97p interacts with the gamma-tubulin of *Saccharomyces cerevisiae* and

functions in microtubule organizationand spindle pole body duplication. *EMBO J.*, 16: 1550-1564

84. Knop M., Schiebel E. (1997) Spc98p and Spc97p of the yeast gamma-tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p. *EMBO J.*, 16: 6985-6995

85. Kratzerová L., Dráberová E., Juliano C., Viklický V., Fiori P.L., Cappuccinelli P., Dráber P. (2001) Cell-cycle-dependent changes in localization of a 21-kDa microtubule-interacting protein in *Leishmania. Exp. Cell Res.*, 266: 270-278

86. Lajoie-Mazenc I., Tollon I., Detravec C., Julian M., Moisand A., Gueth-Hallonet C., Debec A., Salles-Passador I., Puget A., Mazarguil H., Raynaud-Messina B., Wright M. (1994) Recruitment of antigenic gamma-tubulin during mitosis in animal cells: presence of gamma-tubulin in the mitotic spindle. *J. Cell Sci.*, 107: 2825-2837

87. Lajoie-Mazenc I., Detraves C., Rotaru V., Garres M., Tollon Y., Jean C., Julian M., Wright M., Raynaud-Messina B. (1996) A single gamma-tubulin gene and mRNA, but two gamma-tubulin polypeptides differing by their binding to the spindle pole organizing centres. *J. Cell Sci.*, 109: 2483-2492

88. Lambert J.M. (2005) Drug-conjugated monoclonal antibodies for the treatment of cancer. *Curr. Opin. Pharmacol.*, 5: 543-549

89. Lane H.A., Nig E.A. (1996) Dynamic changes in nuclear architecture during mitosis: on the role of protein phosphorylation in spindle assembly and chromosome segregation. *Exp. Cell Res.*, 229: 174-180

90. Larcher J.C., Boucher D., Lazereg S., Gros F., Denoulet P. (1996) Interaction of kinesin motor domains with α- and β-tubulin subunits at a tau-independing binding site. Regulation by polyglutamylation. *J.Biol. Chem.*, 271: 22117-22124

91. Laurent C.E., Delfino F.J., Cheng H.Y., Smithgall T.E. (2004) The human c-Fes tyrosine kinase binds tubulin and microtubules through separate domains and promotes microtubule assembly. *Mol. Cell Biol.*, 24: 9351-9358

92. Leguy R., Melki R., Pantaloni D., Carlier M.F. (2000) Monomeric γ-tubulin nucleates microtubules. *J. Biol. Chem.*, 275: 21975-21980

93. Ley S.C., Marsh M., Bebbington C.R., Proudfoot K., Gaehlen R.L. (1994) Distinct intracellular localization of *lck* and *fyn* protein tyrosine kinases in human T lymphocytes. *J.Cell Biol.*, 125: 639-649

94. L'Hernault S.W., Rosenbaum J.L. (1985) *Chlamydomonas* α–tubulin is posttranslationally modified by acetylation on the ε-amino group of a lysine. *Biochemistry*, 24: 473-478

95. Li H., De Rosier D.J., Nicholson W.V., Nogales E., Downing K.H. (2002) Microtubule structure at 8 Å resolution. *Structure*, 10: 1317-1328

96. Liang A., Ruiz F., Heckmann K., Klotz C., Tollon Y., Beisson J., Wright M. (1996) Gamma-tubulin is permanently associated with basal bodies in ciliates. *Eur. J. Cell Biol.*, 70: 331-338

97. Libusová L., Dráber P. (2006) Multiple tubulin forms in ciliates. *Protoplasma,* in print

98. Linhartová I., Dráber P., Dráberová E., Viklický V. (1992) Immunological discrimination of beta-tubulin isoforms in developing mouse brain. Post-translational modification of non-class-III beta-tubulins. *Biochem. J.*, 288: 919-924

99. Liu B., Marc J., Joshi H.C., Palevitz B.A. (1993) A gamma-tubulin-related protein associated with the microtubule arraysof higher plants in a cell-cycle-dependent manner. *J. Cell Sci.*, 104: 1217-1228

100. Liu B., Joshi H.C., Wilson T.J., Silflow C.D. Palevitz B.A. Snustad D.P. (1994) γ-Tubulin in *Arabidopsis*: gene sequence immunoblot, and immunofluorescence studies. *Plant Cell*, 6: 303-314.

101. Liu B., Joshi H.C., Palevitz B.A. (1995) Experimental manipulation of γ-tubulin distribution in Arabidopsis using anti-microtubule drugs. *Cell Motil. Cytoskel.*, 31: 113-129

102. Llanos R., Chevrier V., Ronjat M., Meurer-Grob P., Martinez P., Frank R., Bornens M., Wade R.H., Wehland J., Job D. (1999) Tubulin binding sites on γ-tubulin: identification and molecular characterization. *Biochemistry*, 38: 15712-15720.

103. Lowe J. Li H., Downing K.H., Nogales E. (2001) Refined structure of αβ-tubulin at 3.5 Å resolution. *J. Mol. Biol.*, 313: 1045-1057

104. Luduena R.F. (1998) Multiple forms of tubulin: different gene products and covalent modifications. *Int. Rev. Cytol.*, 178: 207-275.

105. Mandelkow E.M., Herrmann M., Ruhl U. (1985) Tubulin domains probed by limited proteolysis and subunit-specific antibodies. *J. Mol. Biol.*, 185: 311-327

106. Mandelkow E., Mandelkow E.M. (1995) Microtubules and microtubule-associated proteins. *Curr. Opin. Cell Biol.*, 7: 72-81

107. Marchesi V.T., Nhu Ngo (1993) In vitro assembly of multiprotein complexes containing α, β, and γ tubulin, heat shock protein HSP70, and elongation factor 1α. *Proc. Natl. Acad. Sci. USA*, 90: 3028-3032

108. Martin O.C., Gunawardane R.N., Iwamatsu A., Zheng Y. (1998) Xgrip109: a gamma tubulin-associated protein with an essential role in gamma tubulin ring complex (γ-TuRC) assembly and centrosome function. *J. Cell Biol.*, 141: 675-687

109. Matten W.T., Aubry M., West J., Maness P.F, (1990) Tubulin is phosphorylated at tyrosine by pp60c-src in nerve growth cone membranes. *J. Cell Biol.*, 111: 1959-1970

110. McKean P.G., Vaughan S., Gull K. (2001) The extended tubulin superfamily. *J. Cell Sci.*, 114: 2723-2733

111. Meads T., Schroer T.A. (1995) Polarity and nucleation of microtubules in polarized epithelial cells. *Cell Motil. Cytoskel.*, 32: 273-288

112. Melki R., Vainberg I.E., Chow R.L., Cowan N.J. (1993) Chaperonin-mediated folding of vertebrate actin-related protein and γ-tubulin. *J. Cell Biol.*, 122: 1301-1310

113. Menandez M., Rivas G., Diaz J.F., Andreu J.M. (1998) Control of the structural stability of the tubulin dimer by one high affinity bound magnesium ion at nucleotide N-site. *J. Biol. Chem.*, 273: 167-176

114. Mimori-Kiyosue Y., Grigoriev I., Lansbergen G., Sasaki H., Matsui Ch., Severin F., Galjart N., Grosveld F., Vorobjev I., Tsukita Sh., Akhmanova A. (2005) CLASP1 and CLASP2 bind to EB1 and regulate microtubule plus-end dynamics at the cell cortex. *J. Cell Biol.*, 168: 141-153

115. Mitchison T., Kirschner M. (1984) Dynamic instability of microtubule growth. *Nature*, 312: 237-242.

116. Moritz M., Braunfeld M.B., Sedat J.W.., Alberts B., Agard D.A. (1995) Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature*, 378: 638-640

117. Moritz M., Zheng Y., Alberts B.M., Oegama K. (1998) Recruitment of the gamma-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. J. Cell Biol., 142: 775-786

118. Moritz M., Braunfeld M.B., Guenebaut V., Heuser J., Agard D.A. (2000) Structure of the gamm-tubulin ring complex: a template for microtubule nucleation. *Nature Cell Biol.*, 2: 365-370

119. Moudjou M., Bordes N., Paintrand M., Bornens M. (1996) γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. *J. Cell Sci.*, 109: 875-887

120. Murata T., Sonobe S., Baskin T.I., Hyodo S., Hasezawa S., Nagata T., Horio T., Hasebe M. (2005) Microtubule-dependent microtubule nucleation based on recruitment of gamma-tubulin in higher plants. *Nat. Cell Biol.*, 7: 961-968

121. Muresan V., Joshi H.C., Besharse J.C. (1993) γ-Tubulin in differentiated cell types: localization in the vicinity of basal bodies in retinal photoreceptors and ciliated epithelia. *J. Cell Sci.*, 104: 1229-1237

122. Murphy S.M., Urbani L., Stearns T. (1998) The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components Sps97p and Spc98p. *J. Cell Biol.*, 141: 663-674

123. Murphy S.M., Preble A.M., Patel U.K., O'Connell K.L., Prabha Dias D., Moritz M., Agard D.A., Stults J.T., Stearns T. (2001) GCP5 and GCP6: two new members of the human γ-tubulin complex. Mol. Biol. Cell, 12: 3340-3352

124. Nakamura M., Masuda H., Horii J., Kuma K., Yokoyama N., Ohba T., Nishitani H., Miyata T., Tanaka M., Nishimoto T. (1998) When overexpressed, a novel centrosomal protein, RanBMP, causes ectopic microtubule nucleation. *J. Cell Sci.*, 143: 1041-1052

125. Neff N.F., Thomas J.H., Grisafi P., Botstein D. (1983) Isolation of beta-tubulin gene from yeast and demonstration of its essential function *in vivo*. *Cell*, 33: 211-219

126. Nogales E., Whittaker M., Milligan R.A., Downing K.H. (1999) High-resolution model of the microtubule. *Cell*, 96: 79-88

127. Nohýnková E., Dráber P., Reischig J., Kulda J. (2000) Localization of gamma-tubulin in interphase and mitotic cells of a unicellular eucaryote, *Giardia intestinalis*. Eur. J. Cell Biol., 79: 438-445

128. Nováková M., Dráberová E., Schurmann W., Czihak G., Viklický V., Dráber P. (1996) γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies. *Cell Motil. Cytoskel.*, 33: 38-51

129. Oakley C.E., Oakley B.R. (1989) Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature*, 338: 662-664.

130. Oakley B.R., Oakley C.E., Yoon Y., Jung M. (1990) γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell*, 61: 1289-1301.

131. Oakley B.R. (1994) γ-Tubulin. In: Microtubules. JS Hyams / Ed. C.W.Lloyd – New York: Wiley-Liss. Inc.: 33-45

132. Oakley B.R., Akkari Y.N. (1999) γ-Tubulin at ten: progress and prospects. *Cell Structure and Function*, 24: 365-372

133. Oegema K., Wiese C., Martin O.C., Milligan R.A., Iwamatsu A., Mitchison T.J., Zheng Y. (1999) Characterization of two related *Drosophila* gamma-tubulin complexes that differ in their ability to nucleate microtubules. *J. Cell Biol.*, 144: 721-733

134. Ohlmayer J.T., Kalderon D. (1998) Hedgehog stimulates maturation of Cubitus interruptus into a labile transcriptional activator. *Nature,* 396: 749-753

135. Ozols J., Caron J.M. (1997) Posttranslational modifications of tubulin by palmitoylation: II. Identification of sites of palmitoylation. *Mol. Biol. Cell*, 8: 637-645

136. Paluh J.L., Nogales E., Oakley B.R., McDonald K., Pidoux A.L., Cande W.Z. ((2000) A mutation in gamma-tubulin alters microtubule dynamics and organization and is synthetically lethal with the kinesin-like protein pk11p. *Mol. Biol. Cell.*, 11: 1225-1239

137. Palumbo A., Fiore G., Di Cristo C., Di Cosmo A., d'Ischia M (2002) NMDA receptor stimulation induces temporary a-tubulin degradation by nitric-oxide-mediated tyrosine nitration in the nervous system of Sepia officinalis. *Biochem. Biophys. Res. Commun.*, 293: 1536-1543

138. Panda D., Daijo J.E., Jordan M.A., Wilson L. (1995) Kinetic stabilization of microtubule dynamics at steady state in vitro by substoichiometric concentrations of tubulin-colchicine complex. *Biochemistry*, 34: 9921-9929

139. Paturle-Lafanechere L., Edde B., Denoulet P., Van Dorsselaer A., Mazarguil H., Le Caer J.P., Wehland J., Job D. (1991) Characterization of a major brain tubulin variant which cannot be tyrosinated. *Biochemistry*, 30: 10523-10528

140. Pellegrini F., Budman D.R. (2005) Review: tubulin function, action of antitubulin drugs and new drug development. *Cancer Invest.*, 23: 264-273

141. Prigozhina N.L., Walker R.A., Oakley C.E., Oakley B.R. (2001) Gamma-tubulin and the C-terminal motor domain kinesin-like protein, KLPA, function in the establishment of spindle bipolarity in *Aspergillus nidulans*. *Mol. Biol. Cell*, 12: 3161-3174

142. Quarmby L. (2000) Cellular Samurai: katanin and the severing of microtubules. *J. Cell Sci.*, 113: 2821-2827

143. Raff J.W., Kellogg D.R., Alberts B.M. (1993) *Drosophila* gamma-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.*, 121: 823-835

144. Raynaud-Messina B., Debec A., Tollon Y., Gares M., Wright M. (2001) Differential properties of the two *Drosophila* γ-tubulin isotypes. *E. J. Cell Biol.*, 80: 643-649.

145. Redeker V., Levilliers N., Schmitter J.M., Le Caer J.P., Rossier J., Adoutte A., Bre M.H. (1994) Polyglycylation of tubulin: a postranslational modification in axonemal microtubules. *Science*, 266: 1688-1691

146. Ruiz F., Krzywicka A., Klotz C., Keller A., Cohen J., Koll F., Balavoine G., Beisson J. (2000) The SM19 gene, required for duplication of basal bodies in *Paramecium*, encodes a novel tubulin, eta-tubulin. *Curr. Biol.*, 10: 1451-1454

147. Ruiz F., Dupuis-Williams P., Klotz C., Forquignon F., Bergdoll M., Beisson J., Koll F. (2004) Genetic evidence for interaction between eta- and beta-tubulins. *Eukaryot. Cell*, 3: 212-220

148. Ruthel G., Demmin G.L., Kallstrom G., Javid M.P., Badie S.S., Will A.B., Nelle T., Schokman R., Ngyen T.L., Carra J.H., Bavari S., Aman M.J. (2005) Association of ebola virus matrix protein VP40 with microtubules. *J. Virol.*, 79: 4709-4719

149. Sharp D.J., Rogers G., Scholey J.M. (2000) Microtubule motors in mitosis. *Nature*, 407: 41-47

150. Schuyler S.C. & Pellman D. (2001) Microtubule "plus-end tracking proteins". The end is just the beginning. *Cell*, 105: 421-424

151. Shu H.B., Joshi H.C. (1995) γ-Tubulin can both nucleate microtubule assembly and self-assemble into novel tubular structures in mammalian cells. *J. Cell Biol.*, 130: 1137-1147

152. Scott V., Sherwin T., Gull K. (1997) Gamma-tubulin in trypanosomes: molecular characterization and localization to multiple and diversemicrotubule organizing centres. *J. Cell Sci.*, 110: 157-168

153. Silflow C.D., Liu B., LaVoie M., Richardson E.A., Palevitz B.A. (1999) γ-Tubulin in *Chlamidomonas*: characterization of the gene and localization of the gene product in cells. *Cell Motil. Cytoskel.*, 42: 285-297

154. Smertenko A., Blume Y., Viklicky V., Opatrny Z., Draber P. (1997) Post-translational modifications and multiple tubulin isoforms in *Nicotiana tabacum L.* cells. *Planta*, 201: 349-358

155. Smrzka O.W., Delgehyr N., Bornens M. (2000) Tissue-specific expression and subcellular localization of mammalian delta-tubulin. Curr. Biol., 10: 413-416

156. Sobel S.G., Snyder M. (1995) A highly divergent gamma-tubulin gene is essential for cell growth and proper microtubule organization in *Saccharomyces cerevisiae. J. Cell Biol.*, 131: 1775-1788

157. Solomon F. (1977) Binding sites for calcium on tubulin. *Biochemistry*, 16: 358-363

158. Stearns T., Evans L., Kirschner M.W. (1991) γ-Tubulin is highly conserved component of centrosome. *Cell*, 65: 825-836

159. Stoppin-Mellet V., Peter C., Lambert A.M. (2000) Distribution of γ-tubulin in higher plant cells: cytosolic γ-tubulin is a part of high molecular weight complexes. *Plant Biol.*, 2: 290-296

160. Stumpff J., Kellogg D.R., Krohne K.A., Su T.T. (2005) *Drosophila* Wee1 interacts with members of the g-TuRC and is required for proper mitotic-spindle morphogenesis and positioning. *Curr. Biol.*, 15: 1525-1534

161. Šmejkalová B., Dráber P. (2003) Proteiny organizačních center mikrotubulů. *Biol. Listy*, 68: 313-332

162. Talmor-Cohen A., Tomashov-Matar R., Tsai W.B., Kinsey W.H., Shalgi R. (2004) Fyn kinase-tubulin interaction during meiosis of rat eggs. *Reproduction*, 128: 387-393

163. Tassin A.M., Celati C., Paintrand M., Bornens M. (1997) Identification of an Spc110p-related protein in vertebrates. *J. Cell Sci.*, 110: 2533-2545

164. Tassin A.M., Celati C., Moudjou M., Bornens M. (1998) Characterization of the human homologue of the yeast Spc98p and its association with gamma-tubulin. *J. Cell Biol.*, 141: 689-701

165. Tilney L.G., Bryan J., Bush D.J., Fujiwara K., Mooseker M.S., Murphy D.B., Snyder D.H. (1973) Microtubules: evidence for 13 protofilaments. *J. Cell Biol.*, 59: 267-275

166. Vale R.D., Schnapp B.J., Reese T.S., Sheetz M.P. (1985) Organelle, bead, and microtubule translocations promoted by soluble factor from the squid giant axon. *Cell*, 40: 449-454

167. Vallee R.B., Williams J.C., Varma D., Barnhart L.E. (2004) Dynein: an ancient motor protein involved in multiply modes of transport. *J.Neurobiol.*, 58: 189-200

168. Vaughan S., Attwood T., Navarro M., Scott V., McKean P., Gull K. (2000) New tubulins in protozoal parasites. *Curr. Biol.*, 10: 258-259

169. Vaughn K.C., Harper J.D. (1998) Microtubule organizing centers and nucleating sites in land plants. *Int. Rev. Cytol.*, 181: 75-149

170. Vayssié L., Vargas M., Weber C., Guillén N. (2004) Double-stranded RNA mediates homology-dependent gene silencing of γ-tubulin in the human parasite *Entamoeba hystolitica. Mol.& Biochem. Parasitol.*, 138: 21-28

171. Vernos I., Karsenti E. (1995) Chromosomes take the lead in spindle assembly. *Trends Cell Biol.*, 5: 297-301

172. Vogel J., Drapkin B., Oomen J., Beach D., Bloom K., Snyder M. (2001) Phosporylation of γ-tubulin regulates microtubule organization in buding yeast. *Develop. Cell*, 1: 621-631

173. Walczak C.E., Mitchison T.J., Desai A. (1996) XKCM1: a Xenopus kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. *Cell*, 84: 37-47

174. Webster D.R., Wehland J., Weber K., Borisy G.G. (1990) Detyrosination of α–tubulin does not stabilize microtubules *in vivo. J. Cell Biol.*, 111: 113-122

175. Weisenberg R.C., Deery W.J., Dickinson P.J. (1976) Tubulin-nucleotide interactions during polymerization and depolymerization of microtubules. *Biochemistry*, 15: 4248-4254

176. Wiese C., Zheng Y. (2000) A new function for the gamma-tubulin ring complex as a microtubule minus-end cap. *Nature Cell Biol.*, 2: 358-364

177. Wiche G. (1998) Role of plectin in cytoskeleton organization and dynamics. *J Cell Sci.*, 111: 2477-2486

178. Wilson P.G., Zheng Y., Oakley C.E., Oakley B.R. Borisy G.G., Fuller M.T. (1997) Differential expression of two gamma-tubulin isoforms during gametogenesis and development in *Drosophila. Dev. Biol.*, 184: 207-221.

179. Wise D.O., Krahe R., Oakley B.R. (2000) The gamma-tubulin gene family in humans. *Genomics*, 67: 164-170.

180. Wolff A., Denoulet P., Jeantet C. (1982) High level of tubulin microheterogeneity in the mouse brain. *Neurosci. Lett.*, 31: 323-328

181. Yuba-Kubo A., Kubo A., Hata M., Tsukita S. (2005) Gene knockout analysis of two γ-tubulin isoforms in mice. *Dev. Biol.*, 282: 361-373

182. Zambito A.M., Wolff J. (2001) Plasma membrane localization of palmitoylated tubulin. *Biochem. Biophys. Res. Commun.*, 283: 42-47

183. Zhang Y., Li N., Caron C., Matthias G., Hess D., Khochbin S., Matthias P. (2003) HDAC-6 interacts with and deacetylates tubulin and microtubules *in vivo*. *EMBO J.*, 22: 1168-1179

184. Zheng Y., Jung M.K., Oakley B.R. (1991) Gamma-tubulin is presented in *Drosophila melanogaster* and *Homo sapiens* and is associated with centrosome. *Cell*, 65: 817-823

185. Zheng Y., Wong M.L., Alberts B., Mitchison T. (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature*, 378: 578-583

186. Zhou J., Giannakakou P. (2005) Targeting microtubules for cancer chemotherapy. *Curr. Med. Chem. Anti-Canc. Agents*, 1: 65-71.

VI. PRESENTED PUBLICATIONS

VI.1. Linhartová I., Novotná B., **Sulimenko V.**, Dráberová E., Dráber P.: *Gamma-tubulin in chicken erythrocytes: changes in localization during cell differentiation and characterization of cytoplasmic complexes.* ***Developmental Dynamics*, 223**: 229-240, 2002.

DEVELOPMENTAL DYNAMICS 223:229–240 (2002)

Gamma-Tubulin in Chicken Erythrocytes: Changes in Localization During Cell Differentiation and Characterization of Cytoplasmic Complexes

IRENA LINHARTOVÁ,[1] BOŽENA NOVOTNÁ,[2] VADYM SULIMENKO,[1] EDUARDA DRÁBEROVÁ,[1] AND PAVEL DRÁBER[1]*

Department of Biology of the Cytoskeleton, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Department of Developmental and Genetic Toxicology, Institute of Experimental Medicine, Academy of Sciences of the Czech Republic, Prague, Czech Republic

ABSTRACT **The mechanism of marginal band (MB) formation in differentiating erythroid cells is not fully understood, and the proteins involved in nucleation of MB microtubules are largely unknown. To gain insights into the function of γ-tubulin in MB formation, we have followed its distribution in developing chicken erythrocytes and characterized soluble forms of the protein. In early stages of erythroid cells differentiation, γ-tubulin was present in microtubule-organizing centers, mitotic spindles, as well as on MB. Its subcellular localization changed in the course of differentiation, and in postnatal peripheral erythrocytes γ-tubulin was found only in soluble forms. After cold-induced depolymerization γ-tubulin in erythroid cells formed large clusters that were not observed in matured cells, and re-growth experiments demonstrated that γ-tubulin was not present in distinct nucleation structures at the cell periphery. Soluble γ-tubulin formed complexes of various size and large complexes were prone to dissociation in the presence of high salt concentration. Interaction of γ-tubulin with tubulin dimers was revealed by precipitation experiments. γ-Tubulin occurred in multiple charge variants whose number increased in the course of erythrocyte differentiation and corresponded with decreased binding to MB. The presented data demonstrate for the first time that γ-tubulin is a substrate for developmentally regulated posttranslational modifications and that the binding properties of γ-tubulin or its complexes change during differentiation events.** © 2002 Wiley-Liss, Inc.

Key words: chick embryo; marginal band; nucleated erythrocytes; microtubules; γ-tubulin

INTRODUCTION

Microtubules, assembled from α- and β-tubulin heterodimer, play a decisive role for many cellular functions such as intracellular organization, ordered vesicle transport and cell division, to name but a few. Microtubules exhibit an inherent property of dynamic instability (Mitchison and Kirschner, 1984), and this property is crucial for microtubule functions and their plasticity. In some terminally differentiated cell types such as neurons (Baas and Black, 1990), myocytes (Tassin et al., 1985), polarized epithelial cells (Bre et al., 1990), platelets (Kenney and Linck, 1985), and nucleated erythrocytes of nonmammalian vertebrates (Behnke, 1970) there exist, however, highly stable microtubule configurations. Although the mechanisms and functional significance of dynamic instability are known in great detail, mechanisms governing the conversion of dynamic microtubules into highly ordered structures remain poorly understood.

In mature chicken erythrocytes, the marginal band (MB) consists of a highly ordered and stable microtubule bundle that is located under the plasma membrane at the equatorial plane of the cells (Behnke, 1970; Cohen, 1991). The MB helps maintain the elliptical shape of the cells and helps resist the deformation forces to which the cells are exposed during circulation. Formation of the MB of microtubules represents the most striking event during differentiation of avian spherical erythroblasts to immature flattened discoids and finally to mature ellipsoidal erythrocytes. Changes in shape and size of the nucleus and rearrangements of the cytoskeleton are characteristic for this process. Thus, the formation of MB provides a relatively simple system, allowing studying the morphogenesis of stable microtubule arrays.

The key question in MB biogenesis is whether the nucleation of MB microtubules arises from microtubule organizing centers (MTOCs) or from microtubule-nucleating activity located at the membrane skeleton or at plasma membrane. Although MTOCs in differenti-

Grant sponsor: Czech Academy of Sciences; Grant number: A5052701; Grant sponsor: Czech Republic; Grant number: 304/00/0553; Grant sponsor: Ministry of Education of the Czech Republic; Grant number: LN00A026; Grant sponsor: NATO; Grant number: ENVIR.LG960330.

*Correspondence to: Pavel Dráber, Vídeňská 1083, 14220 Prague 4, Czech Republic. E-mail: paveldra@biomed.cas.cz

Received 10 May 2001; Accepted 22 October 2001

DOI 10.1002/dvdy.10047

ting chicken erythroid cells are present, such structures are missing in mature erythrocytes. However, the MB of mature chicken erythrocytes can reform after old depolymerization followed by rewarming, and it is indistinguishable from that of untreated cells (Miller and Solomon, 1984; Kim et al., 1987). MBs were found to contain the microtubule-associated proteins tau (Murphy and Wallis, 1985; Sanchez and Cohen, 1994), which possibly have microtubule stabilizing or bundling function, and syncolin that might be involved in lateral interactions between microtubules (Feick et al., 1991) or interaction of microtubule bundles with membranes (Sanchez and Cohen, 1994). The mechanism of MB formation in differentiating erythroid cells is not fully understood.

The γ-tubulin (Oakley and Oakley, 1989) is the almost ubiquitous component of MTOC and participates in nucleation of microtubules (Oakley et al., 1990; Joshi et al., 1992). In animal cells, the vast majority of γ-tubulin belongs to cytoplasmic complexes. Large γ-tubulin complexes (γ-tubulin-ring complex; γ-TuRC) were first identified in *Xenopus* eggs (Zheng et al., 1995). They were also found in *Drosophila* embryos (Moritz et al., 1995) and in mammalian cells (Meads and Schroer, 1995; Moudjou et al., 1996; Détraves et al., 1997). In addition to γTuRC, there also exist smaller complexes (γ-tubulin small complex; γTuSC) (Moritz et al., 1998) that compose two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin complex proteins) (Murphy et al., 1998; Tegema et al., 1999), which are homologues of the *Saccharomyces cerevisiae* proteins Spc97p and SPc98p associated with spindle pole bodies (Knop and Schiebel, 1997). The γ-TuRCs are formed by small complexes and by other proteins. Protein composition differs in various cell types. In addition to nucleation from centrosomes, the γ-TuRCs are also involved in regulating the dynamics of the microtubule minus ends (Wiese and Zheng, 2000). Tubulin binding sites on γ-tubulin were identified, and it seems that γ-tubulin itself may, under some circumstances, bind to the side of assembled microtubules (Llanos et al., 1999). The role of γ-tubulin in nucleation of MB microtubules in differentiating and mature chicken erythrocytes is unknown.

Here, we report on changes in the distribution of γ-tubulin during chicken erythrocyte differentiation and characterize the soluble γ-tubulin forms. As a source of embryonic erythrocytes, we took advantage of embryonic peripheral blood, which is easily accessible and contains mainly erythroid cells.

RESULTS

Distribution of Gamma-Tubulin in Erythroid Cells and Mature Erythrocytes

The distribution of γ-tubulin in differentiating erythroid cells was studied by using monoclonal antibodies raised against conservative peptide sequences from human γ-tubulin. Antibodies TU-30, TU-31, and TU-32

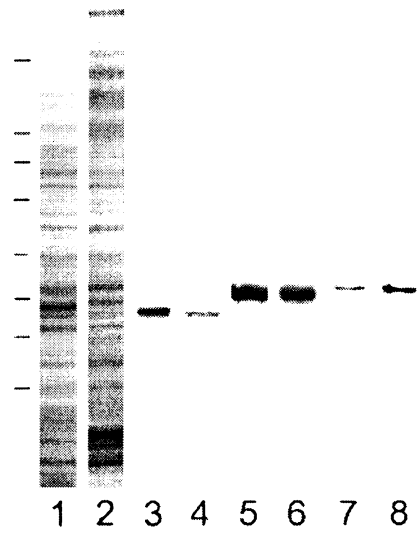


Fig. 1. Immunoblot analysis of differentiating erythrocytes. Whole cell extracts of erythroid cells from 5-day-old embryos (lanes 1, 3, 5, 7) and from erythrocytes of 56-day-old chickens (lanes 2, 4, 6, 8) were separated on 7.5–12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Lanes 1–2, Coomassie Blue staining of separated proteins; lanes 3–8, immunostaining with antibodies TU-32 to γ-tubulin (lanes 3–4), TU-01 to α-tubulin (lanes 5–6), and VI-10 to vimentin (lanes 7–8). The same amounts of proteins were loaded (10 μg/lane). Bars on the left margin indicate positions of molecular mass markers (from the top to the bottom: 205 kDa, 116 kDa, 97.4 kDa, 84 kDa, 66 kDa, 55 kDa, 45 kDa, and 36 kDa).

are directed against a peptide from the C-terminal region of the molecule, the antibody GTU-88 is directed against a peptide from the N-terminal region. The specificity of antibodies was confirmed by immunoblotting on whole cell lysates of erythroid cells isolated from blood of 5-day-old embryos and on lysates of erythrocytes from 56-day-old chickens. An example of immunoblot with antibody TU-32 is shown in Figure 1. Protein with relative electrophoretic mobility corresponding to 48 kDa of γ-tubulin was detected in both samples. However, its amount in older chickens was lower (Fig. 1, lanes 3–4). The same results were obtained with antibodies raised against the C-terminal region of human γ-tubulin as well as with antibody against the N-terminal region of the protein. The observed decrease during erythrocyte development was characteristic for γ-tubulin because the amount of immunodetected α-tubulin was basically unchanged (Fig. 1, lanes 5–6) and the amount of vimentin increased (Fig. 1, lanes 7–8).

To acquire detailed data on the course of γ-tubulin changes during erythrocyte differentiation, its level was determined at various stages of embryonic development. As γ-tubulin is necessary for the formation of mitotic spindle, the number of mitotic cells was estimated. Mitotic cells were most numerous on embryonic day (ED) 3 (4.33 ± 1.59%). The number of mitotic cells decreased to 0.43 ± 0.21% on ED 10, and almost no mitotic cells were detected on postnatal day 1 (0.03 ±

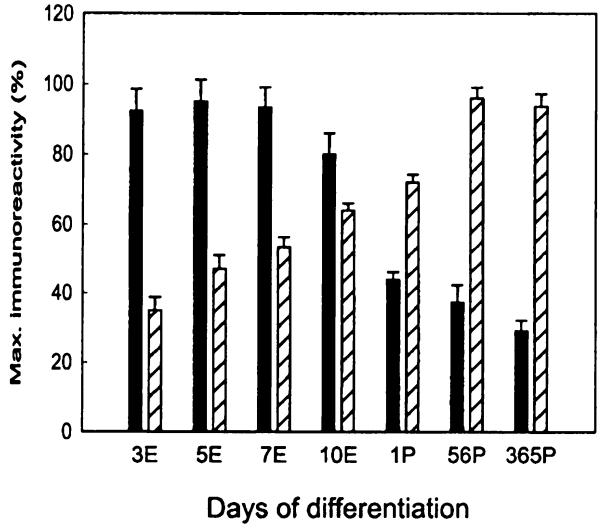


Fig. 2. Comparison of the expression of γ-tubulin and vimentin in differentiating erythrocytes as detected by densitometric measurements of immunoblots. The 3E, 5E, 7E, and 10E denote different days of embryonic development; 1P, 56P, and 365P denote different days of postnatal development. Each column represents the mean ± SE of triplicate readings of three blots. Black columns, γ-tubulin; shaded columns, vimentin.

103%). In the tested time period, the maximal immunoreactivity for γ-tubulin was attained between ED 5-7. Its level was reduced to approximately 45% of the maximum on postnatal day 1 and fell to approximately 30% of the maximum in erythrocytes isolated from adult animals. The level of vimentin increased during the observed period and reached its maximum on postnatal day 56. High level of vimentin was also detected on day 365. A comparison of γ-tubulin and vimentin changes in time is shown in Figure 2. These data show that γ-tubulin is still present in erythrocytes isolated from 1-year-old chickens.

Triple immunofluorescence with polyclonal antibody against αβ-tubulin dimer, monoclonal antibody against γ-tubulin, and DNA-binding dye revealed that γ-tubulin in erythroid cells of 5-day-old embryos was detectable not only in MTOCs in the perinuclear region but also on MB. Diffuse staining in the cytoplasm was also observed. In dividing cells, γ-tubulin was located on spindle poles of mitotic spindle, and also along the length of microtubules in the form of discrete dots. Interphase cells contained MTOCs that nucleated microtubules, which then formed MB under the plasma membrane (Fig. 3A–C). The MB in erythroid cells from 5-day-old embryos was thicker in comparison with later developmental stages. In 10-day-old embryos, the erythroid cells with thick MB, corresponding to primitive erythroid cells as to their shape and size, represented only a minority of erythrocyte population. Cells with MTOC nucleating microtubules were very rare. At that differentiating stage, cells started to appear that apparently did not have MTOC, because no γ-tubulin staining was detected in the perinuclear region of these cells (arrows in Fig. 3D–F). Due to the cell shape and thin MB, such cells represent most probably the final mature forms of the first wave of definitive erythroid cells. γ-Tubulin was also detected on MBs, but the intensity of staining varied among individual cells. An intensive staining of MB was found in cells with prominent labeling of MTOC, whereas the staining was weak in cells without MTOC. Surprisingly, the polyclonal antibody used against αβ-tubulin dimer did not decorate MTOCs that were brightly stained with anti-γ-tubulin antibody. In erythrocytes of 56-day-old chickens, γ-tubulin was not detectable by immunofluorescence in the perinuclear region or on MB (Fig. 3G–I). The same staining pattern of γ-tubulin in differentiating erythrocytes was observed with all monoclonal antibodies used.

To rule out the possibility that the location of γ-tubulin on MB reflects only an unspecific association of soluble cytoskeletal proteins with this prominent cellular structure, or possibly an unspecific sticking of primary and secondary antibodies, erythroid cells of 10-day-old embryos were stained with anti-vimentin antibody. Monoclonal antibody VI-10 (immunoglobulin M [IgM]) decorated only the filamentous network around the nuclei, and no staining of MB was detected (not shown). To learn more about the nature of γ-tubulin association with MB, cells were first extracted with 0.2% Triton X-100 in MSB containing 5 μM Taxol and the resulting cytoskeleton was incubated for 10 min in MSB containing 50, 100, 250, and 500 mM NaCl before fixation. γ-Tubulin staining of MB was observed after incubation of extracted cells in 250 mM NaCl. The majority of extracted cells incubated in 500 mM NaCl were detached from coverslips, but in the remaining cells γ-tubulin was still present on MB.

Redistribution of Gamma-Tubulin After Cold Depolymerization of MB

To investigate whether γ-tubulin is involved in nucleation of MB in erythroid cells from 10-day-old embryos, microtubules were depolymerized by cold treatment and the temperature was subsequently raised to 37°C to induce re-growth of microtubules. Samples were fixed at different time intervals. When cold-treated cells were directly extracted on ice by Triton X-100 and fixed, γ-tubulin was found in large clusters around the nucleus; diffuse staining was also observed. No staining of the cell periphery was detected. Similarly, staining with polyclonal antibody against tubulin dimers revealed tubulin clusters and diffuse staining, but the labeling was less intense than that seen with anti-γ-tubulin antibody (Fig. 4A–C). When cold-treated cells were directly extracted by Triton X-100 at 37°C and then fixed, microtubules started to form in some cells at the periphery. When cold-treated cells were incubated for 1 min at 37°C, different staining patterns were observed. There were cells without microtubules (~20%), cells with microtubules growing at

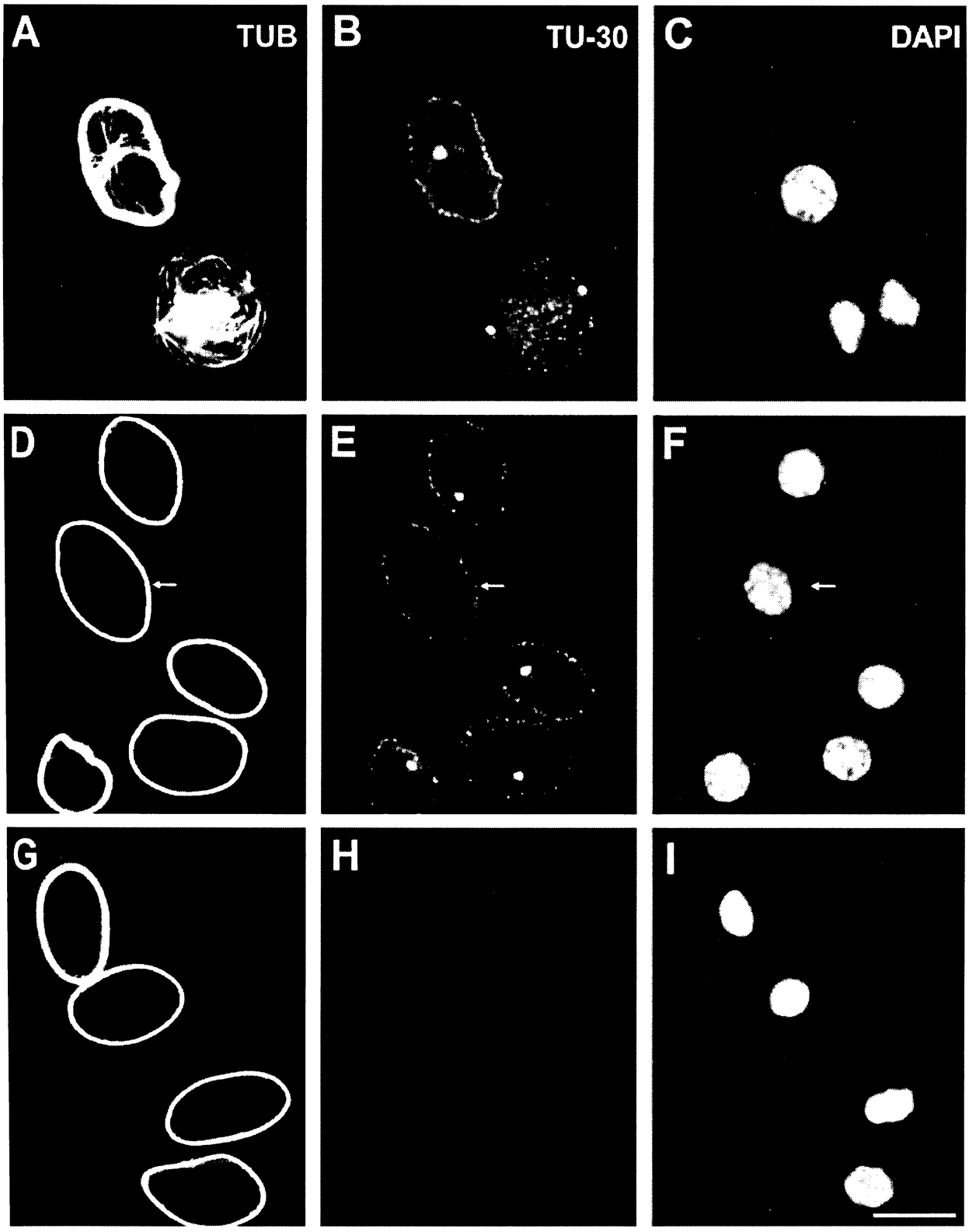


Fig. 3. Immunofluorescence triple-label staining of chicken erythrocytes in various stages of differentiation with anti-γ-tubulin antibody. Erythroid cells from 5-day-old embryos (**A–C**), 10-day-old embryos (D–F), and mature erythrocytes from 56-day-old chickens (**G–I**) were stained with polyclonal antibody TUB against αβ-tubulin dimers (A,D,G), monoclonal antibody TU-30 against γ-tubulin (B, E, H) and DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI; C,F,I). Cells were extracted with Triton X-100, fixed in formaldehyde and post-fixed in cold methanol. Arrows denote the same positions. Scale bar = 10 μm in I (applies to A–I).

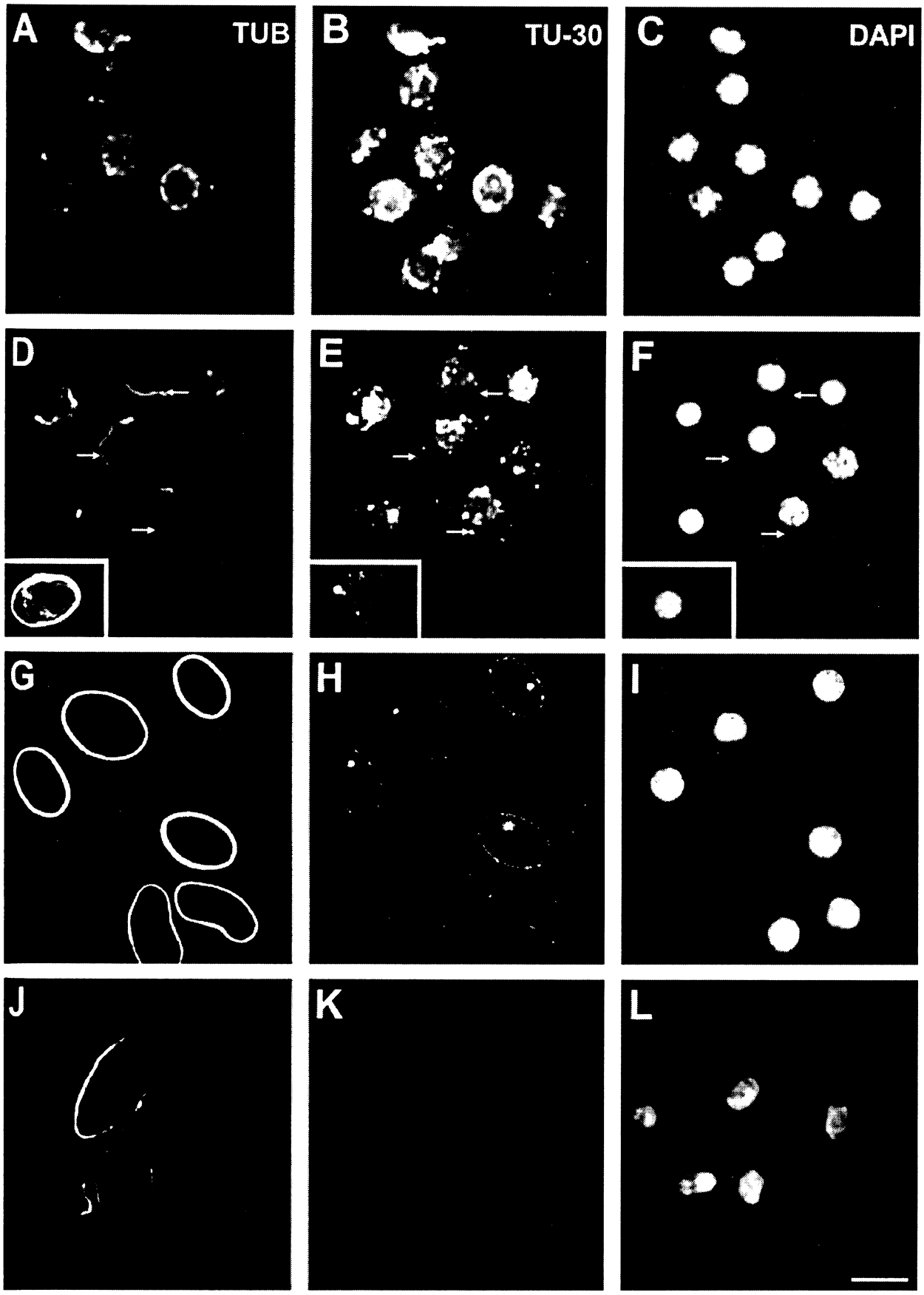


Fig. 4. The distribution of γ-tubulin in cells recovering from cold treatment. **A–I:** Erythroid cells from 10-day-old embryos. **J–L:** Erythrocytes from a 56-day-old chickens. Cells were incubated on ice for 16 hr and then fixed on ice (A–C) or after cold treatment incubated at 37°C for 1 min (D–F,J–L) or for 20 min (G–I) before fixation. Immunofluorescence triple-label staining with polyclonal antibody TUB against αβ-tubulin dimers (A,D,G,J), monoclonal antibody TU-30 against γ-tubulin (B,E,H,K), and DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI; C,F,I,L). Inserts in D–F document the re-growth of microtubules from microtubule organizing centers. Cells were extracted with Triton X-100, fixed in formaldehyde, and post-fixed in cold methanol. Arrows denote the same positions. Scale bar = 10 μm in L (applies to A–L).

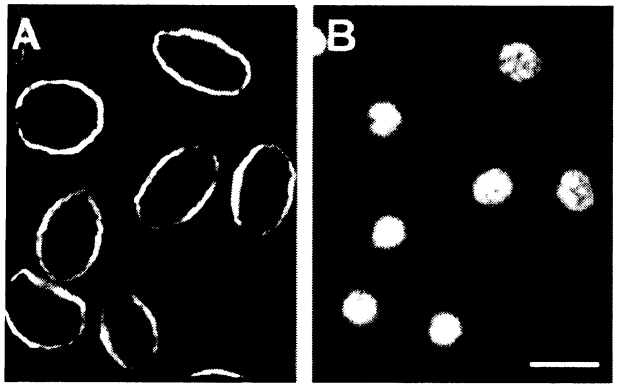


Fig. 5. Immunofluorescence staining of cold-treated erythroid cells)m 10-day-old embryos. Cells were incubated on ice for 16 hr and then ed on ice. Staining of actin with rhodamine-labeled phalloidin (**A**) and \A-binding dye (**B**). Cells were extracted with Triton X-100, fixed in rmaldehyde, and post-fixed in cold methanol. Scale bar = 10 μm in B pplies to A,B).

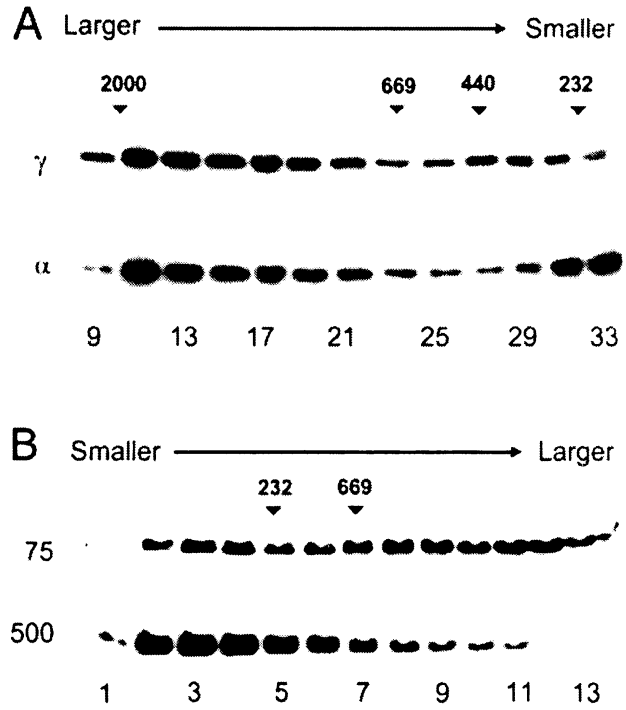


Fig. 6. Behavior of γ-tubulin during Superose-6 gel filtration (A) and sucrose density gradient centrifugation (B) of erythrocyte cell extracts from 3-day-old chickens. **A:** Collected fractions were concentrated, separated, and immunoblotted with antibody against γ-tubulin (γ) and α-tubulin (α). Numbers from 9 to 33 denote individual column fractions. **B:** Samples were sedimented through 5–40% sucrose gradient in the presence of 75 mM (75) or 500 mM (500) NaCl; fractions were separated and immunoblotted for γ-tubulin. Numbers from 1 to 13 denote individual fractions. Proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Calibration standards in kilodaltons are indicated on the top.

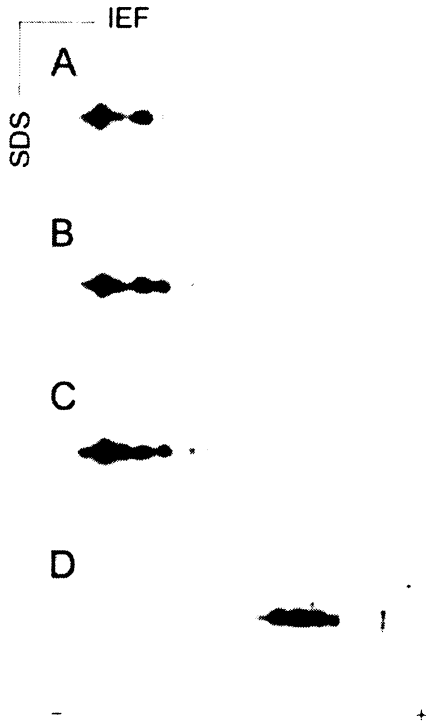
ıe cell periphery (~ 55%), and cells with thin MB ~25%). However, no prominent staining of nucleation ɛnters was detectable with anti–γ-tubulin antibody. 'n the other hand, faint γ-tubulin dots were visible on ɔrmed microtubules (arrows in Fig. 4D–F). Occasionlly, it was possible to find cells that contained MTOC ear the nucleus from which microtubules emanated. n such cells, thick MB had already been formed. γ-Tuulin was then located both on the MTOC and on the ewly formed MB in the form of small spots (inserts in 'ig. 4D–F). After 20 min, typical MBs, indistinguishble from those of untreated cells, were visible in the ıajority of cells (~95%). γ-Tubulin was located both in ITOCs, if present in examined cells, and as dots on IB. No clusters but diffuse staining was detected with nti–γ-tubulin antibody in the cytoplasm (Fig. 4G–I). 'he staining pattern of γ-tubulin in cold-treated cells ras the same with all monoclonal antibodies used.

The formation of γ-tubulin clusters after depolymerzation of microtubules was typical for embryonic eryhroid cells. The same type of experiment performed vith erythrocytes of 56-day-old chickens failed to deect clusters of γ-tubulin and γ-tubulin dots on newly ormed microtubules after 1 min of re-growth at 37°C Fig. 4J–L).

To rule out the possibility that tubulin aggregations eflects potential blebs on the surface of cold-treated ells, the cells were stained with rhodamine-labeled halloidin that reacts with F-actin, which is not depoymerized by cold. F-actin was located on extracted ells on the cell periphery, and no substantial changes n morphology were observed (Fig. 5).

Gamma-Tubulin Complexes

Mature erythrocytes did not exhibit any cytoskeletal structures stained with anti–γ-tubulin antibodies, whereas blots from whole extracts showed the presence of γ-tubulin. Therefore, experiments were performed to determine whether γ-tubulin also exists in soluble cytoplasmic form. Size distribution of γ-tubulin in supernatants after Triton X-100 extraction of erythrocytes from 3-day-old chickens was assessed by gel filtration chromatography on Superose-6 column. Immunoblotting experiments with separated fractions revealed that γ-tubulin was distributed in a large zone and was present in complexes of various sizes. The majority of immunoreactivity was found in complexes with high molecular weight. Similar size fractionation was also found for α-tubulin; however, lower molecular weight complexes were found to prevail here (Fig. 6A). On the other hand, the heat shock protein Hsp70 was absent in high-molecular weight complexes, indicating that not all cytoplasmic proteins had the same size distribution under the used separation conditions (not shown). When Triton X-100 supernatants containing 75 mM NaCl were fractionated by sedimentation in 5–40% sucrose gradients, γ-tubulin was again present in complexes of various sizes. Large complexes, however, were disrupted in samples containing 500 mM NaCl (Fig. 6B). Combined data, thus, indicate that chicken γ-tubulin is present in high-molecular com-



). 7. Immunoblot analysis of erythrocyte cell extracts by two-dimen-
il electrophoresis. Immunostaining of erythroid cells from 5-day-old
yos (**A**), 10-day-old embryos (**B**), and mature erythrocytes from
ay-old chickens (**C,D**) with anti–γ-tubulin antibody GTU-88 (A–C)
anti-α-tubulin antibody TU-01 (D). The basic and acidic ends in the
dimension are marked by minus and plus, respectively. The figures
t the same region in the first dimension. SDS, sodium dodecyl
te-polyacrylamide gel electrophoresis; IEF, isoelectric focusing.

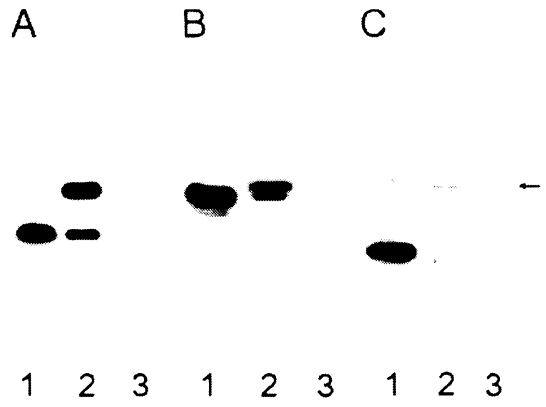


Fig. 8. Immunoprecipitation of erythrocyte cell extracts from 3-day-old chickens with anti–γ-tubulin antibody. Cell extracts were precipitated with anti–γ-tubulin antibody TU-31 (IgG2b) bound to immobilized protein A. Proteins remaining after precipitation (lane 1), immunoprecipitated proteins (lane 2), and proteins bound to protein A without antibody (lane 3) were separated on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. **A:** Immunostaining with anti-γ-tubulin antibody GTU-88. **B:** Immunostaining with anti–α-tubulin antibody TU-01. **C:** Immunostaining with control anti-actin antibody. Arrow indicates the position of heavy chains of immunoglobulin G.

ɛes and that their size is sensitive to salt concentra-
ı.

'wo-dimensional analysis of Triton X-100 extracts ealed that soluble forms of γ-tubulin existed in mul-e charge variants (Fig. 7A–C) that have their iso-:tric points more basic in comparison with α-tubulin 'orms (Fig. 7D). The number of γ-tubulin charge iant increased in the course of differentiation, and least six isoelectric variants of γ-tubulin were de-ted by immunoblotting in mature erythrocytes. In tested differentiation stages, the isoelectric point of major γ-tubulin isoform was 5.88.

mmunoprecipitation experiments showed that γ-tu-lin could be specifically precipitated from Triton 100 extracts of 3-day-old chicken erythrocytes by noclonal antibody TU-31 (IgG2b) (Fig. 8A, lane 2). binding of γ-tubulin to immobilized protein A was tected (Fig. 8A, lane 3), and the control antibody -09 (IgG2b) gave no precipitation of γ-tubulin. Prob-; of immunoprecipitated material with anti–α-tubu-antibody revealed the presence of α-tubulin in γ-tu-lin complexes (Fig. 8B, lane 2). The band rresponding to α-tubulin was just under the band of mobilized TU-31 antibody (arrow in Fig. 8), so it uld potentially reflect proteolytic fragmentation of the antibody. However, no band in this position was detected when the precipitated material was probed only with peroxidase-labeled anti-mouse antibody. When probed with anti–β-tubulin antibody TUB 2.1, specific staining was also detected (not shown). To rule out the possibility of an unspecific attachment of cytoplasmic proteins to the γ-tubulin complexes, immunoprecipitated material was probed with anti-actin antibody. In that case, no actin was detected (Fig. 8C, lane 2). When probed with anti-Hsp 70 antibody, no coprecipitation of this protein was found either (not shown).

Because the above-mentioned experiments indicated that tubulin dimers could be present in γ-tubulin complexes, immunoprecipitation was also performed with immobilized anti–α-tubulin antibody TU-16 (IgM). This antibody specifically precipitated α-tubulin (Fig. 9A, lane 2), and no binding of α-tubulin to immobilized protein L was detected (Fig. 9A, lane 3). Probing of the immunoprecipitated material with anti–γ-tubulin antibody revealed that the α-tubulin complexes also contained γ-tubulin (Fig. 9B, lane 2). Control antibody VI-10 (IgM) gave no precipitation of γ-tubulin. To rule out the possibility that cytoplasmic proteins are unspecifically attached to the α-tubulin complexes, immunoprecipitated material was probed with anti-vimentin antibody. In that case, no vimentin was detected (Fig. 9C, lane 2). Similarly, probing with anti-actin antibody showed no coprecipitation of the relevant protein (not shown). The same immunoprecipitation results were obtained with 3-day-old and 56-day-old chicken erythrocytes.

DISCUSSION

The distribution of γ-tubulin during the development and maturation of MB was investigated by using

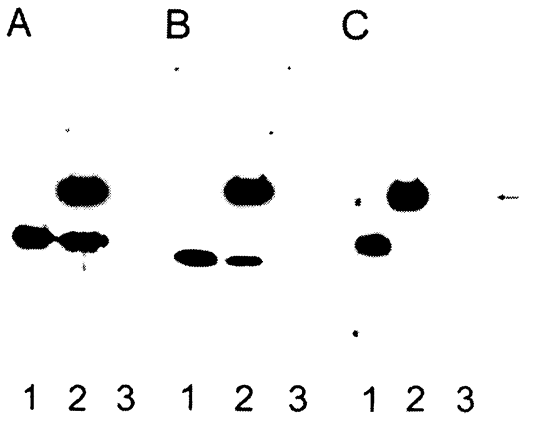


Fig. 9. Immunoprecipitation of erythrocyte cell extracts from 3-day-old ickens with anti–α-tubulin antibody. Cell extracts were precipitated with ti–α-tubulin antibody TU-16 (immunoglobulin M [IgM]) bound to immo- ized protein L. Proteins remaining after precipitation (lane 1), immuno- ecipitated proteins (lane 2), and proteins bound to protein L without ntibody (lane 3) were separated on 7.5% sodium dodecyl sulfate-poly- crylamide gel electrophoresis gels. **A:** Immunostaining with anti–α- ubulin antibody TU-01. **B:** Immunostaining with anti–γ-tubulin antibody TU-88. **C:** Immunostaining with control anti-vimentin antibody VI-10. row indicates the position of heavy chains of IgM.

hicken embryo erythropoiesis as a suitable model sys- em. There are distinct developmental waves of ery- hroid cells distinguishable during chicken embryonic eriod. Cells of the primitive series appear 1.5–2 days fter the start of fertilized egg incubation, and their irst mature forms may be detected around ED 5. At hat time, first immature cells of the definitive ery- hroid series appear in circulation and gradually re- lace the elements of primitive lineage. On ED 10, ainly definitive erythroid cells at different stages of ifferentiation are present in the blood and degenerat- ng primitive erythrocytes constitute only approxi- ately 10% of the blood cell population. Starting from D 15 only cells of definitive series are exclusively resent (Bruns and Ingram, 1973). Previous immuno- luorescence studies indicated that no MTOC were de- ectable among chicken circulating erythroid cells on D 5 and beyond (Kim et al., 1987). Our staining with nti–γ-tubulin antibodies clearly demonstrated the resence of MTOC in the majority of erythroid cells olated from blood of 10-day-old embryos. However, by sing a general polyclonal antibody against αβ-tubulin imer, no such structures were detected. On the other and, antibody against the detyrosinated form of α-tu- ulin, which is found in stable microtubule arrange- ents, was capable of revealing faint staining of TOC at that developmental stage (E. Dráberová, un- ublished results).

The location of γ-tubulin on stable microtubule ar- angements of MB at early stages of development was n unexpected finding. Cold-depolymerization, which isrupted microtubules of MB, also abolished staining f the cell periphery with anti–γ-tubulin antibody, in- icating that γ-tubulin is not associated with periph- eral skeleton components. Differential localization of γ-tubulin on MB in the course of erythrocyte matura- tion suggests that the binding properties of γ-tubulin or it complexes must undergo substantial changes. Al- ternatively, the distinct staining can reflect changes in protein composition of MB proteins during maturation of MB (Murphy and Wallis, 1985; Feick et al., 1991; Zhu et al., 1995). γ-Tubulin has been found to be a microtubule minus-end binding molecule in not-an- chored microtubules (Li and Joshi, 1995; Leguy et al., 2000). On the other hand, in vitro prepared γ-tubulin peptides were capable of binding along the length of assembled microtubules without interference with tu- bulin polymerization (Llanos et al., 1999). Although γ-tubulin in animal cells in vivo was found on micro- tubules of mitotic spindle as well as in midbodies (Julian et al., 1993; Lajoie-Mazenc et al., 1994; Nová- ková et al., 1996) and in plants even on all microtubule structures (Liu et al., 1993; Binarová et al., 1998), its localization on long microtubules in vertebrate cells was not reported. As the association of γ-tubulin with MB has only a transient character, γ-tubulin probably does not play any stabilizing role during MB matura- tion. It also does not reflect the binding to the minus ends of microtubules, as MB in erythrocytes are formed by a single or only a few extremely long microtubules (Cohen, 1991). Staining may, however, signify the stor- age form, inactive form, or both, of γ-tubulin.

The re-growth experiments on erythroid cells from 10-day-old embryos showed that there were no definite initiation sites at the cell periphery that contained γ-tubulin. Although we did not observe any clear γ-tu- bulin signal at the ends of growing microtubules, we cannot rule out that γ-tubulin is located on minus-ends of growing microtubules in erythroid cells or matured erythrocytes, but its detection is below the limit of fluorescence microscopy. Our data also support previ- ous findings that there must be peripheral determi- nants of organization of MB microtubules that are in- dependent of MTOC (Miller and Solomon, 1984; Kim et al., 1987). It is possible that proteins associated with microfilaments, which in erythrocytes colocalize with MB, could be directly or indirectly involved in such organization (Birgbauer and Solomon, 1989; Winckler et al., 1994). The results of re-growth experiments also strengthen the notion that γ-tubulins or their com- plexes from embryonic erythroid cells and from post- natal erythrocytes have different properties.

There were no cytoskeletal structures stained in im- munofluorescence with anti–γ-tubulin antibodies in postnatal erythrocytes, and γ-tubulin was present only in soluble cytoplasmic pools. Soluble γ-tubulin occurred in complexes of various size and high molecular com- plexes dissociated in the presence of high salt concen- tration. Salt dissociation is a general property of γ–tu- bulin complexes in various species (Wiese and Zheng, 1999). Gel filtration also showed that αβ-tubulin dimers were eluted through elution profile of γ-tubulin, indicating heterogeneity in the size of complexes in

which αβ-tubulin dimers could be involved. A wide range of size distribution of tubulin dimers was observed in mammalian cell extracts (Moudjou et al., 1996). Size heterogeneity of erythrocyte γ-tubulin complexes could signify the presence of different complexes characterized by distinct polypeptides or self-association of γ-tubulin complexes (Détraves et al., 1997).

Precipitation experiments with Triton X-100 extracts showed that γ-tubulin indeed formed complexes with αβ-tubulin dimers. The GTU-88 monoclonal antibody directed against amino acid region γ38-53 failed to precipitate γ-tubulin. This finding suggests that this region is not accessible to the antibody binding in contrast to the region γ434-449 recognized by the TU-31 antibody. An association of soluble γ-tubulin with αβ-tubulin dimers was confirmed by precipitation with anti-α-tubulin antibody resulting in a coprecipitation of γ-tubulin. Variable amounts of αβ-tubulin dimers have been reported to coprecipitate with γ-tubulin in preparations from *Xenopus* embryos (Zheng et al., 1995), sheep brain (Détraves et al., 1997), and cells of lymphoblastic cell line KE37 (Moudjou et al., 1996). In other sources, no association of γ-tubulin with tubulin dimers was found (Wiese and Zheng, 1999). It is possible that the interaction of γ-tubulin with αβ-tubulin dimers in cells is weak and can be modified by sample preparations or by procedures used for the assessment of the composition of γ-tubulin complexes. At present, we do not know whether γ-tubulin in erythrocyte lysates interacts with other proteins beside tubulin dimers. Specific elution of proteins precipitated with TU-31 antibody (directly bound to carrier) with excess of the peptide, used for immunization, showed only traces of proteins in the position of tubulins and immunoglobulins when sensitive silver staining of proteins was applied (V. Sulimenko, unpublished results). This finding suggests that the association constant of TU-31 antibody for erythrocyte γ-tubulin is very high.

What the function of soluble γ-tubulin in matured erythrocytes actually is remains to be elucidated. However, the majority of αβ-tubulin dimers (~80%) also appear in adult erythrocytes in unassembled pools (Kim et al., 1987) and they easily form, due to specific properties of tubulin subunits, ring oligomers in vitro (Murphy and Wallis, 1985; Trinczek et al., 1993). It was suggested that tubulin oligomer pools in erythrocytes might be important for maintaining a low dimer concentration and, hence, nucleation rates to generate long microtubules (Murphy and Wallis, 1985). γ-Tubulin or its complexes could then stabilize tubulin oligomers.

The tubulin heterodimers are more homogeneous in avian erythrocytes than in other cell types (Joshi et al., 1987; Pratt and Cleveland, 1988), and posttranslational modifications are also less complex than in other cell types (Rüdiger and Weber, 1993). Surprisingly, multiple charge variants were detected with anti-γ-tubulin antibody, and the number of these isoforms increased during differentiation. Four γ-tubulin isoforms were recognized by two-dimensional electrophoresis in sheep brain (Détraves et al., 1997) and an elongated γ-tubulin signal with two major spots was detected in human lymphoblastic cell line (Moudjou et al., 1996). To date, only two γ-tubulin genes were identified in various species (Wise et al., 2000). These data suggest that γ-tubulin, like the α- and β-tubulin counterparts, could be subject to posttranslational modification(s). We also observed multiple α-tubulin isoforms that had not been previously detected in avian erythrocytes by two-dimensional electrophoresis (Murphy and Wallis, 1983) or by isoelectric focusing (Rüdiger and Weber, 1993). A higher number of α-tubulin isoforms could result from the use of immobilized pH gradient 4–7 in combination with sensitive immunostaining and chemiluminescent detection. An increased modification of γ-tubulin in the course of differentiation could result in the observed lower binding of γ-tubulin or its complexes to MB and in differential formation of γ-tubulin clusters in cold-treated cells.

In conclusion, the presented data indicate that the subcellular localization of γ-tubulin changes in the course of differentiation and that in postmitotic nucleated erythrocytes γ-tubulin persists in soluble form in complexes of various sizes. γ-Tubulin is not present in distinct nucleation structures at the cell periphery, and developmentally regulated posttranslational modification(s) of γ-tubulin could modify its binding properties.

EXPERIMENTAL PROCEDURES

Materials

Immobilized Protein A Plus, Immobilized Protein L Plus, and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce (Rockford, IL). Protease inhibitors and molecular weight markers for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were from Sigma-Aldrich (Prague, Czech Republic). The gel filtration calibration kit contained Blue Dextran 2000, thyroglobulin (M_r = 669 × 10^3), ferritin (M_r = 440 × 10^3), catalase (M_r = 232 × 10^3), and aldolase (M_r = 158 × 10^3). Superose-6, IPG buffer (pH 4–7), and calibration kits for isoelectric focusing were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Antibodies

The following monoclonal antibodies were used: TU-01 (IgG1) against α-tubulin (Viklický et al., 1982); TU-16 (IgM) against α-tubulin (Dráberová and Dráber, 1998); TUB 2.1 (IgG1) against β-tubulin (Sigma Cat. No. T4026); TU-30 (IgG2b), TU-31 (IgG2b), TU-32 (IgG1) anti-peptide antibodies prepared against EYHAATRPDYISWGTQ peptide corresponding to the human γ-tubulin sequence 434-449 (Nováková et al., 1996); GTU-88 (IgG1) anti-peptide antibody prepared against EEFATEGTDRKDVFFY peptide corresponding to the human γ-tubulin sequence 38-53 (Sigma Cat. No. T6557); VI-10 (IgM) against vimentin (Dráberová et al., 1999b); N27F3-4 (IgG1) against heat shock pro-

tein Hsp 70 (StressGen, Cat. No. SPA-820); and NF-09 IgG2a) against neurofilament protein (Dráberová et al., 1999a). Actin was detected by affinity-purified rabbit anti-actin antibody (Sigma Cat. No. A2066) and microtubules were detected by affinity-purified rabbit anti-tubulin antibody (Dráber et al., 1991). Anti-mouse Ig antibodies conjugated with horseradish peroxidase were purchased from Promega Biotec (Madison, WI), lissamine rhodamine-conjugated anti-mouse Ig antibody and fluorescein isothiocyanate-conjugated anti-rabbit Ig antibody was from Jackson Immunoresearch Laboratories (West Grove, PA).

Cells and Preparation of Cell Extracts

Outbred Grey Leghorn chickens (*Gallus domesticus*) were used as a source of chicken blood. During the embryonic period, blood samples were obtained from the dorsal aorta (3- and 5-day-old embryos) and from arteria vitellina (7- and 10-day-old embryos). Heart blood was withdrawn from chicks on day 1, 56, and 365 after hatching. Blood samples were collected into 0.1% BSA in 110 mM NaCl, pH 8.0 (3- and 5-day-old embryos) or into 0.1% BSA in 145 mM NaCl, pH 8.0 (7- and 10-day-old embryos). Citrate-saline solution (final concentration 145 mM NaCl, 34 mM tri-sodium citrate, pH 7.6) was used for collection of blood from hatched chicks. Conventional blood smears were prepared for histochemical evaluation of mitotic cells. The percentage of mitotic cells (mean ± SD) was calculated from four independent samples (3,000 cells/sample).

For preparation of cell extracts, blood samples were spun down at 220 × *g* for 5 min at room temperature, the supernatant and buffy coat were removed by aspiration, cells were resuspended in phosphate-buffered saline (PBS), pelleted again at room temperature, and washed in PBS three times. Washed cells contained more than 99% of erythrocytes as confirmed by conventional hematology examination.

Whole cell extracts for SDS-PAGE and for immunoprecipitation were prepared as follows: pelleted washed cells were resuspended in one volume of cold MEM buffer (0.1 M Mes adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM $MgCl_2$) supplemented with protease (1 μg/ml each of leupeptin, aprotinin, antipain, pepstatin and 1 mM AEBSF) and phosphatase inhibitors (1 mM each of NaF and Na_3VO_4) and with Triton X-100 to final concentration of 0.4%. Cells were extracted 10 min at 4°C, suspension was then spun down (21,000 g, 15 min, 4°C), and supernatant was collected. The remaining pellet was resuspended in cold 0.4% Triton X-100 in MEM buffer supplemented with protease and phosphatase inhibitors and disrupted on ice by sonication for 5 × 10 sec (amplitude 30) with 500-watt Ultrasonic homogenizer (Cole-Parmer, Vernon Hills, IL) equipped with a microtip probe. The suspension was spun down (21,000 × *g*, 15 min, 4°C), supernatant collected and pooled with the supernatant after extraction.

For gel filtration chromatography, pelleted washed cells were resuspended at 4°C in 1 volume of precooled Hepes buffer (50 mM Na-Hepes, pH 7.6, 75 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, and 1 mM 2-mercaptoethanol) supplemented with 2% glycerol and protease and phosphatase inhibitors. Triton X-100 was added to the final concentration of 0.4%, and cells were permeabilized for 10 min at 4°C. Suspensions were spun down (21,000 × *g*, 15 min, 4°C), and supernatants were used for gel filtration.

For sucrose density gradient centrifugation, pelleted washed cells were resuspended at 4°C in 1 volume of precooled Hepes buffer supplemented with protease and phosphatase inhibitors, and with or without 500 mM NaCl. Triton X-100 was added to the final concentration of 0.4%, and cells were permeabilized for 10 min at 4°C. Suspensions were spun down (21,000 × *g*, 15 min, 4°C), and supernatants were used for gradient centrifugation.

Gel Filtration Chromatography

Gel filtration was carried out at 4°C on a 16.6 × 1.6 cm Superose-6 column (Amersham Pharmacia Biotech, Vienna, Austria) equilibrated in Hepes buffer supplemented with 2% glycerol (column buffer). Column buffer as well as the 0.25-ml samples was passed through a 0.22-μm filter. After loading, the column was eluted at 6 ml/hr and 0.5-ml aliquots were collected. Proteins were precipitated with chloroform-methanol (Wessel and Flugge, 1984), and air-dried pellets were then dissolved in 50 μl of SDS-PAGE buffer.

Sucrose Gradient Sedimentation

Sucrose gradient centrifugation was carried out as described (Moritz et al., 1998). The 5–40% sucrose gradients were poured as step density gradients (950-μl steps: 40, 30, 20, 10, and 5% sucrose) and allowed to diffuse into continuous gradients overnight at 4°C before use. The gradients were prepared in Hepes buffer (75 mM NaCl) or in Hepes buffer supplemented with NaCl to final concentration 500 mM. A 75-μl sample aliquot was loaded onto each gradient, and the gradients were centrifuged at 50,000 rpm in a Beckman SW55 rotor for 4 hr at 4°C. Gradients were fractionated from the top into 14 fractions. Protein standards (0.5 mg/ml each) were loaded in an equivalent volume and were run in parallel over identical gradients for each experiment. Samples for SDS-PAGE were prepared by mixing with 5× concentrated SDS-PAGE sample buffer.

Immunoprecipitation

Immunoprecipitation was performed as previously described (Dráberová and Dráber, 1993). Cell extracts were incubated with beads of (1) immobilized protein A saturated with anti–γ-tubulin antibody TU-31, (2) immobilized protein A saturated with control antibody NF-09, (3) immobilized protein A alone, (4) immobilized protein L saturated with anti–α-tubulin antibody

TU-16, (5) immobilized protein L saturated with control anti-vimentin antibody VI-10, or (6) immobilized protein L alone. The antibodies were used in the form of culture supernatants to avoid immobilization of other mouse antibodies. 50 μl of sedimented beads were incubated under rocking at 4°C for 2 hr with 1.2 ml of the corresponding antibody, prepared by mixing 0.4 ml of concentrated supernatant with 0.8 ml of TBST (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20). The beads were pelleted by centrifugation at 5,000 × *g* for 1 min, washed four times (5 min each) in cold TBST, and further incubated under rocking for 3 hr at 4°C with 0.5 ml of cell extract diluted 1:1 with TBST. Thereafter, the beads were pelleted and washed four times (5 min each) in cold TBST before boiling for 5 min in 100 μl of SDS-sample buffer to release the bound proteins.

Gel Electrophoresis and Immunoblotting

SDS-PAGE was performed according to Laemmli (1970). When whole cell extracts were analyzed, boiled samples were centrifuged at 22,000 × *g* for 10 min and separated on 7.5–12.5% gels; Coomassie Blue stained gels were densitometrically scanned. The amount of proteins in samples for subsequent immunoblotting experiments was adjusted, by using BSA as a standard, to 10 μg of proteins per blotted area in each lane. Proteins were electrophoretically transferred from gels onto nitrocellulose sheets and immunostained. Details of the immunostaining procedure are described elsewhere (Dráber et al., 1988). The antibodies TU-01, TU-30, TU-31, TU-32, and VI-10 in the form of spent culture supernatants were used undiluted, the antibody GTU-88, in the form of ascitic fluid, was diluted 1:4,000. The antibody against β-tubulin and antibody against Hsp70 were diluted 1:2,000, and antibody against actin was diluted 1:200. Bound antibodies were detected by incubation of blots with anti-mouse antibody conjugated with horseradish peroxidase diluted 1:10,000, and after washing with chemiluminescent reagents according to the manufacturer's directions. Autoradiography films X-Omat AR (Eastman Kodak, Rochester, NY) were quantified by densitometry using gel documentation system GDS 7500 and GelBase/GelBlot Pro analysis software (UVP, Upland, CA).

In two-dimensional electrophoresis, samples were first separated by isoelectric focusing on rehydrated 11-cm-long Immobiline DryStrip gels, pH gradient 4–7 (Amersham Pharmacia Biotech, Uppsala, Sweden) at 22,000 V hours on Multiphor II apparatus (LKB, Bromma, Sweden). The samples, Triton X-100 extracts in MEM buffer, were diluted 40 times in running solution composed of 7 M urea, 2 M thiourea, 4% CHAPS, 0.5% Triton X-100, 20 mM DTT, and 2% IPG buffer, pH 4–7. Strips were immersed into protein-containing running solution, covered with mineral oil and allowed to equilibrate according to the manufacturer's instruction. The second dimension was performed on 8% SDS-PAGE and separated proteins were electrophoretically transferred onto nitrocellulose.

Immunofluorescence

Blood cells from chicken embryos and hatched chicks were washed three times in, respectively, corresponding collecting buffer and PBS. Washed cells were resuspended 1:1 in the corresponding buffer and thereafter diluted with the buffer 1:2,000 before loading to poly-L-lysine coated coverslips for 10 min at 37°C. In some cases, the attached cells were incubated on ice for 16 hr and then fixed or incubated at 37°C for various time intervals, ranging from 30 sec to 20 min, before cytoskeleton preparation. Extraction and fixation steps were carried out in MEM buffer supplemented with 4% PEG 6,000 (microtubule stabilizing buffer; MSB). Cells on coverslips were extracted for 1 min at 37°C with 0.2% Triton X-100 in MSB and the resulting cytoskeletons were fixed for 20 min at 37°C in 3% formaldehyde in MSB, followed by 10 min fixation in methanol at −20°C. Taxol-stabilized cytoskeleton was prepared by extracting the cells for 4 min at 37°C with 0.2% Triton X-100 in MSB containing 5 μM Taxol. Such preparations were then incubated for 10 min at 37°C with MSB containing 50, 100, 250, and 500 mM NaCl before formaldehyde and methanol fixation. Monoclonal antibodies were used as undiluted supernatants. For double-label immunofluorescence, the coverslips were incubated simultaneously for 45 min with affinity-purified rabbit antibody against the αβ-tubulin heterodimer (dilution 1:10) and with monoclonal antibodies against γ-tubulin. After washing, the slides were incubated simultaneously with fluorescein isothiocyanate–conjugated anti-rabbit Ig and lissamine rhodamine-conjugated anti-mouse Ig antibody, both diluted 1:100. Actin was detected with rhodamine-conjugated phalloidin. The preparations were mounted in Mowiol 4-88 supplemented with 1 μg/ml of 4,6-diamidino-2-phenylindole (DAPI) and n-propylgalate, and examined with an Olympus A70 Provis microscope equipped with 100× oil-immersion objective. Images were recorded with a Life Science Resources KAF 1400 cooled CCD camera. The control antibody NF-09 did not provide staining.

ACKNOWLEDGMENTS

Taxol was a generous gift of the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). Rhodamine-conjugated phalloidin was a generous gift from Dr. T. Wieland.

REFERENCES

Baas PW, Black MM. 1990. Individual microtubules in the axon consist of domains that differ in both composition and stability. J Cell Biol 111:495–509.

Behnke O. 1970. A comparative study of microtubules of disk-shaped blood cells. J Ultrastruct Res 31:61–75.

Binarová P, Doležel J, Hause B, Dráber P. 1998. Association of γ-tubulin with kinetochore/centromeric region of plant chromosomes. Plant J 14:751–757.

auer E, Solomon F. 1989. A marginal band-associated protein properties of both microtubule- and microfilament-associated teins. J Cell Biol 109:1609–1620.
fH, Pepperkok R, Hill AM, Levilliers N, Ansorge W, Stelzer EH, senti E. 1990. Regulation of microtubule dynamics and nucleation ing polarization in MDCK II cells. J Cell Biol 111:3013–3021.
s GA, Ingram VM. 1973. The erythroid cells and haemoglobins of chick embryo. Philos Trans R Soc Lond B Biol Sci 266:225–305.
n WD. 1991. The cytoskeletal system of nucleated erythrocytes. Rev Cytol 130:37–84.
ives C, Mazarguil H, Lajoie-Mazenc I, Julian M, Raynaud-ssina B, Wright M. 1997. Protein complexes containing gamma-ulin are present in mammalian brain microtubule protein prep-itions. Cell Motil Cytoskel 36:179–189.
er P, Lagunowich LA, Dráberová E, Viklický V, Damjanov I. 88. Heterogeneity of tubulin epitopes in mouse fetal tissues. stochemistry 89:485–492.
er P, Dráberová E, Viklický V. 1991. Immunostaining of human ermatozoa with tubulin domain-specific monoclonal antibodies. stochemistry 195:519–524.
erová E, Dráber P. 1993. A microtubule-interacting protein in-lved in coalignment of vimentin intermediate filaments with mi-otubules. J Cell Sci 106:1263–1273.
erová E, Dráber P. 1998. Novel monoclonal antibodies TU-08 and U-16 specific for tubulin subunits. Folia Biol (Praha) 44:35–36.
berová E, Sulimenko V, Kukharskyy V, Dráber P. 1999a. Mono-onal antibody NF-09 specific for neurofilament protein NF-M. olia Biol (Praha) 45:163–165.
berová E, Zíková M, Dráber P. 1999b. Monoclonal antibody VI-10 ecific for vimentin. Folia Biolog (Praha) 45:35–36.
k P, Foisner R, Wiche G. 1991. Immunolocalization and molecular roperties of a high molecular weight microtubule-bundling protein ncolin from chicken erythrocytes. J Cell Biol 112:689–700.
hi HC, Yen TJ, Cleveland DW. 1987. In vivo coassembly of a ivergent beta-tubulin subunit (c beta 6) into microtubules of dif-erent function. J Cell Biol 105:2179–2190.
hi HC, Palacios MJ, McNamara L, Cleveland DW. 1992. γ-Tubulin s a centrosomal protein required for cell cycle-dependent microtu-ule nucleation. Nature 356:80–83.
lian M, Tollon Y, Lajoie-Mazenc I, Moisand A, Mazarguil H, Puget A, Wright M. 1993. γ-Tubulin participates in the formation of mid-body during cytokinesis in mammalian cells. J Cell Sci 105:145–156.
nney DM, Linck RW. 1985. The cytoskeleton of unstimulated blood platelets: structure and composition of the isolated marginal micro-ubular band. J Cell Sci 78:1–22.
in S, Magendantz M, Katz W, Solomon F. 1987. Development of differentiated microtubule structure: formation of the chicken rythrocyte marginal band in vivo. J Cell Biol 104:51–59.
p M, Schiebel E. 1997. Spc98p and Spc97p of the yeast γ-tubulin omplex mediate binding to the spindle pole via their interaction ith Spc110p. EMBO J 16:6985–6995.
mmli UK. 1970. Cleavage of structural proteins during the assem-ly of the head of bacteriophage T_4. Nature 227:680–685.
nie-Mazenc I, Tollon I, Détraves C, Julian M, Moisand A, Gueth-allonet C, Debec A, Salles-Passador I, Puget A, Mazarguil H, et al. 994. Recruitment of antigenic gamma-tubulin during mitosis in nimal cells: presence of gamma-tubulin in the mitotic spindle. J Cell Sci 107:2825–2837.
py R, Melki R, Pantaloni D, Carlier MF. 2000. Monomeric γ-tu-ulin nucleates microtubules. J Biol Chem 275:21975–21980.
Q, Joshi HC. 1995. Gamma-tubulin is a minus end-specific micro-ubule binding protein. J Cell Biol 131:207–214.
iB, Marc J, Joshi HC, Palevitz BA. 1993. γ-Tubulin-related protein ssociated with microtubule arrays of higher plants in cell cycle-dependent manner. J Cell Sci 104:1217–1228.
mos R, Chevrier V, Ronjat M, Meurer-Grob P, Martinez P, Frank R, Bornens M, Wade RH, Wehland J, Job D. 1999. Tubulin binding ites on γ-tubulin: identification and molecular characterization. Biochemistry 38:15712–15720.
ads T, Schroer TA. 1995. Polarity and nucleation of microtubules n polarized epithelial cells. Cell Motil Cytoskel 32:273–288.
Miller M, Solomon F. 1984. Kinetics and intermediates of marginal band reformation: evidence for peripheral determinants of microtubule organization. J Cell Biol 99:S70–S75.
Mitchison T, Kirschner M. 1984. Dynamic instability of microtubule growth. Nature 312:237–242.
Moritz M, Braunfeld MB, Fung JC, Sedat JW, Alberts BM, Agard DA. 1995. Three-dimensional structural characterization of centrosomes from *Drosophila* embryos. J Cell Biol 130:1149–1159.
Moritz M, Zheng Y, Alberts BM, Oegema K. 1998. Recruitment of the gamma-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. J Cell Biol 142:775–786.
Moudjou M, Bordes N, Paintrand M, Bornens M. 1996. γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. J Cell Sci 109:875–887.
Murphy DB, Wallis KT. 1983. Brain and erythrocyte microtubules from chicken contain different β-tubulin polypeptides. J Biol Chem 258:7870–7875.
Murphy DB, Wallis KT. 1985. Erythrocyte microtubule assembly in vitro. J Biol Chem 260:12293–12301.
Murphy SM, Urbani L, Stearns T. 1998. The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components Spc97p and Spc98p. J Cell Biol 141:663–674.
Nováková M, Dráberová E, Schürmann W, Czihak G, Viklický V, Dráber P. 1996. γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies. Cell Motil Cytoskel 33:38–51.
Oakley CE, Oakley BR. 1989. Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mip*A gene of *Aspergillus nidulans*. Nature 338:662–664.
Oakley BR, Oakley CE, Yoon Y, Jung M. 1990. γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. Cell 61:1289–1301.
Oegema K, Wiese C, Martin OC, Milligan RA, Iwamatsu A, Mitchison TJ, Zheng Y. 1999. Characterization of two related *Drosophila* gamma-tubulin complexes that differ in their ability to nucleate microtubules. J Cell Biol 144:721–733.
Pratt LF, Cleveland DW. 1988. A survey of the alpha-tubulin gene family in chicken: unexpected sequence heterogeneity in the polypeptides encoded by five expressed genes. EMBO J 7:931–940.
Rüdiger M, Weber K. 1993. Characterization of the posttranslational modifications in tubulin from the marginal band of avian erythrocytes. Eur J Biochem 218:107–116.
Sanchez I, Cohen WD. 1994. Localization of tau and other proteins of isolated marginal bands. Cell Motil Cytoskel 27:350–360.
Tassin AM, Maro B, Bornens M. 1985. Fate of microtubule-organizing centers during myogenesis in vitro. J Cell Biol 100:35–46.
Trinczek B, Marx A, Mandelkow EM, Murphy DB, Mandelkow E. 1993. Dynamics of microtubules from erythrocyte marginal bands. Mol Biol Cell 4:323–335.
Viklický V, Dráber P, Hašek J, Bártek J. 1982. Production and characterization of a monoclonal antitubulin antibody. Cell Biol Int Rep 6:725–731.
Wessel D, Flugge UI. 1984. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 138:141–143.
Wiese C, Zheng Y. 1999. Gamma-tubulin complexes and their interaction with microtubule- organizing centers. Curr Opin Struct Biol 9:250–259.
Wiese C, Zheng Y. 2000. A new function for the gamma-tubulin ring complex as a microtubule minus-end cap. Nat Cell Biol 2:358–364.
Winckler B, Gonzalez AC, Magendantz M, Solomon F. 1994. Analysis of a cortical cytoskeletal structure: a role for ezrin-radixin-moesin (ERM proteins) in the marginal band of chicken erythrocytes. J Cell Sci 107:2523–2534.
Wise DO, Krahe R, Oakley BR. 2000. The gamma-tubulin gene family in humans. Genomics 67:164–170.
Zheng Y, Wong ML, Alberts B, Mitchison T. 1995. Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. Nature 378:578–583.
Zhu J, Bloom SE, Lazarides E, Woods C. 1995. Identification of a novel $Ca^{(2+)}$-regulated protein that is associated with the marginal band and centrosomes of chicken erythrocytes. J Cell Sci 108:685–698.

VI.2. Libusová L., Sulimenko T., **Sulimenko V.,** Hozák P., Dráber P. *γ-Tubulin in Leishmania: cell cycle dependent changes in subcellular localization and heterogeneity of its isoforms.* ***Experimental Cell Research*, 295**: 375-386, 2004.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Cell Research 295 (2004) 375–386
Experimental Cell Research
www.elsevier.com/locate/yexcr

γ-Tubulin in *Leishmania*: cell cycle-dependent changes in subcellular localization and heterogeneity of its isoforms

nka Libusová,[a,b] Tetyana Sulimenko,[a] Vadym Sulimenko,[a] Pavel Hozák,[c] and Pavel Dráber[a,*]

[a]*Department of Biology of the Cytoskeleton, Institute of Molecular Genetics, Czech Academy of Sciences, CZ-14220 Prague, Czech Republic*
[b]*Department of Animal Physiology and Developmental Biology, Faculty of Sciences, Charles University, CZ-12820 Prague, Czech Republic*
[c]*Department of Cell Ultrastructure and Molecular Biology, Institute of Experimental Medicine, Czech Academy of Sciences, CZ-14220 Prague, Czech Republic*

Received 24 September 2003, revised version received 15 December 2003

stract

A panel of six anti-peptide antibodies recognizing epitopes in different regions of the γ-tubulin molecule was used for the characterization d localization of γ-tubulin during cell cycle in *Leishmania* promastigotes. Immunofluorescence microscopy revealed the presence of γ-ulin in the basal bodies, posterior pole of the cell, and in the flagellum. Furthermore, the antibodies showed punctuate staining in the pellicular microtubule. This complex localization pattern was observed in both interphase and dividing cells, where staining of posterior les and the subpellicular corset was more prominent. In posterior poles, γ-tubulin co-distributed with the 210-kDa microtubule-interacting otein and the 57-kDa protein immunodetected with anti-vimentin antibody. Immunogold electron microscopy on thin sections of isolated gella showed that γ-tubulin was associated with the paraflagellar rod (PFR) that runs adjacent to the axonemal microtubules. Under ferent extraction conditions, γ-tubulin in *Leishmania* was found only in insoluble cytoskeletal fractions, in contrast to tubulin dimers that re both in soluble and cytoskeletal pool. Two-dimensional electrophoresis revealed multiple charge variants of γ-tubulin. Posttranslational odifications of *Leishmania* γ-tubulin might therefore have an important role in the regulation of microtubule nucleation and interaction with her proteins. The complex pattern of γ-tubulin localization and its properties indicate that γ-tubulin in *Leishmania* might have other nction(s) besides microtubule nucleation.

ywords: Antibodies; Cell cycle; Gamma-tubulin; *Leishmania*

troduction

Leishmania are protozoan parasites from the family ypanosomatidae. Members of this family (*Leishmania* p., *Trypanosoma brucei*, *Trypanosoma cruzi*) are deadly man pathogens that affect millions of people in countries rldwide. Leishmaniasis comprises a group of diseases th a wide spectrum of clinical manifestations ranging m self-healing cutaneous ulcers to severe visceral disease d even death. *Leishmania* are digenetic organisms shut-ng between a slender flagellated promastigote in the gut of e dipteran sandfly and an intracellular round-shaped mobile amastigote in the mammalian host. *Leishmania tropica* belongs to the species of the Old World and causes mainly cutaneous leishmaniasis.

Flagellated promastigote of *Leishmania* comprises four major highly organized microtubule arrays: the subpellicular cortical microtubules, the flagellar axoneme, the basal body, and the intranuclear mitotic spindle. Tubulin is also the most abundant protein in *Leishmania* [1]. *Leishmania* therefore represents a particularly interesting model when studying proteins that participate in microtubule nucleation and organization. The microtubular corset of the cell is formed by parallel arrays of microtubules closely apposed to the inner surface of plasma membrane [2]. Promastigotes carry a single flagellum that protrudes from the cell through a flagellar pocket. The flagellum consists of a canonical 9 + 2 microtubule array and a unique cytoskeletal structure called the paraflagellar rod (PFR, also known as paraxonemal rod). The PFR is a complex lattice of filaments that run parallel to the axoneme once the flagellum emerges from the flagellar

* Corresponding author. Department of Biology of the Cytoskeleton, ntute of Molecular Genetics, Czech Academy of Sciences, Building LB, deňská 1083, 142 20 Prague 4, Czech Republic. Fax: +420-241-062-758.
E-mail address: paveldra@biomed.cas.cz (P. Dráber).

14-4827/$ - see front matter
i:10.1016/j.yexcr.2004.01.009

ket. Two major protein components of the PFR (called R 1 and PFR 2 in *Leishmania*) are essential for proper tility and attachment to insect vector [3,4]. Apart from well-characterized major components of PFR, other nstituents have also been described, but their role in the R structure is unknown [5,6]. The basal body of the gellum not only controls the assembly of flagellar axo- ne but is also involved in mitochondrial DNA (called netoplast) segregation. During division, the basal body of flagellum divides first, and a new flagellum is generated m the daughter basal body. Next, the kinetoplast and bsequently the nucleus divide. The basal body as a crotubule-organizing center (MTOC) plays a prominent t in the orchestration of microtubule arrays and regula- n of microtubule assembly. Despite its important role, ly few proteins were so far located to the region of basal dy of trypanosomatids. A homolog of human centrin 2 s localized to the basal body of *Leishmania donovani* [7]. e 210-kDa microtubule-interacting protein and the 57- la protein co-distribute in the region of basal body and on sterior poles of *L. tropica* cells [8]. In *T. brucei*, the γ- bulin, the 170-kDa *T. brucei* basal body component otein (TBBC), and the 43-kDa protein BBA4 closely sociate with the basal body complex [9,10].

γ-Tubulin, a highly conserved member of the tubulin perfamily, is widely accepted as a marker of MTOC in verse eukaryotes [11]. In mammalian cells, the majority of ubulin is associated with other proteins in soluble cyto- smic complexes. Large γ-tubulin-ring complexes (γ- RCs) were identified in various species [12,13]. Smaller mplexes (γ-tubulin small complex; γTuSC) were also und in different cells [14]. The γ-TuRCs are formed by all complexes and by other proteins. In addition to the cleation from MTOC, γ-TuRCs are also involved in the gulation of the dynamics of microtubule (−) ends [15].

γ-Tubulin itself has been recognized as a microtubule)-end binding molecule in non-anchored microtubules 6], and the binding sites for α- and β-tubulin subunits γ-tubulin were identified using synthetic peptides [17]. embrane-bound forms of γ-tubulin with nucleation activ- were also described [18,19]. Phosphorylation of the γ- bulin on tyrosine has been reported [20] and multiple electric variants of γ-tubulin were defined in different ecies [21–23].

In *T. brucei*, γ-tubulin appears to be present as a single py gene per haploid genome. Immunostaining revealed its calization in the basal body, and also in nucleus, on the terior tip of the cell body, along four stable microtubules at underline the flagellum attachment zone and low level nctuate staining over the microtubular corset [9]. RNAi periments implicated γ-tubulin in nucleation of the central ir microtubules during flagellum morphogenesis in *T. ucei* [24]. In comparison with *T. brucei*, *Leishmania* has fferent morphology and different position and attachment the flagellum. We wanted to find out whether γ-tubulin, a y component for microtubule nucleation, is in *Leishmania* also in multiple nucleation sites and whether its distribution changes during cell cycle. Because multiple γ-tubulin isoforms were identified in different species, we searched for such variants in *Leishmania*.

Using a panel of antibodies against different γ-tubulin antigenic determinants, we collected data on complex, cell cycle-dependent distribution patterns of γ-tubulin in *L. tropica*.

Materials and methods

Cell cultures

L. tropica, strain Z-K (MHOM/JO/99/Z-K), an original isolate from human cutaneous leishmaniasis was provided by Dr. E. Nohýnková (Charles University, Prague, Czech Republic). The species identification was done by Dr. C. L. Eisenberger using a permissively primed intergenic PCR [25]. Promastigotes were grown at 28°C in Schneider's *Drosophila* Medium (Sigma-Aldrich, Prague, Czech Republic) supplemented with 10% (v/v) fetal calf serum, penicillin (500 units/ml), and amykacin (200 μg/ml). Cells from the logarithmic phase of growth were used for immunofluorescence and preparation of cell lysates.

Antibodies

The following anti-peptide antibodies against γ-tubulin were used: monoclonal antibodies TU-30 (IgG2b), TU-31 (IgG2b), and TU-32 (IgG1) were prepared against the EYHAATRPDYISWGTQ peptide corresponding to the human γ-tubulin conservative sequence 434–449 [26]; monoclonal antibody GTU-88 (IgG1) prepared against the EEFATEGTDRKDVFFY peptide corresponding to human γ-tubulin sequence 38–53, and affinity-purified rabbit antibody γ-TUB raised against the same peptide. The last two antibodies were bought from Sigma-Aldrich. Rabbit antibody No. 9 was raised against the EEFATEGGDRKDV peptide corresponding to *Aspergillus nidulans* γ-tubulin sequence 38–50 [27]. The antibody was affinity purified on recombinant 6× His-tagged γ-tubulin immobilized on a Ni-NTA agarose column (Qiagen, Hilden, Germany) [28]. The amino acid γ-tubulin sequences in regions where the peptides used for immunizations are located are compared in Fig. 1.

The antibody TU-01 (IgG1) recognizes the epitope that is in phylogenetically highly conserved region (amino acids 65–97) in the N-terminal structural domain of α-tubulin [29]. In double-label immunofluorescence, the microtubule structures were visualized by a rabbit affinity-purified TUB antibody against αβ-tubulin dimer [30]. The MA-01 antibody (IgG1) was raised against porcine brain microtubule-associated proteins MAP2ab and recognizes the 210-kDa protein in different nonneural cells of various species including *L. tropica* [8,31]. The mouse

H.s. 395GIVEEFATEGTDRKDVFFYQA
A.n. 391GNLEEFATEGGDRKDVFFYQS
L.m. 429GVVEPYAVGGEDRKDVFFYQA

H.s. 417QLIDEYHAATRPDYISWGTQEQ
L.m. 453SLVAEYKACESSDYIRNF

1. Comparison of γ-tubulin sequences in regions where the peptides l for immunizations are located. H.s., *Homo sapiens*; L.m., *Leishmania r*; A.n., *Aspergillus nidulans*. Identical amino acid positions are lighted. Full line: human peptide used for preparation of monoclonal ody GTU-88 and polyclonal antibody γ-TUB; dashed line: *A. nidulans* ide used for preparation of polyclonal antibody No. 9; dotted line: an peptide used for preparation of monoclonal antibodies TU-30, TU- and TU-32.

noclonal VI-01 antibody (IgM) is directed against the igenic determinant on vimentin and smooth muscle min [32]. In *L. tropica*, it recognizes the 57-kDa protein . The rabbit affinity-purified antibody M8 was raised inst pericentrin [33]. As negative controls served the bit affinity-purified anti-actin antibody (Sigma-Aldrich; alogue number A2066), mouse monoclonal TU-20 an- ody (IgG1) against neuron-specific class III β-tubulin 4] and mouse monoclonal MT-02 antibody (IgM) against crotubule-associated proteins MAP2ab [35]. Secondary ti-mouse and anti-rabbit antibodies conjugated with rseradish peroxidase were purchased from Promega otec (Madison, WI, USA). Indocarbocyanin 3 (Cy3)- njugated anti-mouse and anti-rabbit Ig antibodies, fluo- scein isothiocyanate (FITC)-conjugated anti-mouse and ti-rabbit Ig antibodies, and immunoglobulin class-specif- antibodies conjugated with Cy3 or FITC were from ckson Immunoresearch Laboratories (West Grove, PA, SA). The 10 nm gold-conjugated anti-mouse Ig antibody as from British BioCell (Cardiff, UK).

bcellular fractionation and preparation of cell extracts

Isolation of flagella was performed according to Bastin et . [36]. Briefly, cells were washed twice in phosphate- uffered saline (PBS) and extracted with 1% NP-40 (v/v) in EME buffer (0.1 M Pipes, pH 6.9, 2 mM EGTA, 1 mM $MgSO_4$, and 0.1 mM EDTA) on ice for 5 min. The insoluble aterial was pelleted, resuspended in 1% NP-40 (v/v) upplemented with 1 M NaCl in PEME buffer, and incu- ated on ice for 20 min. The pelleted material contained a ghly enriched flagellar fraction as confirmed by phase ntrast microscopy.

To prepare total extracts, cells or flagella were washed ice in PBS and solubilized in 6 M guanidine thiocyanate H_2O at room temperature for 10 min. After centrifugation 20,000 × *g* for 15 min at room temperature, the collected pernatant was dialyzed against H_2O. After dialysis the suspension was mixed with twice concentrated SDS-sample buffer and boiled for 5 min.

For the preparation of soluble and insoluble fractions, washed cells were extracted with cold 1% Nonidet P-40 (v/v) in microtubule-stabilizing buffer (MSB; 20 mM MES, pH 6.9, 2 mM EGTA, 2 mM $MgCl_2$) supplemented with protease inhibitor cocktail Complete (Roche Diagnostic, Mannheim, Germany) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). After 10 min incubation at 4°C under mild shaking, the samples were centrifuged at 24,000 × *g* for 15 min at 4°C. One-fourth volume of four times concentrated SDS-sample buffer was added to the supernatant. The pelleted material was washed in cold MSB, resuspended in 1% Nonidet P-40 (v/v) in MSB to the original volume, and one-fourth volume of four times concentrated SDS-sample buffer was added. Samples were boiled for 5 min. Protein quantification in SDS-sample buffer was performed using the colloidal silver method [37].

Gel electrophoresis and immunoblotting

Details of the protein separation on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and the immunostaining procedure are described elsewhere [38]. Primary monoclonal antibodies, in the form of supernatants, were used undiluted. Antibody GTU-88 was diluted 1:5000. Rabbit antibodies against γ-tubulin (γ-TUB) and pericentrin (M8) were diluted 1:2000 and 1:500, respectively. After incubation of the immunoblots with secondary antibodies conjugated with horseradish peroxidase (diluted 1:10,000), bound antibodies were detected by a chemiluminescent reaction (SuperSignal WestPico Chemiluminescent reagents; Pierce, Rockford, IL, USA) according to the manufacturer's recommendation. Autoradiography films X-Omat K were purchased from Eastman Kodak (Rochester, NY, USA).

Two-dimensional PAGE was performed essentially as described previously [39]. The pelleted flagellar fraction was solubilized in sample buffer containing 7 M urea, 2 M thiourea, 20 mM Tris, 4% CHAPS (w/v), 1% Triton X-100 (w/v), and 1% dithiothreitol (w/v) for 30 min at room temperature. Suspension was centrifuged at 24,000 × *g* for 20 min at 24°C and the supernatant was diluted 1:5 in solubilization buffer supplemented with 2% IPG (v/v), pH 4–7 (Amersham Biosciences, Uppsala, Sweden). Immobiline DryStrip gels with linear pH 4–7 gradient, 7 cm long (Amersham Biosciences), were rehydrated using 170 μl of prepared sample containing 30–50 μg of proteins. Each strip was overlaid with mineral oil and left overnight at room temperature. Strips were focused for the total of 22 kV h on Multiphor II apparatus (LKB, Bromma, Sweden). The second dimension was performed using 7.5% SDS-PAGE, and separated proteins were transferred onto nitrocellulose by electroblotting. Markers for 2D electrophoresis were from BioRad Laboratories (München, Germany).

Immunofluorescence microscopy

Cells were harvested by centrifugation, washed three times in PBS, resuspended in PBS, and settled on poly-L-lysine-coated coverslips. Attached cells were treated with 2% Triton X-100 (v/v) in MSB supplemented with 4% PEG 6000 (w/v) for 5 min, washed in MSB, fixed with 3% formaldehyde (w/v) for 30 min, and treated with 0.3% SDS (w/v) in MSB for 10 min. In further text, this extraction–fixation method is called the Triton X-100 extraction and formaldehyde fixation. Alternatively, cells were extracted with 1% Nonidet P-40 (v/v) and fixed in cold methanol. In some experiments, cells extracted with Triton X-100 were incubated for 10 min with MSB containing 150 mM, 250 mM, 500 mM, 750 mM or 1 M NaCl. After washing in MSB, the cells were fixed by formaldehyde. Immunofluorescence staining was then performed as described [40]. In double-label immunofluorescence stainings, the fixed cells were first incubated with the corresponding anti-γ-tubulin antibody for 45 min, washed, and then incubated with the other primary antibody. The anti-γ-tubulin antibodies TU-30, TU-31, and TU-32 were used as undiluted supernatants, GTU-88 was diluted 1:400, polyclonal antibody γ-TUB 1:300 and polyclonal antibody No.9 1:500. The monoclonal antibodies TU-01, MA-01, and VI-01, and negative control antibodies TU-20 and MT-02 were all used as undiluted supernatants. Rabbit antibodies TUB against αβ-tubulin dimer and against pericentrin were diluted 1:5 and 1:500, respectively. The slides were washed three times in PBS and incubated with a mixture of secondary antibodies. The secondary antibodies conjugated with Cy3 were diluted 1:1000 and the secondary antibodies conjugated with FITC were diluted 1:200. Coverslips were mounted in Mowiol 4.88 (Calbiochem AG, Lucerne, Switzerland) containing 2 μg/ml 4,6-diamidino-2-phenylindole (DAPI) and 6.25% propyl gallate (w/v) (Fluka AG, Buchs, Switzerland). The preparations were examined with an AX-70 PROVIS (Olympus) fluorescence microscope equipped with 100/1.35 fluorescence oil-immersion objective. Images were recorded using a Life Science Resources KAF 1400 cooled CCD camera.

In some immunofluorescence experiments, the antibodies TU-32 and GTU-88 were preabsorbed with the peptides used for immunization; that is, the 16 amino acid peptide corresponding to human γ-tubulin sequence 434–449 (TU-30) or 38–53 (GTU-88). Two molar antibody to peptide ratios were used, 1:10 and 1:100. Mixtures of antibodies and peptides were incubated for 30 min at room temperature.

Immunogold electron microscopy

Isolated flagella were washed in Sörensen buffer (SB, 0.1 M Na/K phosphate buffer, pH 7.3), pelleted, fixed for 30 min at room temperature in 3% paraformaldehyde (w/v) with 0.05% glutaraldehyde (w/v) in SB, and washed twice in SB. After centrifugation into 1% agarose (w/v), blocks were incubated for 20 min in 0.02 M glycine in SB, washed in SB, and dehydrated in ethanol. Ethanol was replaced in two steps by LR White acrylic resin (Sigma-Aldrich) and blocks were polymerized by UV light (20 h, 4 °C). Eighty nanometer sections were prepared on Reichert UltraCuts Microtome (Leica, Germany) and nonspecific labelling was blocked for 30 min by 10% normal goat serum (v/v) in PBTB (PBS supplemented with 0.1% Tween

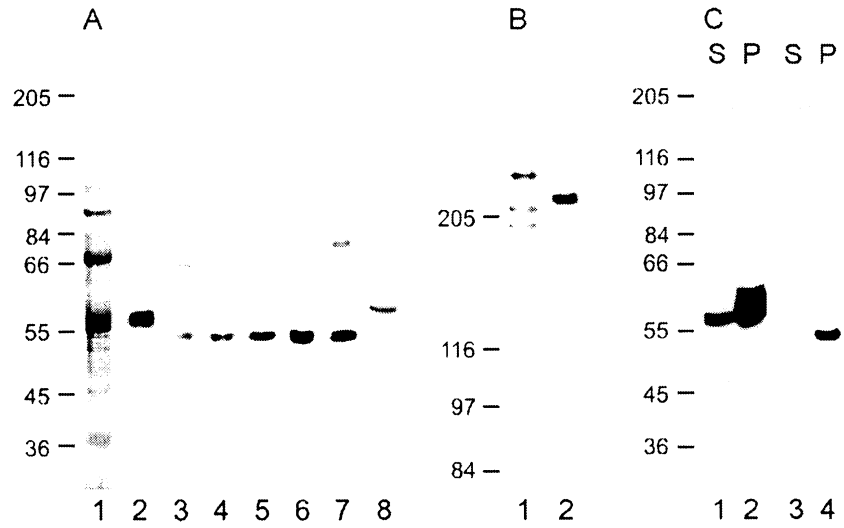


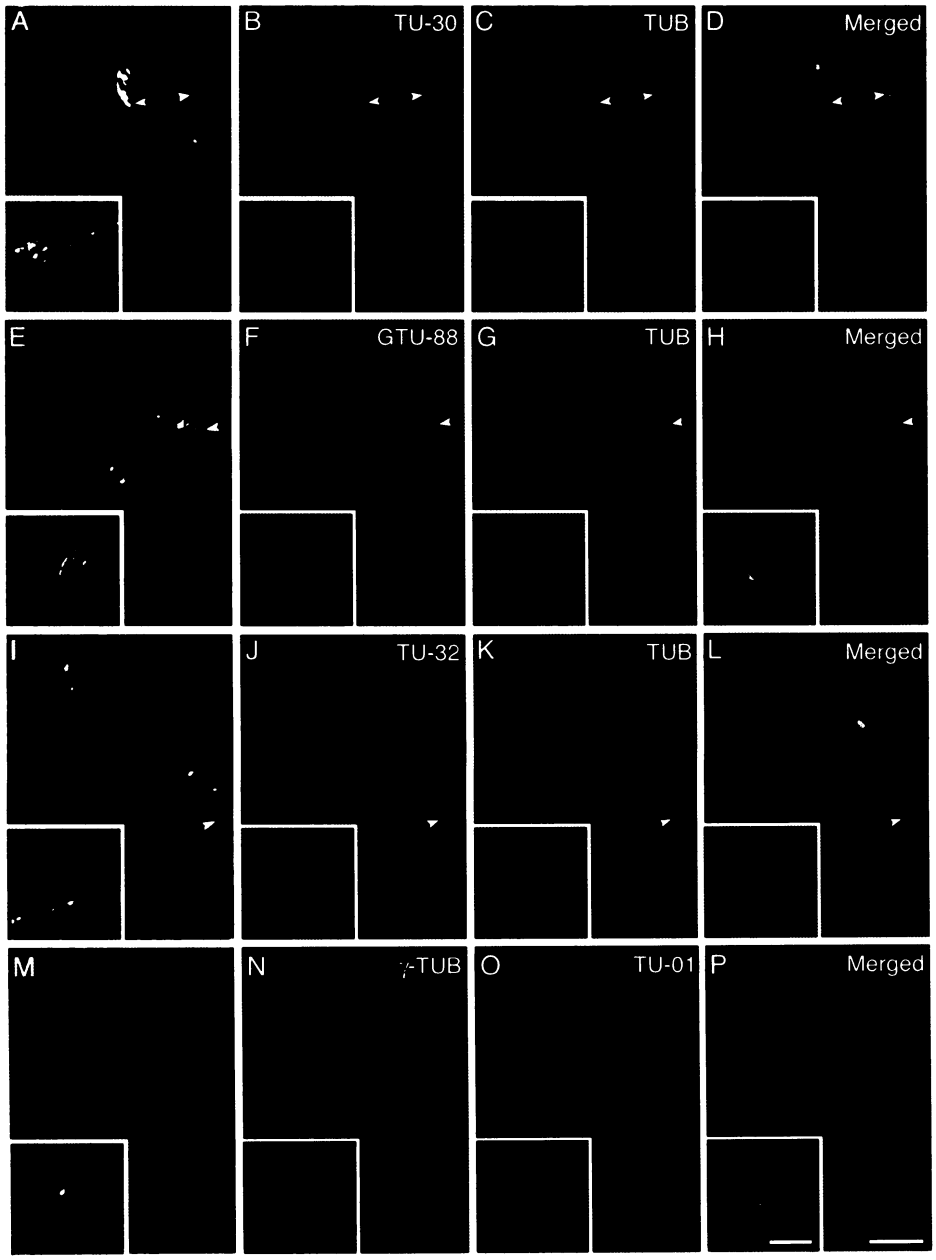
Fig. 2. Immunoblot analysis of *L. tropica* cell extracts with monoclonal antibodies against cytoskeletal proteins. (A and B) Whole cell lysates, (C) insoluble and soluble fractions after 1% NP-40 extraction. (A) Lane 1: Coomassie Blue staining; lanes 2–8: immunostaining with antibodies TU-01, TU-30, TU-31, TU-32, GTU-88, γ-TUB, and VI-01. 7.5% SDS-PAGE. (B) Lanes 1–2: immunostaining with anti-pericentrin antibody and MA-01 antibody. 5% SDS-PAGE. (C) To compare the relative distribution of immunoblotted proteins, the pelleted material was resuspended in a volume equal to that of the corresponding supernatant. Lanes 1–2: antibody TU-01, lanes 3–4: antibody GTU-88. S and P denote supernatant and pellet. 7.5% SDS-PAGE. The same amount of protein (10 μg) was loaded into each lane. Scale bars on the left margins indicate positions of molecular-mass markers in kDa.

20 (w/v) and 1% BSA (w/v)). Sections were incubated 45 min with primary antibodies diluted twice in PBTB, washed three times with PBT (PBS supplemented with 0.005% Tween 20), and incubated for 30 min with 10 nm gold-conjugated secondary antibody diluted 1:30 in PBT. After washing in PBT and water, sections were contrasted 5 min with a saturated solution of uranyl acetate in water and observed in Philips Morgagni electron microscope equipped with Megaview II CCD camera. Control samples were incubated as above except that the primary antibody was omitted.

Results

Antibody characterization

Antibodies were characterized by immunoblotting on whole cell lysates of *L. tropica*. All antibodies directed against the C-terminal (TU-30, TU-31, and TU-32) and N-terminal (GTU-88, γ-TUB) regions of γ-tubulin reacted dominantly with proteins of the same electrophoretic mobility corresponding to the molecular weight around 53 kDa (Fig. 2A, lanes 3–7). Faint cross-reactivity was observed in



1. Distribution of γ-tubulin in interphase and mitotic (inserts) cells of *L. tropica*. Cells were stained with anti-γ-tubulin antibodies (red) TU-30 (B), GTU-), TU-32 (J), or γ-TUB (N); antibodies against tubulin dimer (green) TUB (C, G, K) or TU-01 (O) and DNA-binding dye (blue). Pictures (D, H, L, P) superpositions of stainings in each row. Phase-contrast images (A, E, I, M) correspond to each row of fluorescent images. Arrowheads denote staining of nor poles in cells. Cells were extracted with Triton X-100 and fixed by formaldehyde. Scale bar = 5 μm.

the case of antibodies TU-30 (around 66 kDa) and γ-TUB (around 80 kDa). No reactivity was found in position of tubulin dimers, as demonstrated by staining with anti-α-tubulin antibody TU-01 (Fig. 2A, lane2). The TU-01 antibody recognized a protein with a molecular weight of around 55 kDa and the anti-vimentin antibody VI-01 stained a 57-kDa protein (Fig. 2A, lane 8). The anti-pericentrin antibody reacted mainly with a protein of electrophoretic mobility around 220 kDa and also gave a weaker staining with proteins of lower molecular weight, representing either proteolytic fragments of pericentrin-related protein or cross-reacting protein(s) (Fig. 2B, lane 1). The MA-01 antibody recognized the 210-kDa protein (Fig. 2B, lane 2). No reactivity with blotted proteins was detected when negative control mouse monoclonal antibodies of IgG and IgM class, or negative control rabbit antibody, were applied. To compare the relative distribution of γ-tubulin and tubulin dimers in soluble and insoluble (cytoskeletal) fractions, cells were extracted with 1% Nonidet P-40 and the pelleted material was resuspended in a volume equal to that of the corresponding supernatant. Immunoblotting analysis showed that α-tubulin was present both in soluble and insoluble (cytoskeletal) fractions (Fig. 2C, lanes 1 and 2), whereas γ-tubulin under the same conditions was found only in the insoluble fraction (Fig. 2C, lanes 3 and 4).

Localization of γ-tubulin in interphase and mitotic cells

A panel of anti-γ-tubulin antibodies was used to assign the subcellular localization of γ-tubulin in *L. tropica*. For a better orientation in cell structures, DNA-binding dye was used to visualize the nucleus and kinetoplast. Flagella and subpellicular microtubular arrays were decorated with polyclonal antibody against tubulin dimers (Figs. 3C, G, and K) or with monoclonal antibody TU-01 against α-tubulin (Fig. 3O). Immunofluorescence staining with antibodies TU-30 (C-terminal region of γ-tubulin) and GTU-88 (N-terminal region of γ-tubulin) showed labelling of two major sites: basal body region and posterior pole of the cell opposite the flagellar pocket (Figs. 3B and F). The intensity of labelling at the posterior poles varied in the cell population; some interphase cells were not decorated in this position. However, all mitotic cells were stained at the posterior poles, and the stained area was generally larger than in interphase cells (inserts in Figs. 3B and F). Overexposition of TU-30 labelling revealed a faint decoration of flagella, while no flagellar staining was observed with overexposition of GTU-88 labelling. Staining of the basal body region and posterior pole of the cell was also observed with antibodies TU-32 (C-terminal region of γ-tubulin) and γ-TUB (N-terminal region of γ-tubulin). Again, staining on the posterior pole was cell-cycle dependent with the strongest reaction in mitotic cells (inserts in Figs. 3J and N). In addition, these antibodies gave a strong discontinuous staining along the flagellum in both mitotic and interphase cells (Figs. 3J and N). Similar results were also obtained with antibodies No. 9 (N-terminal region of γ-tubulin) and TU-31 (C-terminal region of γ-tubulin) (not shown). The latter only gave a weak staining of all mentioned structures. Superposition of stainings with anti-γ-tubulin antibodies and antibodies against tubulin dimers revealed that γ-tubulin was not present at the tip of the flagella and did not colocalize with tubulin in this structure (Figs. 3L and P). The described staining patterns with the mentioned anti-γ-tubulin antibodies were similar, no matter which fixation procedure was applied. This was true not only for immunofluorescence data shown in Fig. 3 but also for subsequent immunofluorescence experiments performed on fixed cells. A speckled distribution of γ-tubulin along the whole length of the flagellum, visualized by TU-32 antibody, is shown at higher magnification in Fig. 4A. All anti-γ-tubulin antibodies also gave a weaker punctuate staining over the cell body, as demonstrated at higher magnification in Fig. 4C.

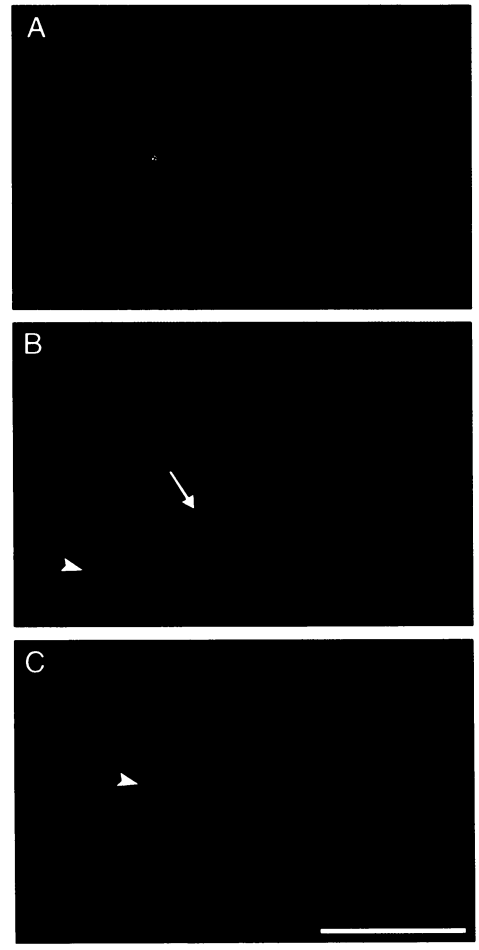


Fig. 4. Details of γ-tubulin localization in *L. tropica* cells. (A) γ-Tubulin staining with TU-32 antibody (red) in flagellum does not match the staining of tubulin dimers with TUB antibody (green). (B) Immunostaining with anti-pericentrin antibody (green) and anti-γ-tubulin antibody TU-30 (red) in the anterior pole of the cell. (C) Dot-like staining of microtubular corset with anti-γ-tubulin antibody TU-30 (red). All cells were stained with DNA-binding dye (blue). Arrowheads denote staining of posterior poles in cells. Arrow shows staining of the basal body region. Cells were extracted with Triton X-100 and fixed by formaldehyde. Scale bar = 5 μm.

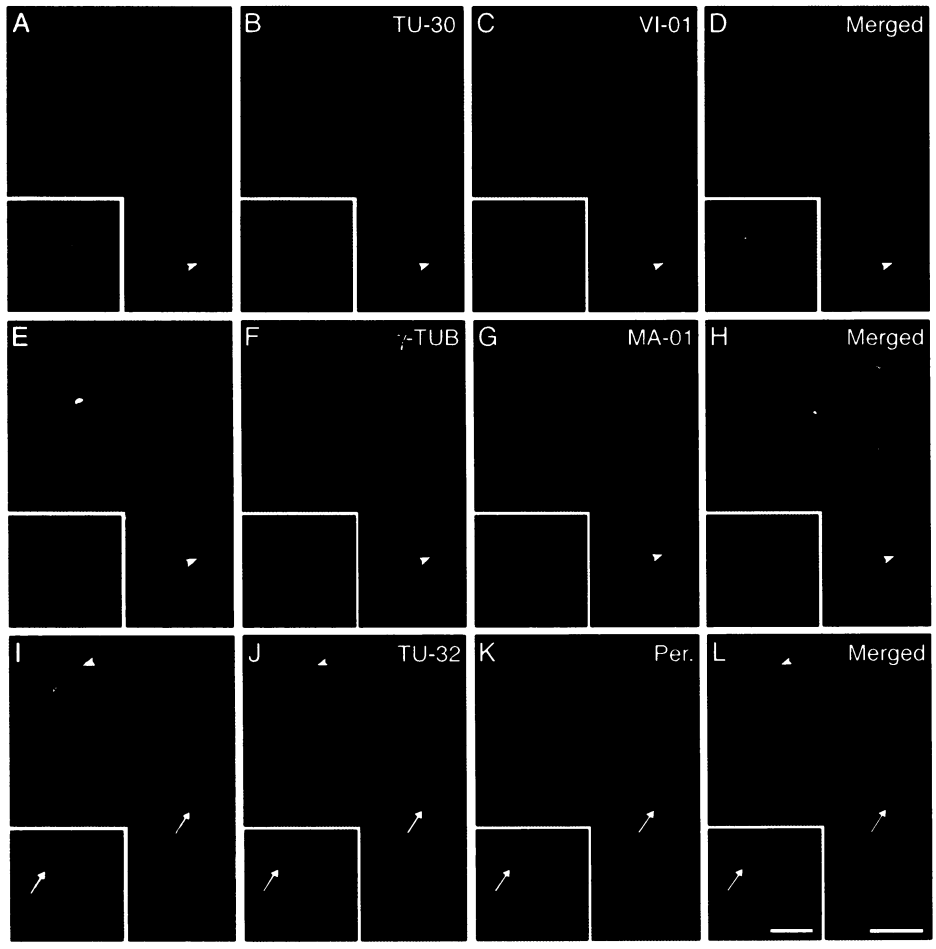
Neither the negative control mouse monoclonal antibodies of the IgG and IgM classes, nor the negative control rabbit antibody or the conjugated secondary antibodies alone gave any staining.

To corroborate the specificity of immunofluorescence staining, antibodies TU-32 and GTU-88 were pre-absorbed with the corresponding peptides which had been used for antibody preparation. No staining of *L. tropica* cells was observed with preabsorbed antibodies. On the other hand, preabsorption of the TU-32 antibody with the peptide used for GTU-88 preparation or preabsorption of GTU-88 antibody with the peptide used for TU-32 preparation caused no reduction in immunofluorescence intensity. The same results were obtained irrespective of the used molar ratio of antibody to peptide (1:10 or 1:100).

To learn more about the nature of γ-tubulin association with the flagellum and posterior pole, cells were first extracted with 2% Triton X-100 in MSB and then incubated for 10 min in MSB containing 150 mM, 250 mM, 500 mM or 1 M NaCl. γ-Tubulin staining at those particular areas was observed after incubation of cells in buffer containing up to 500 mM NaCl. The majority of extracted cells incubated in 1 M NaCl became detached from the coverslips, but in some of the remaining cells, γ-tubulin was still present at the posterior pole and flagellum.

Comparison of γ-tubulin distribution with other cytoskeletal proteins

To compare γ-tubulin distribution with that of other cytoskeletal proteins previously localized at the posterior pole of *L. tropica* cell [8], double-label immunofluorescence was performed with antibodies VI-01 and MA-01. Merged pictures of γ-tubulin stained with TU-30 antibody that decorated only basal bodies and posterior poles of cells, and vimentin-like protein stained with VI-01 antibody, showed a striking colocalization of these two proteins both in the basal body and at the posterior pole of the cell (Fig. 5D). Prominent co-distribution of these proteins was also found in mitotic cells (insert in Fig. 5D). Colocalization of



5. Comparison of γ-tubulin distribution with other cytoskeletal proteins in interphase and mitotic (inserts) cells of *L. tropica*. Cells were stained with anti-ubulin antibodies (red) TU-30 (B), γ-TUB (F), or TU-32 (J); anti-vimentin antibody VI-01 (C; green), antibody against 210-kDa microtubule-interacting ein MA-01 (G; green), anti-pericentrin antibody (K; green), and DNA-binding dye (blue). Pictures (D, H, L) show superpositions of staining in each row. se-contrast images (A, E, I) correspond to each row of fluorescent images. Arrowheads denote staining of posterior poles in cells. Arrows pinpoint the alization of immunostaining with anti-pericentrin antibody. Cells were extracted with Triton X-100 and fixed by formaldehyde. Scale bar = 5 μm.

γ-tubulin, stained with polyclonal γ-TUB antibody, and the 210-kDa microtubule-interacting protein, detected by MA-01 antibody, is shown in Fig. 5H. Again, co-distribution of both proteins was found in posterior poles and basal body regions. The co-distribution was also found in mitotic cells (insert in Fig. 5H). Superposition of staining patterns with either of the two antibodies that decorated the flagella revealed colocalization of γ-tubulin and MA-01 antigen in this region. A comparison of γ-TUB staining in Fig. 3P with that in Fig. 5H indicates that both γ-tubulin and MA-01 antigen are localized outside the axonemal microtubules. Co-distribution was also observed when anti-γ-tubulin antibody TU-32 and biotin-conjugated MA-01 antibody were used for double labelling (not shown).

Pericentrin, like γ-tubulin, is an established marker of MTOCs in different model systems. We therefore performed double-labelling experiments with anti-γ-tubulin and anti-pericentrin antibodies. The anti-pericentrin antibody gave discrete staining at the anterior pole of interphase and mitotic cells (Fig. 5K), but superposition with anti-γ-tubulin staining disclosed a difference in localization (Fig. 5L). The protein immunologically related to pericentrin (pericentrin-related protein) was absent in the basal body, but was found somewhat closer to the anterior tip of the cell. Higher magnification (Fig. 4B) showed that the pericentrin-related protein (green) was located at the anterior pole more distant from the nucleus than γ-tubulin (red), probably in the area of the flagellar pocket opening. No staining at the posterior pole was detected with the used anti-pericentrin antibody.

Flagellar γ-tubulin

To characterize the flagellar γ-tubulin in more detail, we have isolated *L. tropica* flagella. Immunoblotting analysis of flagellar samples containing the same amount of protein demonstrated that it was possible to detect both α-tubulin by antibody TU-01 (Fig. 6A, lane 2) and γ-tubulin by antibody GTU-88 (Fig. 6A, lane 3) or TU-32 antibody (Fig. 6A, lane 4). The two-dimensional analysis of isolated flagella revealed that γ-tubulin existed in multiple charge variants that have their isoelectric points more basic in comparison with α-tubulin (Fig. 6B). Three isoelectric variants of γ-tubulin were identified on immunoblots with antibody GTU-88. The pI value of the most acidic variant was 6.0.

Immunofluorescence and immunogold electron microscopy confirmed the presence of γ-tubulin in isolated flagella. Flagella detected by phase contrast microscopy (Fig. 7A) were discontinuously labelled along the flagellum by anti-γ-tubulin antibody (Fig. 7B). Staining with anti-α-tubulin antibody TU-01 (Fig. 7F) was stronger and more homogeneous. Immunoelectron microscopy with anti-γ-tubulin antibody TU-32 on thin sections of isolated flagella showed that γ-tubulin was present on the paraflagellar rod but absent on microtubular axoneme. Staining of cross-sections (Fig. 7C) and longitudinal sections (Fig. 7D) showed no reactivity with nine outer doublets and central pair of

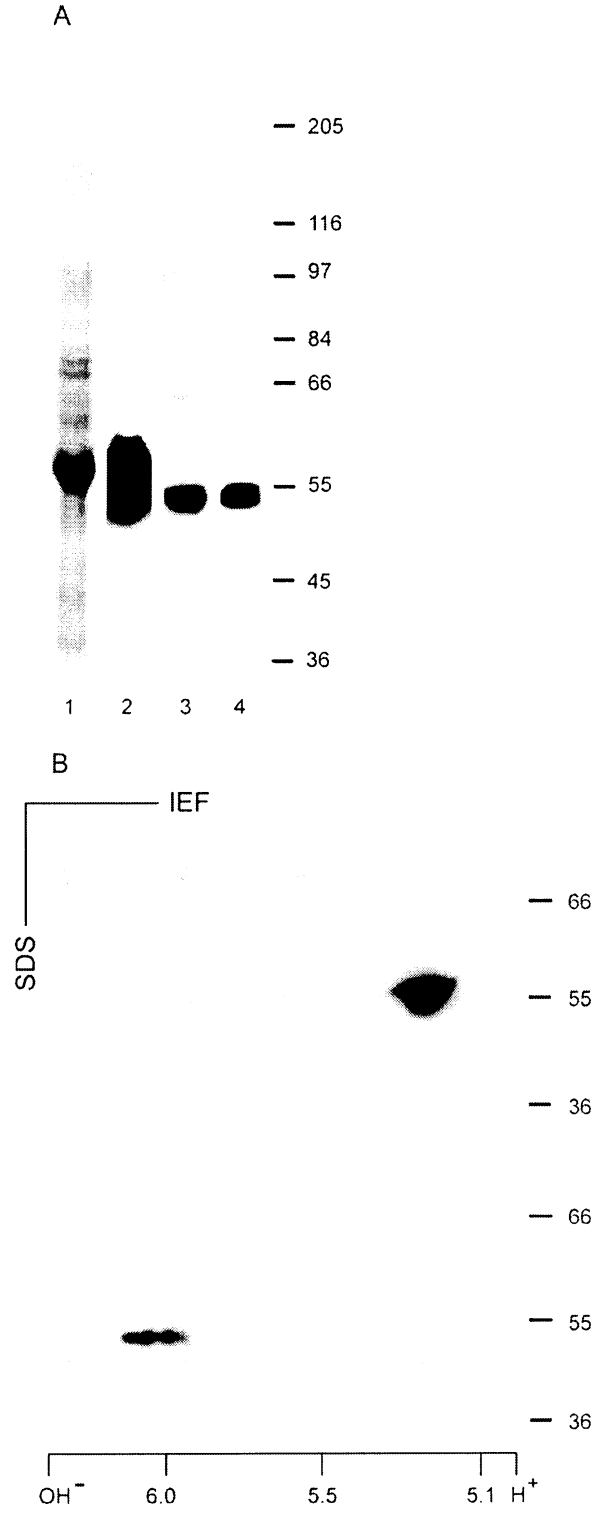
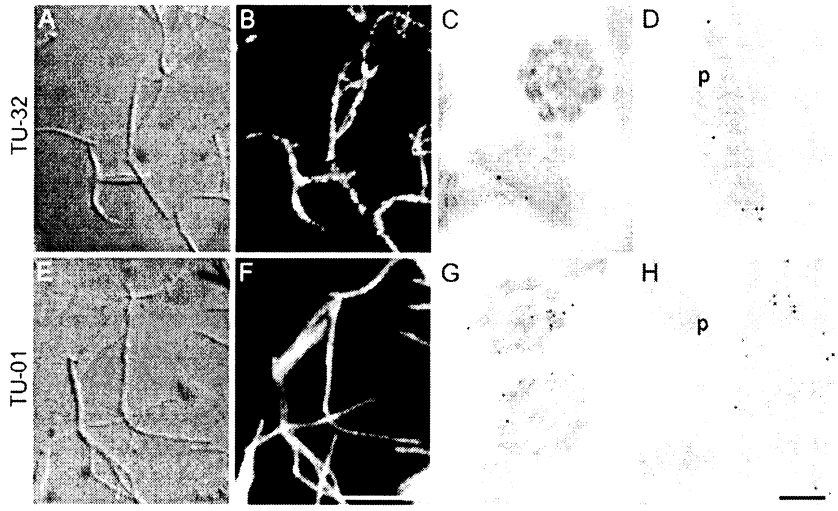


Fig. 6. Immunoblot analysis of *L. tropica*-isolated flagella. (A) Lane 1: Coomassie Blue staining; lanes 2–4: immunostaining with anti-α-tubulin antibody TU-01, anti-γ-tubulin antibody GTU-88, and anti-γ-tubulin antibody TU-32. Protein (10 μg) was loaded into each lane. 7.5% SDS-PAGE. (B) Immunostaining of isolated flagella extract separated by two-dimensional electrophoresis with anti-α-tubulin antibody TU-01 (top) and anti-γ-tubulin antibody GTU-88 (bottom). The pI scale is shown along the bottom of the picture. Scale bars on the right margin indicate positions of molecular-mass markers in kDa.



g. 7. Distribution of γ-tubulin in *L. tropica* flagella. Isolated flagella were stained with anti-γ-tubulin antibody TU-32 (A–D) and anti-α-tubulin antibody U-01 (E–H). Immunofluorescence staining (B, F) and corresponding phase-contrast images (A, E). Flagella were extracted with Triton X-100 and fixed by maldehyde. Scale bar = 5 μm. Immunoelectron microscopy of isolated flagella on cross (C, G) and longitudinal (D, H) sections. p, paraflagellar rod. Scale r = 100 nm.

icrotubules. On the other hand, anti-α-tubulin antibody U-01 decorated axonemal microtubules on cross-sections ig. 7G) and longitudinal sections (Fig. 7H). The paraflagellar rod was not stained. These data are consistent with the indings obtained with fluorescence microscopy (Fig. 4A).

iscussion

To characterize γ-tubulin and its distribution in *L. tropica*, we made use of a panel of anti-peptide antibodies ecognizing epitopes in different regions of the molecule. Monoclonal antibodies TU-30, TU-31, TU-32 are directed gainst the C-terminal region of γ-tubulin, while the monoonal antibody GTU-88 is directed against the N-terminal egion. Both polyclonal antibodies (γ-TUB and No. 9) ecognize the N-terminal region. As anti-peptide antibodies ere used in the study, one can expect that they will ecognize linear epitopes. These can be mapped to small near peptide sequences of 5–20 amino acids [41]. A mparison of the γ-tubulin sequences in *H. sapiens*, *A. dulans*, and *L. major* revealed substantial conservation in e N-terminal region of the molecule. On the other hand, ere is a lower homology between *H. sapiens* and *L. major* i the C-terminal region of the molecule. It is known, owever, that linear epitopes can involve closely spaced d noncontiguous amino acids [41]. Because antibodies ected against the C-terminal and the N-terminal regions γ-tubulin reacted alike on immunoblots with the 53-kDa otein, and the staining patterns in immunofluorescence e generally very similar for both groups of antibodies, believe that monoclonal antibodies directed against the erminal region of human γ-tubulin can detect γ-tubulin *L. tropica*. γ-Tubulin was found only in insoluble toskeletal fraction of cells extracted in 1% NP-40 at 4 °C. On the other hand, tubulin αβ-dimers were found in both soluble and cytoskeletal pools. After extraction in 2% or 0.5% Triton X-100 at 28 °C or 4 °C, γ-tubulin was again detectable only in the insoluble fraction (Libusová, unpublished). Soluble forms of γ-tubulin are therefore either absent in *L. tropica* or their quantities are below the detection limit of the method used. This contrasts with the situation in animal cells, where up to 80% of γ-tubulin participates in large (γTuRCs; 2 MDa) and small (γTuSCs; 240 kDa) soluble complexes [14,42]. Soluble γ-tubulin-containing complexes were also described in plants [43,44], fungi [45], and *Dictyostelium discoideum* [46]. In animal cells, γTuSCs comprise two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin complex protein), which are homologues of *Saccharomyces cerevisiae* proteins Spc97p and Spc98p [47]. An open question still is whether insoluble γ-tubulin in *L. tropica* also exists in the form of such complexes. Up to now γ-tubulin interaction partners from the Spc family were not described in kinetoplastida.

γ-Tubulin in *L. tropica* shows a complex localization pattern as determined by immunofluorescence. In interphase cells, it was located in the basal body region and the posterior pole of the cell. A weak punctuate staining was observed over the whole cell body, and some antibodies also decorated the flagellum. In dividing cells, both old and new basal bodies were decorated, and so were the flagella. There was a very intensive staining at posterior poles. Punctuate labelling of the subpellicular corset was also more intensive. Intensive flagellar staining was seen with both the monoclonal TU-32 antibody against the C-terminal region of γ-tubulin and the polyclonal antibodies against the N-terminal region (γ-TUB, No. 9). Other antibodies directed against the C-terminal region (TU-30, TU-31) also decorated the flagellum, but the signal was far less obvious. Monoclonal

antibody GTU-88, directed against the N-terminal region of γ-tubulin, failed to stain flagellum under the used fixation conditions. Differential staining intensity of the flagellum with diverse antibodies cannot be attributed to an unspecific attachment of antibodies because the negative control monoclonal antibodies of IgG and IgM class, as well as the polyclonal antibody, did not provide any staining of these structures. The entire staining pattern was also preserved after the high salt treatment. Moreover, preabsorption of TU-32 with the peptide used for immunization abolished the staining. On the other hand, evidence exists that antibodies TU-30, TU-31, and TU-32 do not recognize identical epitopes (Dráber, unpublished). Differential staining of flagella could thus reflect the masking of corresponding epitopes by associated proteins, by potential posttranslational modification(s) of the γ-tubulin molecule, or by conformational changes occurring during fixation. The staining pattern in *L. tropica* partially resembled that seen in closely related *T. brucei*. In this case, γ-tubulin was immunodetected in the basal body, on the tip of the cell body, and along the flagellum. γ-Tubulin was also discernible in the form of discrete dots within the nucleus in regions corresponding to the poles of intranuclear spindle. A weak punctuate staining was found over the cell body [9]. It was suggested that staining of the cell body could reflect the presence of γ-tubulin on subpellicular corset microtubules, as new microtubules of the corset are intercalated into the lattice of old microtubules without any obvious MTOC [48].

The localization of γ-tubulin to the basal body is in agreement with previous reports on γ-tubulin presence in the basal body of *Tetrahymena thermophila* [49], *T. brucei* [9], *Chlamydomonas reinhardtii* [50], or *Giardia intestinalis* [51]. In the basal body region of *L. tropica*, γ-tubulin colocalized with the 210-kDa microtubule interacting protein and the 57-kDa vimentin-like protein that had previously been localized in this region [8]. On the other hand, γ-tubulin did not colocalize with the pericentrin-related protein(s) immunodetected with the anti-pericentrin antibody at the anterior pole of the cell in the flagellar pocket area. A special "desmosome-like" thickening has been described in the pocket opening, and a quartet of specialized microtubules run along the flagellar pocket [52]. Whether the pericentrin-related protein interacts with these microtubules remains questionable. In animal cells, pericentrin forms soluble complexes with γ-tubulin, and these are located on centrosomes [53]. The pericentrin-related protein was recently described in amoeba *Naegleria gruberi* during de novo formation of the basal body [54]. The role of γ-tubulin in the formation of kinetoplastid basal body and flagellum axoneme was investigated by the RNAi approach. γ-Tubulin was found to be essential for the formation of the central doublet of microtubules in new flagellum axoneme. Cells with suppressed γ-tubulin expression carried an old motile flagellum and a new immobile flagellum that lacked both of the central pair of microtubules, but possessed nine outer doublet microtubules [24].

γ-Tubulin colocalized with the 210-kDa microtubule-interacting protein and the 57-kDa vimentin-like protein also at the posterior pole of the cell. The role of γ-tubulin at the posterior pole is not clear, but it could be involved in the anchoring or stabilizing of the subpellicular corset of microtubules. A higher number of microtubules are to be anchored in dividing cell and a correspondingly more concentrated γ-tubulin signal is found on the posterior pole. The tapering posterior tip of the cell is the only position of the corset where many microtubules end. The bright dot of γ-tubulin staining at this position could reflect the presence of γ-tubulin on the ends of microtubules rather than a sign of discrete MTOCs. In *T. brucei*, the (+) ends of microtubules are at the same pole of the cell where the basal body of flagellum is located [55], and discrete γ-tubulin staining was found on the opposite cell pole. It remains an open question if the same holds true for *Leishmania*, as the orientation of microtubules has not yet been determined in these protozoa.

The unexpected finding of γ-tubulin along the flagellum was verified by immunoblotting, immunofluorescence, and immunogold electron microscopy on isolated flagella. Immunoelectron microscopy revealed that γ-tubulin was not present on axonemal microtubules but on the paraflagellar rod (PFR). Staining along the whole length of the flagellum in *T. brucei* was the result of decoration of four microtubules underlining the flagellar attachment zone of PFR [9]. However, the flagellar attachment zone is absent in *L. tropica*, and four specialized microtubules are found only in the flagellar pocket region. The filamentous structure of PFR lies alongside the whole length of axoneme under the membrane and consists of three distinct regions—a proximal, intermediate, and distal zone relative to the axoneme [56]. The proximal zone is attached to axonemal microtubules. Detailed localization of γ-tubulin to the zones was not possible because the method of isolation of flagella partially impaired the structure of PFR. Two major protein components, PFR-1 (74 kDa) and PFR-2 (69 kDa), were described in PFR of *Leishmania mexicana* [57]. Secondary structural prediction suggested high alpha-helical content and the potential of these helices to form coiled-coil motifs in all members of the PFR1/PFR2 family [58]. It was reported that noncentrosomal γ-tubulin colocalized with intermediate filaments in CACO-2 epithelial cells, and cytokeratins specifically co-immunoprecipitated with γ-tubulin [59]. It is possible that γ-tubulin interacts with some proteins of the filamentous PFR structure. Alternatively, γ-tubulin could be associated with rafts of proteins that become attached to PFR building components during intraflagellar transport [60]. γ-Tubulin was found in association with membranes both in animal [18] and in plant cells [19].

Two-dimensional electrophoresis revealed the presence of at least three distinguishable isoelectric variants of γ-tubulin in flagella, with isoelectric points more basic when compared to α-tubulin. The pI of the most acidic variant was 6.0, which is close to the pI calculated for the amino acid

ice of *L. major* γ-tubulin (6.39). Although the leish- genome has been almost fully sequenced, only a γ-tubulin gene per haploid genome was found up to databases. This is in contrast to multiple α- and β- genes present in trypanosomatida [61]. Thus, the r of detected γ-tubulin isoforms exceeds the number ubulin genes and suggests the presence of posttrans- l modification. Cell cycle-dependent phosphorylation ubulin on tyrosine was reported in yeast [20]. γ- n was found in association with serine–threonine ke kinase [62] and with protein tyrosine kinase p53/ n [63]. It is possible that phosphorylation or other nslational modification of *L. tropica* γ-tubulin could n important role in the regulation of microtubule tion or its interaction with other proteins.

conclusion, we demonstrate a unique subcellular ation of γ-tubulin in *Leishmania*. γ-Tubulin is local- four distinct structures: basal body, PFR of the ım, posterior end of the cell body, and discrete dots subpellicular microtubular array. The cell cycle- lent distribution of γ-tubulin in the posterior end of ll could imply its important role in microtubule age during interphase and mitosis. γ-Tubulin in *L.* is posttranslationally modified, and these modifica- could have a significant role in the regulation of ubule nucleation or interaction with other proteins.

wledgments

thank Dr. E. Nohýnková (First Faculty of Medicine, s University, Prague, Czech Republic) for providing ll line, Dr. T.H. MacRae (Dalhousie University, , Canada) for the generous gift of anti-γ-tubulin ly, Dr. S.J. Doxsey (University of Massachusetts, al Center, Worcester, MA, USA) for the generous gift -pericentrin antibody, and Dr. B.R. Oakley (The Ohio University, Columbus, OH, USA) for a full-length γ-tubulin cDNA used for expression of 6× His- γ-tubulin. This work was supported in part by grants he Ministry of Education of the Czech Republic 0 and LN00A026).

nces

Fong, M. Wallach, J. Keithly, P.W. Melera, K.P. Chang, Differen- l expression of mRNAs for α- and β-tubulin during differentiation the parasitic protozoan *Leishmania mexicana*, Proc. Natl. Acad. i. U. S. A. 81 (1984) 5782–5786.

Y. Hou, P.F. Pimenta, R.L. Shen, S.P. Da, Stereo views and immu- gold labeling of the pellicular microtubules at the inner surface of plasma membrane of *Leishmania* as revealed by fracture-flip, Histochem. Cytochem. 40 (1992) 1309–1318.

Santrich, L. Moore, T. Sherwin, P. Bastin, C. Brokaw, K. Gull, J.H. Bowitz, A motility function for the paraflagellar rod of *Leishmania* parasites revealed by PFR-2 gene knockouts, Mol. Biochem. Parasitol. 90 (1997) 95–109.

[4] J.A. Maga, J.H. LeBowitz, Unravelling the kinetoplastid paraflagellar rod, Trends Cell Biol. 9 (1999) 409–413.

[5] A. Woods, T. Sherwin, R. Sasse, T.H. MacRae, A.J. Baines, K. Gull, Definition of individual components within the cytoskeleton of *Trypanosoma brucei* by a library of monoclonal antibodies, J. Cell Sci. 93 (1989) 491–500.

[6] D.L. Fouts, G.A. Stryker, K.S. Gorski, M.J. Miller, T.V. Nguyen, R.A. Manning, J.E. Manning, Evidence for four distinct major protein components in the paraflagellar rod of *Trypanosoma cruzi*, J. Biol. Chem. 273 (1998) 21846–21855.

[7] A. Selvapandiyan, R. Duncan, A. Debrabant, S. Bertholet, G. Sreenivas, Negi, N.S. Negi, P. Salotra, H.L. Nakhasi, Expression of a mutant form of *Leishmania donovani* centrin reduces the growth of the parasite, J. Biol. Chem. 276 (2001) 43253–43261.

[8] L. Kratzerová, E. Dráberová, C. Juliano, V. Viklický, P.L. Fiori, P. Cappuccinelli, P. Dráber, Cell cycle-dependent changes in localization of a 210-kDa microtubule-interacting protein in *Leishmania*, Exp. Cell Res. 266 (2001) 270–278.

[9] V. Scott, T. Sherwin, K. Gull, Gamma-tubulin in trypanosomes: molecular characterisation and localisation to multiple and diverse microtubule organising centres, J. Cell Sci. 110 (1997) 157–168.

[10] V. Dilbeck, M. Berberof, C. Van, H. Alexandre, E. Pays, Characterization of a coiled coil protein present in the basal body of *Trypanosoma brucei*, J. Cell Sci. 112 (1999) 4687–4694.

[11] C.E. Oakley, B.R. Oakley, Identification of γ-tubulin, a new member of the tubulin superfamily encoded by mipA gene of *Aspergillus nidulans*, Nature 338 (1989) 662–664.

[12] Y. Zheng, M.L. Wong, B. Alberts, T. Mitchison, Nucleation of microtubule assembly by a γ-tubulin-containing ring complex, Nature 378 (1995) 578–583.

[13] M. Moritz, M.B. Braunfeld, J.C. Fung, J.W. Sedat, B.M. Alberts, D.A. Agard, Three-dimensional structural characterization of centrosomes from early *Drosophila* embryos, J. Cell Biol. 130 (1995) 1149–1159.

[14] M. Moritz, Y. Zheng, B.M. Alberts, K. Oegema, Recruitment of the γ-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds, J. Cell Biol. 142 (1998) 775–786.

[15] C. Wiese, Y. Zheng, A new function for the γ-tubulin ring complex as a microtubule minus-end cap, Nat. Cell Biol. 2 (2000) 358–364.

[16] R. Leguy, R. Melki, D. Pantaloni, M.F. Carlier, Monomeric γ-tubulin nucleates microtubules, J. Biol. Chem. 275 (2000) 21975–21980.

[17] R. Llanos, V. Chevrier, M. Ronjat, G. Meurer, P. Martinez, R. Frank, M. Bornens, R.H. Wade, J. Wehland, D. Job, Tubulin binding sites on γ-tubulin: identification and molecular characterization, Biochemistry 38 (1999) 15712–15720.

[18] K. Chabin-Brion, J. Marceiller, F. Perez, C. Settegrana, A. Drechou, G. Durand, C. Pous, The Golgi complex is a microtubule-organizing organelle, Mol. Biol. Cell 12 (2001) 2047–2060.

[19] D. Dryková, V. Cenklová, V. Sulimenko, J. Volc, P. Dráber, P. Binarová, Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes, Plant Cell 15 (2003) 465–480.

[20] J. Vogel, B. Drapkin, J. Oomen, D. Beach, K. Bloom, M. Snyder, Phosphorylation of gamma-tubulin regulates microtubule organization in budding yeast, Dev. Cell 1 (2001) 621–631.

[21] C. Detraves, H. Mazarguil, I. Lajoie-Mazenc, M. Julian, B. Raynaud-Messina, M. Wright, Protein complexes containing γ-tubulin are present in mammalian brain microtubule protein preparations, Cell Motil. Cytoskeleton 36 (1997) 179–189.

[22] I. Linhartová, B. Novotná, V. Sulimenko, E. Dráberová, P. Dráber, Gamma-tubulin in chicken erythrocytes: changes in localization during cell differentiation and characterization of cytoplasmic complexes, Dev. Dyn. 223 (2002) 229–240.

[23] V. Sulimenko, T. Sulimenko, S. Poznanovic, Z. Nechiporuk, K.J. Böhm, L. Macůrek, E. Unger, P. Dráber, Association of brain γ-tubulins with αβ-tubulin dimers, Biochem. J. 365 (2002) 889–895.

[24] P.G. McKean, A. Baines, S. Vaughan, K. Gull, γ-Tubulin functions in the nucleation of a discrete subset of microtubules in the eukaryotic flagellum, Curr. Biol. 13 (2003) 598–602.
25] C.L. Eisenberger, C.L. Jaffe, *Leishmania*: identification of Old World species using a permissively primed intergenic polymorphic-polymerase chain reaction, Exp. Parasitol. 91 (1999) 70–77.
26] M. Nováková, E. Dráberová, W. Schurmann, G. Czihak, V. Viklický, P. Dráber, γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies, Cell Motil. Cytoskeleton 33 (1996) 38–51.
27] M.A. Walling, G.R. Criel, T.H. MacRae, Characterization of γ-tubulin in Artemia: isoform composition and spatial distribution in polarized cells of the larval epidermis, Cell Motil. Cytoskeleton 40 (1998) 331–341.
28] J. Gu, C.G. Stephenson, M.J. Iadarola, Recombinant proteins attached to a nickel-NTA column: use in affinity purification of antibodies, BioTechniques 17 (1994) 257–262.
29] P. Dráber, E. Dráberová, I. Linhartová, V. Viklický, Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies, J. Cell Sci. 92 (1989) 519–528.
30] P. Dráber, E. Dráberová, V. Viklický, Immunostaining of human spermatozoa with tubulin domain-specific monoclonal antibodies. Recognition of a unique γ-tubulin epitope in the sperm head, Histochemistry 95 (1991) 519–524.
31] E. Dráberová, P. Dráber, V. Viklický, Cellular distribution of a protein related to neuronal microtubule-associated protein MAP-2 in Leydig cells, Cell Biol. Int. Rep. 10 (1986) 881–890.
32] E. Dráberová, P. Dráber, F. Havlíček, V. Viklický, A common antigenic determinant of vimentin and desmin defined by monoclonal antibody, Folia Biol. (Praha) 32 (1986) 295–303.
33] S.J. Doxsey, P. Stein, L. Evans, P.D. Calarco, M. Kirschner, Pericentrin, a highly conserved centrosome protein involved in microtubule organization, Cell 76 (1994) 639–650.
34] E. Dráberová, Z. Lukáš, D. Ivanyi, V. Viklický, P. Dráber, Expression of class III γ-tubulin in normal and neoplastic human tissues, Histochem. Cell Biol. 109 (1998) 231–239.
35] B.M. Riederer, E. Dráberová, V. Viklický, P. Dráber, Changes of MAP2 phosphorylation during brain development, J. Histochem. Cytochem. 43 (1995) 1269–1284.
36] P. Bastin, T.H. MacRae, S.B. Francis, K.R. Matthews, T. Gull, Flagellar morphogenesis: protein targeting and assembly in the paraflagellar rod of trypanosomes, Mol. Cell. Biol. 19 (1999) 8191–8200.
37] P. Dráber, Quantification of proteins in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis using colloidal silver, Electrophoresis 12 (1991) 453–456.
38] P. Dráber, L.A. Lagunowich, E. Dráberová, V. Viklický, I. Damjanov, Heterogeneity of tubulin epitopes in mouse fetal tissues, Histochemistry 89 (1988) 485–492.
39] A. Görg, C. Obermaier, G. Boguth, W. Weiss, Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures, Electrophoresis 20 (1999) 712–717.
40] E. Dráberová, P. Dráber, A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules, J. Cell Sci. 106 (1993) 1263–1273.
41] E. Harlow, D. Lane, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1999.
42] M. Moudjou, N. Bordes, M. Paintrand, M. Bornens, γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms, J. Cell Sci. 109 (1996) 875–887.
43] V. Stoppin-Mellet, C. Peter, B. Buendia, E. Karsenti, A.M. Lambert, Tobacco BY-2 cell-free extracts induce the recovery of microtubule nucleating activity of inactivated mammalian centrosomes, Biochim. Biophys. Acta 1449 (1999) 101–106.
[44] P. Binarová, V. Cenklová, B. Hause, E. Kubátová, M. Lysak, J. Doležel, L. Bögre, P. Dráber, Nuclear γ-tubulin during acentriolar plant mitosis, Plant Cell 12 (2000) 433–442.
[45] M. Knop, E. Schiebel, Spc98p and Spc97p of the yeast γ-tubulin complex mediate binding to the spindle pole body via their interaction with Spc110p, EMBO J. 16 (1997) 6985–6995.
[46] C. Daunderer, R.O. Gräf, Molecular analysis of the cytosolic *Dictyostelium* γ-tubulin complex, Eur. J. Cell Biol. 81 (2002) 175–184.
[47] S. Geissler, G. Pereira, A. Spang, M. Knop, S. Soues, J. Kilmartin, E. Schiebel, The spindle pole body component Spc98p interacts with the γ-tubulin-like Tub4p of *Saccharomyces cerevisiae* at the sites of microtubule attachment, EMBO J. 15 (1996) 3899–3911.
[48] T. Sherwin, K. Gull, Visualization of detyrosination along single microtubules reveals novel mechanisms of assembly during cytoskeletal duplication in trypanosomes, Cell 57 (1989) 211–221.
[49] A. Liang, F. Ruiz, K. Heckmann, C. Klotz, Y. Tollon, J. Beisson, M. Wright, Gamma-tubulin is permanently associated with basal bodies in ciliates, Eur. J. Cell Biol. 70 (1996) 331–338.
[50] C.D. Silflow, B. Liu, M. LaVoie, E.A. Richardson, B.A. Palevitz, Gamma-tubulin in *Chlamydomonas*: characterization of the gene and localization of the gene product in cells, Cell Motil. Cytoskeleton 42 (1999) 285–297.
[51] E. Nohýnková, P. Dráber, J. Reischig, J. Kulda, Localization of γ-tubulin in interphase and mitotic cells of a unicellular eukaryote, *Giardia intestinalis*, Eur. J. Cell Biol. 79 (2000) 438–445.
[52] S.M. Landfear, M. Ignatushchenko, The flagellum and flagellar pocket of trypanosomatids, Mol. Biochem. Parasitol. 115 (2001) 1–17.
[53] J.B. Dictenberg, W. Zimmerman, C.A. Sparks, A. Young, C. Vidair, Y. Zheng, W. Carrington, F.S. Fay, S.J. Doxsey, Pericentrin and gamma-tubulin form a protein complex and are organized into a novel lattice at the centrosome, J. Cell Biol. 141 (1998) 163–174.
[54] M.R. Suh, J.W. Han, Y.R. No, J. Lee, Transient concentration of a gamma-tubulin-related protein with a pericentrin-related protein in the formation of basal bodies and flagella during the differentiation of *Naegleria gruberi*, Cell Motil. Cytoskeleton 52 (2002) 66–81.
[55] D.R. Robinson, T. Sherwin, A. Ploubidou, E.H. Byard, K. Gull, Microtubule polarity and dynamics in the control of organelle positioning, segregation, and cytokinesis in the trypanosome cell cycle, J. Cell Biol. 128 (1995) 1163–1172.
[56] M. Farina, M. Attias, T. Suoto-Padron, W. de Souza, Further studies on organization of the paraxial rod of Trypanosomatids, J. Protozool. 33 (1986) 552–557.
[57] L.L. Moore, C. Santrich, J.H. LeBowitz, Stage-specific expression of the *Leishmania mexicana* paraflagellar rod protein PFR-2, Mol. Biochem. Parasitol. 80 (1996) 125–135.
[58] H.M. Ngo, G.B. Bouck, Heterogeneity and a coiled coil prediction of trypanosomatid-like flagellar rod proteins in Euglena, J. Eukaryotic Microbiol. 45 (1998) 323–333.
[59] P.J. Salas, Insoluble γ-tubulin-containing structures are anchored to the apical network of intermediate filaments in polarized CACO-2 epithelial cells, J. Cell Biol. 146 (1999) 645–657.
[60] J.L. Rosenbaum, D.G. Cole, D.R. Diener, Intraflagellar transport: the eyes have it, J. Cell Biol. 144 (1999) 385–388.
[61] M. Imboden, B. Blum, T. DeLange, R. Braun, T. Seebeck, Tubulin mRNAs of *Trypanosoma brucei*, J. Mol. Biol. 188 (1986) 393–402.
[62] Y. Feng, D.R. Hodge, G. Palmieri, D.L. Chase, D.L. Longo, D.K. Ferris, Association of polo-like kinase with α-, β- and γ-tubulins in a stable complex, Biochem. J. 339 (1999) 435–442.
[63] L. Dráberová, E. Dráberová, Z. Surviladze, P. Dráber, P. Dráber, Protein tyrosine kinase p53/p56(lyn) forms complexes with γ-tubulin in rat basophilic leukemia cells, Int. Immunol. 11 (1999) 1829–1839.

VI.3. **Sulimenko V.,** Sulimenko T., Poznanovic S., Nechiporuk-Zloy V., Bőhm K.J., Macůrek L., Unger E., Dráber P.: *Assotiation of brain γ-tubulin with αβ-tubulin dimers.* ***Biochemical Journal*, 365**: 889-895, 2002.

Association of brain γ-tubulins with αβ-tubulin dimers

Vadym SULIMENKO*, Tetyana SULIMENKO*, Slobodan POZNANOVIC†, Volodymyr NECHIPORUK-ZLOY†, Konrad J. BÖHM†, Libor MACUREK*, Eberhard UNGER† and Pavel DRÁBER*[1]

*Institute of Molecular Genetics, Department of Biology of Cytoskeleton, Academy of Sciences of the Czech Republic, 142 20 Prague 4, Czech Republic and †Institute of Molecular Biotechnology, Department of Molecular Cytology and Electron Microscopy, D 07745 Jena, Germany

γ-Tubulin is necessary for nucleation and polar orientation of microtubules *in vivo*. The molecular mechanism of microtubule nucleation by γ-tubulin and the regulation of this process are not fully understood. Here we show that there are two γ-tubulin forms in the brain that are present in complexes of various sizes. Large complexes tend to dissociate in the presence of a high salt concentration. Both γ-tubulins co-polymerized with tubulin dimers, and multiple γ-tubulin bands were identified in microtubule protein preparations under conditions of non-denaturing electrophoresis. Immunoprecipitation experiments with monoclonal antibodies against γ-tubulin and α-tubulin revealed interactions of both γ-tubulin forms with tubulin dimers, irrespective of the size of complexes. We suggest that, besides small and large γ-tubulin complexes, other molecular γ-tubulin form(s) exist in brain extracts. Two-dimensional electrophoresis revealed multiple charge variants of γ-tubulin in both brain extracts and microtubule protein preparations. Post-translational modification(s) of γ-tubulins might therefore have an important role in the regulation of microtubule nucleation in neuronal cells.

Key words: antibody, charge variant, microtubule protein, tubulin complex.

INTRODUCTION

Microtubules, assembled from α- and β-tubulin heterodimers, are highly dynamic polymers that are indispensable for many cellular functions, such as intracellular organization, ordered vesicle transport and cell division, to name but a few. To accomplish their specialized functions, microtubules are organized into complex arrays by a microtubule-organizing centre (MTOC), which, in animal cells, consists of a pair of centrioles surrounded by electron-dense pericentriolar material. Microtubules are nucleated at the pericentriolar material by their 'minus ends' [1]. One of the key components of MTOCs is γ-tubulin [2].

Although γ-tubulin participates in nucleation of microtubules from MTOCs [3,4], the majority of it is associated with other centrosomal proteins in soluble cytoplasmic complexes. Large γ-tubulin-ring complexes (γTuRCs) were first identified in *Xenopus* eggs [5]. They were also found in *Drosophila* embryos [6] and in various types of mammalian cells [7–9]. Besides γTuRCs, there also exist smaller complexes [γ-tubulin small complexes (γTuSCs)] [10], which comprise two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin-complex proteins) [11,12] and which are homologues of the *Saccharomyces cerevisiae* proteins Spc97p and SPc98p associated with spindle pole bodies [13]. The γTuRCs are formed by small complexes and by other proteins. In addition to nucleation from the MTOC, the γTuRCs are also involved in regulating the dynamics of the microtubule minus ends [14].

The γTuRCs have different sizes, and they also differ in protein composition. The question is still open as to whether αβ-tubulin dimer is an integral component of the large complexes. Although, in the majority of analysed complexes, tubulin dimers were not found [15], they were detectable in frog oocytes [5,16], mammalian brains [9] and in a lymphoblastic cell line [8].

γ-Tubulin itself has been found to be a microtubule minus-end binding molecule in not-anchored microtubules [17], and binding sites for α- and β-tubulin subunits on γ-tubulin were identified using synthetic peptides [18]. Structural models for self-assembly and microtubule interactions suggest that γ-tubulin should be capable of self-assembling into dimer or protofilament-like oligomers, as well as of interacting laterally with α- or β-tubulin [19]. Experiments *in vitro* showed that, under some circumstances, γ-tubulin might bind to surface regions of microtubules without affecting the tubulin polymerization [18]. An association of γ-tubulin along microtubule arrays *in vivo* is well described in plants [20,21].

While a number of studies have concentrated on the characterization of proteins that associate with γ-tubulins, and on positioning of γ-tubulin at the minus end of microtubules, our knowledge about the regulation of nucleation activities of γ-tubulin by post-translational modifications is very limited [22].

Because brain tubulin is used typically to study the microtubule dynamics, and the heterogeneity of αβ-tubulin dimers is known in detail, we have looked for the presence of γ-tubulin forms in pig brain. Here, we report for the first time on two γ-tubulin isoforms, which persist at differently sized complexes and interact with αβ-tubulin heterodimers, irrespective of the size of complexes. Brain γ-tubulin is post-translationally modified, and these modifications could have an important role in the regulation of microtubule nucleation.

EXPERIMENTAL

Materials

Immobilized Protein A Plus, Immobilized Protein L Plus and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce (Rockford, IL, U.S.A.). Protease-inhibitor cocktail tablets ('Complete EDTA-free') were from Roche Molecular Biochemicals (Prague, Czech Republic), molecular-mass markers for SDS/PAGE were obtained from Sigma–Aldrich (Prague, Czech Republic), markers for two-dimensional electrophoresis and Biolyte 3/10 were from Bio-Rad Laboratories (Munich, Germany), CNBr-activated Sepharose 4B, IPG buffer (pH 4–7),

Abbreviations used: MTP-2 and MTP-3, microtubule protein after two or three cycles respectively of assembly and disassembly; MTOC, microtubule-organizing centre; γTuRC, γ-tubulin-ring complex; γTuSC, γ-tubulin small complex.

[1] To whom correspondence should be addressed (e-mail paveldra@biomed.cas.cz).

molecular-mass markers for native electrophoresis and carbamylyte calibration kit for isoelectric focusing were purchased from Amersham Biosciences (Uppsala, Sweden). Peptides were prepared in the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, Prague, as described previously [23].

Antibodies

The following monoclonal antibodies were used: TU-01 (IgG1) [24] and TU-16 (IgM) against α-tubulin [25]; TU-06 (IgM) against β-tubulin [26]; TU-31 (IgG2b) and TU-32 (IgG1) anti-peptide antibodies prepared against the EYHAATRPDYISWGTQ peptide corresponding to human γ-tubulin sequence 434–449 [23]; GTU-88 (IgG1) anti-peptide antibody prepared against the EEFATEGTDRKDVFFY peptide corresponding to human γ-tubulin sequence 38–53 (Sigma cat. no. T6557); VI-01 (IgM) against vimentin [27]; and N27F3-4 (IgG1) against heat-shock protein Hsp70 (StressGen; cat. no. SPA-820). Actin was detected by affinity-purified rabbit anti-actin antibody (Sigma cat. no. A2066). Anti-mouse immunoglobulin and anti-rabbit immunoglobulin antibodies conjugated with horseradish peroxidase, as well as anti-mouse immunoglobulin conjugated with alkaline phosphatase, were purchased from Promega Biotec (Madison, WI, U.S.A.).

Protein preparation

Microtubule protein (MTP) was purified from pig brain by two temperature-dependent cycles of assembly and disassembly [28]. A third polymerization was performed in the presence of DMSO to partially deplete the microtubule-associated proteins [29], before subsequent purification of tubulin by phosphocellulose chromatography [30]. The high-speed extract for gradient centrifugation was prepared by homogenization of washed brains in cold PEM buffer [100 mM Pipes (pH 6.9)/1 mM EGTA/2 mM $MgSO_4$/1 mM 2-mercaptoethanol] supplemented with 1 mM ATP, 1 mM 4-(2-aminoethyl)benzenesulphonyl fluoride and protease inhibitor cocktail. The homogenate was centrifuged at 41000 *g* for 20 min at 4 °C. The supernatant was then re-centrifuged at 280000 *g* for 30 min at 4 °C, and immediately subjected to sucrose-gradient centrifugation. When the presence of salt was required, the high-speed extract was supplemented with 500 mM NaCl. Carboxyamidomethylation of MTP-2 was performed as described previously [31]. Protein concentration was determined by means of the bicinchoninic acid kit (Sigma–Aldrich) with BSA as the standard.

Sucrose-gradient centrifugation

Sucrose-gradient centrifugation was performed as described previously [10]. The sucrose gradients (5–40% in PEM buffer) were poured as step-density gradients (800 μl steps: 40, 30, 20, 10 and 5% sucrose), and allowed to diffuse into continuous gradients overnight at 4 °C before use. In the case of salt-containing extracts, the sucrose gradient was supplemented with NaCl to a final concentration of 500 mM. A 75 μl aliquot of high-speed extract was loaded on to each gradient, and the gradients were centrifuged at 50000 rev./min in a Beckman SW60 rotor for 4 h at 4 °C. Gradients were fractionated from the top into 14 fractions. Protein standards (BSA, bovine liver catalase and mouse IgM) at the same concentration (0.5 mg/ml each) were loaded in an equivalent volume, and were run in parallel over identical gradients. Samples for SDS/PAGE were prepared by mixing with 2× concentrated SDS/PAGE sample buffer.

Immunoprecipitation

Immunoprecipitation was performed as described previously [32], using TBST [10 mM Tris/HCl (pH 7.4)/150 mM NaCl/0.05% (v/v) Tween 20] for dilution of extracts and for washings. High-speed pig brain extracts were incubated with beads of: (i) immobilized Protein A saturated with anti-(γ-tubulin) antibody TU-31; (ii) immobilized Protein A saturated with control antibody NF-09; (iii) immobilized Protein A alone; (iv) immobilized Protein L saturated with anti-(α-tubulin) antibody TU-16; (v) immobilized Protein L saturated with control anti-vimentin antibody VI-01; or (vi) immobilized Protein L alone. The antibodies were used in the form of culture supernatants to avoid immobilization of other mouse antibodies. Sedimented beads (50 μl) were incubated with shaking at 4 °C for 2 h with 1.2 ml of the corresponding antibody, prepared by mixing 0.4 ml of 10× concentrated supernatant with 0.8 ml of the TBST buffer. The beads were pelleted by centrifugation at 5000 *g* for 1 min, washed four times (5 min each wash) in cold TBST, and incubated further with shaking for 3 h at 4 °C with 1 ml of pig brain high-speed supernatant diluted 1:7 with TBST. The beads were then pelleted and washed four times (5 min each) in cold TBST, before boiling for 5 min in 100 μl of SDS sample buffer to release the bound proteins. Alternatively, the TU-31 antibody, purified from ascitic fluid on Protein A, was directly bound to CNBr-activated Sepharose 4B and used for precipitation.

Gel electrophoresis and immunoblotting

SDS/PAGE, electrophoretic transfer of separated proteins on to nitrocellulose and details of the immunostaining procedure are described elsewhere [33]. Under routine conditions, lower-grade SDS (Sigma cat. no. L-5750; 66% SDS) was used throughout the whole procedure, because it facilitates the separation of α- and β-tubulin subunits [34]. Alternatively, high-grade SDS (Sigma cat. no. L-6026; 99% SDS) was used, as indicated in the text. The antibodies TU-01, TU-06 and TU-32 in the form of spent culture supernatants were used undiluted, whereas the antibody GTU-88, in the form of ascitic fluid, was diluted 1:4000. The antibody against Hsp70 was diluted 1:2000, and the antibody against actin was diluted 1:200. After incubating the samples with secondary antibodies conjugated with horseradish peroxidase diluted 1:10000, followed by washing, bound antibodies were detected by a chemiluminescence reaction, according to the manufacturer's instructions. Autoradiography films X-Omat AR were from Eastman Kodak (Rochester, NY, U.S.A.). Alternatively, bound antibodies were detected with secondary antibodies conjugated with alkaline phosphatase diluted 1:7500 and chromogenic substrates [33].

In some experiments, the TU-32 antibody was pre-absorbed with the peptide used for immunization, i.e. the 16-amino-acid peptide EYHAATRPDYISWGTQ that corresponds to amino acid residues 434–449 of human γ-tubulin. The other 16-amino-acid peptide, EEFATEGTDRKDVFFY, corresponding to amino acid residues 38–53 of human γ-tubulin, was used as a negative control. Two molar ratios of antibody to peptide were used: 1:5 and 1:50. Mixtures of antibody and peptides were incubated for 30 min at room temperature.

Non-denaturing PAGE was performed using the Laemmli system [35], except that SDS was completely omitted and electrophoresis was performed at 4 °C. Sample buffer consisted of 62.5 mM Tris/HCl, pH 6.8, 10% (v/v) glycerol and 0.01% (w/v) Bromophenol Blue.

Two-dimensional PAGE was performed essentially as described previously [36]. Samples (30–50 μg of protein) were diluted with IPG sample buffer containing 7 M urea, 2 M

thiourea, 20 mM Tris, 4% (w/v) CHAPS, 1% (w/v) Triton X-100, 1% (w/v) dithiothreitol and 0.8% (w/v) Biolyte 3/10. Immobiline DryStrip gels with a linear pH 4–7 gradient, 11 cm long (Amersham Biosciences), were rehydrated using 250 μl of prepared sample (in IPG sample buffer). Each strip was overlaid with mineral oil, and left overnight at room temperature. Strips were focused for a total of 22.2 kV · h on Multiphor II apparatus (LKB, Bromma, Sweden). The second dimension was performed using SDS/8.5% (w/v) polyacrylamide gels, and separated proteins were transferred on to nitrocellulose by electroblotting.

RESULTS

Distribution of γ-tubulin in brain extracts and MTPs

The distribution of γ-tubulin was monitored during isolation of pig brain MTP by repeated temperature-dependent cycles of polymerization and depolymerization. The third polymerization occurred in the presence of DMSO, which partially depleted the microtubule-associated proteins (Figure 1A, lane 4), before the purification of αβ-tubulin dimers on phosphocellulose (Figure 1A, lane 5). γ-Tubulin was present in supernatants after low-speed (Figure 1B, lane 1) and high-speed (Figure 1B, lane 2) centrifugation of pig brain extract, as well as in MTP prepared by two (Figure 1B, lane 3) and three cycles (Figure 1B, lane 4) of temperature-dependent polymerization/depolymerization (MTP-2 and MTP-3 respectively). Under the conditions employed, which partially stripped away the high-molecular-mass microtubule-associated proteins, γ-tubulin was still detectable (Figure 1B, lane 4). During the isolation, γ-tubulin was not enriched in MTPs, and no γ-tubulin was detected in purified αβ-tubulin dimers separated from microtubule-associated proteins by chromatography on phosphocellulose (Figure 1B, lane 5). It was possible to elute γ-tubulin, like other microtubule-associated proteins, from the column with 1 M KCl. The same staining pattern was obtained with antibodies against the conserved human γ-tubulin amino acid regions 38–53 (GTU-88) and 434–449 (TU-31 and TU-32).

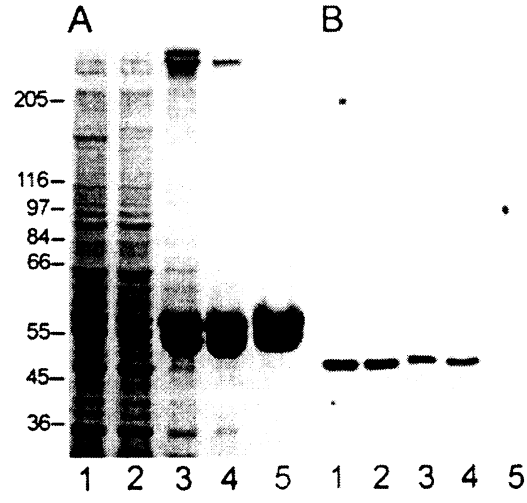


Figure 1 Distribution of γ-tubulin during isolation of MTPs

(**A**) Coomassie Blue staining of separated proteins. Low-speed supernatant of brain extract (lane 1), high-speed supernatant of brain extract (lane 2), MTP-2 (lane 3), MTP-3 (lane 4) and tubulin (lane 5) were separated on SDS/7.5% polyacrylamide gels. (**B**) Immunostaining with anti-(γ-tubulin) antibody TU-32. Aliquots of protein (10 μg) were loaded in lanes 1–4, and 12.5 μg of protein in lane 5. Molecular-mass markers (in kDa) are indicated to the left of (**A**).

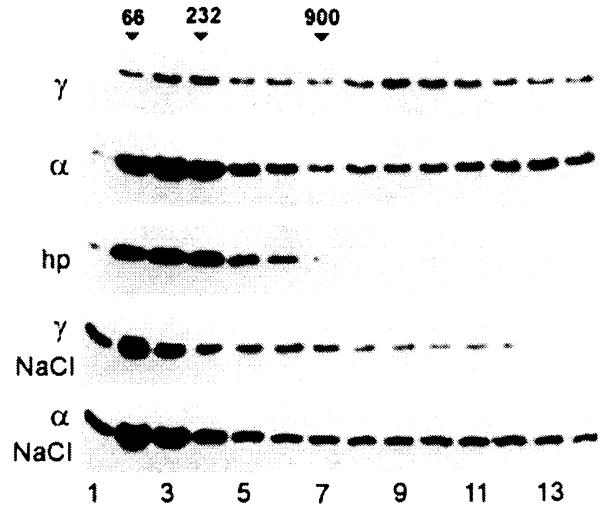


Figure 2 Behaviour of γ-tubulin during sucrose-density gradient centrifugation

Samples were sedimented through a 5–40% sucrose gradient without or with 500 mM NaCl; fractions were then separated on SDS/7.5% polyacrylamide gels and immunoblotted for γ-tubulin (γ), α-tubulin (α) or heat-shock protein Hsp70 (hp). Calibration standards (in kDa) are indicated on the top. Numbers at the bottom denote individual fractions.

Size distribution of γ-tubulin in high-speed extracts was assessed by gradient centrifugation. Immunoblotting experiments with separated fractions revealed that γ-tubulin was distributed in a large zone, and was present in complexes of various sizes (Figure 2, panel labelled 'γ'). Complexes of various sizes were also found for α-tubulin; however, the distribution pattern was different, and lower-molecular-mass complexes were found to prevail there (Figure 2, panel labelled 'α'). The distribution pattern of β-tubulin was similar to that of α-tubulin (results not shown). On the other hand, the heat-shock protein Hsp70 was absent in high-molecular-mass complexes, indicating that not all proteins had the same size distribution under the separation conditions used (Figure 2, panel labelled 'hp'). Large complexes of γ-tubulin were disrupted in samples containing 500 mM NaCl (Figure 2, panel labelled 'γ NaCl'). On the other hand, the same salt concentration had little effect on large complexes containing tubulin dimers (Figure 2, panel labelled 'α NaCl'). This indicates that brain γ-tubulin is present in high-molecular-mass complexes, which are susceptible to a change in salt concentration, and that these complexes differ in their properties from those that contain tubulin dimers.

When MTP-2 and purified tubulin were separated under non-denaturing conditions, multiple tubulin oligomers were detected in both samples, and BSA at the same protein concentration ran primarily as a single band (Figure 3A). The presence of microtubule-associated proteins in MTP-2 was without an observable effect on the mobility of tubulin oligomers, as revealed by the staining with anti-(α-tubulin) antibody (Figure 3B) or anti-(β-tubulin) antibody (results not shown). Multiple resolvable bands were also detected in MTP-2 using the antibody against γ-tubulin (Figure 3C). In contrast with the tubulin oligomers 'ladder', γ-tubulin was not detected at the position corresponding to the fastest migrating species of tubulin subunits, which are primarily monomers [37]. The fastest migrating band stained with anti-(γ-tubulin) antibody was in the position of putative αβ-tubulin heterodimers [37]; the other bands with lower mobilities were in positions different from those occupied by oligomers

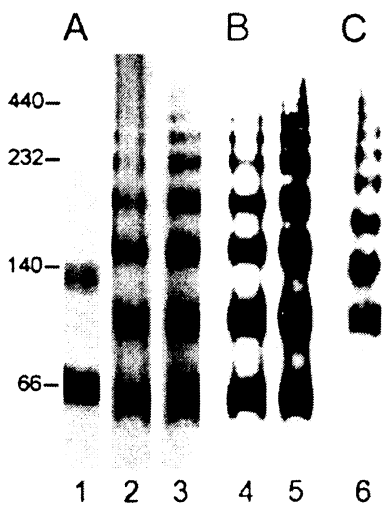


Figure 3 Immunoblot analysis of MTP and purified tubulin separated by native PAGE

BSA (lane 1), MTP-2 (lanes 2, 4 and 6) and tubulin (lanes 3, 5 and 7) were separated on non-denaturing 7% polyacrylamide gels. (**A**) Staining of transferred proteins; (**B**) immunostaining with anti-(α-tubulin) antibody TU-01; (**C**) immunostaining with anti-(γ-tubulin) antibody GTU-88. Aliquots of protein (3 μg) were loaded in lanes 1, 2, 4 and 6, and 5 μg aliquots of protein were loaded in lanes 3, 5 and 7. Molecular-mass markers (in kDa) are indicated on the left.

formed by $\alpha\beta$-tubulin dimers. These data indicate that γ-tubulin itself and/or γ-tubulin with another (unidentified) protein has the ability to form oligomers under conditions of native electrophoresis. The mobilities of these oligomers differ from those formed by $\alpha\beta$-tubulin heterodimers.

Association of γ-tubulin with $\alpha\beta$-tubulin dimers

Immunoprecipitation experiments showed that γ-tubulin could be precipitated specifically from high-speed extracts with monoclonal antibody TU-31 (IgG2b) (Figure 4A, lane 2). When the immobilized antibody was incubated without extract, no staining in the position of γ-tubulin was observed (Figure 4A, lane 3). No binding of γ-tubulin to immobilized Protein A was detected (Figure 4A, lane 4), and the control antibody NF-09 (IgG2b) produced no precipitation of γ-tubulin (results not shown). The possibility of an unspecific attachment of cytoplasmic proteins to possible γ-tubulin complexes was obviated, since no Hsp70 was detectable in the immunoprecipitated material with anti-Hsp70 antibody (Figure 4B, lane 2). Neither was there any co-precipitation of actin when the material was probed with anti-actin antibody (results not shown). γ-Tubulin precipitation was also observed with a low amount of purified antibody TU-31 directly bound covalently to Sepharose 4B (Figure 4C, lane 2). Probing of the precipitated material with anti-tubulin antibody revealed the presence of α-tubulin (Figure 4D, lane 2). The band corresponding to α-tubulin was discernible just under the band pertaining to the immobilized TU-31 antibody (marked with an arrow in Figure 4), so that it could potentially reflect a proteolytic fragmentation of the antibody. When the immobilized antibody alone was incubated without the extract, no band in this position was detected, therefore excluding the presence of a proteolytic fragment of antibody (Figure 4D, lane 3). Moreover, no band in this position was detected when the blot was probed with anti-(γ-tubulin) antibody (Figure 4C, lane 2). The reactivity with a protein in the position of α-tubulin was clearly recognized only when a less sensitive method of immunoglobulin detection with chromogenic substrate for alkaline phosphatase was applied.

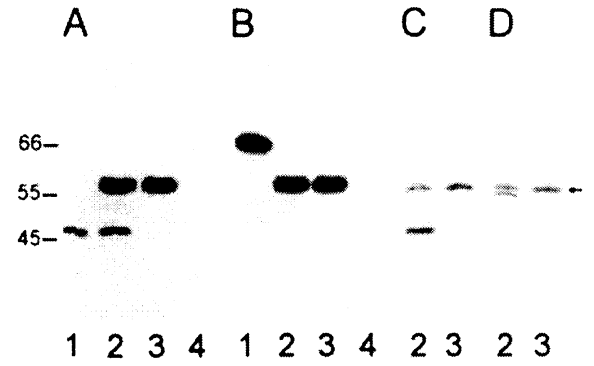


Figure 4 Immunoprecipitation of pig brain extract with anti-(γ-tubulin) antibody

Samples were precipitated with antibody TU-31 (IgG2b) bound to immobilized Protein A (**A**, **B**), or TU-31 covalently bound to Sepharose 4B (**C**, **D**). Proteins remaining after precipitation (lane 1), immunoprecipitated proteins (lane 2), immobilized immunoglobulins not incubated with cell extract (lane 3) and proteins bound to Protein A without antibody (lane 4) were separated on SDS/7.5% polyacrylamide gels. (**A** and **C**) Immunostaining with anti-(γ-tubulin) antibody GTU-88. (**B**) Immunostaining with control antibody raised against heat-shock protein Hsp70. (**D**) Immunostaining with anti-(α-tubulin) antibody TU-01. Bound antibodies were detected by chemiluminescent reagents (**A**, **B**) or by chromogenic substrates for alkaline phosphatase (**C**, **D**). Molecular-mass markers (in kDa) are indicated to the left of (**A**). The arrow on the right indicates the position of heavy chains of IgG.

During chemiluminescent detection, the two closely spaced bands appeared on the film as a single broad band.

Since the described experiments indicated that tubulin dimers could be present in γ-tubulin complexes, immunoprecipitation was also performed with the immobilized anti-(α-tubulin) antibody, TU-16 (IgM). This antibody specifically precipitated α-tubulin (Figure 5A, lane 2), and no binding of α-tubulin to immobilized Protein L was detected (Figure 5A, lane 4). Probing of the immunoprecipitated material with anti-(β-tubulin) antibody TU-06 confirmed the presence of β-tubulin subunits (results not shown). Probing of the immunoprecipitated material with anti-(γ-tubulin) antibody revealed that the precipitated $\alpha\beta$-tubulin dimers also contained γ-tubulin (Figure 5B, lane 2). When the immobilized antibody was incubated without extract, no staining in the position of γ-tubulin was observed (Figure 5B, lane 3). Control antibody VI-01 (IgM) did not elicit precipitation of γ-tubulin (results not shown). To test the possibility that cytoplasmic proteins might be unspecifically attached to the complexes containing $\alpha\beta$-tubulin dimers, the immunoprecipitated material was probed with anti-actin antibody, but no actin was detected (Figure 5C, lane 2). Similarly, probing with anti-Hsp70 antibody showed no co-precipitation of the relevant protein (results not shown). When the pooled fractions from gradient centrifugation corresponding to γ-tubulin complexes of low- (Figure 2, fractions 3 and 4) and high- (Figure 2, fractions 8 and 9) molecular mass were used for precipitation with TU-16 antibody, a large amount of tubulin dimers was precipitated from the low-molecular-mass fraction, whereas a substantially lower amount was precipitated from high-molecular-mass fractions. γ-Tubulin was specifically co-precipitated in both cases, but the yield was again higher with low-molecular-mass fractions. γ-Tubulin was also co-precipitated from fraction 2 (Figure 2). This suggests that tubulin dimers can interact with γ-tubulin irrespective of the size of complexes.

γ-Tubulin isoforms

To compare the electrophoretic behaviour of $\alpha\beta$-tubulin dimers and γ-tubulin, we have used different types of SDS for denatur-

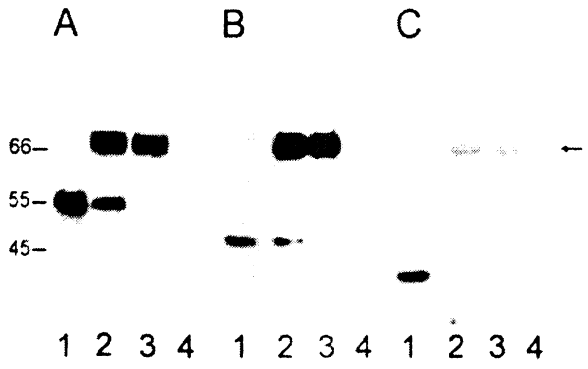


Figure 5 Immunoprecipitation of pig brain extract with anti-(α-tubulin) antibody

Samples were precipitated with antibody TU-16 (IgM) bound to immobilized Protein L. Proteins remaining after precipitation (lane 1), immunoprecipitated proteins (lane 2), immobilized immunoglobulin not incubated with cell extract (lane 3) and proteins bound to Protein L without antibody (lane 4) were separated on SDS/7.5% polyacrylamide gels. (**A**) Immunostaining with anti-(α-tubulin) antibody TU-01. (**B**) Immunostaining with anti-(γ-tubulin) antibody GTU-88. (**C**) Immunostaining with control anti-actin antibody. Molecular-mass markers (in kDa) are indicated on the left of (**A**). The arrow on the right indicates the position of heavy chains of IgM.

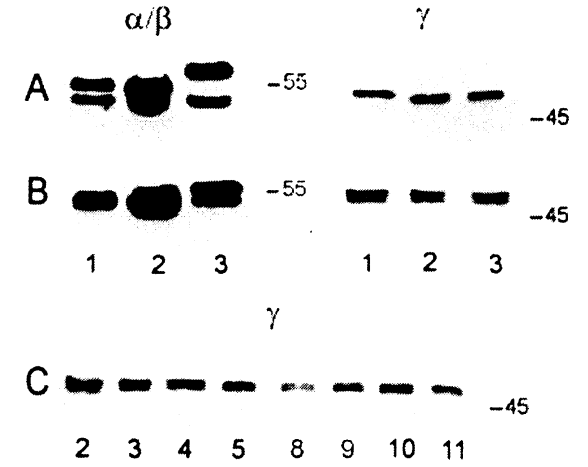


Figure 6 Influence of SDS-composition on electrophoretic properties of γ-tubulin

High-speed supernatant of brain extract (lane 1), MTP-2 (lane 2) and carboxyamidomethylated MTP-2 (lane 3) were separated on SDS/7.5% polyacrylamide gels using (**A**) 66%-purity SDS or (**B**) 99%-purity SDS. 'α/β' denotes immunostaining with a mixture of antibodies TU-01 and TU-06 against α- and β-tubulin respectively, 'γ' denotes immunostaining with anti-(γ-tubulin) antibody TU-32. (**C**) Sucrose-gradient centrifugation fractions separated on SDS/7.5% polyacrylamide gels in the presence of 99% SDS, immunostained with anti-(γ-tubulin) antibody GTU-88. Lanes 2–5 and 8–11 represent fractions shown in Figure 2. Molecular-mass markers (in kDa) are indicated on the right.

ation and gel electrophoresis. For separation of tubulin dimers, we have routinely used lower-grade SDS, from Sigma, that contains hexadecyl and tetradecyl sulphate contaminants [34]. Under such conditions, clear two bands of tubulin dimers could be recognized in blots from a high-speed extract (Figure 6, lane 1), MTP-2 (lane 2) or carboxyamidomethylated MTP-2 (lane 3), where the separation between tubulin subunits was prominent (Figure 6A, panel labelled 'α/β'). On the other hand, in the same samples γ-tubulin provided one band (Figure 6A, panel labelled

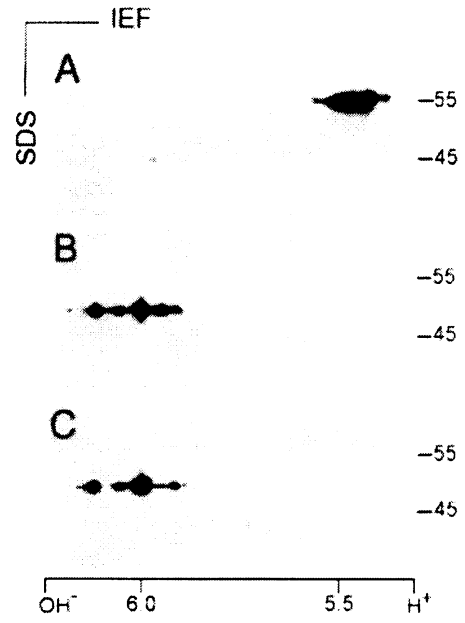


Figure 7 Immunoblot analysis of pig brain extract (A and B) and MTP-2 (C) separated by two-dimensional electrophoresis

Immunostaining with antibodies against α-tubulin (**A**) and γ-tubulin (**B**, **C**). The basic and acidic ends in the first dimension are marked by minus and plus signs respectively. The immunoblots depict the same region in the first dimension. Molecular-mass markers (in kDa) are indicated on the right. The pI scale is shown along the bottom of the Figure. IEF, isoelectric focusing.

'γ'). When high-grade SDS was used for the preparation of samples and following electrophoresis, α- and β-tubulin subunits were not separated in either high-speed extracts or in MTP-2. In carboxyamidomethylated MTP-2, the separation of tubulin subunits was easily distinguishable, but less prominently so (Figure 6B, panel labelled 'α/β'). However, γ-tubulin provided two bands in all samples tested (Figure 6B, panel labelled 'γ'). Carboxymethylation was without any effect on separation of the two γ-tubulin bands. The staining of two γ-tubulins was inhibited after pre-incubation of TU-32 antibody with the peptide used for immunization, but not with a control peptide from the N-terminal region of γ-tubulin. Two γ-tubulins were also detected with antibody GTU-88 against γ-tubulin amino acid region 38–53 (Figure 6C). These experiments eliminated the possibility that the reactivity was with a polypeptide of a similar electrophoretical mobility, but unrelated to γ-tubulin. The double band of γ-tubulins was easily recognizable when the detection method made use of a chromogenic substrate for alkaline phosphatase. With the chemiluminescence detection method, the two closely spaced bands were not so easily discernible. When high-grade SDS (99%) was used in SDS/PAGE during the separation of fractions from gradient centrifugation, doubling of the γ-tubulin band was observed in complexes of both low- and high-molecular mass (Figure 6C). Similarly, under the same conditions, a double γ-tubulin band was also detected in precipitates with anti-(γ-tubulin) and anti-(α-tubulin) antibodies.

The two-dimensional analysis revealed that soluble forms of γ-tubulin existed in multiple charge variants for which isoelectric points were more basic in comparison with α-tubulin isoforms (Figure 7A). At least five isoelectric variants of γ-tubulin were detected by immunoblotting in the high-speed extract (Figure 7B). The relative position of γ-tubulin isoforms with respect

to α-tubulin, and the number of γ-tubulin charge variants, in high-speed extracts were similar to those in MTP-2 (Figure 7C). The pI of the major γ-tubulin isoform was 6.00.

DISCUSSION

Fractionation of high-speed pig brain extracts by gradient centrifugation revealed the presence of γ-tubulin in complexes of different molecular masses. Large complexes dissociated in the presence of a high-salt concentration. The αβ-tubulin dimers were also distributed in a wide range of molecular masses. In the latter case, however, high-salt concentration did not induce a dissociation of large complexes. Non-denaturing PAGE of MTP-2 revealed disparate mobilities of oligomers containing αβ-tubulin dimers and oligomers containing γ-tubulin. In spite of that, γ-tubulin has the ability to interact with αβ-tubulin dimers in brain extracts. This was confirmed by partial cycling of γ-tubulin with tubulin dimers during repeated cycles of polymerization and depolymerization, as shown by Détraves et al. [9] and confirmed in the present study, and by our immunoprecipitation experiments. Our previous failure to detect γ-tubulin in pig brain MTP preparations with monoclonal antibodies [23] can most probably be ascribed to low titres of the used antibody batches.

The co-precipitation of αβ-tubulin dimers and γ-tubulin effected by monoclonal anti-(γ-tubulin) antibody, and co-precipitation of γ-tubulin and αβ-tubulin dimers effected by monoclonal anti-(α-tubulin) antibody, confirmed independently an association of soluble γ-tubulin with αβ-tubulin dimers in cold pig brain high-speed extracts. Multiple control tests have proved that the association is specific, and does not merely reflect a binding of abundant cytoplasmic proteins. The GTU-88 antibody directed against the amino acid region γ38–53 failed to precipitate γ-tubulin. This suggests that this region in brain γ-tubulin is not accessible for binding the antibody, in contrast with the γ434–449 region recognized by TU-31. However, it was reported that GTU-88 was effective in specific precipitation of γ-tubulin from leopard frog (*Rana pipiens*) oocytes [16]. This indicates that this γ-tubulin epitope is exposed differentially in separate model systems.

There are conflicting reports on the presence of αβ-tubulin heterodimers in γTuRC. Variable amounts of αβ-tubulin dimers have been found to co-precipitate with γ-tubulin in preparations from *Xenopus* oocytes [5], *Rana* oocytes [16], sheep brain [9] and cells of the lymphoblastic cell line KE37 [8]. No evidence of the presence of αβ-tubulin heterodimers in γTuRCs could be inferred from other studies [15,38]. One possible explanation of such discrepancies is that the interaction of γ-tubulin with αβ-tubulin dimers in cells is weak, and can be easily modified by sample preparation or by procedures used for the assessment of the composition of γ-tubulin complexes. Alternatively, γ-tubulin can be present in cells in other forms, except for γTuSC and γTuRC, which interact with tubulin dimers. γ-Tubulin dimer was identified under natural conditions in HeLa cells [39]. However, the significance of γ-tubulin interaction with αβ-tubulin dimers outside of γTuRCs remains to be elucidated. Experiments using *in vitro*-translated monomeric γ-tubulin showed that a single γ-tubulin subunit was sufficient to nucleate pig brain microtubules [40]. γ-Tubulin might also stabilize nucleation-competent stable oligomers formed by αβ-tubulin dimers or other types of tubulin oligomers. The capability of tubulin molecules to form various types of oligomers is widely recognized, and these oligomers have been assigned the role of regulators in microtubule dynamics [41].

It appeared relevant in this context to find out whether it would be possible to discriminate between γ-tubulin variants differing in their electrophoretic behaviour. To this end, the effect of SDS purity on the electrophoretic mobility of γ-tubulin was assessed in MTP and carboxyamidomethylated MTP. Although the hexadecyl and tetradecyl sulphate contaminants had a substantial effect on the mobility of α-tubulin subunit, as shown previously [26,34], they were without any effect on γ-tubulin mobility. On the other hand, SDS free of the contaminants led to a reproducible dissociation of γ-tubulin into its slow- and fast-migrating forms. Both polypeptides were detected by antibodies TU-32 and GTU-88 recognizing an epitope in the C-terminal and the N-terminal regions, respectively, of γ-tubulin molecule. The two isoforms were not drawn apart by gradient centrifugation, by cycling of MTP and by immunoprecipitation with anti-(γ-tubulin) or anti-(α-tubulin) antibodies. Two γ-tubulin isoforms were also detected in extracts from adult mouse brain (T. Sulimenko and P. Dráber, unpublished work). Although electrophoretic variants of γ-tubulin that can be discriminated by one-dimensional electrophoresis were not detected up to now in mammalian cells, fast- and slow-migrating γ-tubulin isoforms were distinguished in plants [42], *Physarum* [43] and *Drosophila* [44]. In *Drosophila*, they represent diverse gene products that are differentially expressed during development [45], and a loss of their function results in distinct phenotypes [46]. Two functional γ-tubulin genes were identified in mammals [47]. The observed fast- and slow-migrating γ-tubulin isoforms could represent the relevant proteins. Although both gene products differ in humans only in 12 amino acids [47], one cannot exclude a differential binding of SDS to polypeptides. Alternatively, cell heterogeneity in brain tissue or post-translational modification(s) could be responsible for the observed differences in mobility.

Neuronal α- and β-tubulin subunits are characterized by their high-charge heterogeneity, which stems not only from multiple gene expression, but also from numerous post-translational modifications [48]. Substantial changes in the number of tubulin isoforms were observed during brain development [49,50]. We have detected multiple charge variants of γ-tubulin in brain extracts, as well as in MTP-2. At least five clearly distinguishable isoelectric variants were identified in pig brain extract. This exceeds the number of functional γ-tubulin genes, and points to post-translational modification of γ-tubulin. Two major and two minor γ-tubulin isoforms were recognized by two-dimensional electrophoresis in sheep brain [9]. Phosphorylation of the γ-tubulin residue Tyr^{445}, which is invariant in all γ-tubulins and regulates the microtubule organization in budding yeast, has been reported recently [22]. γ-Tubulin was found in mouse mammary carcinoma cells in association with serine/threonine 'polo-like' kinase [51] and in rat basophilic leukaemia cells in complexes containing protein tyrosine kinase $p53/p56^{lyn}$ [52]. It is possible that phosphorylation or other post-translational modifications of neuronal γ-tubulin modify its interaction with tubulin dimers or other associated proteins.

In conclusion, the presented data demonstrate for the first time that there exist in brain two γ-tubulin isoforms, which persist in differently sized complexes and co-polymerize with tubulin dimers. An interaction of γ-tubulins with tubulin heterodimers irrespective of the size of γ-tubulin complexes reflects the presence of other γ-tubulin forms apart from γTuSC and γTuRC. Brain γ-tubulin is post-translationally modified, and these modifications possibly have an important role in the regulation of microtubule nucleation.

This work was supported in parts by grants from the Grant Agency of the Czech Academy of Sciences (A5052004), Grant Agency of the Czech Republic (312/96/K205) and from the Ministry of Education of the Czech Republic (LN00A026, ME 310).

REFERENCES

1 Kellogg, D. R., Moritz, M. and Alberts, B. M. (1994) The centrosome and cellular organization. Annu. Rev. Biochem. **63**, 639–674
2 Oakley, C. E. and Oakley, B. R. (1989) Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. Nature (London) **338**, 662–664
3 Oakley, B. R., Oakley, C. E., Yoon, Y. and Jung, M. (1990) γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. Cell (Cambridge, Mass.) **61**, 1289–1301
4 Joshi, H. C., Palacios, M. J., McNamara, L. and Cleveland, D. W. (1992) γ-Tubulin is a centrosomal protein required for cell cycle-dependent microtubule nucleation. Nature (London) **356**, 80–83
5 Zheng, Y., Wong, M. L., Alberts, B. and Mitchison, T. (1995) Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. Nature (London) **378**, 578–583
6 Moritz, M., Braunfeld, M. B., Fung, J. C., Sedat, J. W., Alberts, B. M. and Agard, D. A. (1995) Three-dimensional structural characterization of centrosomes from *Drosophila* embryos. J. Cell Biol. **130**, 1149–1159
7 Meads, T. and Schroer, T. A. (1995) Polarity and nucleation of microtubules in polarized epithelial cells. Cell Motil. Cytoskeleton **32**, 273–288
8 Moudjou, M., Bordes, N., Paintrand, M. and Bornens, M. (1996) γ-Tubulin in mammalian cells: the centrosomal and the cytosolic forms. J. Cell Sci. **109**, 875–887
9 Détraves, C., Mazarguil, H., Lajoie-Mazenc, I., Julian, M., Raynaud-Messina, B. and Wright, M. (1997) Protein complexes containing γ-tubulin are present in mammalian brain microtubule protein preparations. Cell Motil. Cytoskeleton **36**, 179–189
10 Moritz, M., Zheng, Y., Alberts, B. M. and Oegema, K. (1998) Recruitment of the γ-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. J. Cell Biol. **142**, 775–786
11 Murphy, S. M., Urbani, L. and Stearns, T. (1998) The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components Spc97p and Spc98p. J. Cell Biol. **141**, 663–674
12 Oegema, K., Wiese, C., Martin, O. C., Milligan, R. A., Iwamatsu, A., Mitchison, T. J. and Zheng, Y. (1999) Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules. J. Cell Biol. **144**, 721–733
13 Knop, M. and Schiebel, E. (1997) Spc98p and Spc97p of the yeast γ-tubulin complex mediate binding to the spindle pole via their interaction with Spc110p. EMBO J. **16**, 6985–6995
14 Wiese, C. and Zheng, Y. (2000) A new function for the γ-tubulin ring complex as a microtubule minus-end cap. Nat. Cell Biol. **2**, 358–364
15 Wiese, C. and Zheng, Y. (1999) γ-Tubulin complexes and their interaction with microtubule-organizing centers. Curr. Opin. Struct. Biol. **9**, 250–259
16 Lessman, C. A. and Kim, H. (2001) Soluble tubulin complexes in oocytes of the common leopard frog, *Rana pipiens*, contain γ-tubulin. Mol. Reprod. Dev. **60**, 128–136
17 Li, Q. and Joshi, H. C. (1995) γ-Tubulin is a minus end-specific microtubule binding protein. J. Cell Biol. **131**, 207–214
18 Llanos, R., Chevrier, V., Ronjat, M., Meurer-Grob, P., Martinez, P., Frank, R., Bornens, M., Wade, R. H., Wehland, J. and Job, D. (1999) Tubulin binding sites on γ-tubulin: identification and molecular characterization. Biochemistry **38**, 15712–15720
19 Inclán, V. M. and Nogales, E. (2000) Structural models for the self-assembly and microtubule interactions of γ-, δ- and ε-tubulin. J. Cell Sci. **114**, 413–422
20 Liu, B., Marc, J., Joshi, H. C. and Palevitz, B. A. (1993) γ-Tubulin-related protein associated with microtubule arrays of higher plants in cell cycle-dependent manner. J. Cell Sci. **104**, 1217–1228
21 Binarová, P., Hause, B., Doležel, J. and Dráber, P. (1998) Association of γ-tubulin with kinetochore/centromeric region of plant chromosomes. Plant J. **14**, 751–757
22 Vogel, J., Drapkin, B., Oomen, J., Beach, D., Bloom, K. and Snyder, M. (2001) Phosphorylation of γ-tubulin regulates microtubule organization in budding yeast. Dev. Cell **1**, 621–631
23 Nováková, M., Dráberová, E., Schürmann, W., Czihak, G., Viklický, V. and Dráber, P. (1996) γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies. Cell Motil. Cytoskeleton **33**, 38–51
24 Viklický, V., Dráber, P., Hašek, J. and Bártek, J. (1982) Production and characterization of a monoclonal antitubulin antibody. Cell Biol. Int. Rep. **6**, 725–731
25 Dráberová, E. and Dráber, P. (1998) Novel monoclonal antibodies TU-08 and TU-16 specific for tubulin subunits. Folia Biol. (Prague) **44**, 35–36
26 Dráber, P., Dráberová, E., Linhartová, I. and Viklický, V. (1989) Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies. J. Cell Sci. **92**, 519–528
27 Dráberová, E., Dráber, P., Havlíček, F. and Viklický, V. (1986) A common antigenic determinant of vimentin and desmin defined by monoclonal antibody. Folia Biol. (Prague) **32**, 295–303
28 Shelanski, M. L., Gaskin, F. and Cantor, C. R. (1973) Microtubule assembly in the absence of added nucleotides. Proc. Natl. Acad. Sci. U.S.A. **70**, 765–768
29 Mandelkow, E. M., Herrman, M. and Ruhl, V. (1985) Tubulin domains probed by limited proteolysis and subunit-specific antibodies. J. Mol. Biol. **185**, 311–327
30 Weingarten, M. D., Lockwood, A. H., Hwo, S. Y. and Kirschner, M. W. (1975) A protein factor essential for microtubule assembly. Proc. Natl. Acad. Sci. U.S.A. **72**, 1858–1868
31 George, H. J., Misra, L., Field, D. J. and Lee, J. C. (1981) Polymorphism of brain tubulin. Biochemistry **20**, 2402–2409
32 Dráberová, E. and Dráber, P. (1993) A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules. J. Cell Sci. **106**, 1263–1273
33 Dráber, P., Lagunowich, L. A., Dráberová, E., Viklický, V. and Damjanov, I. (1988) Heterogeneity of tubulin epitopes in mouse fetal tissues. Histochemistry **89**, 485–492
34 Best, D., Warr, P. J. and Gull, K. (1981) Influence of the composition of commercial sodium dodecyl sulfate preparations on the separation of α- and β-tubulin during polyacrylamide gel electrophoresis. Anal. Biochem. **114**, 281–284
35 Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature (London) **227**, 680–685
36 Görg, A., Obermaier, C., Boguth, G. and Weiss, W. (1999) Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures. Electrophoresis **20**, 712–717
37 Correia, J. J. and Williams, Jr, R. C. (1985) Characterization of oligomers of tubulin by two-dimensional native electrophoresis. Arch. Biochem. Biophys. **239**, 120–129
38 Murphy, S. M., Preble, A. M., Patel, U. K., O'Connell, K. L., Dias, D. P., Moritz, M., Agard, D., Stults, J. T. and Stearns, T. (2001) GCP5 and GCP6: two new members of the human γ-tubulin complex. Mol. Biol. Cell **12**, 3340–3352
39 Vassilev, A., Kimble, M., Silflow, C. D., LaVoie, M. and Kuriyama, R. (1995) Identification of intrinsic dimer and overexpressed monomeric forms of γ-tubulin in Sf9 cells infected with baculovirus containing the *Chlamydomonas* γ-tubulin sequence. J. Cell Sci. **108**, 1083–1092
40 Leguy, R., Melki, R., Pantaloni, D. and Carlier, M. F. (2000) Monomeric γ-tubulin nucleates microtubules. J. Biol. Chem. **275**, 21975–21980
41 Caudron, N., Valiron, O., Usson, Y., Valiron, P. and Job, D. (2000) A reassessment of the factors affecting microtubule assembly and disassembly *in vitro*. J. Mol. Biol. **297**, 211–220
42 Petitprez, M., Caumont, C., Barthou, H., Wright, M. and Alibert, G. (2001) Two γ-tubulin isoforms are differentially expressed during development in *Helianthus annuus*. Physiol. Plant. **111**, 102–107
43 Lajoie-Mazenc, I., Détraves, C., Rotaru, V., Gares, M., Tollon, Y., Jean, C., Julian, M., Wright, M. and Raynaud-Messina, B. (1996) A single γ-tubulin gene and mRNA, but two γ-tubulin polypeptides differing by their binding to the spindle pole organizing centres. J. Cell Sci. **109**, 2483–2492
44 Raynaud-Messina, B., Debec, A., Tollon, Y., Gares, M. and Wright, M. (2001) Differential properties of the two *Drosophila* γ-tubulin isotypes. Eur. J. Cell Biol. **80**, 643–649
45 Wilson, P. G., Zheng, Y., Oakley, C. E., Oakley, B. R., Borisy, G. G. and Fuller, M. T. (1997) Differential expression of two γ-tubulin isoforms during gametogenesis and development in *Drosophila*. Dev. Biol. **184**, 207–221
46 Llamazares, S., Tavosanis, G. and Gonzalez, C. (1999) Cytological characterisation of the mutant phenotypes produced during early embryogenesis by null and loss-of-function alleles of the *γTub37C* gene in *Drosophila*. J. Cell Sci. **112**, 659–667
47 Wise, D. O., Krahe, R. and Oakley, B. R. (2000) The γ-tubulin gene family in humans. Genomics **67**, 164–170
48 Luduena, R. F. (1998) Multiple forms of tubulin: different gene products and covalent modifications. Int. Rev. Cytol. **178**, 207–275
49 Wolff, A., Denoulet, P. and Jeantet, C. (1982) High level of tubulin microheterogeneity in the mouse brain. Neurosci. Lett. **31**, 323–328
50 Linhartová, I., Dráber, P., Dráberová, E. and Viklický, V. (1992) Immunological discrimination of β-tubulin isoforms in developing mouse brain. Posttranslational modification of non-class III β-tubulins. Biochem. J. **288**, 919–924
51 Feng, Y., Hodge, D. R., Palmieri, G., Chase, D. L., Longo, D. L. and Ferris, D. K. (1999) Association of polo-like kinase with α-, β- and γ-tubulins in a stable complex. Biochem. J. **339**, 435–442
52 Dráberová, L., Dráberová, E., Surviladze, Z., Dráber, P. and Dráber, Pa. (1999) Protein tyrosine kinase p53/p56^{lyn} forms complexes with γ-tubulin in rat basophilic leukemia cells. Int. Immunol. **11**, 1829–1839

Received 29 January 2002/8 April 2002; accepted 8 April 2002
Published as BJ Immediate Publication 8 April 2002, DOI 10.1042/BJ20020175

VI.4. Dryková D., Cenklová V., **Sulimenko V.,** Volc J., Dráber P., Binarová P.: *Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes.* ***Plant Cell*, 15**: 465-480, 2003.

The Plant Cell, Vol. 15, 465–480, February 2003, www.plantcell.org © 2003 American Society of Plant Biologists

Plant γ-Tubulin Interacts with αβ-Tubulin Dimers and Forms Membrane-Associated Complexes

Denisa Dryková,[a,1] Věra Cenklová,[b,1] Vadym Sulimenko,[c] Jindřich Volc,[a] Pavel Dráber,[c] and Pavla Binarová[a,2]

[a] Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic
[b] Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Sokolovská 6, 772 00 Olomouc, Czech Republic
[c] Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic

γ-Tubulin is assumed to participate in microtubule nucleation in acentrosomal plant cells, but the underlying molecular mechanisms are still unknown. Here, we show that γ-tubulin is present in protein complexes of various sizes and different subcellular locations in Arabidopsis and fava bean. Immunoprecipitation experiments revealed an association of γ-tubulin with αβ-tubulin dimers. γ-Tubulin cosedimented with microtubules polymerized in vitro and localized along their whole length. Large γ-tubulin complexes resistant to salt treatment were found to be associated with a high-speed microsomal fraction. Blue native electrophoresis of detergent-solubilized microsomes showed that the molecular mass of the complexes was >1 MD. Large γ-tubulin complexes were active in microtubule nucleation, but nucleation activity was not observed for the smaller complexes. Punctate γ-tubulin staining was associated with microtubule arrays, accumulated with short kinetochore microtubules interacting in polar regions with membranes, and localized in the vicinity of nuclei and in the area of cell plate formation. Our results indicate that the association of γ-tubulin complexes with dynamic membranes might ensure the flexibility of noncentrosomal microtubule nucleation. Moreover, the presence of other molecular forms of γ-tubulin suggests additional roles for this protein species in microtubule organization.

INTRODUCTION

The successive replacement of microtubular arrays (cortical microtubules, preprophase band, mitotic spindle, and phragmoplast) during cell cycle progression is unique to higher plants. This flexibility in building microtubule structures at different locations can be achieved in plants because the dominating microtubule organizing centers, which are comparable to those of animal centrosomes, are missing in both somatic and gametic cells. However, centrosomes are not absolutely required for microtubule nucleation even in animal cells. It was shown that microtubules could be nucleated in the absence of centrosomes, presumably by yet undefined cytoplasmic factors (Vorobjev et al., 1997). For an understanding of how microtubules are nucleated and organized without the centrosome, the first step is to identify the molecular composition of the dispersed microtubule nucleation sites.

γ-Tubulin is a highly conserved member of the tubulin superfamily that is located on the minus end of microtubules in microtubule organizing centers, where such structures are present in the cell (Wiese and Zheng, 1999). Although in animal cells, γ-tubulin participates in the nucleation of microtubules from microtubule organizing centers, the majority of this protein is associated with other centrosomal proteins in soluble cytoplasmic complexes. Large (~2.2 MD) γ-tubulin ring complexes (γ-TuRCs) and smaller (~280 kD) γ-tubulin complexes were identified in various species (Moritz et al., 1995; Zheng et al., 1998; Oegema et al., 1999). γ-Tubulin complexes comprise two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin complex proteins), which are homologs of the *Saccharomyces cerevisiae* proteins Spc97p and Spc98p (Geissler et al., 1996). The γ-TuRCs are formed by small complexes and by other proteins. In addition to nucleation from the microtubule organizing center, the large complexes also are involved in regulating the dynamics of the microtubule minus ends (Wiese and Zheng, 2000). Recently, genetic data from *Schizosaccharomyces pombe* and *Aspergillus nidulans* showed that γ-tubulin might play other important roles in the organization of mitotic and cytokinetic microtubules (Hendrickson et al., 2001; Jung et al., 2001).

[1] These authors contributed equally to this article.
[2] To whom correspondence should be addressed. E-mail binarova@biomed.cas.cz; fax 420-2-41062384.
Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.007005.

In plants, γ-tubulin was immunolocalized preferentially on microtubules (Liu et al., 1993). The association of γ-tubulin with kinetochore microtubules and the presence of γ-tubulin in premitotic nuclei suggested its role in microtubule and spindle organization (Binarova et al., 1998, 2000; Petitpren et al., 2001). Immunolocalization studies with different antibodies reported a punctuated labeling for γ-tubulin with nuclear and cortical membranes and with organelle-like structures (McDonald et al., 1993; Liu et al., 1994; Dibbayawan et al., 2001). Soluble cytoplasmic γ-tubulin complexes were identified in fava bean and maize cell extracts (Binarova et al., 2000; Stoppin-Mellet et al., 2000). The latter authors reported a γ-tubulin association with the microsomal fraction. Despite many data suggesting that γ-tubulin is an abundant protein at various locations in acentrosomal plant cells, its role in plant microtubule nucleation and organization is still largely unknown.

Here, we show that plant γ-tubulin is present in the form of protein complexes of various sizes and of different properties. Soluble γ-tubulin interacts with tubulin dimers and cosediments with microtubules in vitro. We report that large γ-tubulin complexes active in microtubule nucleation are associated with membranes. This association of γ-tubulin with membranous structures might ensure the nucleation of microtubule arrays from dispersed sites in acentrosomal cells.

RESULTS

Anti-Peptide Antibodies Raised against Different Parts of the γ-Tubulin Molecule Recognize γ-Tubulins in Several Plant Species

As a prerequisite to the study of γ-tubulin in higher plant cells, plant-specific polyclonal antibody (AthTU) was raised against a 14–amino acid peptide (EYKACESPDYIKWG) corresponding to the Arabidopsis γ-tubulin sequence 437 to 450. Affinity-purified antibody recognized a single band of 56 kD in Arabidopsis and a slightly larger (by ~1 to 2 kD) band in maize, fava bean, and pea (Figures 1A and 1B, AthTU). A similar staining pattern was seen with a polyclonal antibody raised against the human γ-tubulin sequence 38 to 50, but the antibody reactivity was substantially weaker (data not shown). Monoclonal antibody TU-31 raised against a peptide corresponding to the human γ-tubulin sequence 434 to 449 gave similar immunoblot staining in all of the species tested. One extra band of ~60 kD was detected in Arabidopsis (Figures 1A and 1B, TU-31); higher resolution conditions during electrophoresis revealed two corresponding, very close bands in other plant species (data not shown). The same staining pattern was obtained with extracts from seedlings or cell cultures (Arabidopsis) or with extracts from root meristems or whole seedlings (in all other species analyzed). Like the pattern shown previously for TU-31 antibody

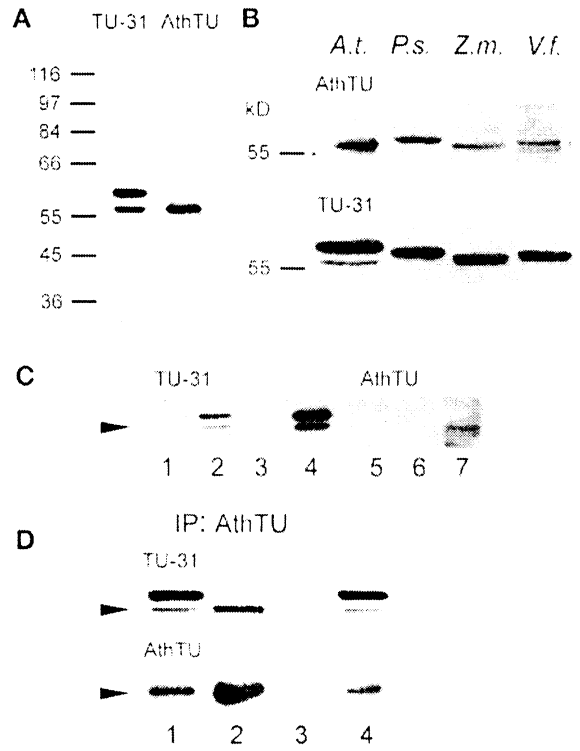


Figure 1. Immunoblot Analysis of Cell Extracts with Polyclonal and Monoclonal Anti-γ-Tubulin Antibodies.

(A) Affinity-purified AthTU antibody recognized one band in Arabidopsis extracts. Two bands were recognized with monoclonal antibody TU-31.

(B) Immunoblot detection of γ-tubulin in several plant species. Cell extracts from Arabidopsis (*A.t.*), pea (*P.s.*), maize (*Z.m.*), and fava bean (*V.f.*) were separated by 10% SDS-PAGE and stained with polyclonal antibody AthTU and monoclonal antibody TU-31.

(C) Immunostaining of γ-tubulin on blots was abolished after preabsorption of antibodies with immunizing peptide. Antibodies TU-31 (lanes 1 to 4) and AthTU (lanes 5 to 7) were preabsorbed with the immunizing peptides and used to probe 10% SDS-PAGE–separated Arabidopsis extracts. Lanes 1 and 6, peptide used to prepare the TU-31 antibody; lanes 2 and 7, no peptide; lanes 3 and 5, peptide used to prepare the AthTU antibody; lane 4; negative control peptide. The arrowhead indicates the position of the 56-kD γ-tubulin.

(D) γ-Tubulin immunoprecipitated (IP) with AthTU antibody was immunodetected with both TU-31 and AthTU antibodies. Lane 1, extract before immunoprecipitation; lane 2, immunoprecipitated and peptide-eluted γ-tubulin; lane 3, control eluate from beads with preimmune serum; lane 4, supernatant after immunoprecipitation. Both antibodies recognized immunopurified γ-tubulin (56 kD). Arrowheads indicate the position of the 56-kD γ-tubulin.

(Binarova et al., 2000), the AthTU antibody recognized γ-tubulin in nuclear extracts (data not shown).

Preabsorption of TU-31 with the peptide used for immunization (Figure 1C, lane 1) or with the Arabidopsis-specific homologous peptide used for the preparation of AthTU anti-

body (Figure 1C, lane 3) prevented the staining of both bands on blots from extracts of Arabidopsis. Both peptides also prevented immunolabeling with AthTU antibody (Figure 1C, lanes 5 and 6). On the other hand, immunolabeling was not influenced by the preabsorption of TU-31 with human γ-tubulin peptide from the N-terminal region of the molecule (Figure 1C, lane 4).

Immunoprecipitation of γ-tubulin from Arabidopsis extracts with AthTU antibody followed by specific elution with corresponding immunizing peptide revealed that the immunoprecipitated 56-kD protein is recognized by both antibodies (Figure 1D, TU-31 [lane 2] and AthTU [lane 2]). When immunoglobulins isolated from preimmune serum were used for precipitation instead of AthTU antibody, no γ-tubulin was eluted with the immunizing peptide (Figure 1D, lane 3). These data demonstrate that both the plant γ-tubulin–specific polyclonal antibody AthTU and the monoclonal antibody TU-31 can be used as markers for γ-tubulin.

Immunofluorescence Analysis Indicates an Association of γ-Tubulin with Microtubules and Membranes

The immunofluorescence staining pattern obtained with affinity-purified AthTU antibody was similar to that described previously for the monoclonal anti-γ-tubulin antibody TU-32 (Binarova et al., 1998, 2000). Punctate γ-tubulin staining was associated with microtubule arrays, localized in the vicinity of nuclei and the cell cortex and in the area of cell plate formation, and provided cell cycle–dependent nuclear staining. Double-label staining of cells with AthTU and anti-α-tubulin antibody showed an association of γ-tubulin with kinetochore microtubules and an accumulation of signal on poles on which acentrosomal spindle is seemingly anchored to the plasma membrane and/or the membranes of polar vacuoles (Figure 2A). To characterize the interaction of γ-tubulin with membranes, antibodies visualizing the *trans*-Golgi network and/or the nuclear membrane were used in double-labeling experiments with anti-γ-tubulin antibodies. Spindle-associated γ-tubulin was on the poles localized close to the Golgi membranes in metaphase (Figure 2B) as well as in anaphase (Figure 2C), when the majority of γ-tubulin accumulated, with the shortening kinetochore fibers focused on the poles. When chromosomes reached the poles, an intensive granular signal of γ-tubulin was associated with remnants of kinetochore microtubules (Figure 2D, arrow). These γ-tubulin–decorated polar structures were localized in the vicinity of the Golgi membranes (Figure 2E, arrow). The inhibitory effect of brefeldin A on Golgi trafficking in plant cells was characterized (Ritzenthaler et al., 2002). Using this pharmacological approach, we observed that 30 min of treatment of fava bean roots with 200 $\mu g \cdot ml^{-1}$ brefeldin A induced the formation of multipolar spindles, with γ-tubulin accumulated with aberrantly located spindle poles (Figure 2F).

γ-Tubulin remained in the kinetochore area oriented to the cell cortex (Figure 2D, arrow) even after kinetochore microtubules disappeared and the anaphase spindle was rearranged into the early phragmoplast. The majority of γ-tubulin in telophase was present in the phragmoplast area, but punctate labeling also was localized around the newly formed nuclear envelope. Double labeling of cells with anti-γ-tubulin antibody and with antibody staining the nuclear envelope marker importin revealed that γ-tubulin is not colocalized with importin on the nuclear envelope but is present in spots mainly in the polar region in the vicinity of the nuclei (Figure 2H).

γ-Tubulin Is Present in the Cytosolic Fraction in the Form of Protein Complexes

In our previous experiments, we determined that γ-tubulin is present in plant extracts in the form of protein complexes (Binarova et al., 2000); here, we analyzed the size distribution of γ-tubulin in cell extracts of fava bean and Arabidopsis using gel filtration chromatography and nondenaturing electrophoresis. After gel filtration, γ-tubulin was distributed in a wide zone, but the first narrow maximum was in fractions 2 to 5, close to the column void volume (~2 MD). This result suggested that γ-tubulin is part of a large complex in Arabidopsis (Figure 3A) as well as in fava bean (Figure 3C). Intermediate-sized γ-tubulin complexes, ranging from ~400 to 900 kD, and smaller complexes than these also were found in extracts from both species. Because it is known from animal cell model systems that the large γ-tubulin complexes disassemble to smaller complexes in the presence of 500 mM NaCl (Oegema et al., 1999), the same separation conditions were used. As shown in Figure 3B, there was no reduction in the amount of γ-tubulin incorporated into large complexes. On the other hand, salt treatment reduced the amount of some types of γ-tubulin intermediate complexes (Figure 3B).

The size and abundance of the intermediate complexes varied among individual experiments, suggesting their dynamics. High-salt treatment also abolished the immunolabeling for γ-tubulin in fractions corresponding in molecular mass to monomers or smaller fractions, resulting from the apparent retention of γ-tubulin in the gel matrix. Similar profiles of γ-tubulin (56 kD) were found in Arabidopsis stained with the polyclonal antibody AthTU and the monoclonal antibody TU-31. However, antibody TU-31 also stained a 60-kD band distributed in fractions corresponding to 120 to 240 kD (Figure 3B, TU-31). αβ-Tubulin dimers were coeluted with γ-tubulin in a broad zone of fractions, with a large molecular mass maximum of ~2 MD (Figure 3A, α-tubulin and β-tubulin). In contrast to γ-tubulin, in which intermediate-sized complexes diminish in the presence of high salt concentrations, intermediate protein complexes containing αβ-tubulin dimers were more stable (Figure 3B).

The presence of γ-tubulin complexes of various sizes also was confirmed by electrophoretic separation of fractions

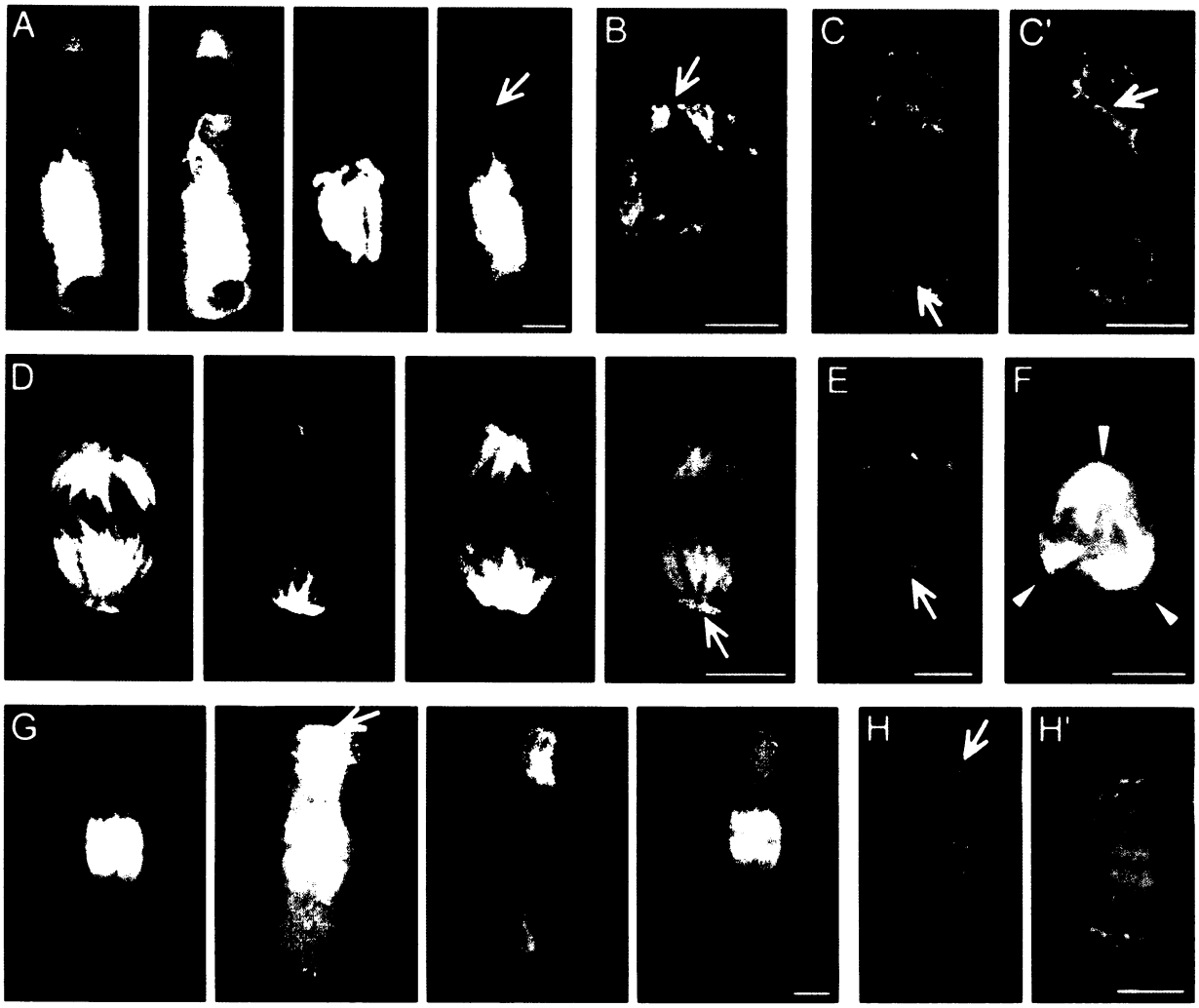


Figure 2. Subcellular Localization of γ-Tubulin in Mitotic Cells.

Root meristem cells of fava bean were stained with rabbit affinity-purified anti-γ-tubulin antibody AthTU (red), mouse monoclonal anti-α-tubulin antibody DMA1 (green), and 4′,6-diamidino-2-phenylindole (blue) for chromatin visualization.

(A), **(D)**, **(F)**, and **(G)** γ-Tubulin was visualized with anti-rabbit Cy3-conjugated antibody, and DMA1 antibody was visualized with anti-mouse fluorescein isothiocyanate (FITC)–conjugated antibody. Golgi membranes in root meristem cells of fava bean were stained with polyclonal rabbit TLG antibody (green) and visualized with anti-rabbit FITC-conjugated antibody.

(B), **(C)**, and **(E)** γ-Tubulin was stained with mouse monoclonal TU-31 antibody (red) and visualized with anti-mouse Cy3-conjugated secondary antibody.

(H) The nuclear envelope marker importin was stained with monoclonal mouse antibody (red) and visualized with anti-mouse Cy3-conjugated antibody. γ-Tubulin was stained with rabbit polyclonal antibody AthTU (green) and visualized with anti-rabbit FITC-conjugated secondary antibody.

(A) Metaphase cell with γ-tubulin anchoring kinetochore spindle microtubules to the vacuolar membrane (arrow).

(B) Confocal laser scanning microscopy (CLSM) single optical section of a metaphase cell with γ-tubulin localized with the metaphase spindle and the Golgi membranes in the area of the spindle poles (arrow).

(C) and **(C′)** Two CLSM optical sections of an anaphase cell with γ-tubulin associated with shortening kinetochore fibers and with the Golgi membranes in the vicinity of the spindle poles (arrows).

(D) A cell later in anaphase shows accumulation of γ-tubulin in the kinetochore region on the spindle poles (arrow).

(E) CLSM single optical sections of a cell in the same stage of mitosis as in **(D)** shows that γ-tubulin–decorated structures in the kinetochore region on poles are associated with the Golgi membranes (arrow).

(F) Multipolar mitosis observed after 30 min of brefeldin A treatment (arrowheads indicate multiple poles with γ-tubulin).

(G) Late anaphase/early telophase cell with remnants of γ-tubulin associated with kinetochores (arrow), whereas kinetochore microtubules had already disappeared.

(H) and **(H′)** CLSM single optical sections **(H)** and reconstitution of image stacks **(H′)** of telophase cells demonstrating that importin decorates the newly formed nuclear envelope and that γ-tubulin labeling is present mainly in the phragmoplast area and in spots in the vicinity of the nuclei.

(A), **(D)**, **(F)**, and **(G)** are images from classic immunofluorescence microscopy. **(B)**, **(C)**, **(E)**, and **(H)** are images from CLSM. Bars = 10 μm.

from gel filtration of Arabidopsis extracts under nondenaturing conditions. Staining of blots with antibody AthTU showed that γ-tubulin is present in both large complexes (gel filtration fractions 3 to 7) and smaller complexes (fractions 15 to 23). In the latter case, γ-tubulin staining appeared in fuzzy broad bands with relative molecular masses corresponding approximately to the sizes of the large and smaller complexes estimated by gel filtration (Figure 4A). The results shown are for the gel filtration performed in the presence of 500 mM NaCl, which should reduce the nonspecific interactions of γ-tubulin with other proteins or γ-tubulin aggregation during sample preparation. To exclude the possibility that broad diffuse bands resulted from the presence of 500 mM NaCl, samples were transferred before electrophoresis to the buffer with 50 mM salt. Fuzzy bands were present; moreover, reconstitution of intermediate-sized complexes was observed (data not shown). The fuzzy bands probably reflect the heterogeneity of γ-tubulin complexes or γ-tubulin oligomers, because other cytoplasmic proteins migrated under the same conditions as distinct bands.

The monoclonal antibody TU-31, like the AthTU antibody, recognized γ-tubulin in large complexes (Figure 4B). Intense staining of bands in the region 120 to 220 kD probably reflects the presence of complexes containing a 60-kD protein recognized by the TU-31 antibody (Figure 3B, TU-31). Therefore, both antibodies provide similar staining in high molecular mass fractions, but they clearly differ in staining in lower molecular mass fractions. Collectively, these data indicate that in cytosolic fractions there are tubulin protein complexes of different properties.

γ-Tubulin Is Associated with Membrane Microsomal Fractions

To analyze the interaction of plant γ-tubulin with membranes (as indicated by immunofluorescence analysis), total cell extracts of fava bean and Arabidopsis were separated by differential centrifugation into cytosolic and membrane fractions. The data obtained for fava bean and Arabidopsis were very similar; therefore, only Arabidopsis data are presented. In Figure 5A, the relative distribution of γ-tubulin in

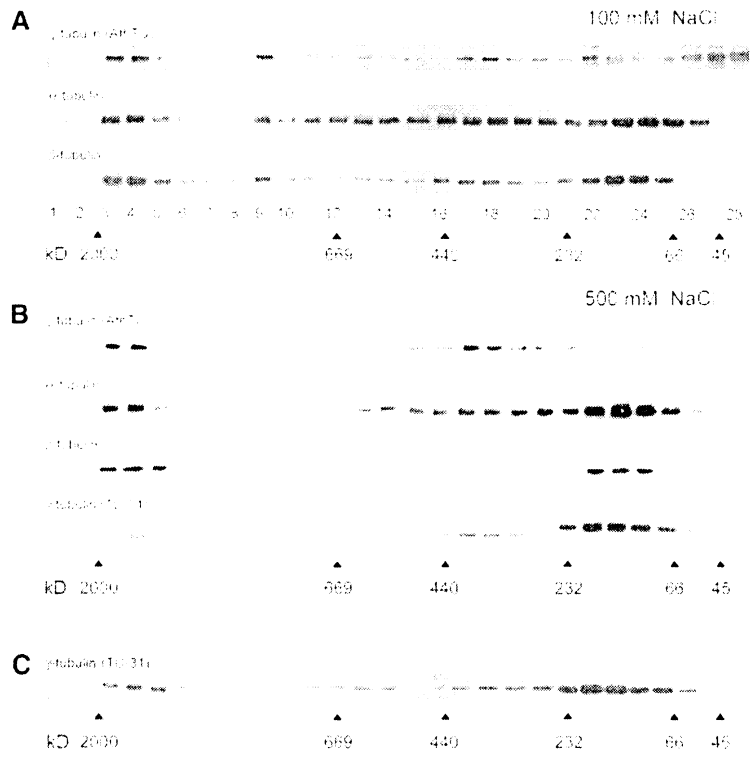


Figure 3. Gel Filtration of Cytoplasmic Extracts Followed by SDS-PAGE Analysis.

(A) and **(B)** Fractionations from gel filtration of Arabidopsis extracts in the presence of 100 **(A)** or 500 **(B)** mM NaCl.
(C) Fractionations from gel filtration of fava bean extract.
Fractions were analyzed by 10% SDS-PAGE, immunoblotted with antibodies AthTU and TU-31 against γ-tubulin and DM1A against α-tubulin, and reblotted with TUB 2.1 antibody against β-tubulin. Calibration standards (in kD) are indicated with arrowheads. Numbers from 1 to 28 denote individual fractions.

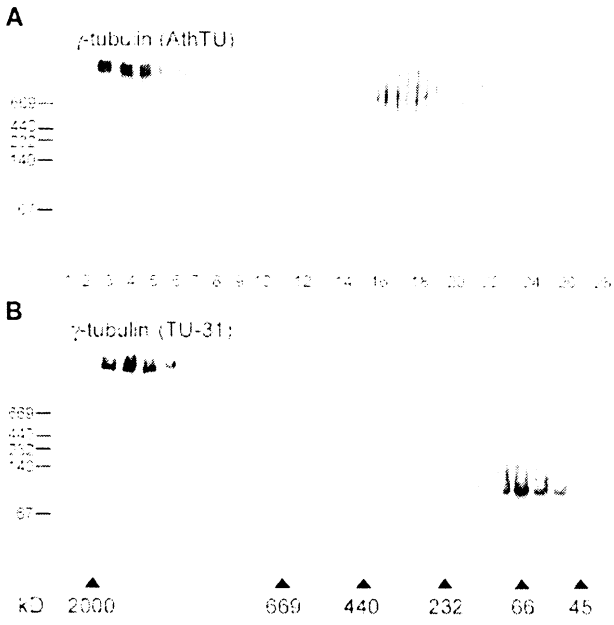


Figure 4. Gel Filtration of Cytoplasmic Extracts Followed by Nondenaturing PAGE Analysis.

Arabidopsis extracts were subjected to gel filtration chromatography in the presence of 500 mM NaCl. Fractions were analyzed by 3 to 10% native PAGE and immunoblotted with antibodies AthTU **(A)** and TU-31 **(B)** against γ-tubulin. Molecular mass standards (in kD) are indicated with arrowheads. Numbers from 1 to 28 denote individual fractions.

cytosolic and membrane fractions is shown; in Figure 5B, the same amount of protein was loaded from each fraction. After low-speed centrifugation, γ-tubulin was present in the pellet (P4) as well as in the supernatant (S4). After further spinning (27,000*g* for 60 min) of the supernatant (S4), a smaller amount of γ-tubulin was detected in the pellet (P27) containing mainly mitochondria, plastids, and larger microsomes. The majority of γ-tubulin remained in the supernatant (S27), from which a high-speed microsomal pellet (P100) was obtained by further centrifugation (100,000*g* for 60 min). P100 contained small vesicles with diameters of ~100 to 300 nm. The composition of P27 and P100 was checked by electron microscopy (data not shown).

As shown in Figure 5A, ~50% of γ-tubulin from supernatant S27 was spun down to pellet P100. γ-Tubulin was solubilized almost completely from P100 by the detergent 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid (CHAPS) (Figures 5A and 5B, P100 S_{CH} for solubilized membrane proteins and P100 P_{CH} for insoluble membrane material). The detergents CHAPS and β-D-lauryl maltoside solubilized almost all γ-tubulin from the microsomal pellet (P100), whereas other detergents (0.1% Triton X-100 or 0.1% Nonidet P-40) were less effective. Treatment of the pellet (P100) with Na_2CO_3, pH 11, used to strip extrinsic or absorbed proteins, was much less efficient in the solubilization of γ-tubulin than the detergents used (data not shown). With TU-31 antibody, the pattern of fractionation of the 56-kD γ-tubulin was similar to that seen with AthTU antibody. However, the 60-kD protein detected by TU-31 was enriched in high-speed supernatant (S100), and only a very small amount of this protein was found in the microsomal membrane fraction (P100 S_{CH}) (Figure 5B, TU-31). This result indicates a different subcellular localization of the 56-kD γ-tubulin and the 60-kD protein. Homogenization with liquid nitrogen and in cold buffer using a prechilled blender gave the same results, excluding the effect of the homogenization procedure on γ-tubulin distribution.

The distribution of tubulin dimers among fractions was similar to that of γ-tubulin, and α- and β-tubulin were solubilized together with γ-tubulin from the microsomal fraction (Figure 5A). On the other hand, the small GTPase Ran, an abundant cytosolic protein used as a control, was found only in the soluble cytosolic fraction but not in the fraction containing microsomal membranes (Figure 5A, Ran). We conclude that γ-tubulin and αβ-tubulin dimers are associated with plant membranes, mainly in the high-speed microsomal fraction.

Blue Native PAGE Confirms That γ-Tubulin Is Associated with Membranes in the Form of Large Protein Complexes

Nondenaturing blue native PAGE (BN-PAGE) was developed for the isolation of membrane-associated protein complexes (Caliebe et al., 1997). To further examine the association of γ-tubulin with membranes, samples from cell fractionation were subjected to BN-PAGE in the first dimension and SDS-PAGE in the second dimension. Antibody AthTU strongly reacted with material electrophoretically separated from the supernatant (S27) with protein complexes of >1 MD (Figure 6, S27). Intermediate complexes also were detected, and there was no γ-tubulin in the monomeric form. Reprobing of membranes with anti-α-tubulin antibody revealed a large distribution of α-tubulin, with molecular masses corresponding to large complexes and to monomers (Figure 6, S27). A similar distribution was found for β-tubulin (data not shown). Further fractionation of S27 to the high-speed supernatant and pellet showed that smaller γ-tubulin complexes remained in the supernatant (Figure 6, S100). The majority of large γ-tubulin complexes were pulled down to the high-speed microsomal pellet, from which they were solubilized by lauryl maltoside (Figure 6, $P100S_L$). α-Tubulin (Figure 6, $P100S_L$) and β-tubulin (data not shown) also were detected in the large membrane-associated complexes. Similar data were obtained regardless of the differing extraction procedures (frozen cells ground in liquid nitrogen or fresh cells ground with a blender) or detergents (CHAPS or lauryl maltoside) used for solubilization of the membrane protein. These data indicate that stable large

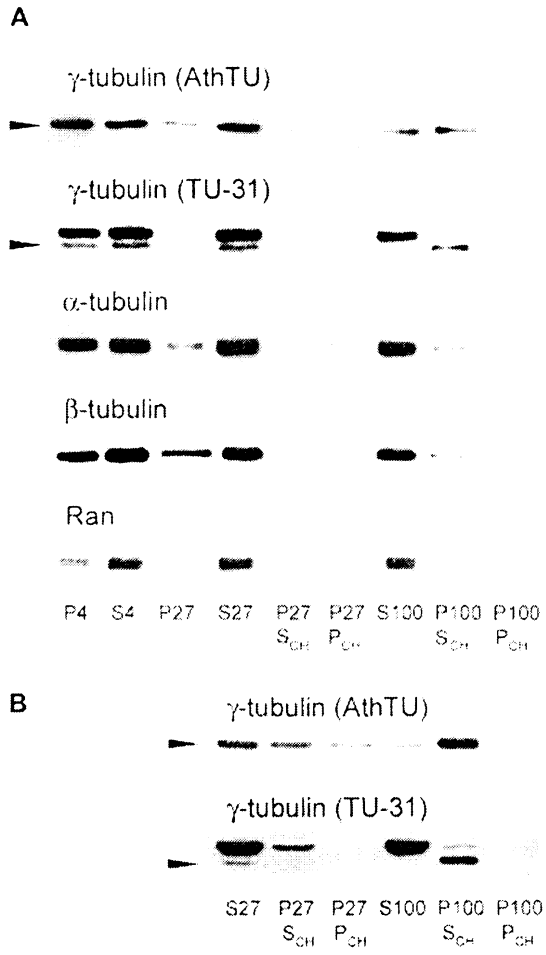


Figure 5. Distribution of γ-Tubulin in Cytoplasmic and Membrane Fractions.

Cell extracts from Arabidopsis were differentially centrifuged, and pellets were extracted with CHAPS.
(A) To compare the relative distribution of immunoblotted proteins, pelleted material was resuspended in a volume equal to the volume of the corresponding supernatant.
(B) Samples loaded at the same protein content (10 μg/lane).
Samples were analyzed by 10% SDS-PAGE and immunoblotted with antibodies AthTU and TU-31 against γ-tubulin, DM1A against α-tubulin, TUB 2.1 against β-tubulin, and anti-Ran antibody. P4 and S4, low-speed pellet and supernatant (4000*g* for 10 min); P27 and S27, pellet and supernatant after centrifugation of the S4 supernatant (27,000*g* for 1 h); P27 S_{CH} and P27 P_{CH}, supernatant and pellet after solubilization of the P27 pellet with CHAPS; S100, high-speed supernatant after centrifugation of the S27 supernatant (100,000*g* for 1 h); P100 S_{CH} and P100 P_{CH}, supernatant and pellet after solubilization of the P100 pellet with CHAPS. Arrowheads indicate the position of the 56-kD γ-tubulin.

γ-tubulin complexes containing tubulin dimers are associated with microsomal membranes.

Large γ-Tubulin Complexes Are Potent Microtubule Nucleators

The functional assay–cover slip nucleation assay (Oegema et al., 1999), developed originally to test the nucleation activity of γ-tubulin complexes in *Drosophila*, was modified and used to determine the microtubule nucleation activity of fractions from gel filtration of the Arabidopsis cell extract. Purified bovine brain tubulin at a concentration of 0.5 mg/mL, which was shown to be subcritical for spontaneous tubulin polymerization, was used in all nucleation assays. The results are shown in Figure 7, in which all of the micrographs were made under the same exposure conditions. Maximal tubulin polymerization was observed with fractions 3 to 4,

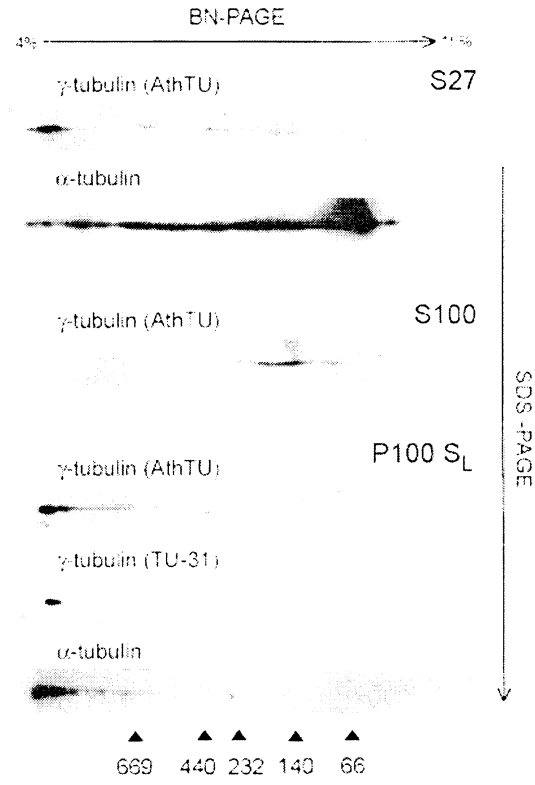


Figure 6. BN-PAGE of Cell Extracts and Solubilized Microsomes.

S27, S100, and lauryl maltoside–solubilized microsomal proteins (P100 S_L), as described in the legend to Figure 5, were separated in the first dimension by 4 to 15% BN-PAGE and in the second dimension by 10% SDS-PAGE. Separated proteins were immunoblotted with antibodies AthTU and TU-31 against γ-tubulin and DM1A against α-tubulin. Molecular mass standards (in kD) for the first dimension are indicated with arrowheads at bottom.

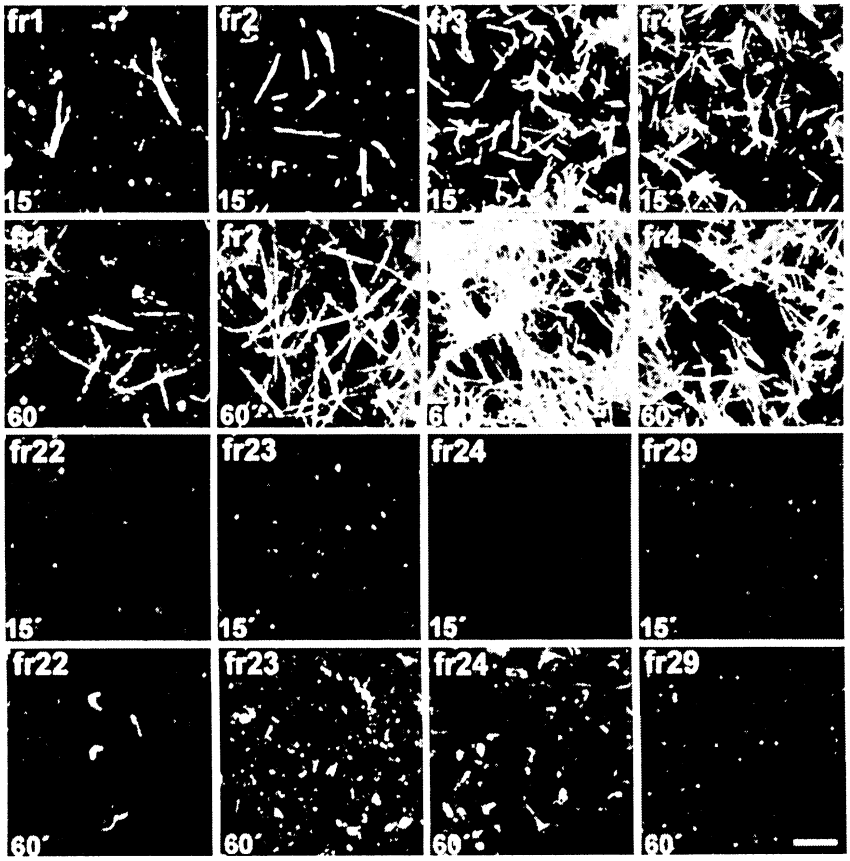


Figure 7. Analysis of Fractions from Gel Filtration Using the Cover Slip Nucleation Assay.

Selected fractions from gel filtration of Arabidopsis (S27) extracts were used for the cover slip microtubule nucleation assay performed as described in Methods. The top two rows represent fractions containing large γ-tubulin complexes (fr1 to fr4) after incubation with brain tubulin for 15 min (15′) or 60 min (60′). The bottom two rows represent fractions containing small γ-tubulin complexes (fr22 to fr24) and a control without γ-tubulin (fr29) after incubation with brain tubulin for 15 min (15′) or 60 min (60′). Microtubules were visualized by immunofluorescence microscopy with anti-α-tubulin antibody DM1A. Equivalent exposures are presented. Bar = 10 μm.

corresponding to the large γ-tubulin complexes. Microtubules were found after 15 min of polymerization, and their number increased after 1 h of polymerization (Figure 7, top two rows). No microtubule polymerization was observed with fractions 22 to 24, which contained γ-tubulin in the form of smaller complexes (Figure 7, bottom two rows), or with fraction 29, in which γ-tubulin was not detected and which served as a negative control. Data from several independent nucleation experiments with gel filtration samples from Arabidopsis and fava bean cell extracts confirmed that the number of microtubules formed in the presence of fractions 2 to 5 correlated with the amount of γ-, α-, and β-tubulins immunodetected in these fractions. Little (or no) nucleation activity was observed when fractions from gel filtration of high-speed supernatants (S100), which were largely depleted in large γ-tubulin complexes, were tested in the assays.

Coprecipitation of γ-Tubulin with αβ-Tubulin Dimers

Because our data showed a codistribution of tubulin dimers with γ-tubulin during fractionation procedures, we tried to determine whether γ-tubulin in the soluble cytoplasmic pool interacts with tubulin dimers. As shown in Figure 1C, immunoprecipitation of γ-tubulin from Arabidopsis extracts with AthTU antibody followed by specific elution with the immunizing peptide revealed that a 56-kD protein was recognized by both TU-31 and AthTU antibodies. γ-Tubulin (56 kD) was precipitated specifically from Arabidopsis extracts with immobilized anti-γ-tubulin antibody TU-31, as demonstrated by the staining of blots with the plant-specific anti-γ-tubulin antibody AthTU (Figure 8A, lane 5). When the immobilized antibody was incubated without extract, no staining in the position of γ-tubulin was observed and no binding of γ-tubulin to immobilized protein L was detected (Figure 8A,

lanes 4 and 6). The control antibody NF-09 gave no precipitation of γ-tubulin (Figure 8A, lane 3). The faint staining in lanes 2 to 5 of Figure 8A represents the heavy chains of immunoglobulins. When the TU-31–precipitated material was probed with mouse antibodies against tubulin subunits, the heavy chains of mouse IgG obscured the detection of coprecipitated tubulin dimers. Therefore, immobilized anti-β-tubulin antibody TU-06 (IgM) was used, which specifically precipitated tubulin dimers, as documented by staining with anti-α-tubulin antibody (Figure 8B, α-tubulin, lane 2). No binding of α-tubulin to immobilized protein L was detected (Figure 8B, α-tubulin, lane 4).

Probing of the immunoprecipitate with anti-β-tubulin antibody confirmed the presence of β-tubulin subunits (data not shown). Probing of the immunoprecipitate with anti-γ-tubulin antibody (TU-31) revealed that the precipitated αβ-tubulin dimers also contained γ-tubulin (Figure 8B, TU-31, lane 2). Closer inspection of the immunoblots revealed that only the 56-kD protein was coprecipitated. The same band was stained with the anti-γ-tubulin antibody AthTU. When the immobilized antibody was incubated without extract, no staining in the position of γ-tubulin was observed (Figure 8B, TU-31, lane 3). Control antibody VI-10 (IgM) gave no precipitation of γ-tubulin and tubulin dimers (data not shown). Coprecipitation of γ-tubulin with tubulin dimers also was confirmed on extracts prepared from fava bean (Figure 8C). These results strongly suggest that tubulin dimers can interact directly or indirectly with γ-tubulins in plant extracts.

γ-Tubulin Cosedimented with Plant Microtubules Polymerized in Vitro and Was Localized along Their Entire Length

After showing that γ-tubulin interacts with αβ-tubulin dimers, we wanted to elucidate the interaction of γ-tubulin with polymerized microtubules in a spin-down assay we developed previously (Weingartner et al., 2001). Pelleted microtubules, polymerized from Arabidopsis and fava bean extracts, were analyzed for the presence of α-, β-, and γ-tubulin. Because the data for microtubules polymerized from S27 or S100 were similar, only data for S100 are shown. Both of the anti-γ-tubulin antibodies revealed an association of γ-tubulin (56 kD) with polymerized microtubules, as shown for Arabidopsis (Figure 9A, lanes 2 and 3). The 60-kD protein recognized by TU-31 also was associated with microtubules, but compared with γ-tubulin (56 kD), it was not enriched in the microtubular pellet. Control extracts without taxol or DMSO did not provide a tubulin pellet, which excluded a possible nonspecific sedimentation of clustered tubulins (Figure 9A, lanes 4 and 5). The absence of abundant cytoplasmic Ran GTPase in sedimented microtubules (Figure 9A) confirmed the affinity association of γ-tubulin with microtubules.

Because the supernatant S100 contained only a minor part of the large γ-tubulin complexes and no monomeric γ-tubulin (Figure 6), it is likely that soluble smaller γ-tubulin complexes or γ-tubulin oligomers were bound to microtubules. When microtubules were polymerized on slides and, after extensive washing, immunolabeled with anti-γ-tubulin antibodies, both antibodies stained microtubules in a dot-like manner along their entire length (Figure 9B, AthTU and TU-31). The staining pattern was similar to the punctuate staining of γ-tubulin observed along microtubules in cells (Figure 2) (Liu et al., 1993). No such staining was observed when preimmune serum was used as a negative control (Figure 9B, preimmune serum). These results suggest that γ-tubulin or its forms laterally associate with plant microtubules polymerized in vitro.

DISCUSSION

Antibody Tools

The newly prepared polyclonal anti-peptide antibody AthTU against the Arabidopsis γ-tubulin sequence recognizes, on immunoblots of various plant species, a single band in the range 56 to 58 kD. In Arabidopsis, all anti-γ-tubulin antibodies we have used (AthTU, TU-31, and N38-53) stained a 56-kD protein. It is 2 to 3 kD larger than the predicted molecular masses for two Arabidopsis-expressed γ-tubulin genes (53.3 and 53.4 kD), as calculated from their amino acid sequences. γ-Tubulin might run on gels in positions corresponding to relative molecular masses greater than those predicted previously (Ovechkina and Oakley, 2001). Because of this fact, the determination of whether the protein recognized with anti-γ-tubulin antibodies is γ-tubulin or another protein containing the recognized epitope is crucial for further experimental work. Our immunoprecipitation experiments provide direct evidence that the antibodies AthTU and TU-31 recognized the identical 56-kD γ-tubulin protein in Arabidopsis. Moreover, staining of the 56-kD γ-tubulin with AthTU antibody was abolished by preabsorption of the antibody with γ-tubulin peptides used to generate AthTU and/or TU-31 antibodies.

The TU-31 antibody reacted in Arabidopsis extracts not only with the 56-kD protein but also with a 60-kD immunoreactive protein, and corresponding very close bands also were immunodetected in other plant species. The fact that the staining of both protein bands in Arabidopsis was abolished after preabsorption of the TU-31 antibody with each of the two immunizing peptides suggests that the epitope recognized on the SDS-denatured 60-kD protein by TU-31 is similar to or identical with the phylogenetically conserved γ-tubulin amino acid sequence from the C-terminal region of the molecule. A database search showed that no other proteins containing the peptide sequence used to generate the TU-31 antibody or a peptide close to it are found in the Arabidopsis genome. Both the 56-kD γ-tubulin and the 60-kD protein were spun down with microtubules polymerized

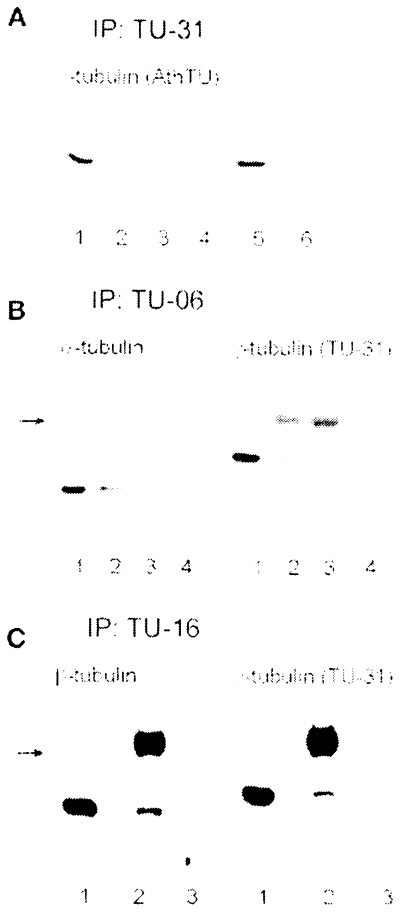


Figure 8. Immunoprecipitation of Cell Extracts with Anti-Tubulin Antibodies.

Proteins were separated by 7.5% SDS-PAGE and immunoblotted. IP, immunoprecipitant.

(A) Immunoblots of Arabidopsis cell extracts precipitated by monoclonal anti-γ-tubulin antibody TU-31 and immunoblotted with polyclonal anti-γ-tubulin antibody AthTU. Lane 1, proteins remaining after precipitation; lane 2, NF-09 antibody (negative control) not incubated with the protein extract; lane 3, immunoprecipitated proteins with NF-09 antibody (negative control); lane 4, TU-31 antibody not incubated with the protein extract; lane 5, immunoprecipitated proteins with TU-31 antibody; lane 6, proteins bound to protein L without antibody.

(B) Immunoblots of Arabidopsis cell extracts precipitated by monoclonal anti-β-tubulin antibody TU-06 and immunoblotted with antibodies TU-01 against α-tubulin and monoclonal antibody TU-31 against γ-tubulin. Lane 1, proteins remaining after precipitation; lane 2, immunoprecipitated proteins; lane 3 immobilized immunoglobulin not incubated with protein extract; lane 4, proteins bound to protein L without antibody. The arrow indicates the position of the IgM heavy chain of the precipitating antibody.

(C) Immunoblots of fava bean extracts precipitated by monoclonal anti-α-tubulin antibody TU-16 and immunoblotted with TUB 2.1 against β-tubulin and anti-γ-tubulin antibody TU-31. Lane 1, proteins remaining after precipitation; lane 2, immunoprecipitated proteins; lane 3, proteins bound to protein L without antibody. The arrow indicates the position of the IgM heavy chain of the precipitating antibody.

from Arabidopsis extracts, and the immunofluorescence staining patterns with TU-31 and AthTU in cells were very similar. Therefore, one cannot exclude completely the possibility that the antibody recognizes two γ-tubulin forms. There are two highly homologous γ-tubulin genes present in the Arabidopsis genome database, but they have identical C-terminal sequences. On the other hand, the 60-kD protein might represent a post-translational modified form of γ-tubulin that has different electrophoretic mobility.

Forms of γ-tubulin differing in their electrophoretic mobility have been distinguished in *Physarum* (Lajoie-Mazenc et al., 1996), *Drosophila* (Raynaud-Messina et al., 2001), brain cells (Sulimenko et al., 2002), and plants (Petitpren et al., 2001). In the latter study, it was found that the 58-kD form was present in all sunflower tissues tested and also was associated with the nucleus, whereas the smaller γ-tubulin (52-kD) form was present only in meristematic and dedifferentiated cells. The subcellular distribution of the 60-kD protein differed from that of the 56-kD γ-tubulin in our experiments as well. Its amount was very limited in the high-speed microsomal fraction; consequently, it was not detected in large γ-tubulin complexes in microsomes. Instead, the 60-kD protein was abundant in the high-speed supernatant in the form of small (120- to 220-kD) complexes or oligomers. The 60-kD protein was not coprecipitated with 56-kD γ-tubulin using AthTU and/or anti-β-tubulin antibody. Therefore, the characteristics of the putative two forms of γ-tubulin (56 and 60 kD) are different. Alternatively, the 60-kD protein represents a protein, sharing the same sequencing epitope as γ-tubulin, that is associated with microtubules. Further protein purification and sequencing is needed for conclusive protein identification.

Heterogeneity of γ-Tubulin Complexes

The presence of γ-tubulin in the form of protein complexes of various sizes in fava bean extracts has been postulated previously (Binarova et al., 2000). Gel filtration analysis, nondenaturing PAGE, and BN-PAGE of the cytosolic fraction from Arabidopsis and fava bean followed by detection with plant-specific antibody revealed the presence of large γ-tubulin complexes (>1 MD) and intermediate-sized complexes. Similar large and intermediate γ-tubulin protein complexes have been reported in different organisms, including fungi (Akashi et al., 1997) and plants (Stoppin-Mellet et al., 2000). The well-characterized large γ-tubulin complexes in animal

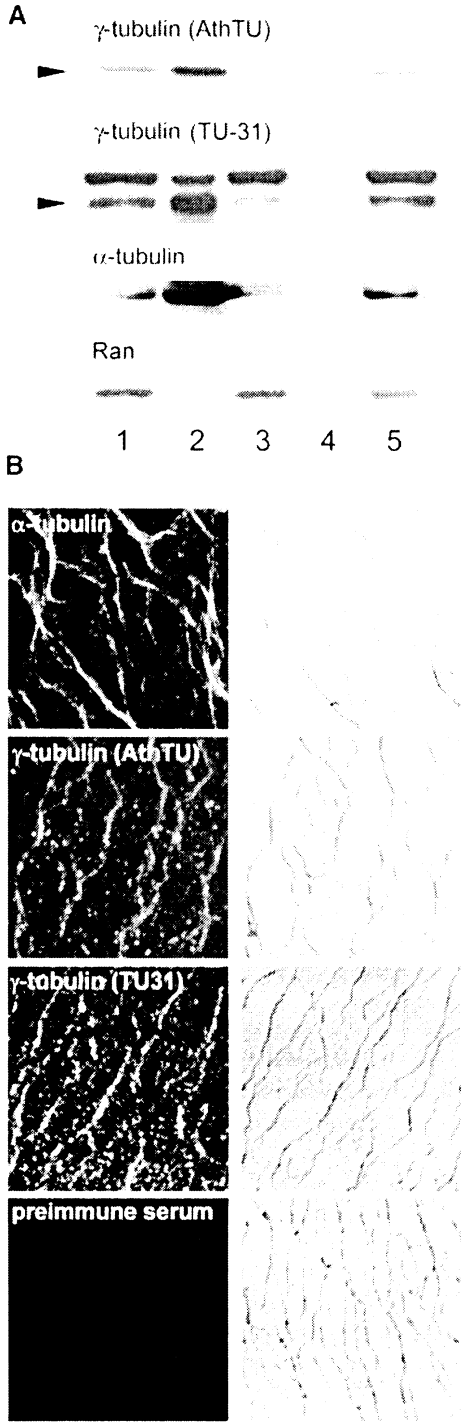


Figure 9. Association of γ-Tubulin with in Vitro–Polymerized Microtubules.

(A) Microtubules were prepared from Arabidopsis extracts (S100) by taxol-driven polymerization and analyzed by immunoblotting with antibodies AthTU and TU-31 against γ-tubulin, DM1A against α-tubulin, and anti-Ran antibody (control). Lane 1, extract; lane 2, microtubule pellet; lane 3, supernatant after taxol polymerization; lanes 4 and 5, as in lanes 2 and 3 but without taxol. Arrowheads indicate the position of the 56-kD γ-tubulin.

(B) Immunofluorescence staining of polymerized microtubules with antibodies AthTU and TU-31 against γ-tubulin, DM1A against α-tubulin, and preimmune serum (control). Slides were observed by fluorescence microscopy (left) and differential interference contrast (right). Bar = 10 μm.

models are γ-TuRCs, with a molecular mass of 2.2 MD (Zheng et al., 1998; Oegema et al., 1999). In contrast to the reported salt sensitivity of γ-TuRC, large γ-tubulin complexes of Arabidopsis and fava bean did not dissociate to smaller complexes in the presence of 500 mM NaCl. Cover slip nucleation assays revealed that only fractions with large complexes affect nucleation activity strongly, whereas salt-sensitive intermediate and smaller complexes basically had no effect on microtubule nucleation. In this respect, large complexes resemble the animal γ-TuRCs, which are the most potent soluble microtubule nucleators (Oegema et al., 1999), whereas variable intermediate-sized plant complexes might present another functional type of tubulin complex.

Association of γ-Tubulin Complexes with Membranes

Solubilization of the microsomal fraction with detergents and BN-PAGE demonstrated that γ-tubulin was associated with membranes in the form of large complexes (>1 MD). Coomassie blue and aminocaproic acid used during BN-PAGE made it possible to estimate the molecular mass of detergent-solubilized, membrane-bound γ-tubulin complexes while avoiding the problem of detergent interference, which appeared during gel filtration of solubilized membrane proteins. Although γ-tubulin itself does not have a consensus membrane binding motif, it is possible that other proteins of the complex mediate its association with membranes. Our data indicate that αβ-tubulin dimers are associated with large membrane-bound γ-tubulin complexes. Interestingly, palmitoylation of the α-tubulin molecule has been described (Caron, 1997). Moreover, an association of γ-tubulin with the protein Tyr kinase p53/p56^{lyn}, whose significant fraction is located in membrane microdomains, has been reported (Draberova et al., 1999b).

Nuclear and cortical membranes are believed to be sites of microtubule nucleation, and studies of both green fluorescent protein–tubulin dynamics (Kumagai et al., 2001) and γ-tubulin immunolocalization (Vaughn and Harper, 1998) support this hypothesis. The plant homolog of Spc98p, a protein that interacts with γ-tubulin in complexes in a broad range of eukaryotes, colocalizes with γ-tubulin on the nuclear envelope in tobacco. Antibodies against Spc98p and

γ-tubulin decreased the ability of isolated nuclei to nucleate purified brain tubulin. However, no direct biochemical evidence for the presence of an Spc98p homolog in plant γ-tubulin complexes was provided (Erhardt et al., 2002). Our results show that large membrane-associated γ-tubulin complexes are active in microtubule nucleation. Also in accordance with biochemical and functional data are our microscopic observations showing that γ-tubulin associated with membranes. The accumulation of γ-tubulin–decorated vesicle-like structures at the spindle anchoring sites on poles in mitosis, in the area of the phragmoplast in cytokinesis, suggests γ-tubulin interaction with membranes. The localization of Golgi membranes in the vicinity of γ-tubulin–decorated spindle poles and results from brefeldin A treatment indicated that correct endomembrane trafficking is a prerequisite for spindle pole organization, and the interaction of γ-tubulin with membranes might be involved directly or indirectly in this process.

In acentriolar early mouse oocytes, γ-tubulin–positive membranous aggregates containing a variety of vesicular structures are suggested to be the centrosomal precursors of a unique ultrastructure (Calarco, 2000). It is possible that similar membrane vesicular structures containing γ-tubulin complexes exist in acentrosomal plant cells. The majority of large γ-tubulin complexes with nucleation activity were present in the high-speed microsomal fraction in which some of the small vesicles were immunogold labeled for γ-tubulin (electron microscopy data not shown). Further characterization of membranous vesicles is in progress. Recently emerging data show that membranous organelles are not organized solely in a passive manner by the cytoskeleton but participate actively in the organization of cytoskeleton structures. It was reported that Golgi-derived vesicles acted as microtubule-nucleating and -organizing sites, with γ-tubulin participating in the process (Chabin-Brion et al., 2001). In plants, evidence for the presence of a signaling molecule (phospholipase D) associated with membranes and microtubules was provided (Gardiner et al., 2001).

Association of γ-Tubulin with αβ-Tubulin Dimers

The colocalization of γ-tubulin along the entire length of plant microtubules with no preference for possible minus ends and its presence with tubulin paracrystals suggested a more complex role of γ-tubulin related to αβ-tubulins than simple microtubule nucleation (Binarova et al., 1998; Panteris et al., 2000). Our present data on the coprecipitation of αβ-tubulin dimer and γ-tubulin confirmed independently an association of soluble γ-tubulin with tubulin dimers in Arabidopsis and fava bean. This finding might reflect the presence of tubulin dimers in γ-tubulin complexes; alternatively, γ-tubulin could be present in cells in some other forms capable of interacting with αβ-tubulin dimers. Tubulin dimers were not found in the majority of animal γ-TuRCs analyzed (Wiese and Zheng, 1999), but variable amounts of tubulin dimers have been reported to coprecipitate with γ-tubulin in preparations from oocytes (Zheng et al., 1995; Lessman and Kim, 2001) and erythrocytes (Linhartova et al., 2002). γ-Tubulin dimers were identified under natural conditions in HeLa cells (Vassilev et al., 1995), and it was shown that γ-tubulin in the brain could form oligomers (Sulimenko et al., 2002).

Our data indicate that a similar situation might exist in plants. Structural models for self-assembly suggest that γ-tubulin should be capable of self-assembling into dimers or protofilament-like oligomers and of interacting laterally with α- or β-tubulin (Inclan and Nogales, 2001). γ-Tubulin was bound to microtubules polymerized from both low-speed and high-speed supernatant (S100). Because monomeric tubulin as well as the large γ-tubulin complexes are almost completely absent in S100, γ-tubulin most likely is bound to microtubules in the form of small complexes or γ-tubulin oligomers. Dot-like γ-tubulin staining of microtubules along their entire length and its accumulation with microtubular bundles suggest a lateral association of γ-tubulin forms. It was shown that γ-tubulin peptides did not interfere with microtubule assembly in vitro and were associated with microtubules along the polymer length (Llanos et al., 1999).

The massive association of γ-tubulin with kinetochore microtubules in cells with regular mitosis, as well as its presence in the vicinity of kinetochores in monopolar mitosis (Binarova et al., 1998), imply a role for γ-tubulin in the organization of the bipolar spindle. Several mutants of *S. pombe* and *A. nidulans* (Hendrickson et al., 2001; Jung et al., 2001) as well as *Caenorhabditis elegans* with γ-tubulin depleted by RNA interference (Strome et al., 2001) showed that in the absence of γ-tubulin, the function of kinetochore microtubules and anaphase chromosome separation were more affected than microtubule nucleation. Moreover, the overlapping roles of γ-tubulin and kinesin in the organization of kinetochore microtubules and in the establishment of spindle bipolarity were proven genetically in *S. pombe* and *A. nidulans* (Paluh et al., 2000; Prigozhina et al., 2001).

Heterogeneity of γ-Tubulin Forms and Microtubule Organization in Plants

The distribution of γ-tubulin protein complexes among various species might underscore the specific need for an organism to regulate its microtubule dynamics and spindle function. In plants, all somatic and gametic cells are acentrosomal; nuclear and cortical membranes are important sites for organizing the microtubular arrays during cell cycle progression. The large γ-tubulin complexes associated with membranes are potent in vitro nucleators of microtubules. Membranes, being dynamic self-organizing systems, can provide flexible microtubule nucleation activity. More generally, an association of γ-tubulin with membranous structures might ensure the nucleation of microtubule arrays not only in plants but also in other eukaryotic cell types with noncen-

trosomal microtubules. The abundance of γ-tubulin in plant cells and the presence of γ-tubulin in protein complexes of various sizes, different properties, and subcellular locations, as well as γ-tubulin interactions with tubulin heterodimers and the association of γ-tubulin complexes or oligomers with microtubules, all could reflect the specific needs of the plant cells. The precise functions of different γ-tubulin forms in the nucleation and organization of plant microtubules remain to be elucidated.

METHODS

Cells

Seedlings of *Arabidopsis thaliana*, fava bean (*Vicia faba*), pea (*Pisum sativum*), and maize (*Zea mays*) were grown, and whole seedlings or root meristems were collected as described previously (Binarova et al., 2000). Cell suspension cultures of Arabidopsis (ecotype Landsberg *erecta*), described by May and Leaver (1993), were obtained from L. Bogre (Royal Holloway University of London). The sample was cultured in 1× Murashige and Skoog (1962) solution (Sigma) and 3% saccharose supplemented with the growth regulators 1-naphthalene acetic acid (2.5 μM) and kinetine (0.25 μM). Cells were cultured with shaking at 24°C with a 16-h photoperiod and 1-week subculture intervals.

Antibodies

γ-Tubulin was detected with the mouse monoclonal antibody TU-31 (IgG2b) (Novakova et al., 1996) prepared against the conserved 16–amino acid peptide EYHAATRPDYISWGTQ corresponding to the human γ-tubulin sequence 434 to 449. To generate rabbit polyclonal antibody AthTU against the 14–amino acid peptide EYKACESPDYIKWG corresponding to the Arabidopsis γ-tubulin sequence 437 to 450, peptide was coupled to keyhole limpet hemocyanin and antibodies were purified on protein A or affinity purified on peptide-coupled Sulfo-Link beads (Pierce). Polyclonal antibody against human γ-tubulin sequence 38 to 53 (EEFATEGTDRKDVFFYN) from the N-terminal region of the molecule was purchased from Sigma. α-Tubulin and β-tubulin were detected using the mouse monoclonal antibodies DM1A and TUB 2.1 (IgG1) (Sigma). For the precipitation of tubulin dimers, mouse monoclonal antibodies TU-06 (IgM) against β-tubulin (Draber et al., 1989) and TU-16 (IgM) against α-tubulin (Draberova and Draber, 1998) were used. As negative controls for immunoprecipitation experiments, the mouse monoclonal antibodies NF-09 (IgG2a) and VI-10 (IgM) against vimentin were used (Draberova et al., 1999a). Polyclonal anti-Ran antibody was obtained from Babco (Richmond, CA), and monoclonal mouse anti-importin antibody and polyclonal rabbit antibody against the *trans*-Golgi network protein TLG were obtained from Affinity BioReagents (Golden, CO) and Secant Chemicals (Winchendon, MA), respectively. Fluorescein isothiocyanate (FITC)– and indocarbocyanate (Cy3)-conjugated anti-mouse and anti-rabbit antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). The anti-mouse Ig antibody and anti-rabbit antibody conjugated with horseradish peroxidase were purchased from Amersham Biosciences (Uppsala, Sweden) or from Promega (Madison, WI).

Preparation of Protein Extracts and Solubilization of Membrane Proteins

Root meristems, seedlings, and suspension cells were collected, ground in liquid nitrogen, and thawed in 1 to 2 volumes of extraction buffer (50 mM K-Hepes, pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, 100 mM NaCl, 1 mM DTT, and 0.05% [v/v] Nonidet P-40) supplemented with protease [5 μg/mL each of leupeptin, aprotinin, antipain, soybean trypsin inhibitor, and pepstatin and 10 μg/mL 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride] and phosphatase [1 mM NaF, 0.5 mM Na_3VO_4, and 15 mM β-glycerophosphate] inhibitors. Alternatively, plant material was ground in extraction buffer using a prechilled blender. Crude extracts were centrifuged at 4000*g* for 10 min at 4°C to sediment cell walls, nuclei, and cell debris (P4). Supernatants (S4) were centrifuged subsequently at 27,000*g* for 1 h at 4°C to separate cytosolic fractions (S27) from pellet (P27).

The cytosolic fraction (S27) from Arabidopsis cells and fava bean root meristems was centrifuged further at 100,000*g* for 1 h at 4°C to obtain a high-speed microsomal pellet (P100) and a high-speed supernatant (S100). The pellet (P27) and the microsomal membrane fraction (P100) were resuspended in extraction buffer supplemented with one of the following detergents: 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid, 1% β-D-lauryl maltoside, 0.1% Triton X-100, and 0.1% Nonidet P-40. Alternatively, pellets P27 and P100 were treated with 0.1 M Na_2CO_3, pH 11. After 30 min of incubation at 4°C, detergent-insoluble material was centrifuged at 48,000*g* for 20 min at 4°C.

Gel Filtration Chromatography

Gel filtration of cell extracts (fractions S27 or S100) was performed using a fast protein liquid chromatography system with a Superose 12 HR 10/30 column (Amersham Pharmacia). Column equilibration and chromatography were performed in column buffer (50 mM K-Hepes, pH 7.4, 1 mM EGTA, 100 mM NaCl, 2% glycerol, and 1 mM DTT) supplemented with protease and phosphatase inhibitors (see extraction buffer). In some experiments, the column buffer was supplemented with 500 mM NaCl. Fractions (0.25 mL) were collected, and aliquots were used for the preparation of samples for SDS-PAGE or nondenaturing PAGE. The column was calibrated for each particular chromatography condition used. The following molecular mass standards were used: thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD) (Gel Filtration Calibration Kit; Amersham Pharmacia), alcohol dehydrogenase (150 kD), BSA (66 kD), and ovalbumin (45 kD) (Sigma). Dextran blue (Amersham Pharmacia) was used to determine the column void volume.

Electrophoresis and Immunoblotting

Proteins separated by SDS-PAGE (Laemmli, 1970) were transferred onto nitrocellulose membranes by wet electroblotting. Details of the immunostaining procedure are described elsewhere (Draber et al., 1989). Immunoreactive bands were visualized using either the enhanced chemiluminescence detection system (Pierce) or the alkaline phosphatase detection system (Promega).

In some experiments, anti-γ-tubulin antibodies were preabsorbed with the peptides used for immunization. Both human γ-tubulin peptide (EEFATEGTDRKDVFFYN) and Arabidopsis γ-tubulin peptide (EYKACESPDYIKWG) were used for preabsorption of TU-31 and

AthTU antibody. Peptide corresponding to amino acid residues 38 to 53 of human γ-tubulin was used as a negative control. Two molar ratios of antibody to peptide were used: 1:5 and 1:50. Mixtures of antibodies and peptides were incubated for 30 min at room temperature.

Nondenaturing PAGE was performed using the Laemmli system (Laemmli, 1970), except that SDS was omitted completely and electrophoresis was performed at 4°C. Sample buffer consisted of 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, and 0.01% (w/v) bromphenol blue. Proteins were separated on 3 to 10% linear gradient gels with 3% stacking gels. After electrophoresis, proteins were electroblotted onto nitrocellulose for protein gel blot analysis.

Blue native PAGE (BN-PAGE) was performed using a slightly modified method of Schagger and von Jagow (1991). Microsomal membranes were resuspended in solubilization buffer (50 μM bis-Tris-HCl, pH 7.0, 750 mM aminocaproic acid, and 1% dodecyl-β-D-maltoside). After 20 min of centrifugation at 48,000*g*, the supernatants were supplemented with Coomassie Brilliant Blue G to a final concentration 0.25% and applied to 4 to 15% gradient polyacrylamide gels. In two-dimensional BN-PAGE/SDS-PAGE, strips of lines were cut, soaked in five-times-concentrated SDS sample buffer for 5 min, and mounted on 10% denaturing SDS gels for separation in the second dimension. After electrophoresis, proteins were electroblotted to nitrocellulose. For nondenaturing PAGE and BN-PAGE, the High Molecular Mass Calibration Kit (Amersham Pharmacia) was used.

Immunoprecipitation and Peptide Elution

The supernatant (S27) at a total protein concentration of 3 mg/mL was precleaned using protein A–agarose beads (Pierce) to reduce the nonspecific binding of proteins. Beads saturated with affinity-purified AthTU antibody were washed three times with washing buffer (extraction buffer supplemented with 150 mM NaCl), added to the precleaned extract (50 μL/mL extract), and incubated with rocking for 2 h at 4°C. After washing five times for 10 min with washing buffer, the beads were incubated with immunogenic peptide (1 mg/mL washing buffer) with rocking for 18 h at 4°C to elute γ-tubulin. As a control, immunoglobulins from preimmune serum, or irrelevant antibodies instead of AthTU antibody, were used.

Immunoprecipitation with TU-31 and anti-α-tubulin and anti-β-tubulin antibodies was performed according to Draberova and Draber (1993). Cell extracts (S27) were incubated with beads of protein L (Pierce) saturated with anti-γ-tubulin antibody TU-31, anti-α-tubulin antibody TU-16, anti-β-tubulin antibody TU-06, negative control IgM antibody VI-10, negative control IgG antibody NF-09, or protein L alone. Antibodies were used in the form of 10-times-concentrated spent culture supernatants from hybridoma cells. Sedimented beads (50 μL) with immobilized protein L were incubated with rocking at 4°C for 2 h with 1 mL of corresponding antibody in 0.4 mL of concentrated supernatant (mixed with 0.8 mL of TBST: 10 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20). The beads were pelleted, washed four times in TBST, and incubated further with rocking for 3 h at 4°C with 0.5 mL of cell extract diluted 1:1 with TBST. The beads were pelleted and washed four times for 5 min each before boiling for 5 min in 100 μL of SDS sample buffer to release the bound proteins.

Cover Slip Nucleation Assay

A modified cover slip nucleation assay (Oegema et al., 1999) was used to test the microtubule nucleation activity of fractions from gel filtration. Poly-L-Lys–coated cover slips were blocked for 5 min with 1% BSA in blocking buffer (50 mM K-Hepes, 100 mM KCl, 1 mM $MgCl_2$, and 1 mM EGTA, pH 7.6). Blocking solution was replaced with 20 μL of samples (fractions from gel filtration), and after 10 min, the cover slips were washed with buffer BrB80 (80 mM K-Pipes, 1 mM $MgCl_2$, and 1 mM EGTA) supplemented with 10% glycerol and 1 mM GTP. Samples were incubated with 0.5 mg/mL purified bovine brain tubulin (Molecular Probes, Leiden, The Netherlands) in BrB80 buffer. Spontaneous polymerization of purified tubulin on slides without tested samples occurred at tubulin concentrations of 1.5 mg/mL and higher. Therefore, a subcritical tubulin concentration of 0.5 mg/mL was used in all nucleation assays. Cover slips with the samples to be tested attached were incubated for 15 or 60 min; then, the tubulin was removed by aspiration and replaced with 1% glutaraldehyde for 3 min, followed 5 min after fixation by −20°C methanol. The cover slips were rehydrated, and the microtubules were visualized by indirect immunofluorescence with the anti-α-tubulin antibody DM1A.

Microtubule Sedimentation Assay

The microtubule cosedimentation experiments were performed as described previously (Weingartner et al., 2001) with slight modifications. Arabidopsis cells or fava bean root meristems were homogenized in BrB80 buffer and supplemented with protease and phosphatase inhibitors, as described for the extraction buffer. Polymerizations were performed from both the S27 and the high-speed extracts (S100). GTP was added to 1 mM, and taxol was added to 20 μM. Alternatively, DMSO at a final concentration 7.5% was used instead of taxol. After 15 min of polymerization at 37°C, the extracts were loaded onto a 40% Suc cushion in BrB80 buffer and spun down. Microtubules were washed twice by the resuspension of pellets in 10 volumes of BrB80 buffer supplemented with GTP and taxol followed by centrifugation. In control experiments, taxol or DMSO was omitted from the reaction mixture. Washed microtubules were resuspended in SDS sample buffer. For immunofluorescent visualization of in vitro–prepared microtubules, the polymerization mixture was laid on cover slips coated with poly-L-Lys and blocked by BSA, as described for the cover slip nucleation assays. After 15 min of polymerization at room temperature, the excess extracts were removed by aspiration and the cover slips were washed extensively in BrB80 buffer. Samples then were processed for immunofluorescence examination as described for the cover slip nucleation assays.

Immunofluorescence

Root tips or cultured cells were fixed for 1 h in 3.7% paraformaldehyde and processed for immunofluorescence as described previously (Binarova et al., 1993). In some experiments, seedlings were treated with brefeldin A at 200 $\mu g \cdot ml^{-1}$ for 30 min before root tip fixation and immunolabeling. A stock solution of brefeldin A (10 mg/mL) in DMSO was used. Affinity-purified antibody AthTU was used at a dilution 1:100, antibody DM1A was used at a dilution 1:1000, and antibody TU-31 was used as an undiluted supernatant. For double-label immunofluorescence with rabbit and mouse monoclonal antibodies, slides were incubated with the polyclonal antibody, washed, and incubated with the monoclonal antibody. Samples then were incubated simultaneously with a mixture of anti-mouse FITC-conjugated and anti-rabbit Cy3-conjugated secondary antibodies. After 4′,6-diamidino-2-phenylindole staining of DNA and mounting, slides were examined with a confocal laser scanning microscope (TCS/SP;

Leica, Wetzlar, Germany). Laser scanning was performed using the sequential multitrack mode to avoid bleed through. Excitation and emission wavelengths were 488 nm and 505 to 532 nm for FITC and 543 nm and 566 to 600 nm for Cy3. Alternatively, a Provis AX70 optical microscope (Olympus, Tokyo, Japan) equipped with a 100 × 1.4 oil-immersion objective and a Sensi Cam cooled charge-coupled device camera (Kelheim, Germany) coupled with Micro Image Olympus optical software were used. To avoid filter cross-talk, fluorescence was detected using HQ 480/40 exciter and HQ 510/560 emitter filter cubes for FITC and HQ 545/30 exciter and HQ 610/75 emitter filter cubes for Cy3 (both AHF Analysen Technique, Tubingen, Germany). Digital images were processed using Adobe Photoshop 5.5 (Adobe Systems, San Jose, CA).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

ACKNOWLEDGMENTS

The authors thank Laczlo Bogre for the gift of the cell suspension of Arabidopsis. This work was supported in part by Grant LN00A081 from the Ministry of Education of the Czech Republic and Wellcome Trust Collaborative Research Initiative Grant 067411/Z/02/Z to P.B., by Grant LN00A026 from the Ministry of Education of the Czech Republic to P.D., and by a postdoctoral grant (204/02/D06S) from the Grant Agency of the Czech Republic to V.C.

Received August 21, 2002; accepted October 29, 2002.

REFERENCES

Akashi, T., Yoon, Y., and Oakley, B.R. (1997). Characterization of gamma-tubulin complexes in *Aspergillus nidulans* and detection of putative gamma-tubulin interacting proteins. Cell Motil. Cytoskeleton **37,** 149–158.

Binarova, P., Cenklova, V., Hause, B., Kubatova, E., Lysak, M., Dolezel, J., Bogre, L., and Draber, P. (2000). Nuclear gamma-tubulin during acentriolar plant mitosis. Plant Cell **12,** 433–442.

Binarova, P., Cihalikova, J., and Dolezel, J. (1993). Localization of MPM-2 recognized phosphoproteins and tubulin during cell cycle progression in synchronized *Vicia faba* root meristem cells. Cell Biol. Int. **17,** 847–856.

Binarova, P., Dolezel, J., Draber, P., Heberle-Bors, E., Strnad, M., and Bogre, L. (1998). Treatment of *Vicia faba* root tip cells with specific inhibitors to cyclin-dependent kinases leads to abnormal spindle formation. Plant J. **16,** 697–707.

Calarco, P.G. (2000). Centrosome precursors in the acentriolar mouse oocyte. Microsc. Res. Tech. **49,** 428–434.

Caliebe, A., Grimm, R., Kaiser, G., Lubeck, J., Soll, J., and Heins, L. (1997). The chloroplastic protein import machinery contains a Rieske-type iron-sulfur cluster and a mononuclear iron-binding prótein. EMBO J. **16,** 7342–7350.

Caron, J.M. (1997). Posttranslational modification of tubulin by palmitoylation. I. In vivo and cell-free studies. Mol. Biol. Cell **8,** 621–636.

Chabin-Brion, K., Marceiller, J., Perez, F., Settegrana, C., Drechou, A., Durand, G., and Pous, C. (2001). The Golgi complex is a microtubule-organizing organelle. Mol. Biol. Cell **12,** 2047–2060.

Dibbayawan, T.P., Harper, J.D., and Marc, J. (2001). A gamma-tubulin antibody against a plant peptide sequence localises to cell division-specific microtubule arrays and organelles in plants. Micron **32,** 671–678.

Draber, P., Draberova, E., Linhartova, I., and Viklicky, V. (1989). Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies. J. Cell Sci. **92,** 519–528.

Draberova, E., and Draber, P. (1993). A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules. J. Cell Sci. **106,** 1263–1273.

Draberova, E., and Draber, P. (1998). Novel monoclonal antibodies TU-08 and TU-16 specific for tubulin subunits. Folia Biol. **44,** 35–36.

Draberova, E., Zikova, M., and Draber, P. (1999a). Monoclonal antibody VI-10 specific for vimentin. Folia Biol. **45,** 35–36.

Draberova, L., Draberova, E., Surviladze, Z., and Draber, P. (1999b). Protein tyrosine kinase p53/p56(lyn) forms complexes with gamma-tubulin in rat basophilic leukemia cells. Int. Immunol. **11,** 1829–1839.

Erhardt, M., Stoppin-Mellet, V., Campagne, S., Canaday, J., Mutterer, J., Fabian, T., Sauter, M., Muller, T., Peter, C., Lambert, A.M., and Schmit, A.C. (2002). The plant Spc98p homologue colocalizes with gamma-tubulin at microtubule nucleation sites and is required for microtubule nucleation. J. Cell Sci. **115,** 2423–2431.

Gardiner, J.C., Harper, J.D., Weerakoon, N.D., Collings, D.A., Ritchie, S., Gilroy, S., Cyr, R.J., and Marc, J. (2001). A 90-kD phospholipase D from tobacco binds to microtubules and the plasma membrane. Plant Cell **13,** 2143–2158.

Geissler, S., Pereira, G., Spang, A., Knop, M., Soues, S., Kilmartin, J., and Schiebel, E. (1996). The spindle pole body component Spc98p interacts with the gamma-tubulin-like Tub4p of *Saccharomyces cerevisiae* at the sites of microtubule attachment. EMBO J. **15,** 3899–3911.

Hendrickson, T.W., Yao, J., Bhadury, S., Corbett, A.H., and Joshi, H.C. (2001). Conditional mutations in gamma-tubulin reveal its involvement in chromosome segregation and cytokinesis. Mol. Biol. Cell **12,** 2469–2481.

Inclan, Y.F., and Nogales, E. (2001). Structural models for the self-assembly and microtubule interactions of gamma-, delta- and epsilon-tubulin. J. Cell Sci. **114,** 413–422.

Jung, M.K., Prigozhina, N., Oakley, C.E., Nogales, E., and Oakley, B.R. (2001). Alanine-scanning mutagenesis of Aspergillus gamma-tubulin yields diverse and novel phenotypes. Mol. Biol. Cell **12,** 2119–2136.

Kumagai, F., Yoneda, A., Tomida, T., Sano, T., Nagata, T., and Hasezawa, S. (2001). Fate of nascent microtubules organized at the M/G1 interface, as visualized by synchronized tobacco BY-2 cells stably expressing GFP-tubulin: Time-sequence observations of the reorganization of cortical microtubules in living plant cells. Plant Cell Physiol. **42,** 723–732.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227,** 680–685.

Lajoie-Mazenc, I., Detraves, C., Rotaru, V., Gares, M., Tollon, Y., Jean, C., Julian, M., Wright, M., and Raynaud-Messina, B. (1996). A single gamma-tubulin gene and mRNA, but two gamma-tubulin

polypeptides differing by their binding to the spindle pole organizing centres. J. Cell Sci. **109,** 2483–2492.

Lessman, C.A., and Kim, H. (2001). Soluble tubulin complexes in oocytes of the common leopard frog, *Rana pipiens*, contain gamma-tubulin. Mol. Reprod. Dev. **60,** 128–136.

Linhartova, I., Novotna, B., Sulimenko, V., Draberova, E., and Draber, P. (2002). Gamma-tubulin in chicken erythrocytes: Changes in localization during cell differentiation and characterization of cytoplasmic complexes. Dev. Dyn. **223,** 229–240.

Liu, B., Joshi, H.C., Wilson, T.J., Silflow, C.D., Palevitz, B.A., and Snustad, D.P. (1994). γ-Tubulin in Arabidopsis: Gene sequence, immunoblot, and immunofluorescence studies. Plant Cell **6,** 303–314.

Liu, B., Marc, J., Joshi, H.C., and Palevitz, B.A. (1993). A gamma-tubulin-related protein associated with the microtubule arrays of higher plants in a cell cycle-dependent manner. J. Cell Sci. **104,** 1217–1228.

Llanos, R., Chevrier, V., Ronjat, M., Meurer-Grob, P., Martinez, P., Frank, R., Bornens, M., Wade, R.H., Wehland, J., and Job, D. (1999). Tubulin binding sites on gamma-tubulin: Identification and molecular characterization. Biochemistry **38,** 15712–15720.

May, M., and Leaver, C. (1993). Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension culture. Plant Physiol. **103,** 621–627.

McDonald, A.R., Liu, B., Joshi, H.C., and Palevitz, B.A. (1993). Gamma-tubulin is associated with a cortical-microtubule-organizing zone in the developing guard cells of *Allium cepa* L. Planta **191,** 357–361.

Moritz, M., Braunfeld, M.B., Sedat, J.W., Alberts, B., and Agard, D.A. (1995). Microtubule nucleation by gamma-tubulin-containing rings in the centrosome. Nature **378,** 638–640.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Novakova, M., Draberova, E., Schurmann, W., Czihak, G., Viklicky, V., and Draber, P. (1996). γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies. Cell Motil. Cytoskeleton **33,** 38–51.

Oegema, K., Wiese, C., Martin, O.C., Milligan, R.A., Iwamatsu, A., Mitchison, T.J., and Zheng, Y. (1999). Characterization of two related Drosophila gamma-tubulin complexes that differ in their ability to nucleate microtubules. J. Cell Biol. **144,** 721–733.

Ovechkina, Y., and Oakley, B.R. (2001). Gamma tubulin in plant cells. Methods Cell Biol. **67,** 195–212.

Paluh, J.L., Nogales, E., Oakley, B.R., McDonald, K., Pidoux, A.L., and Cande, W.Z. (2000). A mutation in gamma-tubulin alters microtubule dynamics and organization and is synthetically lethal with the kinesin-like protein pkl1p. Mol. Biol. Cell **11,** 1225–1239.

Panteris, E., Apostolakos, P., Graf, R., and Galatis, B. (2000). Gamma-tubulin colocalize with microtubule arrays and tubulin paracrystals in dividing vegetative cells of higher plants. Protoplasma **210,** 179–187.

Petitpren, M., Caumont, C., Barthou, H., Wright, M., and Alibert, G. (2001). Two γ-tubulin isoforms are differentially expressed during development in *Helianthus annuus*. Physiol. Plant. **111,** 102–107.

Prigozhina, N.L., Walker, R.A., Oakley, C.E., and Oakley, B.R. (2001). γ-Tubulin and the C-terminal motor domain kinesin-like protein, KLPA, function in the establishment of spindle bipolarity in *Aspergillus nidulans*. Mol. Biol. Cell **12,** 3161–3174.

Raynaud-Messina, B., Debec, A., Tollon, Y., Gares, M., and Wright, M. (2001). Differential properties of the two Drosophila gamma-tubulin isotypes. Eur. J. Cell Biol. **80,** 643–649.

Ritzenthaler, C., Nebenfuhr, A., Movafegi, A., Stussi-Garaud, C., Behnia, L., Staehlin, L.A., and Robinson, D.G. (2002). Reevaluation of the effect of brefeldin A on plant cells using tobacco Bright Yellow 2 cells expressing Golgi-targeted green fluorescent protein and COPI antisera. Plant Cell **14,** 237–261.

Schagger, H., and von Jagow, G. (1991). Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form. Anal. Biochem. **199,** 223–231.

Stoppin-Mellet, V., Peter, C., and Lambert, A.M. (2000). Distribution of γ-tubulin in higher plant cells: Cytosolic γ-tubulin is a part of high molecular weight complexes. Plant Biol. **2,** 290–296.

Strome, S., Powers, J., Dunn, M., Reese, K., Malone, C.J., White, J., Seydoux, G., and Saxton, W. (2001). Spindle dynamics and the role of gamma-tubulin in early *Caenorhabditis elegans* embryos. Mol. Biol. Cell **12,** 1751–1764.

Sulimenko, V., Sulimenko, T., Poznanovic, S., Nechiporuk-Zloy, V., Bohm, K., Macurek, L., Unger, E., and Draber, P. (2002). Association of brain tubulin with alpha/beta-tubulin dimers. Biochem. J. **365,** 889–895.

Vassilev, A., Kimble, M., Silflow, C.D., LaVoie, M., and Kuriyama, R. (1995). Identification of intrinsic dimer and overexpressed monomeric forms of gamma-tubulin in Sf9 cells infected with baculovirus containing the Chlamydomonas gamma-tubulin sequence. J. Cell Sci. **108,** 1083–1092.

Vaughn, K.C., and Harper, J.D. (1998). Microtubule-organizing centers and nucleating sites in land plants. Int. Rev. Cytol. **181,** 75–149.

Vorobjev, I.A., Svitkina, T.M., and Borisy, G.G. (1997). Cytoplasmic assembly of microtubules in cultured cells. J. Cell Sci. **110,** 2635–2645.

Weingartner, M., Binarova, P., Drykova, D., Schweighofer, A., David, J.P., Heberle-Bors, E., Doonan, J., and Bogre, L. (2001). Dynamic recruitment of Cdc2 to specific microtubule structures during mitosis. Plant Cell **13,** 1929–1943.

Wiese, C., and Zheng, Y. (1999). Gamma-tubulin complexes and their interaction with microtubule-organizing centers. Curr. Opin. Struct. Biol. **9,** 250–259.

Wiese, C., and Zheng, Y. (2000). A new function for the gamma-tubulin ring complex as a microtubule minus-end cap. Nat. Cell Biol **2,** 358–364.

Zheng, Y., Wong, M.L., Alberts, B., and Mitchison, T. (1995). Nucleation of microtubule assembly by a gamma-tubulin-containing ring complex. Nature **378,** 578–583.

Zheng, Y., Wong, M.L., Alberts, B., and Mitchison, T. (1998). Purification and assay of gamma tubulin ring complex. Methods Enzymol. **298,** 218–228.

VI.5. Kukharskyy V., **Sulimenko V.,** Macurek L., Sulimenko T., Dráberová E, Dráber P. *Complexes of γ-tubulin with non-receptor protein tyrosine kinases Src and Fyn in differentiating P19 embryonal carcinoma cells.* ***Experimental Cell Research*, 298**: 218-228, 2004.

Available online at www.sciencedirect.com

SCIENCE DIRECT

Experimental Cell Research 298 (2004) 218–228

Experimental Cell Research

www.elsevier.com/locate/yexcr

Complexes of γ-tubulin with nonreceptor protein tyrosine kinases Src and Fyn in differentiating P19 embryonal carcinoma cells

Vitaliy Kukharskyy, Vadym Sulimenko, Libor Macůrek, Tetyana Sulimenko, Eduarda Dráberová, and Pavel Dráber*

Institute of Molecular Genetics, Department of Biology of Cytoskeleton, Academy of Sciences of the Czech Republic, Prague 4, Czech Republic

Received 2 October 2003, revised version received 8 April 2004
Available online 10 May 2004

Abstract

Nonreceptor protein tyrosine kinases of the Src family have been shown to play an important role in signal transduction as well as in regulation of microtubule protein interactions. Here we show that γ-tubulin (γ-Tb) in P19 embryonal carcinoma cells undergoing neuronal differentiation is phosphorylated and forms complexes with protein tyrosine kinases of the Src family, Src and Fyn. Elevated expression of both kinases during differentiation corresponded with increased level of proteins phosphorylated on tyrosine. Immunoprecipitation experiments with antibodies against Src, Fyn, γ-tubulin, and with anti-phosphotyrosine antibody revealed that γ-tubulin appeared in complexes with these kinases. In vitro kinase assays showed tyrosine phosphorylation of proteins in γ-tubulin complexes isolated from differentiated cells. Pretreatment of cells with Src family selective tyrosine kinase inhibitor PP2 reduced the amount of phosphorylated γ-tubulin in the complexes. Binding experiments with recombinant SH2 and SH3 domains of Src and Fyn kinases revealed that protein complexes containing γ-tubulin bound to SH2 domains and that these interactions were of SH2-phosphotyrosine type. The combined data suggest that Src family kinases might have an important role in the regulation of γ-tubulin interaction with tubulin dimers or other proteins during neurogenesis.

Keywords: Antibodies; Fyn kinase; Gamma-tubulin; Neuronal differentiation; P19 cells; Src kinase

Introduction

It is well established that the development of nervous system is regulated by a variety of protein tyrosine kinases. While the neural functions of receptor tyrosine kinases are sufficiently characterized, the functions of nonreceptor tyrosine kinases, including kinases $pp60^{src}$ (Src) and $p59^{fyn}$ (Fyn) that belong to the family of Src kinases, are not fully understood. Src family kinases have been implicated in events regulating neuronal differentiation and function of neuronal cells [1–3]. The kinases could modulate microtubule dynamics during neurite outgrowth as a fraction of tubulin associated with plasma membrane serves as a substrate for Src kinase [4,5]. Both Src and Fyn kinases were found in complexes containing cell surface receptors and tubulin [6].

Embryonal carcinoma cell line P19 is a suitable model system for studying the molecular mechanisms underlying differentiation and early embryonic development. P19s are murine multipotent cells that can differentiate in culture into neural cells when aggregated and subsequently cultured in the presence of a nontoxic concentration of all-*trans*-retinoic acid (RA) [7]. There are substantial changes in the expression of microtubule proteins during neuronal differentiation of P19 cells [8–10], and both Src and Fyn kinases are present in differentiated cells [11].

Abbreviations: EC, embryonal carcinoma; Fyn, protein tyrosine kinase $p59^{fyn}$; GST, glutathione *S*-transferase; MTOC, microtubule organizing center; MSB, microtubule-stabilizing buffer; PBS, phosphate buffered saline; PP2, Src-family selective tyrosine kinase inhibitor; PP3, negative control for inhibitor PP2; P-Tyr, protein phosphorylated on tyrosine; RA, all-*trans*-retinoic acid; SDS-PAGE, one-dimensional SDS-polyacrylamide gel electrophoresis; 2D-PAGE, two-dimensional electrophoresis; Src, protein tyrosine kinase $pp60^{src}$; SH2 and SH3, Src homology 2 and 3 domains; α-Tb, α-tubulin; Acα-Tb, acetylated α-tubulin; βIII-Tb, β-tubulin class III; γ-Tb, γ-tubulin; γTuRC, γ-tubulin-ring complex; γTuSC, γ-tubulin small complex; TBST, 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20.

* Corresponding author. Institute of Molecular Genetics, Department of Biology of Cytoskeleton, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic. Fax: +420-241-062-758.
E-mail address: paveldra@biomed.cas.cz (P. Dráber).

0014-4827/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexcr.2004.04.016

Microtubules, known to play an essential role during neurite outgrowth, are dynamic polymers assembled from α- and β-tubulin heterodimers. γ-Tubulin (γ-Tb) [12] is a highly conserved member of the tubulin superfamily that is located on the minus end of microtubules in microtubule organizing centers (MTOCs) [13]. The majority of γ-tubulin is, however, associated with other proteins in soluble cytoplasmic complexes. Large γ-tubulin-ring complexes (γTuRCs) were identified in various species [14,15]. Besides γTuRC, there also exist smaller complexes (γ-tubulin small complex; γTuSC) [16] that comprise two molecules of γ-tubulin and one molecule each of GCP2 and GCP3 (γ-tubulin complex proteins) [17,18]. The γ-TuRCs are formed by small complexes and by other proteins. In addition to nucleation from MTOC, γ-TuRCs are also involved in the regulation of the dynamics of microtubule minus ends [19].

γ-Tubulin itself has been recognized as a microtubule minus end binding molecule in not anchored microtubules [20], and the binding sites for α- and β-tubulin subunits on γ-tubulin were identified using synthetic peptides [21]. In brain, soluble γ-tubulin associated with αβ-tubulin dimers irrespective of the size of γ-tubulin complexes [22]. Membrane-bound forms of γ-tubulin with nucleation activity were also described [23,24].

While it is well known that the αβ-tubulin heterodimers are subject to a large number of posttranslational modifications that are essential for regulation of their functions [25], knowledge about posttranslational modifications of γ-tubulin and its interaction partners is limited. Phosphorylation of γ-tubulin on tyrosine has so far been reported in budding yeast [26], and we found γ-tubulin in rat basophilic leukemia cells in complexes containing protein tyrosine kinase $p53/p56^{lyn}$ belonging to the family of Src kinases [27]. We therefore wanted to find out whether γ-tubulin also interacts with other Src family kinases that are expressed during neuronal differentiation. During this process, substantial changes in cell morphology and microtubule arrangement are known to occur.

Here we report for the first time that γ-tubulin in differentiating P19 cells is phosphorylated and forms complexes with nonreceptor protein tyrosine kinases Src and Fyn. Consequently, the Src family kinases might have an important role in the regulation of γ-tubulin interaction with tubulin dimers or other proteins during neuronal differentiation.

Materials and methods

Materials

Immobilized Protein A Plus, Immobilized Protein L Plus, and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce (Rockford, IL, USA) and SYPRO Ruby Protein Gel Stain from Molecular Probes (Leiden, The Netherlands). Protease-inhibitor cocktail tablets ("Complete EDTA-free") were from Roche Molecular Biochemicals (Mannheim, Germany), and bovine serum albumin was from Serva Feinbiochemica (Heidelberg, Germany). All-*trans*-retinoic acid (RA), alkaline phosphatase from *Escherichia coli* (type III), cytosine β-D-arabinofuranoside, phenyl phosphate, phosphoserine, bicinchoninic acid kit for protein determination, and molecular-mass markers for SDS-polyacrylamide gel electrophoresis (PAGE) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Glutathione *S*-transferase (GST)-tagged fusion proteins containing amino acid residues 145–247 (SH2 domain) and 85–139 (SH3 domain) of Fyn were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Constructs encoding the GST-tagged fusion proteins containing amino acid residues 146–251 (SH2 domain) and 87–146 (SH3 domain) of Src [28] in pGEX-2TKX vector were obtained from Dr. V. Sovová (Institute of Molecular Genetics, Prague) and expressed in *Escherichia coli* strain BL21 by isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The Src family selective tyrosine kinase inhibitor 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP2) and 4-amino-7-phenylpyrazol[3,4-d]pyrimidine (PP3; negative control for inhibitor PP2) were obtained from Calbiochem (La Jolla, CA, USA). Stock solutions of PP2 and PP3 (10 mM) were prepared in dimethylsulfoxide. [γ-^{32}P]ATP (110 TBq/mmol; 370 MBq/ml) and glutathione Sepharose 4B were from Amersham Biosciences (Little Chalfont, England).

Cells

P19.X1 cells, a subclone of mouse EC cells P19 [29], were cultured in basic culture medium that was prepared by mixing Dulbecco's modified Eagle's medium and RPMI 1640 medium (1/1, vol/vol) containing 10% (v/v) fetal bovine serum, penicillin (100 units/ml), and streptomycin (0.1 mg/ml). Cells were grown at 37 °C in 5% CO_2 in air and passaged every 2 days using 0.25% (w/v) trypsin/0.01% (w/v) EDTA in PBS, pH 7.5. Cell aggregates were formed by plating 10^5 cells/ml into bacteriological-grade Petri dishes. After 2 days, the cell aggregates were gently spun down, resuspended in a medium containing 10^{-6} M RA, and transferred into tissue culture dishes (diameter 6 cm) in aliquots of 10^5 cells/dish. Medium containing RA was changed every 2 days. In some cases, cells before extraction were incubated for 60 min at 37 °C with Src family selective tyrosine kinase inhibitor PP2 or with PP3 (negative control for PP2) at a concentration 20 μM.

For immunofluorescence visualization on differentiated cells, a modified culturing procedure was applied [11] that made it possible to better discriminate neuron-like cells by eliminating proliferating nonneuronal cells. Briefly, cells in bacteriological-grade Petri dishes were aggregated 2 days in a medium containing 10^{-6} M RA. The aggregates were then dispersed into single cell suspension by treatment with 0.25% (w/v) trypsin/0.01% (w/v) EDTA in PBS, pH 7.5. The suspension (10^6 cells) in medium with RA was replated onto a culture dish (diameter 6 cm) containing poly-L-

lysine-coated glass coverslips. After 3 days, fresh medium with RA supplemented with cytosine arabinoside at concentration 5 μg/ml was added and culturing continued for an additional 4 days before fixation of the samples for immunofluorescence staining.

Antibodies

The following antibodies against protein tyrosine kinases were used: monoclonal antibody v-src (Ab-1) (IgG1, clone 327) against $pp60^{src}$ (Oncogene Research products, Boston, MA, USA); monoclonal antibody fyn (IgG2b, clone 25) against $p59^{fyn}$ (Transduction Laboratories, Lexington, KY, USA), and affinity-purified rabbit antibody Fyn (FYN3) against $p59^{fyn}$ (Santa Cruz Biotechnology). The monoclonal anti-phosphotyrosine antibody 4G10 (IgG1) and affinity-purified rabbit antibody against phosphotyrosine were from Upstate Laboratories (Lake Placid, NY, USA). The following monoclonal antibodies against tubulins were used: TU-31 (IgG2b) anti-peptide antibody prepared against the EYHAATRPDYISWGTQ peptide corresponding to human γ-tubulin sequence 434–449 [30], GTU-88 (IgG1) anti-peptide antibody prepared against the EEFA-TEGTDRKDVFFY peptide corresponding to human γ-tubulin sequence 38–53 (Sigma), TU-01 (IgG1) against α-tubulin (α-Tb) [31], TU-20 (IgG1) against neuron-specific class III β-tubulin [32], and 6-11B-1 (IgG2b) against acetylated α-tubulin (Acα-Tb; Sigma). For double-label immunofluorescence was used rabbit affinity-purified antibody TUB against αβ-tubulin dimer [33]. Actin was detected by rabbit affinity-purified antibody against the C-terminal peptide (Sigma). Monoclonal antibody NF-09 (IgG2a) against neurofilament protein NF-M [34] and rabbit antibody against nonmuscle myosin (Biomedical Technologies Inc., Stoughton, MA, USA) were used as negative controls in immunoprecipitation experiments. Rabbit antibody against GST was from Dr. Petr Dráber (Institute of Molecular Genetics, Prague, Czech Republic). Anti-mouse Ig antibodies and anti-rabbit antibodies conjugated with horseradish peroxidase were purchased from Promega Biotec (Madison, WI, USA); indocarbocyanate (Cy3)-conjugated anti-mouse Ig antibody and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit Ig antibody were from Jackson Immunoresearch Laboratories (West Grove, PA, USA).

Preparation of cell extracts

Whole-cell extracts for SDS-PAGE were prepared as follows: cells on 6-cm Petri dishes were rinsed twice in cold MEM buffer (100 mM MES adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM $MgCl_2$), solubilized in hot SDS-sample buffer [35] without bromphenol blue (0.7 ml/dish), and boiled for 5 min.

When preparing the extract for immunoprecipitation and for binding to immobilized GST-fusion proteins, cells were rinsed twice in cold MEM buffer and extracted 10 min at 4 °C with MEM buffer (0.6 ml/dish) supplemented with 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), protease inhibitor cocktail, phosphatase inhibitors (1 mM Na3VO4, 1 mM NaF), and 1% (v/v) NP-40. The suspension was then spun down (20.000 × *g*, 15 min, 4 °C), and supernatant collected.

Protein quantifications in lysates and SDS-PAGE-samples were performed, respectively, with bicinchoninic acid assay and silver dot assay [36] using bovine serum albumin as a standard.

Protein phosphatase treatment of extracts

For phosphatase treatment, 1% (v/v) NP-40 protein extracts were prepared in Tris buffer (100 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $ZnCl_2$) supplemented with protease inhibitor cocktail. The extracts were incubated with alkaline phosphatase (1 unit/10 μg protein) or with buffer at 37 °C for 60 min. The reaction was quenched by adding of the sample buffer for two-dimensional electrophoresis (2D-PAGE).

Immunoprecipitation

Immunoprecipitation was performed as described [37] using TBST (10 mM Tris–HCl adjusted to pH 7.4 with HCl, 150 mM NaCl, 0.05% (v/v) Tween 20) for dilution of extracts and for washings. For comparison of immunoprecipitation from unstimulated and stimulated cells, protein extracts from both stages were of the same protein concentration. Cell extracts were incubated with beads of protein A saturated with (I) rabbit antibody against kinase Fyn, (II) rabbit antibody against phosphotyrosine, (III) negative control rabbit antibody against nonmuscle myosin, (IV) mouse antibody TU-31 (IgG2b) against γ-tubulin, (V) negative control mouse antibody NF-09 (IgG2a), or with (VI) immobilized protein A alone. Alternatively, cell extracts were incubated with beads of protein L saturated with (I) mouse antibody against kinase Src (IgG1), (II) negative control mouse antibody NF-09 (IgG2a), and (III) immobilized protein L alone. Sedimented beads (30 μl) were incubated for 2 h at 4 °C under constant shaking with 1 ml of the corresponding antibody in TBST. Antibodies against Src, Fyn, myosin, and phosphotyrosine were used at immunoglobulin concentration 4 μg/ml. Antibody TU-31 and control antibody NF-09 were prepared by mixing 0.4 ml of 10× concentrated supernatant with 0.8 ml of the TBST buffer. The beads were pelleted by centrifugation at 5000 × *g* for 1 min, washed four times (5 min each) in cold TBST, and incubated under rocking for 2 h at 4 °C with 1 ml of cell extract, prepared by diluting the extract with TBST to final protein concentration 1.7 mg/ml. The beads were pelleted and washed four times (5 min each) in cold TBST, followed by boiling for 5 min in 100 μl of SDS-sample buffer to release the bound proteins. Alternatively, beads were washed twice in TBST and further processed in the kinase assay (see below).

Binding of cell extracts to GST fusion proteins

GST fusion proteins (5–15 μg) were non-covalently coupled to glutathione Sepharose beads (50 μl of sedimented beads) and used after washing in TBST for binding analysis. Sedimented beads were incubated under rocking for 1 h at 4 °C with 0.5 ml of cell extract diluted with TBST to final concentration 1.7 mg/ml. Unbound material was removed by four washes in cold TBST, and bound proteins were eluted from the beads by boiling for 5 min in 80 μl of SDS-sample buffer. In competitive inhibition experiments, phenyl phosphate or phosphoserine was added to cell extracts at concentrations of 4.5 or 45 mM before adding the beads, and the mixtures were incubated for 1 h followed by washing and elution as indicated above.

In vitro kinase assay

Beads with immunoprecipitated material were washed twice in kinase buffer (25 mM HEPES adjusted to pH 7.2 with NaOH, 3 mM $MnCl_2$, 5 mM $MgCl_2$, 1 mM NaF, 0.1% (v/v) NP-40), and pelleted beads were resuspended in 30 μl of kinase buffer supplemented with 185 kBq of [γ-^{32}P]ATP. After incubation for 15 min at 37 °C, the reaction was stopped by washing the beads three times in kinase buffer and the labeled immunocomplexes were solubilized by boiling for 5 min in 80 μl of SDS-sample buffer. The samples (10 μl) were resolved by SDS-PAGE, transferred to nitrocellulose, and the ^{32}P-labeled proteins were detected by autoradiography. Alternatively, the labeled immunocomplexes were solubilized in 80 μl of sample buffer for 2D-PAGE, and 70-μl aliquots were used for 2D-PAGE. After electrotransfer to nitrocellulose, the ^{32}P-labeled proteins were detected by autoradiography and blots were then immunostained.

Gel electrophoresis and immunoblotting

One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% or 10% gels, electrophoretic transfer of separated proteins onto nitrocellulose, and details of the immunostaining procedure are described elsewhere [38]. SDS-sample buffer was supplemented with 0.2 mM Na_3V0_4. The two-dimensional electrophoresis (2D-PAGE) was performed essentially as described [39]. The unlabeled samples containing about 30 μg protein were diluted with sample buffer containing 7 M urea, 2 M thiourea, 20 mM Tris, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% (w/v) Triton X-100, 1% (w/v) DL-dithiothreitol, and 2% (v/v) IPG buffer, pH 4–7 (Amersham Pharmacia Biotech, Uppsala, Sweden). After kinase assay Immobiline DryStrip gels with linear pH 4–7 gradient, 7 or 11 cm long (Amersham Pharmacia Biotech), were rehydrated using 250 μl of prepared sample (in IPG sample buffer). Each strip was overlaid with mineral oil and left overnight at room temperature. Strips were focused for a total of 22 kV h on Multiphor II apparatus (LKB, Bromma, Sweden). The second dimension was performed on 8.5% SDS-PAGE, and separated proteins were transferred onto nitrocellulose by electroblotting. The anti-tubulin monoclonal antibodies TU-01, TU-20, and TU-32, in the form of spent culture supernatants, were used undiluted, whereas the antibodies 6-11B-1 and GTU-88 were diluted 1:2000 and 1:5000, respectively. Monoclonal antibodies against kinase Src, kinase Fyn, and phosphotyrosine were diluted 1:300, 1:150, and 1:1500, respectively. Rabbit antibody against actin was diluted 1:200, and rabbit antibody against GST 1:30,000. Bound antibodies were detected after incubation of the blots with secondary antibodies diluted 1:10,000 and after washing with chemiluminescence reagents in accordance with the manufacturer's directions. Exposed autoradiography films X-Omat AR (Eastman Kodak, Rochester, NY, USA) were quantified by densitometry using gel documentation system GDS 7500 and GelBase/GelBlot Pro analysis software (UVP, Upland, CA, USA).

Immunofluorescence

Immunofluorescence microscopy was performed on fixed cells as described [37]. Shortly, cells were rinsed briefly with microtubule-stabilizing buffer (MSB; MEM buffer supplemented with 4% (w/v) polyethylene glycol 6000), extracted for 1 min with 0.2% Triton X-100, and fixed for 20 min in 3% formaldehyde in MSB. Preparations were incubated for 45 min at room temperature with primary antibodies. Polyclonal anti-tubulin antibody TUB was diluted 1:5, TU-20 monoclonal antibody against neuron-specific class III β-tubulin was used as undiluted supernatant, and GTU-88 monoclonal antibody against γ-tubulin was diluted 1:1000. Cy3-conjugated anti-mouse Ig antibody and FITC-conjugated anti-rabbit Ig antibody were diluted 1:500 and 1:200, respectively. For double-label immunofluorescence staining, the coverslips were incubated simultaneously with TU-20 and polyclonal anti-tubulin antibody, and after washing were incubated simultaneously with the secondary fluorochrome-conjugated antibodies. The preparations were mounted in MOWIOL 4–88 (Calbiochem AG, Lucerne, Switzerland) and examined with Olympus A70 Provis microscope equipped with 60× and 40× water-immersion objectives. Images were recorded with a Life Science Resources KAF 1400 cooled CCD camera. Conjugates alone did not give any detectable staining.

Results

Expression of kinases and tubulins in differentiating P19 cells

To compare the expression of protein tyrosine kinases of Src family and tubulins during neuronal differentiation of P19 cells, blots of whole cell extracts from unstimulated cells and cells stimulated with RA for 1–12 days were

probed with antibodies against kinases Src and Fyn and with antibodies against tubulins. Neuron-specific class III β-tubulin was used as a marker of neuronal differentiation and acetylated α-tubulin as a marker of stable microtubules in newly formed neuronal projections. Typical results are shown in Fig. 1A. Day 0 stands for cells that were aggregated but not incubated in the presence of RA. The same amount of proteins was loaded at each time interval. An increased expression of Src, Fyn, and acetylated α-tubulin appeared after 48-h incubation of cells with RA and reached its maximum after 12 days. Densitometric measurements of immunoblots showed that within the time interval between day 0 and day 12, the amounts of Src kinase increased approximately six times, Fyn 11 times and acetylated α-tubulin 16 times. The class III β-tubulin was not detected in unstimulated cells and began to appear after 3 days of incubation with RA. At the same time, the amount of α-tubulins rose only moderately. The used TU-01 anti-tubulin antibody reacts with an epitope located in the conservative N-terminal region and recognizes all α-tubulin isotype classes and their posttranslational modifications [31]. The amount of detected γ-tubulin did not change during differentiation and the same was true for actin (not shown). Elevated expression of protein tyrosine kinases during neuronal differentiation corresponded with the increased level of proteins phosphorylated on tyrosine (Fig. 1B). Both qualitative and quantitative differences were observed between unstimulated and stimulated cells when

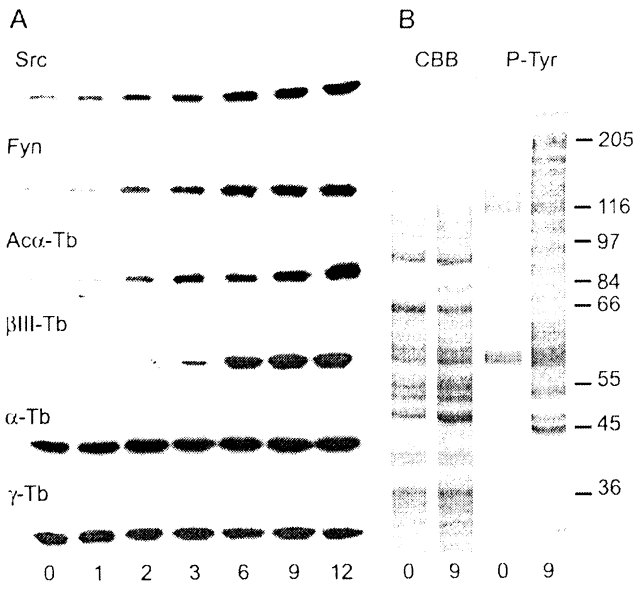


Fig. 1. Immunoblot analysis of whole cell extracts from unstimulated cells (day 0) and cells stimulated with RA for 1–12 days. (A) Immunostaining with antibodies against kinase Src (Src), kinase Fyn (Fyn), acetylated α-tubulin (Acα-Tb), neuron-specific β-tubulin class III (βIII-Tb), α-tubulin (α-Tb), and γ-tubulin (γ-Tb). (B) Coomassie blue staining (CBB) and immunostaining with anti-phosphotyrosine antibody (P-Tyr). Numbers 0–12 indicate days of incubation of cells with RA. The same amount of proteins (10μg) was loaded into each lane. Molecular mass markers (in kDa) are indicated to the left of B.

stained with the monoclonal antibody 4G10 against phosphorylated tyrosine.

Stimulation of P19 cells with RA was accompanied with substantial changes in cell morphology and in distribution of γ-tubulin. Double-label immunostaining with polyclonal antibody against αβ-tubulin dimer and monoclonal antibody against neuron-specific class III β-tubulin proved that neuron-specific tubulin was absent in unstimulated cells (Figs. 2A and B), but was detectable in neurite projections of neuron-like cells after 9 days stimulation with RA (Figs. 2C and D). Immunofluorescence staining with anti-γ-tubulin antibody GTU-88 revealed that γ-tubulin in unstimulated cells was accumulated in MTOC, but diffuse staining was also observed (Fig. 2E). In stimulated cells, a dot-like staining was found both in cell bodies and in neurite projections (Fig. 2F). A similar staining was obtained with TU-31 antibody.

γ-Tubulin is phosphorylated in vivo

Previous studies using 2D-PAGE revealed in brain tissue multiple isoforms of γ-tubulin. We therefore investigated whether γ-tubulin in P19 cells stimulated to neuronal differentiation was also modified in vivo. Two-dimensional analysis of 1% NP-40 extracts followed by immunoblotting analysis of separated proteins with anti-γ-tubulin antibody GTU-88 confirmed that soluble forms of γ-tubulin did exist in multiple isoforms. As shown in Fig. 3 (upper panel), multiple isoforms were detectable, and the isoelectric point of the major γ-tubulin isoform was 6.00. Repeated experiments with varying protein loadings and with different exposure times of autoradiography films revealed the presence of five isoforms. To determine whether γ-tubulin is modified by phosphorylation, the extracts were treated with alkaline phosphatase. Under these conditions, the two most acidic isoforms were not observed (Fig. 3, lower panel). The decrease in isoform number was not observed in samples incubated without phosphatase (not shown). These data document that γ-tubulin is phosphorylated in vivo.

Kinases form complexes with γ-tubulin

Immunoprecipitation experiments showed that γ-tubulin could be precipitated from resting and stimulated cells specifically with monoclonal anti-Src antibody (IgG1) immobilized on protein L (Fig. 4A, panel "γ-Tb", lanes 1 and 3). Stimulation enhanced the amount of precipitated material. No staining in the position of γ-tubulin was observed when the immobilized anti-Src antibody was incubated without the extract (Fig. 4A, panel "γ-Tb", lane 2), when the negative control antibody NF-09 (IgG2a) was used for immunoprecipitation of extract from stimulated cells (Fig. 4A, panel "γ-Tb", lane 4), or when protein L without the antibody was incubated with extract from stimulated cells (Fig. 4A, panel "γ-Tb", lane 5). Staining of the precipitated material with anti-Src antibody confirmed

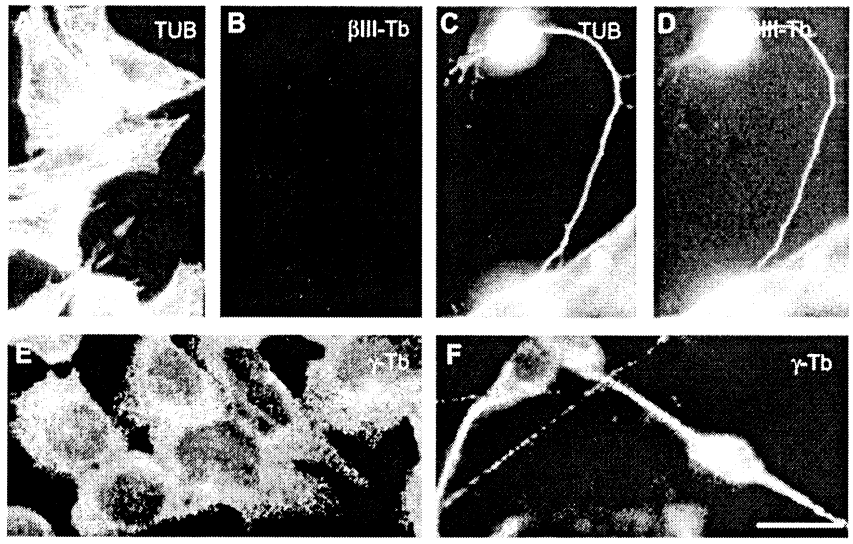


Fig. 2. Immunofluorescence localization of tubulins in resting and activated P19 cells. Staining of unstimulated cells (A, B, and E) and cells stimulated with RA for 9 days (C, D, and F). Double-label staining with polyclonal antibody (TUB) against αβ-tubulin dimers (A and C) and monoclonal antibody (βIII-Tb) against neuron-specific β-tubulin class III (B and D). Each pair (A–B and C–D) represents the same cells. Distribution of γ-tubulin (γ-Tb) is shown in E and F. Cells were extracted with Triton X-100 and fixed in formaldehyde. Scale bar, 20 μm. Comparable magnifications are in A–F.

the presence of Src kinase in unstimulated and stimulated cells (Fig. 4A, panel "Src", lanes 1 and 3). Staining with anti-phosphotyrosine antibody revealed that protein phosphorylated on tyrosine abounded in a position corresponding to relative electrophoretic mobility of Src kinase in both unstimulated and stimulated cells (Fig. 4A, panel "P-Tyr, lanes 1 and 3). No co-precipitation of Fyn was observed (not shown).

A similar set of immunoprecipitation experiments with rabbit anti-Fyn antibody immobilized on protein A is shown in Fig. 4B. γ-Tubulin could be precipitated specifically from resting and stimulated cells (Fig. 4B, panel "γ-Tb", lanes 1 and 3). But again, the amount of precipitated γ-tubulin was higher if stimulated cells were used. No staining in the position of γ-tubulin was observed when immobilized anti-Fyn antibody was incubated without the extract (Fig. 4B, panel "γ-Tb", lane 2), when rabbit negative control anti-

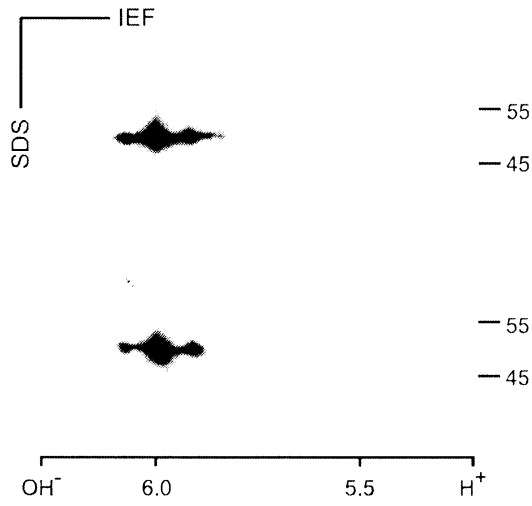


Fig. 3. Immunoblot analysis of extracts from cells stimulated with RA for 9 days after two-dimensional electrophoresis. Extracted proteins (upper panel) or extracted proteins treated with alkaline phosphatase were separated by 2D-PAGE and immunoblotted with anti-(γ-tubulin) antibody GTU-88. Molecular mass markers (in kDa) are indicated on the right. The pI scale is shown along the bottom of the figure. IEF, isoelectric focusing.

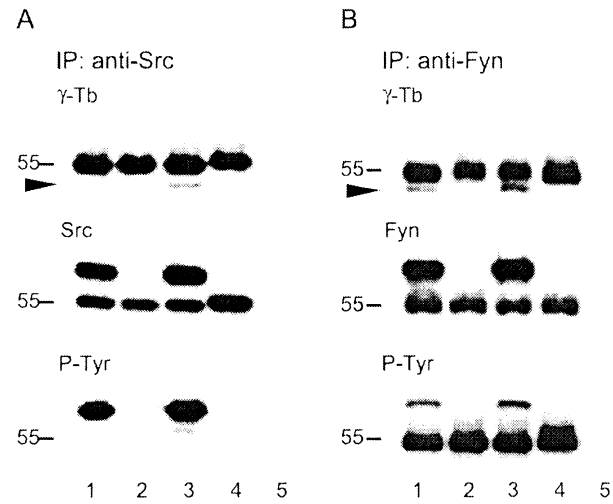


Fig. 4. Immunoprecipitation of extracts from resting and activated P19 cells with anti-(Src) and anti-(Fyn) antibodies. Samples were precipitated with mouse monoclonal anti-(Src) antibody bound to immobilized Protein L (A) or with rabbit anti-(Fyn) antibody bound to immobilized Protein A (B). Immunoprecipitation from unstimulated cells (lane 1) or cells stimulated with RA for 9 days (lanes 3–5). Immunoblots were probed with antibodies against γ-tubulin (γ-Tb), kinase Src (Src), kinase Fyn (Fyn), and phosphotyrosine (P-Tyr). Proteins immunoprecipitated from unstimulated cells (lane 1), immobilized immunoglobulin not incubated with cell extract (lane 2), proteins immunoprecipitated from stimulated cells (lane 3), proteins immunoprecipitated from stimulated cells with negative control antibody (lane 4), and proteins from stimulated cells bound to carrier without antibody (lane 5). Molecular mass markers (in kDa) are indicated to the left. Arrowheads indicate the position of γ-tubulin.

body against myosin was used for immunoprecipitation of extract from stimulated cells (Fig. 4B, panel "γ-Tb", lane 4), or when protein A without the antibody was incubated with extract from stimulated cells (Fig. 4B, panel "γ-Tb", lane 5). Staining of precipitated material with anti-Fyn antibody confirmed the presence of Fyn kinase in unstimulated and stimulated cells (Fig. 4B, panel "Fyn", lanes 1 and 3). Staining with anti-phosphotyrosine antibody showed that the protein phosphorylated on tyrosine was present in a position corresponding to relative electrophoretic mobility of Fyn kinase in both unstimulated and stimulated cells (Fig. 4B, panel "P-Tyr, lanes 1 and 3). No co-precipitation of Src was observed (not shown). When samples precipitated from stimulated cells with anti-Src or anti-Fyn antibodies were separated by SDS-PAGE and gels were subsequently stained with sensitive SYPRO Ruby Protein Gel Stain, no other proteins were detectable except for immunoglobulin heavy and light chains.

Because antibodies against Src or Fyn kinase co-precipitated larger amounts of γ-tubulin from stimulated cells than from unstimulated cells, and because tyrosine-phosphorylated forms of kinases were present in such complexes, precipitation with rabbit anti-phosphotyrosine antibody bound to immobilized Protein A was carried out. The resulting precipitates contained γ-tubulin (Fig. 5A, panel "γ-Tb", lane 1), kinase Src (Fig. 5A, panel "Src", lane 1), and kinase Fyn (Fig. 5A, panel "Fyn", lane 1). No staining in corresponding positions was observed when rabbit negative control antibody against myosin was used for immunoprecipitation or when protein A without the antibody was incubated with cell extract.

To determine whether proteins phosphorylated by Src kinases are involved in the formation of γ-tubulin complexes, precipitation with anti-protein phosphorylated on tyrosine (P-Tyr) antibody was performed in the presence of Src family selective tyrosine kinase inhibitor PP2. As control, cells untreated with PP2 or cells treated with negative control inhibitor PP3 were used. In PP2-treated cells, the amount of co-precipitated γ-tubulin was smaller (Fig. 5B, panel "γ-Tb", lane 2) than in untreated cells (Fig. 5B, panel "γ-Tb", lane 1) or cells treated with PP3 (Fig. 5B, panel "γ-Tb", lane 3). Staining with anti-phosphotyrosine antibody showed a substantially lower level of proteins phosphorylated on tyrosine in PP2-treated cells (Fig. 5B, panel "P-Tyr", lane 2) than in untreated cells (Fig. 5B, panel "P-Tyr", lane 1) or in cells treated with PP3 (Fig. 5B, panel "P-Tyr", lane 3). The possibility of an unspecific attachment of cytoplasmic proteins to complexes containing γ-tubulin can be eliminated because no actin was detectable in the immunoprecipitated material with anti-actin antibody (not shown).

Combined data from immunoprecipitation experiments indicate that soluble γ-tubulin in P19 cells appears in complexes with protein tyrosine kinases of Src family. A larger amount of γ-tubulin is present in complexes from stimulated cells, with higher expression of kinases Src and Fyn.

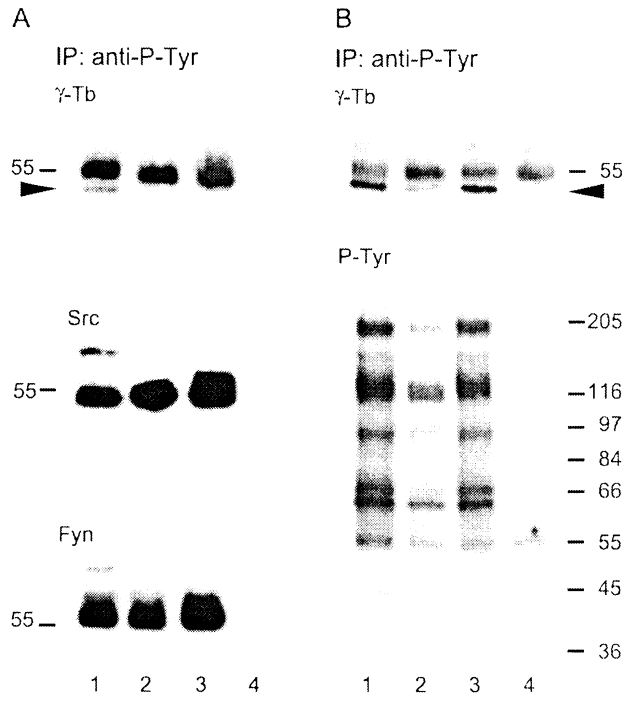


Fig. 5. Immunoprecipitation of extracts from activated P19 cells with anti-(phosphotyrosine) antibody. Samples from cells stimulated with RA for 9 days (A) or from stimulated cells treated with Src family selective inhibitor PP2 (B) were precipitated with rabbit anti-phosphotyrosine antibody bound to immobilized Protein A. Immunoblots were probed with antibodies against γ-tubulin (γ-Tb), kinase Src (Src), kinase Fyn (Fyn), and phosphotyrosine (P-Tyr). (A) Immunoprecipitated proteins (lane 1), immobilized immunoglobulin not incubated with cell extract (lane 2), proteins immunoprecipitated from cells with negative control antibody (lane 3), and proteins bound to carrier without antibody (lane 4). (B) Proteins precipitated from untreated cells (lane 1), from PP2-treated cells (lane 2), and from PP3-treated cells (lane 3), and immobilized immunoglobulin not incubated with cell extract (lane 4). Molecular mass markers (in kDa) are indicated to the left in A and to the right in B. Arrowheads indicate the position of γ-tubulin.

γ-Tubulin complexes have kinase activity dependent on Src kinases

A possible association of γ-tubulin with kinase substrates was examined with the immunocomplex kinase assay. Stimulated cells were precipitated with monoclonal anti-(γ-tubulin) antibody TU-31 or with negative control monoclonal antibody NF-09 immobilized on protein A. Immunocomplexes were then subjected to the in vitro kinase assay, and proteins separated by SDS-PAGE or 2D-PAGE were electroblotted and visualized by autoradiography. γ-Tubulin in the extract from stimulated cells was associated with several kinase substrates (Fig. 6A, lane 1), whereas no kinase activity was detected after precipitation with the control antibody (Fig. 6A, lane 3). When the cells were cultured in the presence of Src family selective tyrosine kinase inhibitor PP2 before precipitation, a lower level of phosphorylation was clearly detected in proteins with relative molecular weights of approximately 75, 65, 60, and 50 kDa (Fig. 6A, lane 2). These proteins are thus substrates for

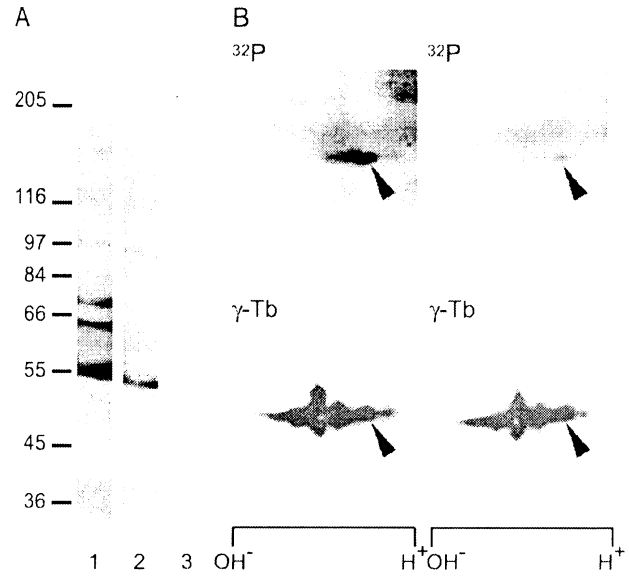


Fig. 6. The kinase activity of immunocomplexes containing γ-tubulin. Samples from cells stimulated with RA for 9 days or from stimulated cells treated with Src family selective inhibitor PP2 were precipitated with anti-(γ-tubulin) antibody TU-31 or negative control antibody NF-09 bound to immobilized Protein A. Immunocomplexes were subjected to in vitro kinase assay and electrophoretically separated. (A) Autoradiography after SDS-PAGE. Precipitation with TU-31 from untreated (lane 1) or PP2-treated cells (lane 2). Precipitation with NF-09 from untreated cells (lane 3). Molecular mass markers (in kDa) are indicated to the left. (B) Autoradiography (^{32}P) and immunoblotting with anti-(γ-tubulin) antibody GTU-88 (γ-Tb) after 2D-PAGE. Precipitation with TU-31 from untreated (left column) or PP2-treated cells (right column). Arrowheads indicate the same positions.

Src family kinases. When solubilized labeled immunocomplexes were analyzed by 2D-PAGE, autoradiography revealed a signal in the region of acidic γ-tubulin isoforms. This was confirmed by staining the same blot with anti-γ-tubulin antibody GTU-88 (Fig. 6B, left panels "^{32}P" and "γ-Tb"). When the cells were treated with the PP2 inhibitor, a weaker autoradiography signal (Fig. 6B, right panel "^{32}P") was detected in the region of acidic γ-tubulin isoforms (Fig. 6B, right panel "γ-Tb"). These data demonstrate that active Src family kinases are part of γ-tubulin complexes and that γ-tubulin could be a substrate for Src family kinases.

γ-Tubulin complexes bind to SH2 domains of kinases

Guided by the fact that there is an association of γ-tubulin with active Src family kinases, we investigated whether the SH2 or SH3 kinase domains, which are important in the assembly of protein complexes, participate in this interaction. We have performed in vitro binding experiments with immobilized GST fusion proteins containing the corresponding domains of Src and Fyn kinases. γ-Tubulin bound to GST-Fyn-SH2 and GST-Src-SH2 fusion proteins, but not to GST-Fyn-SH3 and GST-Src-SH3 fusion proteins or GST alone. Under identical conditions, more γ-tubulin was bound to GST-Fyn-SH2 than to GST-Src-SH2 (Fig. 7A, panel "γ-Tb"). Similarly, proteins phosphorylated on tyrosine were also bound only to GST-Fyn-SH2 and GST-Src-SH2 fusion proteins, and more proteins phosphorylated on tyrosine were bound to GST-Fyn-SH2 (Fig. 7A, panel "P-Tyr"). The amount of immobilized fusion proteins is shown by staining with anti-GST antibody (Fig. 7A, panel "GST"). Anti-actin antibody did not detect any binding of actin to SH2 or SH3 domains of kinases.

To determine whether the observed interaction of γ-tubulin with SH2 domains of kinases is an SH2-phosphotyrosin type interaction, we performed a competition experiment with phenyl phosphate, an analogue of phosphotyrosine. Phenyl phosphate (45 mM) inhibited the binding of γ-tubulin to GST-Fyn-SH2 and GST-Src-SH2

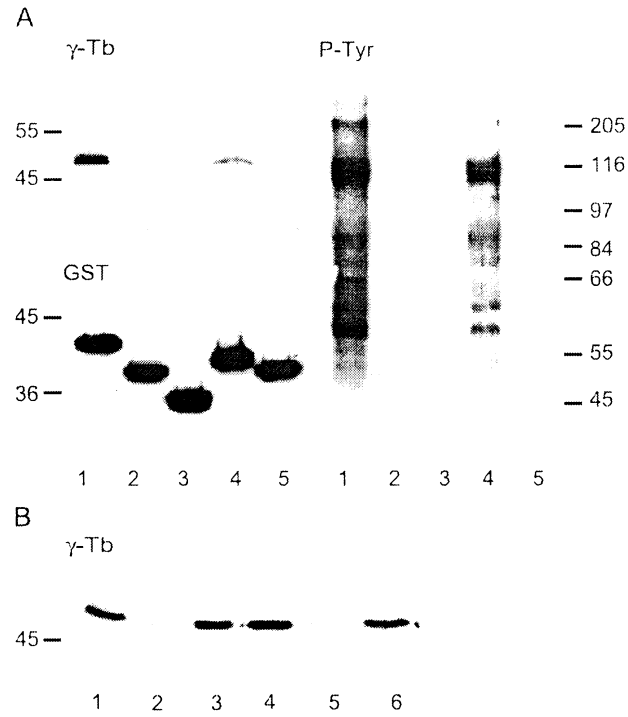


Fig. 7. Binding of γ-tubulin to SH2 domains of Fyn and Src kinases. Samples from cells stimulated with RA for 9 days were incubated with GST-fusion proteins or GST alone (negative control) immobilized to glutathione Sepharose beads. Bound proteins were eluted into SDS-sample buffer and fractionated on SDS-PAGE. Immunoblots were probed with antibodies against γ-tubulin (γ-Tb), GST (GST), or phosphotyrosine (P-Tyr). (A) Comparison of γ-tubulin binding to SH2 and SH3 domains of kinases Src and Fyn. GST-SH2 domain of Fyn kinase (lane 1), GST-SH3 domain of Fyn kinase (lane 2), GST alone (lane 3), GST-SH2 domain of Src kinase (lane 4), and GST-SH3 domain of Src kinase (lane 5). Fusion proteins (10 μg) were immobilized to Sepharose beads. (B) Effect of phenyl phosphate and phosphoserine on the binding of γ-tubulin to SH2 domains of kinases Src and Fyn. GST-SH2 domain of Fyn kinase (lanes 1–3) and GST-SH2 domain of Src kinase (lanes 4–6) were incubated with cell extract in the absence (lanes 1 and 4) or presence of 45 mM phenyl phosphate (lanes 2 and 5) or 45 mM phosphoserine (lanes 3 and 6). GST-SH2 domain (5 μg) of Fyn kinase or 15 μg of GST-SH2 domain of Src kinase was immobilized to Sepharose beads. Molecular mass markers (in kDa) are indicated on the left in panels γ-Tb and GST, and on the right in panel P-Tyr.

fusion proteins as can be seen in Fig. 7B (lanes 2 and 5). With GST-Src-SH2, 4.5 mM phenyl phosphate was also tested; it still had some inhibitory effect on γ-tubulin binding (not shown). This inhibition was specific, because the same concentrations of phosphoserine had no effect on binding of γ-tubulin to SH2 domains of kinases (Fig. 7B, lanes 3 and 6).

Discussion

Quantitative immunoblotting of P19 cells stimulated with retinoic acid revealed that the expression of Src family kinases Src and Fyn was substantially increased during neuronal differentiation documented by typical changes in cell morphology. Elevated expression of kinases corresponded with the increased level of proteins phosphorylated on tyrosine. Although the level of γ-tubulin did not vary during differentiation, substantial changes were observed in its subcellular localization. Immunofluorescence microscopy revealed in stimulated cells an association of γ-tubulin with detergent-resistant fraction in cell bodies and neurite projections. Because the greater part of γ-tubulin in animal cells is not associated with polymerized microtubules, its binding to the fraction not extractable by detergent indicates an association with intracellular membranous components under the extraction conditions used (0.2% Triton X-100 in MSB, at 37°C). A significant association of γ-tubulin with intracellular components was observed in various cell lines.

Immunoprecipitation experiments with antibodies against Src kinase, Fyn kinase, and phosphotyrosine revealed that γ-tubulin in stimulated cells was associated with kinases. Binding experiments with recombinant SH2 and SH3 domains of these kinases revealed that γ-tubulin or protein complexes containing γ-tubulin bound to SH2 domains, and that this interaction was of the SH2-phosphotyrosine type. The presence of Src family kinases in complexes with γ-tubulin was also confirmed by the in vitro kinase assay with material precipitated with anti-γ-tubulin antibody from stimulated P19 cells, which were either treated or not with Src family selective inhibitor PP2. As the Fyn kinase did not co-precipitate with Src kinase and, reciprocally, the Src kinase did not co-precipitate with Fyn kinase, it is probable that the two kinases form different types of complexes with γ-tubulin. Sucrose gradient centrifugation, which effectively separates γTuRC from γTuSC in P19 cells, revealed that kinases Src and Fyn did not associate with γTuRC (L. Macůrek, unpublished). In contrast, the serine–threonine polo-like kinase (Plk) that plays an important role in the regulation of cell division co-eluted with γ-tubulin during gel filtration on Superose 6 column in the range of large complexes. Plk also phosphorylated γ-tubulin [40]. Moreover, it has been reported that lipid phosphoinositide 3-kinase (PI 3-kinase) binds to γ-tubulin in response to insulin [41], and that the 55-kDa regulatory subunit of PI 3-kinase interacts with γ-tubulin [42]. An association of protein tyrosine kinase p53/p56^{lyn}, another member of the Src family kinases, with γ-tubulin was reported in rat basophilic leukemia cells [27]. Collectively, these data suggest that γ-tubulin could participate in the formation of protein complexes whose activity might be regulated by phosphorylation.

While there are only two functional γ-tubulin genes in mammals [43], multiple isoelectric variants of γ-tubulin were detected by two-dimensional electrophoresis in differentiated P19 cells. γ-Tubulin charge variants were already described in brain [22,44], nucleated erythrocytes [45], and budding yeast [26]. In the last case, phosphorylation of the γ-tubulin residue Tyr 445, which is invariably present in all γ-tubulins, was described [26]. Mutation of this residue changed the microtubule dynamics, and it was suggested that γ-tubulin could play a role in the dynamics of the plus ends of microtubules [26,46]. In vitro kinase assay with material precipitated with anti-(γ-tubulin) antibody followed by 2D-PAGE revealed the presence of labeled protein in the position of the acidic variant of γ-tubulin. The labeling was weaker when cells were pretreated with an inhibitor of Src kinases. This indicates that γ-tubulin could be the substrate for Src family kinases. When monoclonal anti-phosphotyrosine antibody was used for probing the blotted proteins after two-dimensional electrophoresis, staining in the position of acidic γ-tubulin isoforms was detectable, even though it was weak (V. Sulimenko unpublished). On the other hand, when polyclonal or monoclonal anti-phosphotyrosine antibodies were used for precipitation of differentiated P19 cells, γ-tubulin was present in immunocomplexes, but no accumulation of protein phosphorylated on tyrosine was detected in the position of γ-tubulin. This suggests that only a small fraction of γ-tubulin is phosphorylated on tyrosine in activated P19 cells compared to other co-precipitated proteins.

In conclusion, the presented data demonstrate for the first time that in P19 cells induced to neuronal differentiation, the Src family protein tyrosine kinases Src and Fyn form complexes with γ-tubulin by an interaction with their SH2 domains. Activity of these kinases might regulate the interaction of γ-tubulin with tubulin dimers or other proteins during neurogenesis.

Acknowledgments

We thank Dr. H.E. Varmus (Memorial Sloan-Kettering Cancer Center, New York, USA) and Dr. V. Sovová (Institute of Molecular Genetics, Prague, Czech Republic) for providing constructs encoding GST-tagged fusion proteins containing SH2 and SH3 domains of Src. We thank Dr. Petr Dráber (Institute of Molecular Genetics, Prague, Czech Republic) for antibody against GST. This work was supported in part by grants from the Grant Agency of the Czech Academy of Sciences (A5052004), Grant Agency of the Czech Republic (304/00/0553, 304/04/

1273), and from the Ministry of Education of the Czech Republic (LN00A026).

References

[1] P.F. Maness, M. Aubry, C.G. Shores, I. Frame, K.H. Pfenninger, c-src gene product in developing rat brain is enriched in nerve growth cone membranes, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 5001–5005.

[2] H.E. Beggs, P. Soriano, P.F. Maness, NCAM-dependent neurite outgrowth is inhibited in neurons from Fyn-minus mice, J. Cell Biol. 127 (1994) 825–833.

[3] M.A. Ignelzi Jr., D.R. Miller, P. Soriano, P.F. Maness, Impaired neurite outgrowth of src-minus cerebellar neurons on the cell adhesion molecule L1, Neuron 12 (1994) 873–884.

[4] W.T. Matten, M. Aubry, J. West, P.F. Maness, Tubulin is phosphorylated at tyrosine by pp60c-src in nerve growth cone membranes, J. Cell Biol. 111 (1990) 1959–1970.

[5] J.R. Atashi, S.G. Klinz, C.A. Ingraham, W.T. Matten, M. Schachner, P.F. Maness, Neural cell adhesion molecules modulate tyrosine phosphorylation of tubulin in nerve growth cone membranes, Neuron 8 (1992) 831–842.

[6] T. Kinnunen, M. Kaksonen, J. Saarinen, N. Kalkkinen, H.B. Peng, H. Rauvala, Cortactin-Src kinase signaling pathway is involved in *N*-syndecan-dependent neurite outgrowth, J. Biol. Chem. 273 (1998) 10702–10708.

[7] E.M. Jones-Villeneuve, M.W. McBurney, K.A. Rogers, V.I. Kalnins, Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells, J. Cell Biol. 94 (1982) 253–262.

[8] Y. Tanaka, K. Kawahata, T. Nakata, N. Hirokawa, Chronological expression of microtubule-associated proteins (MAPs) in EC cell P19 after neuronal induction by retinoic acid, Brain Res. 596 (1992) 269–278.

[9] A.R. Vaillant, D.L. Brown, Accumulation of microtubule-associated protein 1A (MAP1A) in differentiating P19 embryonal carcinoma cells, Biochem. Cell Biol. 73 (1995) 695–702.

[10] M. Ziková, V. Sulimenko, P. Dráber, E. Dráberová, Accumulation of 210 kDa microtubule-interacting protein in differentiating P19 embryonal carcinoma cells, FEBS Lett. 473 (2000) 19–23.

[11] Y. Takayama, S. Nada, K. Nagai, M. Okada, Role of Csk in neural differentiation of the embryonic carcinoma cell line P19, FEBS Lett. 406 (1997) 11–16.

[12] C.E. Oakley, B.R. Oakley, Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*, Nature 338 (1989) 662–664.

[13] B.R. Oakley, C.E. Oakley, Y. Yoon, M. Jung, γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*, Cell 61 (1990) 1289–1301.

[14] Y. Zheng, M.L. Wong, B. Alberts, T. Mitchison, Nucleation of microtubule assembly by a γ-tubulin-containing ring complex, Nature 378 (1995) 578–583.

[15] M. Moritz, M.B. Braunfeld, J.C. Fung, J.W. Sedat, B.M. Alberts, D.A. Agard, Three-dimensional structural characterization of centrosomes from *Drosophila* embryos, J. Cell Biol. 130 (1995) 1149–1159.

[16] M. Moritz, Y. Zheng, B.M. Alberts, K. Oegema, Recruitment of the γ-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds, J. Cell Biol. 142 (1998) 775–786.

[17] S.M. Murphy, L. Urbani, T. Stearns, The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components Spc97p and Spc98p, J. Cell Biol. 141 (1998) 663–674.

[18] K. Oegema, C. Wiese, O.C. Martin, R.A. Milligan, A. Iwamatsu, T.J. Mitchison, Y. Zheng, Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules, J. Cell Biol. 144 (1999) 721–733.

[19] C. Wiese, Y. Zheng, A new function for the γ-tubulin ring complex as a microtubule minus-end cap, Nat. Cell Biol. 2 (2000) 358–364.

[20] R. Leguy, R. Melki, D. Pantaloni, M.F. Carlier, Monomeric γ-tubulin nucleates microtubules, J. Biol. Chem. 275 (2000) 21975–21980.

[21] R. Llanos, V. Chevrier, M. Ronjat, P. Meurer-Grob, P. Martinez, R. Frank, M. Bornens, R.H. Wade, J. Wehland, D. Job, Tubulin binding sites on γ-tubulin: identification and molecular characterization, Biochemistry 38 (1999) 15712–15720.

[22] V. Sulimenko, T. Sulimenko, S. Poznanovic, V. Nechiporuk-Zloy, J.K. Böhm, L. Macůrek, E. Unger, P. Dráber, Association of brain γ-tubulins with αβ-tubulin dimers, Biochem. J. 365 (2002) 889–895.

[23] K. Chabin-Brion, J. Marceiller, F. Perez, C. Settegrana, A. Drechou, G. Durand, C. Pous, The Golgi complex is a microtubule-organizing organelle, Mol. Biol. Cell 12 (2001) 2047–2060.

[24] D. Dryková, V. Sulimenko, V. Cenklová, J. Volc, P. Dráber, P. Binarová, Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes, Plant Cell 15 (2003) 465–480.

[25] S. Westermann, K. Weber, Post-translational modifications regulate microtubule function, Nat. Rev. 4 (2003) 938–945.

[26] J. Vogel, B. Drapkin, J. Oomen, D. Beach, K. Bloom, M. Snyder, Phosphorylation of γ-tubulin regulates microtubule organization in budding yeast, Dev. Cell 1 (2001) 621–631.

[27] L. Dráberová, E. Dráberová, Z. Surviladze, P. Dráber, Pa. Dráber, Protein tyrosine kinase $p53/p56^{lyn}$ forms complexes with γ-tubulin in rat basophilic leukemia cells, Int. Immunol. 11 (1999) 1829–1839.

[28] K.B. Bibbins, H. Boeuf, H.E. Varmus, Binding of the Src SH2 domain to phosphopeptides is determined by residues in both the SH2 domain and the phosphopeptides, Mol. Cell. Biol. 13 (1993) 7278–7287.

[29] P. Dráber, P. Malý, Mutants of embryonal carcinoma cells defective in the expression of embryoglycan, Proc. Natl. Acad. Sci. U. S. A 84 (1987) 5798–5802.

[30] M. Nováková, E. Dráberová, W. Schürmann, G. Czihak, V. Viklický, P. Dráber, γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies, Cell Motil. Cytoskeleton 33 (1996) 38–51.

[31] P. Dráber, E. Dráberová, I. Linhartová, V. Viklický, Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies, J. Cell Sci. 92 (1989) 519–528.

[32] E. Dráberová, Z. Lukáš, D. Ivanyi, V. Viklický, P. Dráber, Expression of class III β-tubulin in normal and neoplastic human tissues, Histochem. Cell Biol. 109 (1998) 231–239.

[33] P. Dráber, E. Dráberová, V. Viklický, Immunostaining of human spermatozoa with tubulin domain-specific monoclonal antibodies, Histochemistry 195 (1991) 519–524.

[34] E. Dráberová, V. Sulimenko, V. Kukharskyy, P. Dráber, Monoclonal antibody NF-09 specific for neurofilament protein NF-M, Folia Biol. (Praha) 45 (1999) 163–165.

[35] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T_4, Nature 227 (1970) 680–685.

[36] P. Dráber, Quantitation of proteins in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis using colloidal silver, Electrophoresis 12 (1991) 453–456.

[37] E. Dráberová, P. Dráber, A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules, J. Cell Sci. 106 (1993) 1263–1273.

[38] P. Dráber, L.A. Lagunowich, E. Dráberová, V. Viklický, I. Damjanov, Heterogeneity of tubulin epitopes in mouse fetal tissues, Histochemistry 89 (1988) 485–492.

[39] A. Görg, C. Obermaier, G. Boguth, W. Weiss, Recent developments in two-dimensional gel electrophoresis with immobilized pH gradients: wide pH gradients up to pH 12, longer separation distances and simplified procedures, Electrophoresis 20 (1999) 712–717.

[40] Y. Feng, D.R. Hodge, G. Palmieri, D.L. Chase, D.L. Longo, D.K. Ferris, Association of polo-like kinase with α-, β- and γ-tubulins in a stable complex, Biochem. J. 339 (1999) 435–442.

[41] R. Kapeller, A. Toker, L.C. Cantley, C.L. Carpenter, Phosphoinositide 3-kinase binds constitutively to α/β-tubulin and binds to γ-tubulin in response to insulin, J. Biol. Chem. 270 (1995) 25985–25991.

[42] K. Inukai, M. Funaki, M. Nawano, H. Katagiri, T. Ogihara, M. Anai, Y. Onishi, H. Sakoda, H. Ono, Y. Fukushima, M. Kikuchi, Y. Oka, T. Asano, The N-terminal 34 residues of the 55 kDa regulatory subunits of phosphoinositide 3-kinase interact with tubulin, Biochem. J. 346 (2000) 483–489.

[43] D.O. Wise, R. Krahe, B.R. Oakley, The γ-tubulin gene family in humans, Genomics 67 (2000) 164–170.

[44] C. Détraves, H. Mazarguil, I. Lajoie-Mazenc, M. Julian, B. Raynaud-Messina, M. Wright, Protein complexes containing gamma-tubulin are present in mammalian brain microtubule protein preparations, Cell Motil. Cytoskeleton 36 (1997) 179–189.

[45] I. Linhartová, B. Novotná, V. Sulimenko, E. Dráberová, P. Dráber, Gamma-tubulin in chicken erythrocytes: changes in localization during cell differentiation and characterization of cytoplasmic complexes, Dev. Dyn. 223 (2002) 229–240.

[46] D. Job, O. Valiron, B. Oakley, Microtubule nucleation, Curr. Opin. Cell Biol. 15 (2003) 111–117.

VI.6. **Sulimenko V.,** Dráberová E., Sulimenko T., Macurek L., Richterová V., Dráber Pe., Dráber P. Regulation of microtubule formation in activated mast cells by complexes of γ-tubulin with Fyn and Syk kinases. ***Journal of Immunology*, 176**: 7243-7253, 2006.

Regulation of Microtubule Formation in Activated Mast Cells by Complexes of γ-Tubulin with Fyn and Syk Kinases[1]

Vadym Sulimenko,* Eduarda Dráberová,* Tetyana Sulimenko,* Libor Macůrek,* Věra Richterová,* Petr Dráber,† and Pavel Dráber[2]*

Aggregation of the high-affinity IgE receptors (FcεRIs) on the surface of granulated mast cells initiates a chain of signaling events culminating in the release of allergy mediators. Although microtubules are involved in mast cell degranulation, the molecular mechanism that controls microtubule rearrangement after FcεRI triggering is poorly understood. In this study, we show that the activation of bone marrow-derived mast cells (BMMCs) induced by FcεRI aggregation or treatment with pervanadate leads to a rapid polymerization of microtubules. This polymerization was not dependent on the presence of Lyn kinase as determined by experiments with BMMCs isolated from Lyn-negative mice. One of the key regulators of microtubule polymerization is γ-tubulin. Immunoprecipitation experiments revealed that γ-tubulin from activated cells formed complexes with Fyn and Syk protein tyrosine kinases and several tyrosine phosphorylated proteins from both wild-type and $Lyn^{-/-}$ BMMCs. Pretreatment of the cells with Src-family or Syk-family selective tyrosine kinase inhibitors, PP2 or piceatannol, respectively, inhibited the formation of microtubules and reduced the amount of tyrosine phosphorylated proteins in γ-tubulin complexes, suggesting that Src and Syk family kinases are involved in the initial stages of microtubule formation. This notion was corroborated by pull-down experiments in which γ-tubulin complex bounds to the recombinant Src homology 2 and Src homology 3 domains of Fyn kinase. We propose that Fyn and Syk kinases are involved in the regulation of binding properties of γ-tubulin and/or its associated proteins, and thus modulate the microtubule nucleation in activated mast cells. *The Journal of Immunology*, **2006, 176: 7243–7253.**

Granulated mast cells play a pivotal role in allergy and inflammation. Their granules contain inflammatory mediators such as histamine, proteases, lipid mediators, and cytokines. Mast cells express on their surfaces receptors with a high affinity for IgE (FcεRI). An aggregation of the FcεRI by multivalent Ag-IgE complexes triggers a series of biochemical events leading to fusion of cytoplasmic granules with the plasma membrane and release of the inflammatory mediators (1). The first defined steps in FcεRI signaling are activation of protein tyrosine kinases of the Src family (Lyn and Fyn) and Syk/Zap family and phosphorylation of their substrates (2). Two signaling pathways have been discovered in FcεRI-activated mast cells. One involves sequential activity of Lyn and Syk kinases and tyrosine phosphorylation of the FcεRI and the linker for activation of T cells. Phosphorylated linker for activation of T cells then serves as an anchor for binding of phospholipase Cγ, which is crucial in generating increased levels of intracellular calcium (3). The second pathway uses Fyn kinase, which is required for FcεRI-induced phosphorylation of Gab2 and for mast cell degranulation, but not for a rapid enhancement of intracelular calcium concentration (4). Mast cells can also be activated by an exposure to pervanadate, a compound that inhibits protein tyrosine phosphatases; such activation also leads to the secretion of inflammatory mediators (5).

Microtubules play an important role in mast cell degranulation, as the movement of secretory granules depends on intact microtubules (6) and agents inhibiting tubulin polymerization suppress the degranulation (7–9). Recently, Nishida et al. (10) documented that FcεRI stimulation triggered the formation of microtubules and that drugs affecting microtubule dynamics effectively suppressed the FcεRI-mediated translocation of granules to the plasma membrane and the degranulation. Furthermore, the translocation of granules to the plasma membrane occurred in a calcium-independent manner, whereas the release of mediators and granule-plasma membrane fusion were completely dependent on calcium. Thus, the degranulation process can be dissected into two events: the calcium-independent microtubule-dependent translocation of granules to the plasma membrane and calcium-dependent membrane fusion and exocytosis. The same authors also showed that the Fyn/Gab2/RhoA (but not Lyn/SLP-76) signaling pathway played a critical role in the calcium-independent microtubule-dependent pathway (10). Although these data confirmed that a dynamic microtubule network is required for mast cell degranulation, the precise roles of tyrosin kinases and the molecular mechanisms controlling microtubule rearrangements in this process are still unknown.

One of the key components required for microtubule formation is γ-tubulin (11), a highly conserved member of the tubulin superfamily that is located on the minus end of microtubules in microtubule organizing center (12). Interestingly, the majority of γ-tubulin is, however, associated with other proteins in soluble cytoplasmic complexes. Large γ-tubulin-ring complex (γ-TuRC)[3]

*Department of Biology of Cytoskeleton and †Department of Signal Transduction, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic

Received for publication October 24, 2005. Accepted for publication March 24, 2006.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by Grants 304/04/1273 and 204/05/2375 from the Grant Agency of Czech Republic, Grants LC545 and 1M6837805001 from Ministry of Education, Youth and Sports of the Czech Republic, and by the Institutional Research Support (AVOZ 50520514).

[2] Address correspondence and reprint requests to Dr. Pavel Dráber, Department of Biology of Cytoskeleton, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, 142 20 Prague 4, Czech Republic. E-mail address: paveldra@biomed.cas.cz

[3] Abbreviations used in this paper: γ-TuRC, γ-tubulin-ring complex; RBL, rat basophilic leukemia; BMMC, bone marrow-derived mast cell; SH, Src homology; TNP,

0022-1767/06/$02.00

(13, 14) is formed by small complexes (γ-tubulin small complex) (15), comprising two molecules of γ-tubulin, one molecule each of γ-tubulin complex proteins 2 and 3 (16, 17), and some other proteins. In addition to the nucleation from microtubule organizing center, γ-TuRCs are also involved in the regulation of the dynamics of microtubule minus ends (18). γ-Tubulin itself has been recognized as a microtubule minus-end binding molecule in nonanchored microtubules (19). Soluble γ-tubulin can associate with αβ-tubulin dimers irrespective of the size of γ-tubulin complexes (20). γ-Tubulin has also been found to bind to membranous components of the cell (21–23). Several reports indicated that kinases might be involved in the regulation of γ-tubulin interactions (24–28).

We have previously shown that γ-tubulin formed complexes with tyrosine-phosphorylated proteins and Lyn kinase in rat basophilic leukemia (RBL) cells activated by FcεRI aggregation or by an exposure to pervanadate (29). To decide whether Lyn kinase is indispensable for the formation of such complexes, we analyzed interactions of γ-tubulin with kinases and their substrates in resting and activated bone marrow-derived mast cells (BMMCs) isolated from wild-type or Lyn-deficient mice. The data indicate that fully functional microtubule nucleation is attained even in the absence of Lyn kinase. We have found that γ-tubulin in $Lyn^{-/-}$ BMMCs formed complexes with Fyn and Syk kinases, which could thus have a key role in γ-tubulin-mediated microtubule nucleation in activated mast cells.

Materials and Methods

Reagents

Immobilized Protein A Plus and SuperSignal WestPico Chemiluminescent reagents were bought from Pierce and protease-inhibitor mixture tablets ("Complete EDTA-free") were from Roche Molecular Biochemicals. Phenyl phosphate, phosphoserine, the Syk kinase-selective inhibitor piceatannol, the Src-family selective tyrosine kinase inhibitor SU6656 and isopropyl β-D-1-thiogalactopyranoside were obtained from Sigma-Aldrich. The Src-family selective tyrosine kinase inhibitor PP2 and the PP3 (negative control for inhibitor PP2) were obtained from Calbiochem. [γ-^{32}P]ATP (110 TBq/mmol; 370 MBq/ml) and glutathione Sepharose 4B were from Amersham Biosciences. Synthetic peptides were prepared at the Institute of Biochemistry and Organic Chemistry, Czech Academy of Sciences or at Sigma-Genosys. The primers were from Genetica. Tubulin was prepared from porcine brain (30), and associated proteins were removed by phosphocellulose chromatography (31).

DNA constructs

Total cellular mRNA was obtained from mouse Neuro2a cells by the acid guanidium thiocyanate-phenol-chloroform extraction method (32). Reverse transcription was performed with random hexamers (Amersham Biosciences) and Moloney murine leukemia virus-reverse transcriptase (Invitrogen Life Technologies). The fragment coding both Src homology (SH)3 and SH2 domains (aa 80–245) of mouse Fyn kinase (EMBL, accession no. M27266) was amplified by PCR using forward 5′-GAACTC CTCCTCTCACACTGGGACC-3′ and reverse 5′-CTTTAGCCAATCCA GAAGTTTGTGGG-3′ primers and total cell cDNA as a template. Isolated fragment was directly ligated into pCR3.1 vector (Invitrogen Life Technologies) and isolated DNA was subsequently used as a template for preparation of constructs encoding the GST-tagged fusion proteins. pFYSH2 vector was constructed by PCR amplification of SH2 domain of Fyn kinase (aa 80–143) using forward 5′-GACGGATCCTGGTACTTTGGAA AACTTG-3′ and reverse 5′-AGCCTCGAGTCATGAAACCACAGTT AAG-3′ primers, restriction by *Bam*HI and *Xho*I and ligation into pGEX-6P-1 vector (Amersham Biosciences). pFYSH3 vector was constructed by PCR amplification of SH3 domain of Fyn kinase (aa 148–245) using forward 5′-GCAGAATTCGGGACAGGAGTGACAC-3′ and reverse 5′-AT ACCTCGAGATTCAGATGGAGTCAACTGG-3′ primers, restriction by *Eco*RI and *Xho*I and subsequent ligation into pGEX-6P-1 vector. All constructs were verified by restriction analyze and bidirectional sequencing with universal pGEX5′ and pGEX3′ primers (Amersham Biosciences). GST-tagged fusion proteins were expressed in *Escherichia coli* strain BL21 after isopropyl β-D-1-thiogalactopyranoside induction.

Cells

Mouse BMMCs and $Lyn^{-/-}$ BMMCs were provided by M. Hibbs (Ludwig Institute for Cancer Research, Melbourne, Australia) (33). The cells were incubated in suspension cultures in freshly prepared culture medium (RPMI 1640 supplemented with 20 mM HEPES (pH 7.5), 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μM MEM nonessential amino acids, 1 mM sodium pyruvate) supplemented with 10% FCS and 10% WEHI-3 cell supernatant as a source of IL-3. Cells were grown at 37°C in 5% CO_2 in air and passaged every 2 days. No discernible differences in growth properties and morphology were detected between BMMCs and $Lyn^{-/-}$ BMMCs. In some cases, cells intended for preparation of extracts for immunoprecipitation were pretreated for 60 min at 37°C with Src family selective tyrosine kinase inhibitors SU6656, PP2, and/or PP3 (negative control for PP2) at concentrations of 5–20 μM, and piceatannol at concentration of 10–50 μM.

Antibodies

Polyclonal Abs to p53/p56^{lyn} (Lyn-44), 59^{fyn} (FYN3), Fgr, and Zap were from Santa Cruz Biotechnology. mAbs to p59^{fyn} (clone 25, IgG2b,), Lck (IgG2a), Yes (IgG1), and phosphotyrosine (PY-20, IgG2b,) were obtained from BD Transduction Laboratories. Polyclonal Abs to Hck, phosphotyrosine and mAb to phosphotyrosine (4G10, IgG1) labeled with HRP were from Upstate Laboratories. mAb to pp60^{src} (clone 327, IgG1) was from Oncogene Research Products. Abs Lyn-01/Pr (IgG1) to p53/p56^{lyn} (34), Syk-01 (IgG1), and rabbit Ab against Syk (35) were described previously. mAbs to human γ-tubulin peptide 38-53 (GTU-88, IgG1), α-tubulin (DM1A, IgG1), β-tubulin (TUB 2.1, IgG1), phosphoserine (PSR-45, IgG1) phosphothreonine (PTR-8, IgG2b), and polyclonal Ab to actin were from Sigma-Aldrich. Abs TU-31 (IgG2b) and TU-32 (IgG1) to human γ-tubulin peptide 434–449 (36), TU-01 (IgG1) to α-tubulin and TU-06 (IgM) were specified previously (37). Immunofluorescence was conducted with polyclonal Ab TUB to αβ-tubulin dimer (38) and Ab TUB 2.1 labeled with indocarbocyanate (Cy3). α-Tubulin on immunoblots was detected with polyclonal Ab to tyrosinated α-tubulin (39). Ab IGEL b4 I (IgE), specific for 2,4,6-trinitrophenyl (TNP) (40), was used to sensitize the cells. Abs NF-09 (IgG2a) (41), VI-01 (IgM) (42) and polyclonal Ab to nonmuscle myosin (Biomedical Technologies) served as negative controls. Ab against GST was prepared by immunizing rabbits with GST. Anti-mouse Abs and anti-rabbit Abs conjugated with HRP were purchased from Promega Biotec. Cy3-conjugated anti-mouse and FITC-conjugated anti-rabbit Abs were from Jackson ImmunoResearch Laboratories. FITC-conjugated anti-mouse Ab specific for IgG was from Sigma-Aldrich.

Cell activation and preparation of cell extracts

Cells were harvested, resuspended in culture medium at concentration 10×10^6 cells/ml, and sensitized in suspension with TNP-specific mAb IGEL b4 (IgE; ascitic fluid diluted 1/1000) for 60 min at 37°C. The cells were then centrifuged, washed twice in buffered saline solution (BSS) containing 20 mM HEPES (pH 7.4), 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 5.6 mM glucose, 1 mM $MgCl_2$, and 0.1% BSA (BSS-BSA), and activated for 1–6 min with the cross-linking reagent TNP-BSA at a final concentration of 1 μg/ml. Alternatively, the cells were activated by pervanadate. Pervanadate solution was freshly made by mixing sodium orthovanadate solution with hydrogen peroxide to get a 10 mM final concentration of both components. The pervanadate solution was incubated at room temperature for 15 min and then diluted 1/100 into cell suspension in BSS-BSA. Cells were incubated for 3 or 15 min at 37°C. Cell activation was stopped by transferring the tubes on ice and pelleting the cells by a brief centrifugation. For immunofluorescence experiments, cells were attached to poly-L-lysine-covered coverslips and thereafter sensitized with IgE Ab and activated with Ag for 3 min. Alternatively, attached cells were incubated with pervanadate solution for 3 or 15 min.

Whole cell extracts for SDS-PAGE were prepared by washing the cells in cold MES buffer (100 mM MES adjusted to pH 6.9 with KOH, 2 mM EGTA, 2 mM $MgCl_2$), solubilizing them in hot SDS-sample buffer (43) without bromphenol blue and boiling for 5 min.

For evaluation of protein distribution into soluble and detergent-resistant fractions under microtubule depolymerizing conditions, 6.5×10^6 cells were rinsed twice in cold MES buffer and then extracted with 0.4 ml of cold MES buffer supplemented with protease inhibitor mixture, phosphatase inhibitors (1 mM Na_3VO_4, 1 mM NaF) and 1% Nonidet P-40. After 30 min incubation at 4°C, the suspension was spun down at 20,000 × *g* for 15 min at 4°C, and the same volume of 2× SDS-PAGE sample buffer was added

2,4,6-trinitrophenyl; BSS, buffered saline solution; MSB, microtubule-stabilizing buffer.

to the supernatant. The insoluble material in the pellet was gently rinsed twice with MES buffer containing inhibitors, resuspended in 0.4 ml of MES buffer with inhibitors and mixed with 0.4 ml of 2× SDS-PAGE sample buffer.

For analysis of microtubule polymer in resting and activated cells, 6.5×10^6 cells were rinsed twice in MES buffer at 37°C and then extracted with 0.4 ml of MES buffer supplemented with protease and phosphatase inhibitors, 2 M glycerol and 0.2% Triton X-100. After a 2-min incubation at 37°C, the suspension was spun down at $8,000 \times g$ for 15 min at 25°C; the nuclear pellet containing cytoskeleton was resuspended in SDS-PAGE sample buffer.

When preparing the extract for immunoprecipitation and for binding to immobilized GST-fusion proteins, cells were rinsed twice in cold MES buffer and extracted at a concentration 15×10^6 cells/ml for 10 min at 4°C with MEM buffer supplemented with protease inhibitor mixture, phosphatase inhibitors and 1% Nonidet P-40. The suspension was then spun down ($20,000 \times g$, 15 min, 4°C), and supernatant collected. Protein quantification in SDS-PAGE-samples was performed by silver dot assay (44) using BSA as a standard.

Immunoprecipitation

Immunoprecipitation was performed as described (45), using TBST (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween 20) for dilution of extracts and for washings. Cell extracts were incubated with beads of protein A saturated with: 1) rabbit Ab against Fyn kinase, 2) rabbit Ab against phosphotyrosine, 3) rabbit Ab against Syk kinase, 4) negative control rabbit Ab against nonmuscle myosin, 5) mouse Ab TU-31 against γ-tubulin, 6) negative control mouse Ab NF-09, or with 7) immobilized protein A alone. Abs against Fyn, myosin and phosphotyrosine were used at Ig concentration 4 μg/ml. Ab against Syk was used at dilution 1/250. Ab TU-31 and control Ab NF-09 were prepared by mixing 0.1 ml of 10× concentrated hybridoma supernatant with 0.9 ml of the TBST buffer. The washed beads with bound Abs were incubated under rocking for 2 h at 4°C with 1 ml of sample, prepared by diluting the cell extract with TBST at a ratio 1:1. The beads were washed, followed by boiling in SDS-sample buffer to release the bound proteins. Alternatively, beads were washed twice in TBST and further processed in the kinase assay (see below).

Binding of cell extracts to GST fusion proteins

GST fusion proteins were noncovalently coupled to glutathione Sepharose beads (50 μl of sedimented beads) and used after washing in TBST for binding analysis. Sedimented beads were incubated under rocking for 2 h at 4°C with 1 ml of sample, prepared by diluting the cell extract with TBST in the ratio 1:1. Unbound material was removed by four washes in cold TBST, and bound proteins were eluted by boiling in SDS-sample buffer. In competitive inhibition experiments, phenyl phosphate or phosphoserine were added to cell extracts at concentrations varying from 2 to 40 mM before adding the beads, and the mixtures were incubated for 1 h followed by washing and elution as indicated above. When synthetic peptides were used in inhibition experiments, they were used at a concentration ranging from 0.005 up to 5 mM.

In vitro kinase assay

Beads with immunoprecipitated material were washed twice in kinase buffer (25 mM HEPES (pH 7.2), 5 mM $MgCl_2$, 1 mM NaF, 0.1% Nonidet P-40), and resuspended in 30 μl of kinase buffer supplemented with 370 kBq of [γ-^{32}P]ATP. After incubation for 30 min at 37°C, the reaction was stopped by washing the beads four times in cold kinase buffer and the labeled immunocomplexes were solubilized by boiling for 5 min in 50 μl of SDS-sample buffer. The samples (10 μl) were resolved by SDS-PAGE, transferred to nitrocellulose, and the ^{32}P-labeled proteins were detected using the bioimaging analyzer BAS-5000 (Fuji Photo Film). In some experiments, 5 μg of porcine brain tubulin or 5 μg of BSA were added to the immunocomplexes before kinase assay.

Gel electrophoresis and immunoblotting

SDS-PAGE on 7.5% gels, electrophoretic transfer of separated proteins onto nitrocellulose and details of the immunostaining procedure have been described elsewhere (46). The anti-tubulin Abs TU-01 and TU-32, in the form of spent culture supernatants, were diluted 1/10, whereas GTU-88 was diluted 1/5,000. mAbs against kinases Src, Fyn, Lyn, Syk, and phosphotyrosine (4G10-HRP) were diluted 1/300, 1/250, 1/1,000, 1/1,000 and 1/30,000, respectively. Rabbit Abs against phosphotyrosine, actin, and GST were diluted 1/2,000, 1/2,000 and 1/10,000, respectively. Bound Abs were detected after incubation of the blots with secondary Abs diluted 1/10,000, and after washing with chemiluminiscence reagents in accordance with the manufacturer's directions. Exposed autoradiography films were quantified by densitometry.

Immunofluorescence

Immunofluorescence microscopy was performed on fixed cells as described (45). Shortly, cells were attached to poly-L-lysine covered coverslips, rinsed briefly with microtubule-stabilizing buffer (MSB; MES buffer supplemented with 4% polyethylene glycol 6000), fixed for 20 min in 3% formaldehyde in MSB and extracted for 4 min with 0.5% Triton X-100 in MSB. Ab TUB against $\alpha\beta$-tubulin dimer was diluted 1/10 and Cy3-conjugated TUB2.1 Ab against β-tubulin was diluted 1/500. Anti-Lyn Ab Lyn-01/Pr and anti-Syk Ab Syk-01 were used as ascitic fluids diluted 1/200, anti-Fyn mAb and anti-γ-tubulin Ab GTU-88 were diluted 1/50 and 1/500, respectively. Anti-phosphotyrosine Ab PY-20 was used at concentration of 2.5 μg/ml. Cy3-conjugated and FITC-conjugated anti-mouse Abs were diluted, respectively, 1/1,000 and 1/100. FITC-conjugated rabbit Ab was diluted 1/200. For double-label staining of microtubules and γ-tubulin, the coverslips were incubated simultaneously with GTU-88 and polyclonal TUB Ab. After washing, the coverslips were incubated simultaneously with the secondary fluorochrome-conjugated Abs. For double-label staining of microtubules and tyrosine phosphorylated proteins, the coverslips were incubated with PY-20 Ab, followed by incubation with FITC-conjugated anti-mouse Ab specific for IgG. The remaining binding sites on FITC-conjugated Ab were blocked by incubation with normal mouse serum (diluted 1/10) before incubation with Cy3-conjugated TUB2.1 Ab. The preparations were mounted in MOWIOL 4–88 (Calbiochem) and examined with Olympus A70 Provis microscope. Conjugates alone did not give any detectable staining.

Results

Distribution of kinases and tubulins in Lyn$^{-/-}$ BMMCs

To compare the expression profiles of protein tyrosine kinases of the Src and Syk/Zap families and tubulins in wild-type and Lyn$^{-/-}$ BMMCs, blots of whole cell extracts were probed with Abs against Src family kinases Lyn, Fyn, Src, Yes, Fgr, Hck, and Lck, Abs against kinases Syk and Zap, and with Abs against tubulins. In both wild-type and Lyn$^{-/-}$ BMMCs, kinases Fyn, Src, and Syk were easily detectable, while Yes, Lck, and Zap kinases were not detected. As expected, Lyn kinase was found only in wild-type cells. A substantially lower signal was detected for Src kinase in comparison to Fyn kinase. From the remaining tested Src family kinases, Fgr and Hck were stained very faintly only after a longer exposure of the film. When Abs against Syk kinase, α-tubulin (55 kDa) and γ-tubulin (48 kDa) were used, similar signal was observed in wild-type and Lyn$^{-/-}$ BMMCs, and the same was true for actin and vinculin. The absence of Lyn kinase in Lyn$^{-/-}$ BMMCs was also confirmed by immunofluorescence microscopy. Dot-like staining concentrated often in pericentrosomal region was detected in wild-type cells with anti-Lyn Ab (Fig. 1*A*), while no specific staining was detected in Lyn$^{-/-}$ BMMCs (Fig. 1*B*). In both wild-type (data not shown) and Lyn$^{-/-}$ BMMCs, Fyn kinase (Fig. 1*C*) and Syk kinase (Fig. 1*D*) also exhibited dot-like distribution. Double-labeling in Lyn$^{-/-}$ BMMCs revealed that Ab against $\alpha\beta$-tubulin dimer stained a typical network of microtubules originating from centrosomes (Fig. 1*E*), while the Ab against γ-tubulin stained centrosomes and diffusely the cytoplasm (Fig. 1*F*). It should be noted that a comparable staining pattern of γ-tubulin was observed with two different mAbs, GTU-88 and TU-31 directed against peptides from N-terminal domain and C-terminal domain of γ-tubulin, respectively, and with polyclonal Ab.

Because γ-tubulin, Fyn kinase, and Syk kinase exhibited a dot-like staining pattern suggesting an association with membrane components, we investigated their distribution in detergent-soluble and insoluble fractions. Extraction of wild-type BMMCs with 1% Nonidet P-40 at 4°C for 30 min showed that there were differences in solubility of Fyn kinase, Syk kinase, and tubulins (Fig. 2). Although Fyn kinase was present in soluble and insoluble fractions in

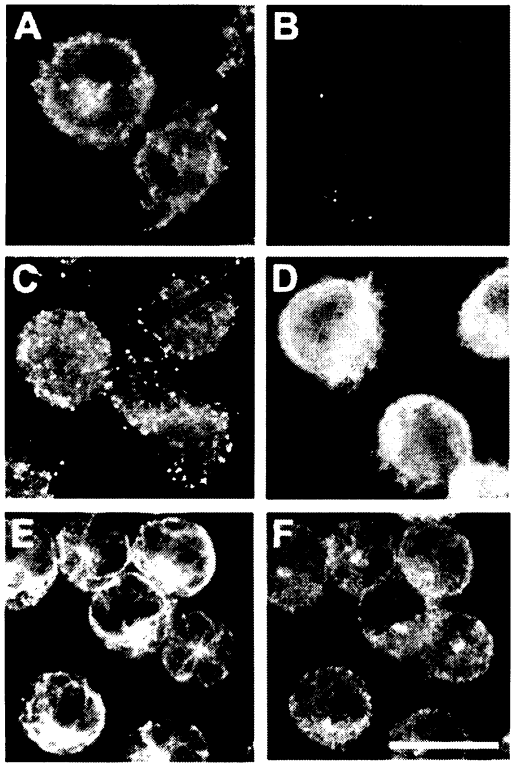


FIGURE 1. Immunofluorescence localization of kinases and tubulins in wild-type (*A*) and Lyn$^{-/-}$ BMMCs (*B–F*). The cells were stained with Abs specific for Lyn (*A* and *B*), Fyn (*C*), and Syk (*D*). Double-label staining with a polyclonal Ab against αβ-tubulin dimers (*E*) and mAb against γ-tubulin (*F*). Pair (*E* and *F*) represents the same cells. The cells were fixed in formaldehyde and extracted in Triton X-100 before labeling. Scale bar, 10 μm. Comparable magnifications are in *A–F*.

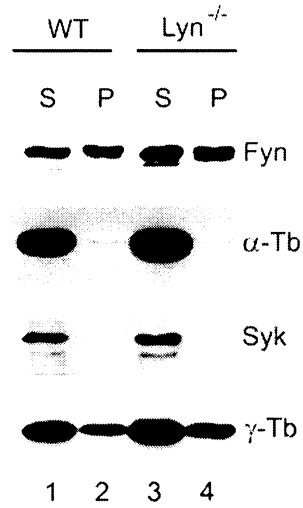


FIGURE 2. Immunoblot analysis of soluble and insoluble fractions from wild-type and Lyn$^{-/-}$ BMMCs. To compare the relative distribution of various proteins in wild-type (WT; *lanes 1–2*), and Lyn$^{-/-}$ BMMCs (*lanes 3–4*), the cells were solubilized in lysis buffer with 1% Nonidet P-40, and after centrifugation the supernatant (S) and pellet (P) were separated. Pelleted material was resuspended in a volume equal to the volume of the supernatant. Immunostaining of two identical blots with Abs against Fyn kinase (Fyn) and α-tubulin (α-Tb), and Syk kinase (Syk) and γ-tubulin (γ-Tb). A typical result from three experiments performed.

similar amounts. Syk kinase was found almost exclusively in soluble fraction. α-Tubulin was also highly abounding in soluble fraction. The relative distribution of γ-tubulin resembled that of Fyn kinase, and 38 ± 2% (mean ± SD; $n = 6$) of γ-tubulin was present in insoluble form. A similar distribution pattern of the proteins was found in Lyn$^{-/-}$ BMMCs. These data demonstrate that cells extracted under conditions favoring depolymerization of microtubules contain a significant fraction of both Fyn kinase and γ-tubulin in the detergent-resistant fraction. Thus, based on Nonidet P-40 detergent solubility, γ-tubulin differs from α-tubulin.

Tyrosine phosphorylation of γ-tubulin-associated proteins in activated cells

To evaluate the effect of cell activation on microtubule distribution, wild-type and Lyn$^{-/-}$BMMCs were stimulated by FcεRI aggregation or pervanadate exposure before immunofluorescence double labeling with anti-β-tubulin and anti-phosphotyrosine Abs. Fig. 3*A* shows double-label immunofluorescence experiment on resting and FcεRI-activated Lyn$^{-/-}$ BMMCs with Abs against phosphotyrosine and β-tubulin. In resting cells, tyrosine-phosphorylated proteins were stained very faintly and diffusely in cytoplasm (Fig. 3*Aa*), whereas in cells activated for 3 min with Ag they were stained strongly and were concentrated in the perinuclear region (Fig. 3*Ad*). After activation more intense staining of microtubules was detected (Fig. 3*Ae*) when compared with resting cells (Fig. 3*Ab*). Similar changes in the distribution pattern of tyrosine-phosphorylated proteins and microtubules were observed in wild-type BMMCs (data not shown). Fig. 3*B* shows a similar experiment using Lyn$^{-/-}$ BMMCs activated by pervanadate. Again, in cells activated for 3 min, phosphorylated proteins were concentrated in perinuclear region (Fig. 3*Bd*). After 15-min activation, a substantial increase in the staining of proteins phosphorylated on tyrosine was detected at the cell periphery (Fig. 3*Bg*). As to microtubules, 3-min activation resulted in enhanced accumulation of microtubules in cell periphery (Fig. 3*Be*). Longer stimulation (15 min) reduced the staining of microtubules when compared with activation for shorter time (Fig. 3*Bh*). When the cells were pretreated before activation with Src family specific inhibitor PP2, phosphorylated proteins were stained faintly, comparably to nonactivated cells, and staining of microtubules was not increased (data not shown). Quantitative immunoblotting revealed that the amount of polymerized tubulin was increasing with a peak at 3–5 min activation, while the amount of polymerized vimentin was unchanged (Fig. 3*C*). The distribution of γ-tubulin examined by means of specific Abs was found basically unchanged during activation and was located both on centrosomes and in the cytoplasm (Fig. 1*F*). Collectively, these data demonstrate that early stages of cell activation, when microtubule formation is stimulated, are characterized by tyrosine-phosphorylated proteins concentrating in the centrosomal region of the cell, where γ-tubulin is accumulated.

To determine whether γ-tubulin forms de novo complexes with tyrosine-phosphorylated proteins in activated cells, immunoprecipitation experiments were performed with cell lysates prepared from cells activated by FcεRI aggregation or by pretreatment with pervanadate. Using anti-γ-tubulin Ab TU-31 immobilized on protein A, and cell lysate from nonactivated cells, only small amount of tyrosine-phosphorylated proteins coprecipitated with γ-tubulin. (Fig. 4, *panel* P-Tyr, *lane 1*). However, in cells activated with Ag for 1 min the amounts of coprecipitated and phosphorylated proteins increased and weres further enhanced after another 2 min (Fig. 4, *panel* P-Tyr, *lanes 2* and *3*). The amount of coprecipitated proteins decreased after 6 min activation (data not shown). No staining was seen when protein A alone was incubated with extracts from activated cells (Fig. 4, *panel* P-Tyr, *lane 4*) or when the immobilized Ab was incubated without the extract (Fig. 4, *panel*

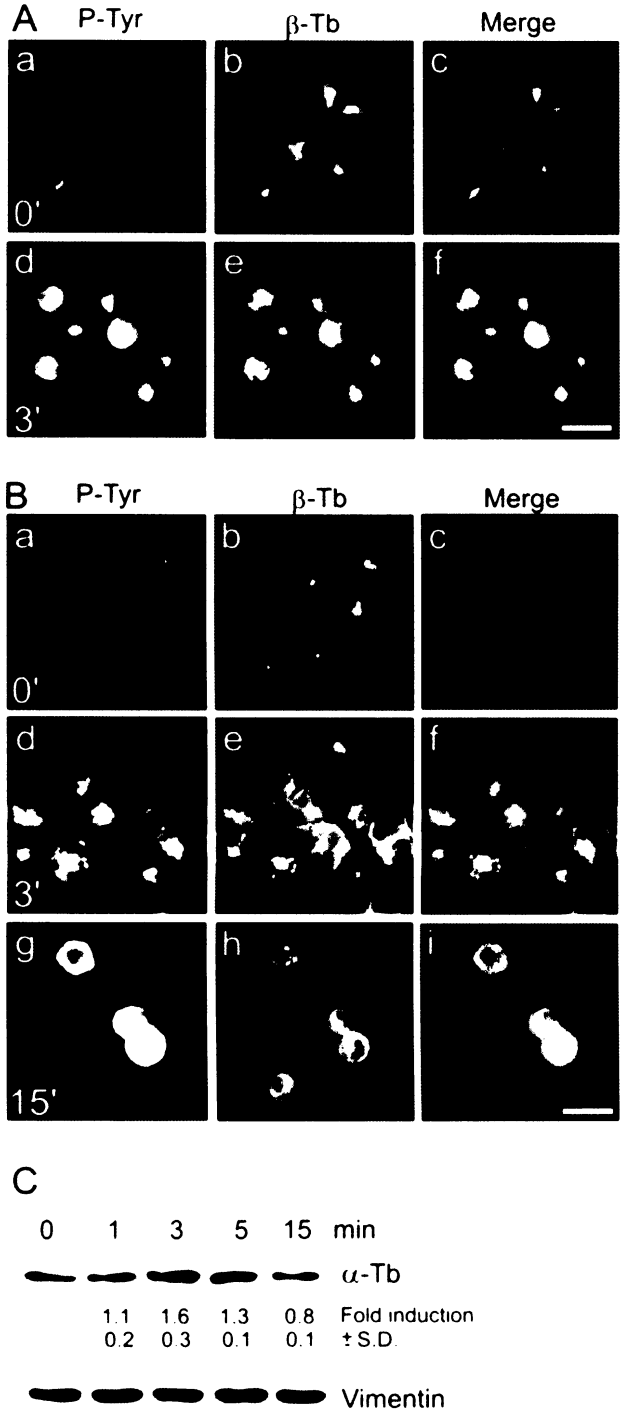


FIGURE 3. Immunofluorescence localization of tyrosine-phosphorylated proteins and β-tubulin in resting and activated $Lyn^{-/-}$ BMMCs. *A*, The resting cells (*a–c*) or cells activated for 3 min by FcεRI aggregation (*d–f*) were stained by double labeling with Abs specific for phosphotyrosine (*a* and *d*; green) and β-tubulin (*b* and *e*; red). *c* and *f*, Superpositions of stainings in each row. All photographs were taken under the same exposure conditions. *B*, The resting cells (*a–c*) or cells treated with pervanadate for 3 min (*d–f*) or 15 min (*g–i*) were stained by double labeling with Abs specific for phosphotyrosine (*a*, *d*, and *g*; green) and β-tubulin (*b*, *e*, and *h*; red). *c*, *f*, and *i*, Superpositions of stainings in each row. All photographs were taken under the same exposure conditions. Scale bars, 10 μm. *C*, The resting cells or cells stimulated for various time intervals (1–15 min) by pervanadate were extracted in 0.2% Triton X-100, and detergent-insoluble fractions were analyzed by immunoblotting using Ab against α-tubulin. Anti-vimentin Ab was used as loading control. Numbers under the blot indicate relative amount of α-tubulin normalized to unstimulated control. Means ± SD were calculated from three experiments.

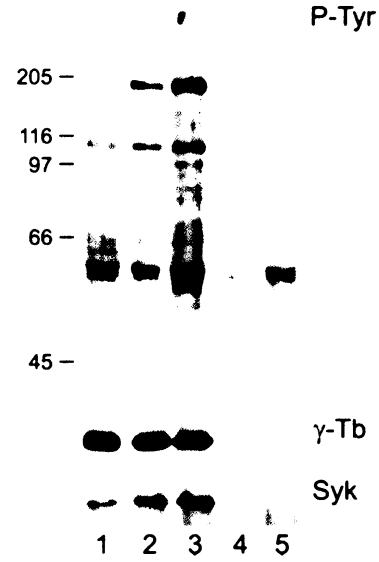


FIGURE 4. γ-Tubulin-associated proteins in resting and FcεRI-activated $Lyn^{-/-}$ BMMCs. γ-Tubulin was precipitated with TU-31 Ab immobilized to protein A beads and the blots were probed with Abs against phosphotyrosine (P-Tyr), γ-tubulin (γ-Tb), and Syk kinase (Syk). Immunoprecipitated proteins from resting cells (*lane 1*), cells activated by FcεRI aggregation for 1 min (*lane 2*) or 3 min (*lane 3*). Negative control precipitations from activated cells (3 min) using protein A beads without Ab (*lane 4*) or protein A beads with anti-γ-tubulin Ab but without cell extract (*lane 5*). Positions of molecular mass markers (in kilodaltons) are indicated on the *left*. A typical results from four experiments performed.

P-Tyr, *lane 5*). Staining of the precipitated material with anti-γ-tubulin Ab confirmed the presence of γ-tubulin in both unstimulated and stimulated cells (Fig. 4, *panel* γ-Tb, *lanes 1–3*). Similar experiments with pervanadate-activated cells yielded complexes of comparable properties but formation of γ-tubulin assemblies and the extent of tyrosine phosphorylation were more pronounced. From nonactivated cells, only a small amount of tyrosine-phosphorylated proteins coprecipitated with γ-tubulin. (Fig. 5, *panel* P-Tyr, *lane 1*). However, after a 3-min stimulation, the amount of coprecipitated proteins increased and was further enhanced after another 12 min (Fig. 5, *panel* P-Tyr, *lanes 2* and *3*). Close inspection showed that γ-tubulin associated with tyrosine-phosphorylated proteins with relative molecular weights around 50, 60, 70, 80–97, 110, and 200 kDa. Similar staining pattern was observed with different Abs against phosphotyrosine proteins (mAbs 4G10 or PY-20 and polyclonal Ab; data not shown). No such staining was observed with negative controls (Fig. 5, *panel* P-Tyr, *lanes 4* and *5*). When a negative control Ab NF-09 (IgG2a) was used, no phosphotyrosine proteins were detected (data not shown), proving the specificity of the observed reactivity. γ-Tubulin was present in both unstimulated and stimulated cells (Fig. 5, *panel* γ-Tb, *lanes 1–3*). When the immunoprecipitated proteins were probed with polyclonal Ab against α-tubulin, a distinct faint band was detected in control as well as in activated cells in the position of α-tubulin (Fig. 5, *panel* α-Tb, *lanes 1–3*). It is unlikely that the observed associations of the proteins reflect unspecific interactions because a number of other cytoplasmic proteins, including actin, showed no such association. A comparison of tyrosine-phosphorylated proteins associated with γ-tubulin from wild-type with those from $Lyn^{-/-}$ BMMC failed to reveal any substantial qualitative and quantitative differences, except a more intense staining of ~70-kDa protein in wild-type BMMC (data not shown).

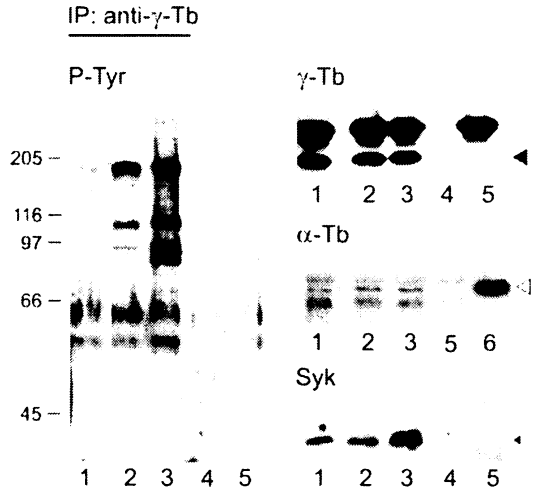


FIGURE 5. γ-Tubulin-associated proteins in resting and pervanadate-activated Lyn$^{-/-}$ BMMCs. γ-Tubulin was precipitated with TU-31 Ab immobilized to protein A beads, and the blots were probed with Abs against phosphotyrosine (P-Tyr), γ-tubulin (γ-Tb), α-tubulin (α-Tb), and Syk kinase (Syk). Immunoprecipitated proteins from resting cells (*lane 1*), cells treated with pervanadate for 3 min (*lane 2*) or 15 min (*lane 3*). Negative control precipitations from pervanadate activated cells (15 min) using protein A beads without Ab (*lane 4*) or protein A beads with anti-γ-tubulin Ab but without cell extract (*lane 5*). Position of tubulin in cell extract is shown in *lane 6*. Positions of molecular mass markers (in kilodaltons) are indicated on the *left* in panel P-Tyr. Black arrowhead, white arrowhead, and small arrowhead point to γ-tubulin, α-tubulin, and phosphorylated Syk, respectively. A typical result from four experiments performed.

Association of γ-tubulin with kinases

To find out whether γ-tubulin forms complexes with kinases, γ-tubulin was precipitated from nonactivated or FcεRI-activated cells. Activation of the cells was alternatively induced with pervanadate which gave somewhat stonger signal. Data presented in Fig. 4, *panel* Syk, *lanes 1–3*, clearly show that Syk kinase associates with γ-tubulin in FcεRI-activated cells and that the amount of Syk immunoprecipitated with γ-tubulin correlates with the extent of protein tyrosine phosphorylation. Similar results were observed with pervanadate (Fig. 5, *panel* Syk). Additional experiments showed that γ-tubulin could be precipitated specifically with anti-Fyn-, anti-Syk-, and anti-P-Tyr-specific Abs (Fig. 6, *A–C*, *lanes 2* and *3*). In precipitates with anti-phosphotyrosine Ab, more γ-tubulin was observed in activated than in resting cells. No staining in the position of γ-tubulin was observed when immobilized Abs were incubated without the extract (Fig. 6, *A–C*, *lane 1*) or when protein A without the Ab was incubated with extracts from stimulated cells (data not shown). Immunostaining with anti-Fyn Ab confirmed the presence of Fyn kinase in the precipitate (Fig. 6*D*). Labeling with anti-phosphotyrosine Ab showed that a protein phosphorylated on tyrosine was present in a position corresponding to Fyn kinase in both resting and activated cells, however, in activated cells Fyn showed an enhanced phosphorylation (Fig. 6*G*). In precipitates with anti-Syk Ab, nonphosphorylated and phosphorylated forms of Syk kinase were observed in activated cells (Fig. 6*E*). This was confirmed by labeling with anti-phosphotyrosine Ab (Fig. 6*H*). In precipitates with anti-phosphotyrosine Ab, both phosphorylated forms of Syk kinase (Fig. 6*F*) and Fyn kinase (Fig. 6*I*) were detected in enhanced amounts in activated cells. When negative control rabbit Ab against myosin was used for immunoprecipitation of the extract from stimulated cells, no γ-tubulin was detected (data not shown). Basically, the same results were obtained with lysates from wild-type BMMCs (data not shown). To rule out the possibility that the association of γ-tubulin with kinases is only due to indirect protein associations within large phosphoprotein aggregates, precipitation experiments with anti-Fyn and anti-Syk Abs were also performed with FcεRI-activated cells. In cells activated with Ag, the amount of γ-tubulin coprecipitated with anti-Fyn Ab increased with a peak 3 min after triggering (Fig. 6*J*, *panel* γ-Tb, *lanes 1–4*). Similarly, a peak in the amount of γ-tubulin coprecipitated with Syk was observed at 3 min in FcεRI-activated cells (Fig. 6*K*, *panel* γ-Tb, *lanes 1–4*). The combined data indicate that soluble γ-tubulin in activated cells appears in complexes with Fyn and Syk kinases and several other proteins phosphorylated on tyrosine. Importantly, the formation of these complexes is not dependent on the presence of Lyn kinase.

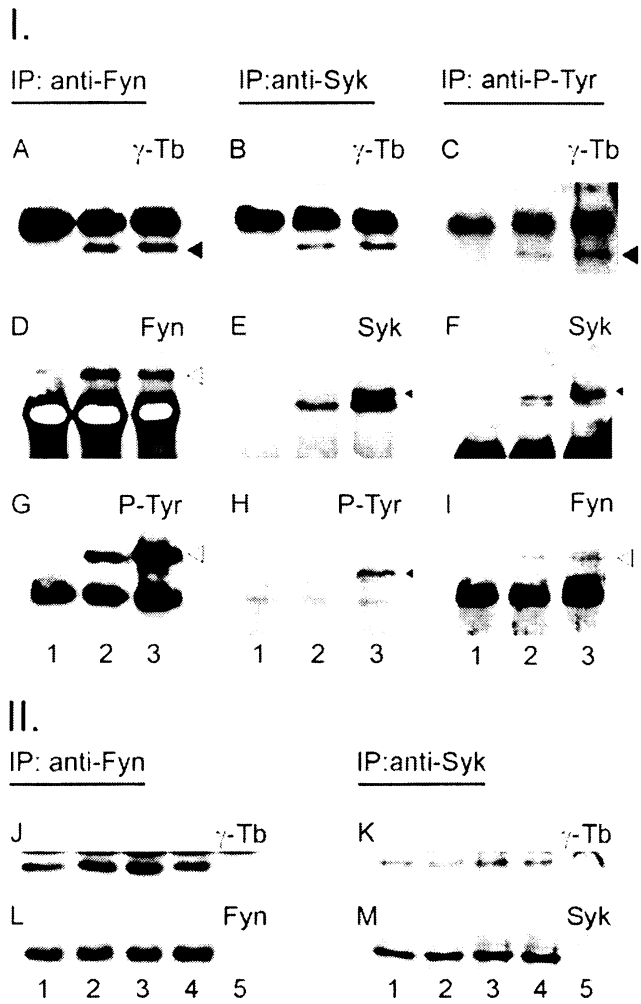


FIGURE 6. Immunoprecipitation of Fyn and Syk from Lyn$^{-/-}$ BMMCs stimulated by pervanadate (*I*) or FcεRI aggregation (*II*). *I*. Cell extracts were precipitated with protein A immobilized Abs specific to Fyn kinase (*A*, *D*, and *G*), Syk kinase (*B*, *E*, and *H*), or phosphotyrosine (*C*, *F*, and *I*). Blots were probed with Abs against γ-tubulin (γ-Tb), kinase Fyn (Fyn), kinase Syk (Syk), or phosphotyrosine (P-Tyr). Immobilized Igs not incubated with cell extract (negative control, *lane 1*), immunoprecipitated proteins from resting cells (*lane 2*), and from cells treated with pervanadate for 15 min (*lane 3*). Black arrowhead, white arrowhead, and small arrowhead point to γ-tubulin, Fyn kinase, and phosphorylated Syk kinase, respectively. *II*. Cell extracts were precipitated with protein A immobilized Abs specific to Fyn kinase (*J* and *L*) and Syk kinase (*K* and *M*). Blots were probed with Abs against γ-tubulin (γ-Tb), kinase Fyn (Fyn), and kinase Syk (Syk). Immunoprecipitated proteins from resting cells (*lane 1*), cells activated by FcεRI aggregation for 1 min (*lane 2*), 3 min (*lane 3*), or 6 min (*lane 4*). Immobilized Igs not incubated with cell extract (negative control, *lane 5*). A typical result from four experiments performed.

Binding of γ-tubulin complexes to the regulatory domains of Fyn kinase

Guided by our finding that γ-tubulin in Lyn$^{-/-}$ BMMCs forms complexes with Fyn kinase, we further investigated whether the SH2 and/or SH3 domains of Fyn participate in these interactions. Data presented in Fig. 7 show that γ-tubulin complex binds to GST-Fyn-SH2 as well as GST-Fyn-SH3 fusion proteins, but not to GST alone. Under identical conditions, more γ-tubulin was bound to GST-Fyn-SH2 than to GST-Fyn-SH3. In activated cells, more γ-tubulin complex was bound to GST-Fyn SH2, but there was no difference in binding of γ-tubulin complex from resting and activated cells to the GST-Fyn-SH3 (Fig. 7*A*, *panel* γ-Tb). The same distribution pattern of γ-tubulin was observed with anti-γ-tubulin Abs GTU-88 and TU-32 that are directed against different epitopes on γ-tubulin molecule (data not shown). The amount of immobilized GST fusion proteins was similar as detected by staining with anti-GST Ab (Fig. 7*A*, *panel* GST). Using two different Abs against α-tubulin (DM1A and TU-01) and two different Abs against β-tubulin (TUB 2.1 and TU-06), we failed to detect the binding of αβ-tubulin dimers to GST-fusion proteins, and the same holds true for negative-control anti-actin Ab (data not shown). Interestingly, strong binding of phosphorylated Syk kinase to GST-Fyn-SH2 was observed in activated cells (Fig. 7*B*, *panels* Syk); however, no binding of Syk to GST-Fyn-SH3 was detected under identical conditions (data not shown). More γ-tubulin was also found in GST-Fyn-SH2 pull-down complexes when lysates from FcεRI-activated cells were used (data not shown).

To determine whether the observed interactions of γ-tubulin complex with the SH2 domain of Fyn kinase reflect an SH2-phosphotyrosine type interaction, we performed competition experiments with extracts from activated Lyn$^{-/-}$ BMMCs and phenyl phosphate, an analog of phosphotyrosine. Phenyl phosphate inhibited in a concentration-dependent manner the binding of γ-tubulin complex to GST-Fyn-SH2; IC_{50} was attained at 7.5 mM phenyl phosphate. This inhibition was specific, because phosphoserine had no effect on the binding (data not shown). Similarly, the binding of Syk to GST-Fyn-SH2 was inhibited by phenyl phosphate (IC_{50} at 9 mM), while phosphoserine (up to 40 mM) was without effect. The relatively high concentration of phenyl phosphate required to get IC_{50} implies that the inhibitor has low specificity. Additional experiments, therefore, made use of the peptide containing the most preferable motif (pY-E-E-I) for binding to Fyn-SH2 domain (47). The results showed that the phosphorylated peptide (PQpYEEIPI) reduced the binding of γ-tubulin complex to GST-Fyn-SH2 domain (Fig. 7*B*, *panel* γ-Tb). The IC_{50} was attained at the concentration of 14 μM (Fig. 7*C*). The unphosphorylated form of the same peptide was without effect. The phosphorylated oligopeptide also inhibited the binding of Syk to GST-Fyn-SH2 (Fig. 7*B*, *panel* Syk) with IC_{50} attained at 21 μM. These data suggest that the bindings were mediated by an interaction of Fyn-SH2 domain with tyrosine-phosphorylated residues present in adaptor proteins associated with the complexes. Experiments repeated with FcεRI-activated cells gave similar results (data not shown). Treatment of the cells with Src family specific inhibitor SU6656 before activation resulted in a lower amount of γ-tubulin and phosphorylated Syk associated with GST-Fyn-SH2 as revealed by pull-down experiments (Fig. 8). Another Src family inhibitor PP2 had a similar effect (data not shown).

To decide whether the observed interactions of γ-tubulin with Fyn-SH2 and Fyn-SH3 domains are direct or indirect, we searched the ubiquitously expressed (48) mouse γ-tubulin (Swiss-prot accession no. P83887) for consensus sequences that could be involved directly in binding to Fyn SH2 or SH3 domains. The data

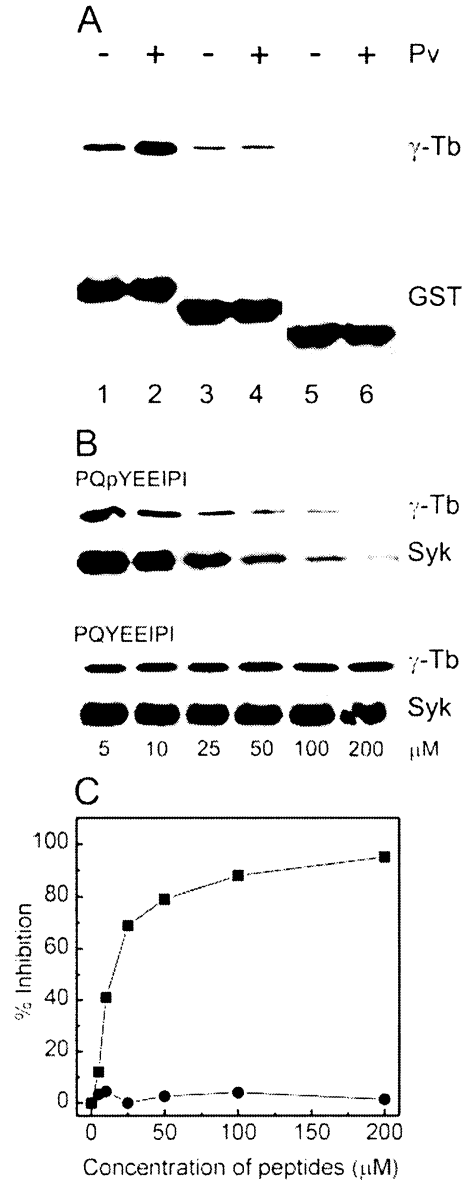


FIGURE 7. Binding of γ-tubulin and Syk kinase to SH2 or SH3 domains of Fyn kinase. *A*, Postnuclear supernatants from resting (−) or pervanadate-activated cells (+; 15 min) were incubated with GST fusion proteins or GST alone (negative control) immobilized to glutathione Sepharose beads. Bound proteins were eluted into SDS-sample buffer and fractionated on SDS-PAGE. Blots were probed with Ab against γ-tubulin (γ-Tb) or Ab against GST. GST-SH2 domain of Fyn kinase (*lanes 1* and *2*), GST-SH3 domain of Fyn kinase (*lanes 3* and *4*), GST alone (*lanes 5* and *6*). *B*, Effect of tyrosine-phosphorylated oligopeptide (PQpYEEIPI) and its nonphosphorylated form (PQYEEIPI) on binding of γ-tubulin (γ-Tb) and Syk kinase (Syk) to SH2 domain of Fyn kinase. GST-SH2 domain of Fyn kinase was incubated with cell extract from pervanadate-activated cells in the absence (0 mM) or presence of tyrosine-phosphorylated oligopeptide (PQpYEEIPI) and its nonphosphorylated form at concentrations 5–200 μM. *C*, Quantification of data for γ-tubulin presented in (*B*). Extent of binding of γ-tubulin complexes to the GST-SH2 domain of Fyn kinase in the presence of PQpYEEIPI oligopeptide (■—■) or PQYEEIPI oligopeptide (●—●) was determined by densitometric analysis.

show that γ-tubulin possesses 15 tyrosine residues, but none of them is within the most preferable motif (Y-E-E-I) for binding to Fyn-SH2 domain. However, one tyrosine (residue 186) fits to the general amino acid consensus sequence (Y-hydrophilic-hydrophilic-hydrophobic) recognized by SH2 domains of the Src family kinases (47). Moreover, γ-tubulin contains two consensus motifs

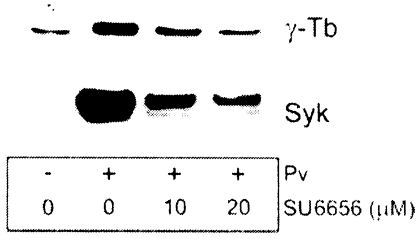


FIGURE 8. Effect of Src-family specific inhibitor on the binding of γ-tubulin or Syk kinase to the SH2 domain of Fyn kinase. $Lyn^{-/-}$ BMMCs were incubated with SU6656 inhibitor at final concentration 10 and 20 μM or without inhibitor and then activated (+) or not (−) with pervanadate (15 min). Postnuclear supernatants were incubated with GST-Fyn-SH2 fusion proteins immobilized to glutathione Sepharose beads. Bound proteins were eluted into SDS-sample buffer and fractionated on SDS-PAGE. Blots were probed with Ab against γ-tubulin (γ-Tb) or Ab against Syk kinase (Syk).

for binding to SH3 domains, P-X-X-P (49). In further competition experiments, we therefore used the oligopeptides FIPWGPAS covering the sequence 348–355 of γ-tubulin and KSPYLPSA covering the sequence 363–370. The P-X-X-P sequences are underlined. However, even at the highest concentration of the tested peptides used (5 mM) there was no inhibition of binding to Fyn SH3 domain. We also used the oligopeptide PYNSLL, covering the sequence 185–190 of mouse γ-tubulin and its tyrosine-phosphorylated form, in competition experiments with the SH2 domain of Fyn. Unphosphorylated oligopeptide was without effect and its phosphorylated form gave only 30% inhibition at a concentration 3 mM (data not shown), indicating a low specificity of inhibition reflecting just the presence of phosphotyrosine in the peptide. Collectively, these data suggest that the binding of γ-tubulin to SH2 and SH3 domains of Fyn kinase is indirect.

Phosphorylation of tubulin dimers by protein tyrosine kinases associated with γ-tubulin complexes

Possible associations of γ-tubulin with kinases and their substrates were also examined by immunocomplex kinase assays. Lysates from resting $Lyn^{-/-}$ BMMCs or cells stimulated with pervanadate or FcεRI aggregation were precipitated with anti-γ-tubulin Ab (TU-31) or a negative control Ab NF-09. Immunocomplexes were then subjected to the in vitro kinase assays and analyzed by SDS-PAGE followed by electroblotting and autoradiography. γ-Tubulin in the extract from resting cells was associated with several kinase substrates ranging from 40 to 200 kDa (Fig. 9*A*, *lane 1*). When activated, the pattern of labeled proteins was similar, but the amount of ^{32}P-labeled proteins increased (Fig. 9*A*, *lanes 2* and *3*). No kinase activity was detected after precipitation with the control Ab (Fig. 9*A*, *lane 4*). The labeling patterns using lysate from resting and pervanadate-stimulated (15 min) wild-type BMMCs are shown in Fig. 9*A* (*lanes 5–6*). Compared with $Lyn^{-/-}$ BMMCs, stronger signals were detected, namely at a region of ~70 kDa. The staining of size-separated ^{32}P-labeled proteins in γ-tubulin immunocomplexes, from $Lyn^{-/-}$ BMMCs, subjected to the in vitro kinase assay with anti-phosphotyrosine Ab revealed that the dominant ^{32}P-labeled proteins of 50, 80–97, 110, and 200 kDa contained phosphotyrosine. Staining with Ab against phosphoserine showed only a faint staining of 110- and 200-kDa proteins. In contrast, no staining was detected with Ab specific for phosphothreonine (data not shown).

When cells before activation were cultured in the presence of Src family selective tyrosine kinase inhibitor PP2, a lower level of phosphorylation was detected (Fig. 9*B*, *lane 2*). In contrast, the presence of PP3 (negative control to PP2) had no effect (data not shown). Similarly, when the cells were cultured in the presence of Syk selective tyrosine kinase inhibitor piceatannol (dissolved in DMSO), a clear inhibition of phosphorylation was detected (Fig. 9*C*, *lane 2*). No inhibition was observed when DMSO alone was used. Staining of parallel blots with anti-phosphotyrosine and anti-Syk Abs revealed that PP2 pretreatment inhibited the phosphorylation of Syk kinase (data not shown).

Because γ-tubulin forms a complex with tubulin dimers (Fig. 5), we wanted to find out whether tubulin dimers could serve as a substrate for kinases present in the γ-tubulin immunocomplexes. When exogenous αβ-tubulin dimers (5 μg) were added to γ-tubulin immunocomplexes, phosphorylation of this extra tubulin was observed in kinase assay (Fig. 9*D*, *lane 3*). In cells pretreated with PP2, the extent of tubulin labeling was lower (Fig. 9*D*, *lane 4*). The results of similar experiments with extracts from cells pretreated with piceatannol also showed lower phosphorylation of exogenous tubulin (Fig. 9*E*, *lanes 3* and *4*). When BSA (5 μg) was added to kinase mixture no labeling of this protein was observed. These data demonstrate that both active Src family kinase(s) and active Syk kinase are part of the γ-tubulin complexes in $Lyn^{-/-}$ BMMCs and that these kinases can be involved in phosphorylation of tubulin dimers.

To prove that an enhanced kinase activity in γ-tubulin complexes is also present in cells activated by FcεRI aggregation, the cells were stimulated with Ag and the kinase activity of γ-tubulin immunocomplexes was evaluated by kinase assay and autoradiography. Enhanced kinase activity in γ-tubulin immunocomplexes detected in FcεRI-activated cells (Fig. 9*F*) proved that an association of γ-tubulin with kinases also occurred also under more physiological conditions (Fig. 9*F*). When the cells were exposed to Src kinase inhibitor PP2 (20 μM) and then activated by FcεRI aggregation (3 min), a lower level of phosphorylation of proteins associated with γ-tubulin immunocomplexes was detected (data not shown).

Discussion

FcεRI aggregation in mast cells and basophiles leads to rapid cytoskeleton rearrangements that are important for cell activation and degranulation. Both actin filaments and microtubules play a critical role in this process (7, 8, 10, 50, 51). Data presented in this study show that stimulation of mast cells through FcεRI aggregation or pervanadate exposure triggers the formation of microtubules in both wild-type and $Lyn^{-/-}$ BMMCs, as documented by immunofluorescence microscopy. In our previous study, we have found that Lyn kinase, a major Src family kinase in RBL-2H3 cells (52), forms complexes containing γ-tubulin and phosphotyrosine proteins, and we proposed that Lyn might be involved in microtubule formation (29). To shed more light on the role of Lyn kinase in the formation of γ-tubulin-based complexes, we have primarily analyzed the properties of γ-tubulin immunocomplexes isolated from Lyn-deficient BMMCs. Wild-type BMMCs served as controls. The first evidence that Lyn kinase is dispensable for the formation of functional γ-tubulin complexes was our finding of normal topography of microtubules in $Lyn^{-/-}$ BMMCs. Importantly, FcεRI-induced cell activation resulted in an enhanced microtubule formation, and no difference was observed between wild-type and $Lyn^{-/-}$ BMMCs. More intense formation of microtubules was observed in pervanadate-stimulated cells, supporting the concept that an enhanced activity of kinases and/or shift in balance between kinases and phosphatases is required to accomplish this process.

Several lines of evidence indicated that γ-tubulin extracted from mast cells with nonionic detergent Nonidet P-40-formed complexes with signal transduction molecules that could modulate the

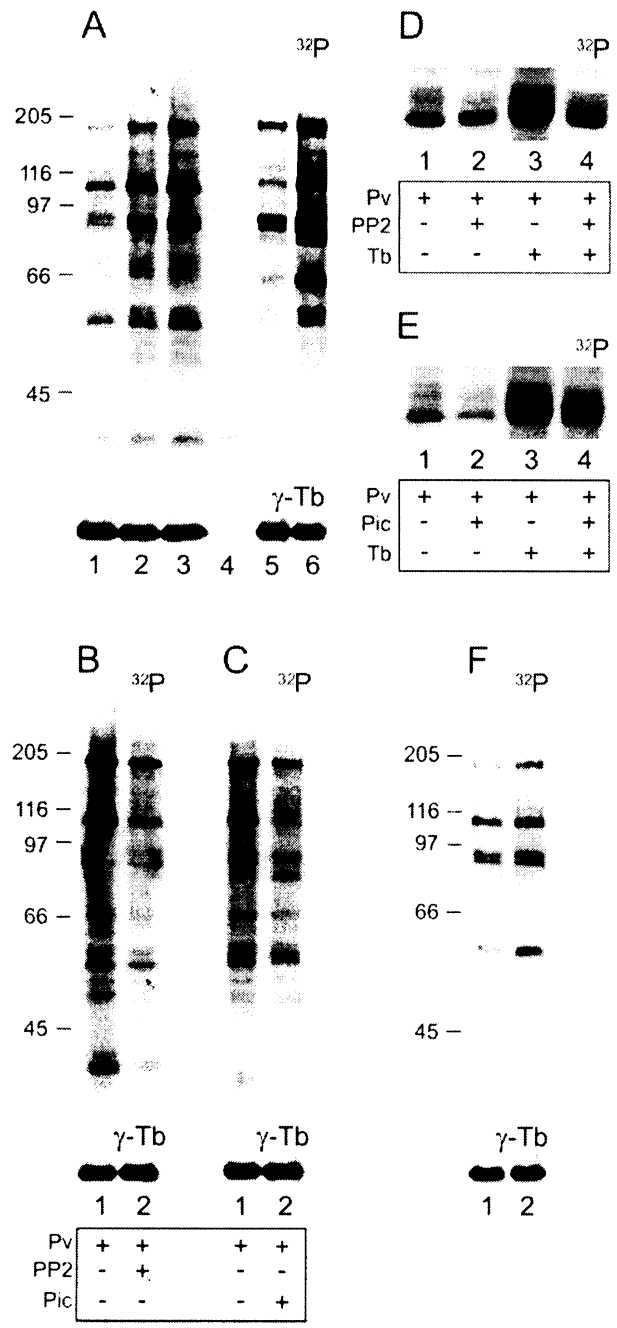


FIGURE 9. The kinase activity in γ-tubulin immunocomplexes from Lyn $^{-/-}$ BMMCs. Samples were prepared from pervanadate-activated (*A* and *E*) or from FcεRI-activated (*F*) cells. Cell lysates were precipitated with anti-γ-tubulin Ab TU-31 or negative control Ab NF-09 bound to immobilized protein A. Immunocomplexes were subjected to in vitro kinase assay, electrophoretically separated, and detected by autoradiography (^{32}P). The presence of γ-tubulin in immunocomplexes was confirmed by immunoblotting with anti-γ-tubulin Ab (γ-Tb). *A*, Precipitation from resting cells (*lane 1*), cells treated with pervanadate for 3 min (*lane 2*) or 15 min (*lane 3*). Precipitation with negative control Ab NF-09 from 15 min pervanadate-treated cells (*lane 4*). For comparison, the kinase activity after precipitation from resting (*lane 5*) and pervanadate-activated (15 min) wild-type BMMCs (*lane 6*) is shown. *B*, Comparison of kinase activity from pervanadate-activated cells pretreated with DMSO alone (*lane 1*, control) or Src kinase family selective inhibitor PP2 (20 μM) before activation (*lane 2*). *C*, Comparison of kinase activity from pervanadate-activated cells pretreated with DMSO alone (*lane 1*) or Syk kinase inhibitor piceatannol (Pic, 50 μM) (*lane 2*). *D*, Kinase activity from pervanadate-activated cells without (*lanes 1* and *3*) or with PP2 pretreatment (*lanes 2* and *4*). Exogenous αβ-tubulin dimers were added to immunocomplexes before kinase assay (*lanes 3* and *4*). *E*, Kinase activity from pervanadate-activated cells without (*lanes 1* and *3*) or with piceatannol pretreatment (*lanes 2* and *4*). Exogenous αβ-tubulin dimers were added to immunocomplexes (*lanes 3* and *4*). *F*, Precipitation from resting cells (*lane 1*) and from cells stimulated by FcεRI aggregation for 3 min (*lane 2*). Molecular mass markers (in kilodaltons) are indicated on the *left* in *A*, *B*, and *F*.

microtubule arrays. First, tyrosine-phosphorylated proteins were found to be associated with immunoprecipitated γ-tubulin in resting cells and the amount of these proteins increased after activation. Second, in vitro kinase assays revealed that γ-tubulin formed complexes containing kinases and their substrates. Increased ^{32}P labeling of proteins was observed when kinase assays were performed with lysates from cells exposed to pervanadate or cells activated by FcεRI aggregation. Third, the activity of γ-tubulin-associated kinases was inhibited by pretreatment of the cells with Src-family specific (PP2) and Syk-specific (piceatannol) inhibitors; this suggests that Src and Syk family kinases have an important role in the formation of γ-tubulin-signaling complexes. Fourth, association of Fyn kinase with γ-tubulin complexes was confirmed by immunoprecipitation experiments, and the same holds true for Syk kinase. Finally, kinases in γ-tubulin complexes were capable of using tubulin dimer as a substrate, and its phosphorylation was inhibited by both PP2 and piceatannol. Whether other kinases become associated with γ-tubulin complexes in the course of cell activation remains to be determined.

Interaction of γ-tubulin complexes with Fyn was confirmed by pull-down experiments, in which γ-tubulin complex bound to SH2 domain of Fyn kinase in a phosphotyrosine-dependent manner. Similar binding has already been described in embryonal carcinoma P19 cells during neuronal differentiation (28). However, in contrast to P19 cells, Fyn from Lyn$^{-/-}$ BMMC bound to γ-tubulin complexes not only through the SH2 domain, but also the SH3 domain. Although γ-tubulin possesses two potential binding sites for SH3 domains and one for SH2 domain, experiments with phenyl phosphate and synthetic peptide inhibitors failed to confirm a direct binding of γ-tubulin to these domains. Moreover, when purified GST-Fyn-SH2 and GST-Fyn-SH3 fusion constructs were used to probe size-fractionated proteins from activated Lyn$^{-/-}$ BMMCs on nitrocellulose membranes, no specific staining was detected with anti-GST Ab in the γ-tubulin region. Thus, the association with the SH2 domain is probably mediated via adaptor-like tyrosine-phosphorylated protein(s). The binding of γ-tubulin complex with the SH2 domain-containing proteins is probably not mediated by tyrosine-phosphorylated tubulin dimers as we were unable to detect them in pull-down experiments with Fyn SH2 domains using various anti-tubulin Abs. Yet, a binding of α-tubulin to SH2 domain of Fyn was observed in activated human T lymphocytes (53). Either different cell type used or binding of very low amount of tubulin dimer, under the detection limit of our assay, might account for the discrepancy. Concerning γ-tubulin immunocomplexes, further studies are in progress to elucidate their composition, mode of interaction and functions of individual components in mast cell signaling. Fyn kinase, like other Src family kinases, is commonly involved in the formation of multiprotein complexes engaged in interaction with the SH2 and SH3 domains. It is therefore likely that the association of Fyn with γ-tubulin is mediated through other proteins that are also important for microtubule nucleation. Currently, we are verifying whether some of the phosphotyrosine proteins of γ-tubulin immunocomplexes in activated cells belong to the class of proteins of the large γ-TuRC.

The molecular mechanism of the association of Syk kinase with γ-tubulin is unclear. Our study shows that phosphorylated Syk is among the proteins bound to the SH2 domain of Fyn kinase. This

suggests a direct binding of Fyn-SH2 with the phosphotyrosine of Syk. Direct binding of phosphorylated Syk to the SH2 domain of Lyn kinase was described in RBL-2H3 cells (5). Syk has one general amino acid consensus sequence that is recognized by SH2 domains of Src family kinases (5). In RBL as well as in BMMCs, Syk is one of the preferable substrates for Lyn kinase (54). However, even in $Lyn^{-/-}$ BMMCs there is still some phosphorylation of Syk on tyrosine, which is dependent on FcεRI activation (4). Because PP2 inhibited phosphorylation of Syk in pervanadate-activated $Lyn^{-/-}$ BMMCs and phosphorylated Syk bound to GST-Fyn-SH2 in a phosphotyrosine-dependent manner, it is likely that Fyn participates in the phosphorylation of Syk in these cells. These data, together with our finding that piceatannol diminished phosphorylation of proteins in γ-tubulin immunocomplexes, suggest that it is the cross-talk between Fyn and Syk which is responsible for tyrosine phosphorylation of proteins associated with γ-tubulin immunocomplexes in $Lyn^{-/-}$ BMMCs. Whether Fyn can directly phosphorylate Syk in FcεRI-activated $Lyn^{-/-}$ BMMCs remains to be elucidated. Association of Lyn kinase with γ-tubulin was described in RBL cells (29). One might speculate that Fyn kinase could substitute for Lyn in $Lyn^{-/-}$ BMMCs in phosphorylation of those proteins that are important for microtubule nucleation in the course of the activation process. An important regulator of γ-tubulin functions could be the Syk kinase. In wild-type cells Syk kinase is phosphorylated predominantly by Lyn kinase, whereas in $Lyn^{-/-}$ BMMCs, the Fyn kinase could assume this role.

Tubulin has been shown to serve as a substrate for Syk kinase in vivo (55). Syk can phosphorylate both soluble tubulin (56) and tubulin in microtubules (57). Syk phosphorylates α-tubulin on the conserved tyrosine residue (Tyr^{432}) and Syk-selective inhibitor blocks receptor-stimulated tubulin phosphorylation in B lymphocytes (55). However, phosphorylation of tubulin by Syk did not have any profound effect on microtubule assembly in pervanadate-treated cells (57). Furthermore, phosphorylation of tubulin by Src kinase did not cause any significant changes in microtubule polymer (58). It is, therefore, unlikely that phosphorylation of tubulin dimers plays a key role in the increase of microtubule formation.

The formation of microtubules can be regulated either by stabilizing the plus ends of microtubules or by regulating the microtubule nucleation where γ-tubulin plays a key role. It has been shown that the Fyn/Gab2/RhoA-signaling pathway plays a critical role in microtubule-dependent degranulation of mast cells, and that RhoA kinase could be involved in stabilization of the plus ends of microtubules (10). Our data suggest that Fyn also might be involved in phosphorylation of proteins that regulate γ-tubulin interactions. As we saw no evidence of Gab2 association with γ-tubulin immunocomplexes (V. Sulimenko, unpublished data), it seems unlikely that γ-tubulin directly participates in the Fyn/Gab2/RhoA-signaling pathway. However, as we and others have found, γ-tubulin is phosphorylated (27, 28, 59). Phosphorylation of the γ-tubulin residue Tyr^{445}, which is invariably present in all γ-tubulins, was described in budding yeast; a mutation of this residue changed the microtubule dynamics (27). There are other data that point to an association of γ-tubulin with kinases. PI3K binds to γ-tubulin in response to insulin (25), and the 55-kDa regulatory subunit of PI3K interacts with γ-tubulin (26). Besides, we have found that Src family kinases appear in complexes with γ-tubulin in RBL cells (29). Collectively, these data suggest that kinases take part in the regulation of γ-tubulin function(s). This could lead to changes in nucleation properties of centrosomes or alternatively to an enhancement of noncentrosomal microtubule nucleation.

Our data show that both Fyn kinase and γ-tubulin are associated in detergent-resistant fraction of BMMCs. Interestingly, there are several reports pointing to the localization of Fyn and Syk kinases to centrosomal region. Fyn kinase was localized in different cell types in centrosomes and in microtubule bundles radiating from the centrosome (53, 60, 61). Furthermore, Fyn kinase was also found to be associated with microtubules of meiotic cells (62). Finally, Syk was located at the centrosomes in B lymphocytes (63). Thus, tyrosine phosphorylation of centrosomal proteins by Fyn and Syk kinases could be the process linking microtubules to early activation events in BMMCs.

In conclusion, it appears that activation of BMMCs leads to a rapid formation of microtubules and that γ-tubulin forms complexes with Fyn and Syk kinases and other signal transduction molecules in the process of cell activation. We propose that Fyn and Syk kinases are involved in the regulation of binding properties of γ-tubulin and/or its associated proteins and thus modulate the microtubule nucleation in activated mast cells.

Acknowledgments

We thank Dr. J. C. Bulinski (Columbia University, New York, NY) for providing Ab against tyrosinated α-tubulin. We thank Dr. J. Truksa for help with preparation of cDNA.

Disclosures

The authors have no financial conflict of interest.

References

1. Kawakami, T., and S. Galli. 2002. Regulation of mast-cell and basophil function and survival by IgE. *Nat. Rev. Immunol.* 2: 773–786.
2. Blank, U., and J. Rivera. 2004. The ins and outs of IgE-dependent mast-cell exocytosis. *Trends Immunol.* 25: 266–273.
3. Nadler, M. J. S., S. A. Matthews, H. Turner, and J. P. Kinet. 2001. Signal transduction by the high-affinity immunoglobulin E receptor FcεRI: coupling form to function. *Adv. Immunol.* 76: 325–355.
4. Parravicini, V., M. Gadina, M. Kovarova, S. Odom, C. Gonzalez-Espinosa, Y. Furumoto, S. Saitoh, L. E. Samelson, J. J. O'Shea, and J. Rivera. 2002. Fyn kinase initiates complementary signals required for IgE-dependent mast cell degranulation. *Nat. Immunol.* 3: 741–748.
5. Amoui, M., L. Draberova, P. Tolar, and P. Dráber. 1997. Direct interaction of Syk and Lyn protein tyrosine kinases in rat basophilic leukemia cells activated via type I Fcε receptors. *Eur. J. Immunol.* 27: 321–328.
6. Smith, A. J., J. R. Pfeiffer, J. Zhang, A. M. Martinez, G. M. Griffiths, and B. S. Wilson. 2003. Microtubule-dependent transport of secretory vesicles in RBL-2H3 cells. *Traffic* 4: 302–312.
7. Tasaka, K., M. Mio, K. Fujisawa, and I. Aoki. 1991. Role of microtubules on Ca^{2+} release from the endoplasmic-reticulum and associated histamine-release from rat peritoneal mast-cells. *Biochem. Pharmacol.* 41: 1031–1037.
8. Marti-Verdeaux, S., I. Pombo, B. Iannascoli, M. Roa, N. Varin-Blank, J. Rivera, and C. Blank. 2003. Evidence of a role for Munc18-2 and microtubules in mast cell granule exocytosis. *J. Cell Sci.* 116: 325–334.
9. Urata, C., and R. P. Siraganian. 1985. Pharmacologic Modulation of the IgE or Ca^{2+} ionophore A23187 mediated Ca^{2+} influx, phospholipase activation, and histamine-release in rat basophilic leukemia-cells. *Int. Arch. Allergy Appl. Immunol.* 78: 92–100.
10. Nishida, K., S. Yamasaki, Y. Ito, K. Kabu, K. Hattori, T. Tezuka, H. Nishizumi, D. Kitamura, R. Goitsuka, R. S. Geha, et al. 2005. FcεRI-mediated mast cell degranulation requires calcium-independent microtubule-dependent translocation of granules to the plasma membrane. *J. Cell Biol.* 170: 115–126.
11. Oakley, C. E., and B. R. Oakley. 1989. Identification of γ-tubulin, a new member of the tubulin superfamily encoded by mipA gene of *Aspergillus nidulans*. *Nature* 338: 662–664.
12. Oakley, B. R., C. E. Oakley, Y. Yoon, and M. Jung. 1990. γ-Tubulin is a component of the spindle pole body that is essential for microtubule function in *Aspergillus nidulans*. *Cell* 61: 1289–1301.
13. Zheng, Y., B. Alberts, and T. Mitchison. 1995. Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* 378: 578–583.
14. Moritz, M., M. B. Braunfeld, J. C. Fung, J. W. Sedat, B. M. Alberts, and D. A. Agard. 1995. Three-dimensional structural characterization of centrosomes from *Drosophila* embryos. *J. Cell Biol.* 130: 1149–1159.
15. Moritz, M., Y. Zheng, B. M. Alberts, and K. Oegema. 1998. Recruitment of the γ-tubulin ring complex to *Drosophila* salt-stripped centrosome scaffolds. *J. Cell Biol.* 142: 775–786.
16. Murphy, S. M., L. Urbani, and T. Stearns. 1998. The mammalian γ-tubulin complex contains homologues of the yeast spindle pole body components Spc97p and Spc98p. *J. Cell Biol.* 141: 663–674.
17. Oegema, K., C. Wiese, O. C. Martin, R. A. Milligan, A. Iwamatsu, T. J. Mitchison, and Y. Zheng. 1999. Characterization of two related *Drosophila* γ-tubulin complexes that differ in their ability to nucleate microtubules. *J. Cell Biol.* 144: 721–733.
18. Wiese, C., and Y. Zheng. 2000. A new function for the γ-tubulin ring complex as a microtubule minus-end cap. *Nat. Cell Biol.* 2: 358–364.

19. Leguy, R., R. Melki, D. Pantaloni, and M. F. Carlier. 2000. Monomeric γ-tubulin nucleates microtubules. *J. Biol. Chem.* 275: 21975–21980.
20. Sulimenko, V., T. Sulimenko, S. Poznanovic, V. Nechiporuk-Zloy, J. K. Böhm, L. Macúrek, E. Unger, and P. Dráber. 2002. Association of brain γ-tubulins with αβ-tubulin dimers. *Biochem. J.* 365: 889–895.
21. Chabin-Brion, K., J. Marceiller, F. Perez, C. Settegrana, A. Drechou, G. Durand, and C. Pous. 2001. The Golgi complex is a microtubule-organizing organelle. *Mol. Biol. Cell.* 12: 2047–2060.
22. Dryková, D., V. Sulimenko, V. Cenklová, J. Volc, P. Dráber, and P. Binarová. 2003. Plant γ-tubulin interacts with αβ-tubulin dimers and forms membrane-associated complexes. *Plant Cell* 15: 465–480.
23. Rios, R. M., A. Sanchis, A. M. Tassin, C. Fedriani, and M. Bornens. 2004. GMAP-210 recruits γ-tubulin complexes to *cis*-Golgi membranes and is required for Golgi ribbon formation. *Cell* 118: 323–335.
24. Feng, Y., D. R. Hodge, G. Palmieri, D. L. Chase, D. L. Longo, and D. K. Ferris. 1999. Association of polo-like kinase with α-, β- and γ-tubulins in a stable complex. *Biochem. J.* 339: 435–442.
25. Kapeller, R., A. Toker, L. C. Cantley, and C. L. Carpenter. 1995. Phosphoinositide 3-kinase binds constitutively to αβ-tubulin and binds to γ-tubulin in response to insulin. *J. Biol. Chem.* 270: 25985–25991.
26. Inukai, K., M. Funaki, M. Nawano, H. Katagiri, T. Ogihara, M. Anai, Y. Onishi, H. Sakoda, H. Ono, Y. Fukushima, et al. 2000. The N-terminal 34 residues of the 55 kDa regulatory subunits of phosphoinositide 3-kinase interact with tubulin. *Biochem. J.* 346: 483–489.
27. Vogel, J., B. Drapkin, J. Oomen, D. Beach, K. Bloom, and M. Snyder. 2001. Phosphorylation of γ-tubulin regulates microtubule organization in budding yeast. *Dev. Cell.* 1: 621–631.
28. Kukharskyy, V., V. Sulimenko, L. Macúrek, T. Sulimenko, E. Dráberová, and P. Dráber. 2004. Complexes of γ-tubulin with non-receptor protein tyrosine kinases Src and Fyn in differentiating P19 embryonal carcinoma cells. *Exp. Cell Res.* 298: 218–228.
29. Dráberová, L., E. Dráberová, Z. Surviladze, Pe. Dráber, and P. Draber. 1999. Protein tyrosine kinase p53/p56^{lyn} form complexes with γ-tubulin in rat basophilic leukemia cells. *Int. Immunol.* 11: 1829–1839.
30. Shelanski, M. L., F. Gaskin, and C. R. Cantor. 1973. Microtubule assembly in the absence of added nucleotides. *Proc. Natl. Acad. Sci. USA* 70: 765–768.
31. Weingarten, M. D., A. H. Lockwood, S. Y. Hwo, K., and M. W. Kirschner. 1975. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* 72: 1858–1868.
32. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal. Biochem.* 162: 156–159.
33. Hibbs, M. L., D. M. Tarlinton, J. Armes, D. Grail, G. Hodgson, R. Maglitto, S. A. Stacker, and A. R. R. Dunn. 1995. Multiple defects in the immune-system of Lyn-deficient mice, culminating in autoimmune-disease. *Cell* 83: 301–311.
34. Dráberová, L., M. Amoui, and P. Dráber. 1996. Thy-1 mediated activation of rat mast cells: the role of Thy-1 membrane microdomains. *Immunology* 87: 141–148.
35. Tolar, P., L. Dráberová, and P. Dráber. 1997. Protein tyrosine kinase Syk is involved in Thy-1 signaling in rat basophilic leukemia cells. *Eur. J. Immunol.* 27: 3389–3397.
36. Nováková, M., E. Dráberová, W. Schürmann, G. Czihak, V. Viklicky, and P. Dráber. 1996. γ-Tubulin redistribution in taxol-treated mitotic cells probed by monoclonal antibodies. *Cell Motil. Cytoskel.* 33: 38–51.
37. Dráber, P., E. Dráberova, I. Linhartová, and V. Viklický. 1989. Differences in the exposure of C- and N-terminal tubulin domains in cytoplasmic microtubules detected with domain-specific monoclonal antibodies. *J. Cell Sci.* 92: 519–528.
38. Dráber, P., E. Dráberová, and V. Viklický. 1991. Immunostaining of human spermatozoa with tubulin domain-specific monoclonal antibodies. *Histochemistry* 195: 519–524.
39. Gundersen, G., M. H. Kalnoski, and J. C. Bulinski. 1984. Distinct populations of microtubules: tyrosinated and nontyrosinated α tubulin are distributed differently in vivo. *Cell* 38: 779–789.
40. Rudolph, A. K., P. D. Burrows, and M. R. Wabl. 1981. Thirteen hybridomas secreting hapten-specific immunoglobulin E from mice with Ig^a or Ig^b heavy-chain haplotype. *Eur. J. Immunol.* 11: 527–529.
41. Dráberová, E., V. Sulimenko, V. Kukharskyy, and P. Dráber. 1999. Monoclonal antibody NF-09 specific for neurofilament protein NF-M. *Folia Biol.* 45: 163–165.
42. Dráberová, E., P. Dráber, F. Havlíček, and V. Viklický. 1986. A common antigenic determinant of vimentin and desmin defined by monoclonal antibody. *Folia Biol.* 32: 295–303.
43. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227: 680–685.
44. Dráber, P. 1991. Quantitation of proteins in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis using colloidal silver. *Electrophoresis* 12: 453–456.
45. Dráberová, E., and P. Dráber. 1993. A microtubule-interacting protein involved in coalignment of vimentin intermediate filaments with microtubules. *J. Cell Sci.* 106: 1263–1273.
46. Dráber, P., L. A. Lagunowich, E. Dráberová, V. Viklický, and I. Damjanov. 1988. Heterogeneity of tubulin epitopes in mouse fetal tissues. *Histochemistry* 89: 485–492.
47. Songyang, Z., S. E. Shoelson, M. Chaudhuri, G. Gish, T. Pawson, W. G. Haser, F. King, T. Roberts, S. Ratnofsky, R. J. Lechleider, et al. 1993. SH2 domains recognize specific phosphopeptide sequences. *Cell* 72: 767–778.
48. Yuba-Kubo, A., A. Kubo, M. Hata, and S. Tsukita. 2005. Gene knockout analysis of two γ-tubulin isoforms in mice. *Dev. Biol.* 282: 361–373.
49. Ren, R. B., B. J. Mayer, P. Cicchetti, and D. Baltimore. 1993. Identification of a 10-amino acid proline-rich SH3 binding-site. *Science* 259: 1157–1161.
50. Frigeri, L., and J. R. Apgar. 1999. The role of actin microfilaments in the down-regulation of the degranulation response in RBL-2H3 mast cells. *J. Immunol.* 162: 2243–2250.
51. Nielsen, E. H., and T. Johansen. 1986. Effects of dimethylsulfoxide (DMSO), nocodazole, and taxol on mast-cell histamine-secretion. *Acta Pharmacol. Toxicol.* 59: 214–219.
52. Eiseman, E., and J. B. Bolen. 1992. Engagement of the high-affinity IgE receptor activates Src protein-related tyrosine kinases. *Nature* 355: 78–80.
53. MarieCardine, A., H. Kirchgessner, C. Eckerskorn, S. C. Mener, and B. Schraven. 1995. Human T lymphocyte activation induces tyrosine phosphorylation of α-tubulin and its association with the SH2 domain of the p59^{fyn} protein tyrosine kinase. *Eur. J. Immunol.* 25: 3290–3297.
54. Jouvin, M. H. E., M. Adamczewski, R. Numerof, O. Letourneur, A. Valle, and J. P. Kinet. 1994. Differential control of the tyrosine kinases Lyn and Syk by the 2 signaling chains of the high-affinity immunoglobulin-e receptor. *J. Biol. Chem.* 269: 5918–5925.
55. Peters, J. D., M. T. Furlong, D. J. Asai, M. L. Harrison, and R. L. Geahlen. 1996. Syk, activated by cross-linking the B-cell antigen receptor, localizes to the cytosol where it interacts with and phosphorylates a-tubulin on tyrosine. *J. Biol. Chem.* 271: 4755–4762.
56. Fernandez, J. A., L. M. Keshvara, J. D. Peters, M. T. Furlong, M. L. Harrison, and R. L. Geahlen. 1999. Phosphorylation- and activation-independent association of the tyrosine kinase Syk and the tyrosine kinase substrates Cbl and Vav with tubulin in B-cells. *J. Biol. Chem.* 274: 1401–1406.
57. Faruki, S., R. L. Geahlen, and D. J. Asai. 2000. Syk-dependent phosphorylation of microtubules in activated B-lymphocytes. *J. Cell Sci.* 113: 2557–2565.
58. Simon, J. R., R. D. Graff, and P. F. Maness. 1998. Microtubule dynamics in a cytosolic extract of fetal rat brain. *J. Neurocytol.* 27: 119–126.
59. Stumpff, J., T. Duncan, E. Homola, S. D. Campbell, and T. T. Su. 2004. *Drosophila* Wee1 kinase regulates Cdk1 and mitotic entry during embryogenesis. *Curr. Biol.* 14: 2143–2148.
60. Katagiri, K., T. Katagiri, K. Kajiyama, T. Yamamoto, and T. Yoshida. 1993. Tyrosine-phosphorylation of tubulin during monocytic differentiation of HL-60 cells. *J. Immunol.* 150: 585–593.
61. Ley, S. C., M. Marsh, C. R. Bebbington, K. Proudfoot, and P. Jordan. 1994. Distinct intracellular-localization of Lck and Fyn protein-tyrosine kinases in human T-lymphocytes. *J. Cell Biol.* 125: 639–649.
62. Talmor-Cohen, A., R. Tomashov-Matar, W. B. Tsai, W. H. Kinsey, and R. Shalgi. 2004. Fyn kinase-tubulin interaction during meiosis of rat eggs. *Reproduction* 128: 387–393.
63. Navara, C. S., A. O. Vassilev, H. E. Tibbles, B. Marks, and F. M. Uckun. 1999. The spleen tyrosine kinase (Syk) is present at the centrosome in cultured B-cells. *Blood* 94: 9A.