Charles University in Prague
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The role of the exocyst in development and maintaining of cell migration structures

Bachelor´s thesis

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Declaration

I declare that I elaborated the bachelor´s thesis „The role of the exocyst in development and maintaining of cell migration structures“ independently, guided by my supervisor. I also declare that I have mentioned all the used literature and other sources.

Prague, April 2010

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Abstract

The exocyst is a hetero-octameric protein complex which mediates tethering secretory vesicles to specific sites of plasma membrane for polarized exocytosis. The exocyst was long known to contribute to processes such as yeast budding, cytokinesis, epithelia polarization and neurite outgrowth. Recently, the role of the exocyst in regulation of actin cytoskeleton and cell migration was discovered.

It was shown, that the exocyst is important for formation of cell migration structures such as lamellipodia and filopodia in motile cells and invadopodia in invasive cancer cells. These structures are all actin-based membrane protrusions and the exocyst can through its Exo70 subunit interact with the Arp2/3 complex, the activator of actin nucleation. By binding and activating the Arp2/3 complex, the exocyst mediates actin polymerization resulting in formation of these membrane protrusions. Furthermore, the exocyst probably targets the Arp2/3 complex to specific sites of plasma membrane that are intended to become membrane protrusions. In addition, the exocyst mediates secretion of matrix metalloproteinases (MMPs) in invadopodia. MMPs are important for degradation of the extracellular matrix, an essential process in cancer cell invasion.

The exocyst seems to be part of the cascade downstream of cytokines TNF-α and IL-1 leading to filopodia formation. TNF-α and IL-1 activate Cdc42 GTPase, which in turn activates RalA GTPase. Activated RalA GTPase consequently interacts with exocyst subunit Sec5 and induces filopodia formation. For invadopodia formation and function the interaction between exocyst subunits Sec3/8 and IQGAP1 under the control of Cdc42 and RhoA GTPases is needed.

Key words: the exocyst, cell migration, tumor cell invasiveness, lamellipodia, filopodia, invadopodia, actin polymerization, the Arp2/3 complex

Abstrakt

Exocyst je hetero-oktamérny proteínový komplex, ktorý sprostredkováva cielenie sekretorických vačkov na špecifické miesta plazmatickej membrány pre polarizovanú exocytózu. Dlho bolo známe, že exocyst je dôležitý v procesoch ako pučanie kvasiniek, cytokinéza, polarizácia epitélií a vetvenie neuritov. Nedávno sa však ukázalo, že exocyst hrá rolu aj v regulácii aktínového cytoskeletu a v bunkovej migrácii.

Exocyst je dôležitý pre tvorbu štruktúr, ktoré bunka tvorí, aby mohla migrovať. Takýmito štruktúrami sú lamelipódiá a filopódiá v pohyblivých bunkách a invadopódiá v invazívnych rakovinových bunkách. Všetky tieto štruktúry sú výčnelky plazmatickej membrány a ich základom sú aktínové vlákna. Podjednotka exocystu Exo70 interaguje s Arp2/3 komplexom, ktorý katalyzuje vetvenie aktínových filamentov. Väzbou a aktivovaním Arp2/3 komplexu exocyst sprostredkováva polymerizáciu aktínu, ktorá má za následok tvorbu vyššie spomínaných membránových výbežkov. Exocyst tiež sprostredkováva špecifickú lokalizáciu Arp2/3 komplexu na miesta, ktoré sa majú stať membránovými výčnelkami. Ďalšou úlohou exocystu je sekrécia matrixových metalloproteáz (MMPs), ktoré degradujú extracelulárnu matrix, čo je proces nevyhnutný v invazivite nádorových buniek.

Exocyst je súčasťou signálnej kaskády vedúcej od cytokínov TNF-α a IL-1 k tvorbe filopódií. TNF-α a IL-1 aktivujú malú GTPázu Cdc42, ktorá následne aktivuje malú GTPázu RalA. Aktivovaná RalA GTPáza potom interaguje so Sec5 podjednotkou exocystu a indukuje formáciu filopódií. Pre tvorbu invadopódií je potrebná interakcia medzi Sec3/8 podjednotkami exocystu a proteínom IQGAP1. Táto interakcia je kontrolovaná GTPázami Cdc42 a RhoA.

Kľúčové slová: exocyst, bunková migrácia, invazivita nádorových buniek, lamelipódiá, filopódiá, invadopódiá, polymerizácia aktínu, Arp2/3 komplex

Contents

1. Introduction

Cell migration is important in many biological processes in multicellular organisms including developmental processes, wound healing and immune responses. In addition to this, cell migration could participate in pathological processes during cancer progression. If a few cells within primary tumor gain the increased ability to migrate through the extracellular matrix, they can invade into other parts of organism and establish here secondary tumors. The importance of studying the process of metastasis is critical, because metastatic cancer is often connected to poor prognosis for cancer patients.

Recently, it was shown, that the exocyst complex, a protein complex essential for polarized exocytosis by tethering secretory vesicles to specific sites of the plasma membrane, is also implicated in cell migration. The aim of this work is to summarize the contribution of the exocyst to cell migration, specifically the exocyst role in formation and function of cell migration structures such as lamellipodia and filopodia in motile cells and invadopodia in invasive tumor cells. The understanding of mechanism by which the exocyst is promoting cell migration could provide a number of new targets for anti-metastatic therapy.

2. The exocyst

The exocyst is also known as Sec6/8 complex. It is a multi-protein complex evolutionary conserved from yeasts to humans and consists of 8 subunits: Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84.

The exocyst was first discovered in budding yeast *Saccharomyces cerevisieae*, where it was shown to be essential for exocytosis (TerBush et al., 1996). Mutations in exocyst subunits resulted in inhibition of secretion and accumulation of secretory vesicles in the cell.

The main role of the exocyst is thought to be targeting of secretory vesicles from Golgi apparatus to the specific sites of their fusion on plasma membrane. The exocyst can therefore mediate polarized exocytosis, which is required in many cellular processes such as yeast budding, cytokinesis, epithelia polarization and neurite outgrowth. Its importance can be demonstrated by the fact, that exocyst subunits knockouts in multicellular organisms cause their early embryonic lethality.

Recently, it was also shown that the exocyst is involved in formation and regulation of actin-based membrane protrusions such as lamellipodia, filopodia or invadopodia and in cellular migration.

2.1 The structure of the exocyst

It is known, that the exocyst is 734 kDa hetero-octameric protein complex, but its inner structure and organization of its subunits is still under investigation.

So far, there are only 4 exocyst subunits better characterized - nearly full-length yeast and mouse Exo70, the C-terminal domains of Drosophila Sec15 and yeast Exo84 and Sec6. Although sequence identity of exocyst proteins is less than 10%, these subunits show very similar structure – they are all rod-like proteins consisting of two or more packed helical bundles composed of three to five α-helices linked by loops (He and Guo, 2009). Croteau et al. validated the presumption that also other exocyst subunits show similar conformation (Croteau et al., 2009). Differences occur in details, mainly in the surface residues, but conserved helical bundles composition suggests that these proteins evolved from a common ancestor protein (He and Guo, 2009; Croteau et al., 2009).

According to studies of binding sites of these subunits, we can presume their associations with other exocyst subunits. So there is an assumption, that the exocyst assembly includes its rod-shaped subunits to package in a side-to-side fashion (He and Guo, 2009).

Further structure analysis is needed for better understanding of exocyst organization and assembly. This could be crucial for determination of molecular mechanisms of exocyst function.

2.2 The localization of the exocyst and its assembly

Different exocyst subunits are localized in different cell compartments. There is a subcomplex associated with plasma membrane, which creates so-called targeting patch for vesicles. Another group of subunits is linked to secretory vesicles, but also interacts with exocyst subunits in targeting patch. The assembly of the whole complex therefore tethers vesicles to the specific sites of their fusion. This step precedes the fusion of vesicles with plasma membrane, which is mediated by SNARE complexes.

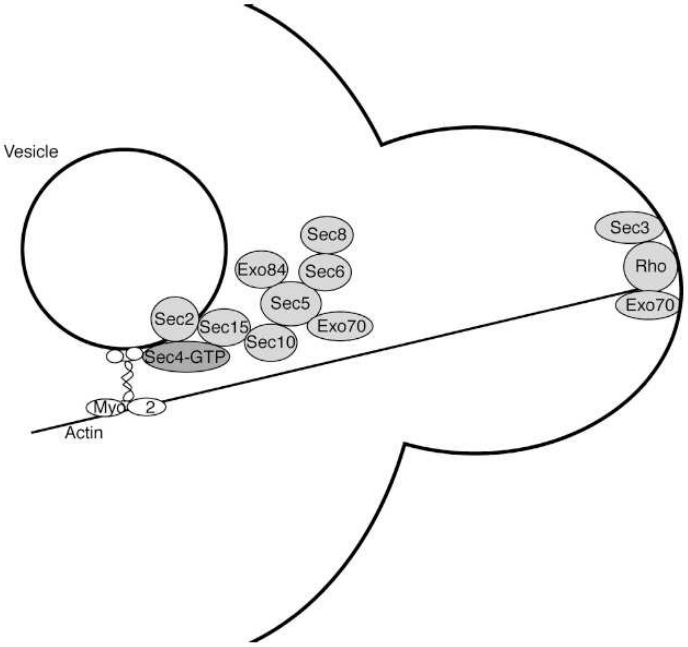

Figure 1. The model of targeting secretory vesicles according to Boyd et al. Subcomplex associated with vesicles contain Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84 exocyst subunits. Sec3 and Exo70 are membrane-associated. In yeast, assembly of the whole complex is mediated by small GTPase Sec4. (Adapted from Boyd, C., Hughes, T., Pypaert, M. and Novick, P. (2004). The Journal of Cell Biology 167, 889-901. Vesicles carry most exocyst subunits to exocytic sites marked by the remaining two subunits, Sec3p and Exo70p).

According to Boyd et al., a subcomplex associated with vesicle contains Sec5, Sec6, Sec8, Sec10, Sec15 and Exo84 exocyst subunits (Boyd et al., 2004; Fig. 1). Localization of these proteins is dependent on actin cables, along which vesicle is trafficking. Rab GTPase Sec4 is also localized on secretory vesicles and because Sec15 is shown to be its direct effector, Sec4 is supposed to regulate the assembly of exocyst complex in yeast (Guo et al., 1999). On the other hand, localization of Sec3 and Exo70 subunits is not actin-dependent. These proteins bind to phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) situated in the inner leaflet of the plasma membrane and this interaction probably targets the exocyst to particular membrane compartments (Exo70 likely utilizes both ways of targeting and therefore it is also partly associated with secretory vesicles). Localization of Sec3 is also controlled by Rho GTPases Cdc42 and Rho1, Exo70 interacts with Rho3 (reviewed in (He and Guo, 2009)).

On the other hand, Moskalenko et al. claim, that vesicle-associated are just Sec15, Sec10 and Exo84 exocyst subunits, other subunits (Sec3, Sec5, Sec6, Sec8 and Exo70) are said to be localized in the plasma membrane (Moskalenko et al., 2003; Fig. 2). Their studies also proved that exocyst assembly is in mammalian cells regulated by Ral GTPases interacting with both Sec5 and Exo84. This connection between two different exocyst subcomplexes results in assembly of the whole complex.

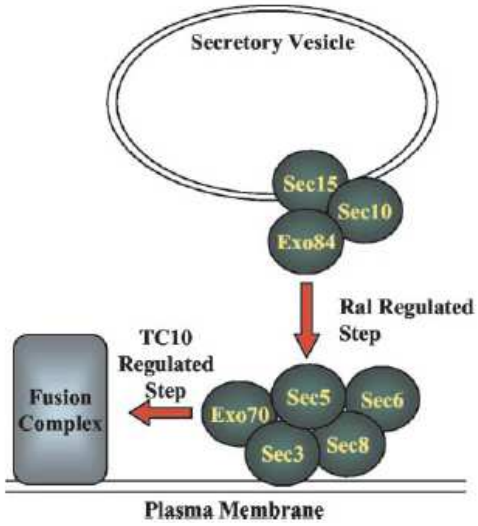

Figure 2. The model of targeting secretory vesicles according to Moskalenko et al. Exocyst subunits Sec15, Sec10 and Exo84 are vesicle-associated, exocyst subunits Sec3, Sec5, Sec6, Sec8 and Exo70 are membrane-associated. In mammalian cells, assembly of the exocyst is mediated by Ral GTPase interacting with both Exo84 and Sec5. (adapted from Moskalenko, S., Tong, C., Rosse, C., Mirey, G., Formstecher, E., Daviet, L., Camonis, J. and White, M. A. (2003). The Journal of Biological Chemistry 278, 51743-51748. Ral GTPases regulate exocyst assembly through dual subunit interactions).

A few studies tried to identify interactions between all exocyst subunits, nevertheless, their observations are not consistent. Therefore we are still far from understanding of exact mechanism of exocyst assembly.

2.3 The regulation of exocyst function

Exocyst is known to directly interact with several small GTPases which may regulate its function.

First identified GTPase interacting with the exocyst was Sec4 GTPase, member of the Rab family. As mentioned before, Sec4 binds to Sec15 exocyst subunit and controls the assembly of exocyst complex in yeast (Guo et al., 1999). In higher eukaryotes, another Rab GTPase, Rab11, was found to bind Sec15 and thus regulate vesicle delivery to the plasma membrane either from recycling endosomes or endocytic vesicles during transcytosis. In mammalian cells, GTPase Arf6 was found to interact with Sec10 in process of recycling membranes to sites of intense membrane remodeling mediated by exocyst complex (reviewed in (He and Guo, 2009)).

Other exocyst regulators belong to Rho family GTPases. In yeast, Sec3 interacts with Cdc42 and Rho1 GTPases and these are responsible for Sec3 localization, probably at different stages of cell cycle. Exo70 is a downstream effector of Rho3 GTPase (which is implicated in exocytosis), but also Cdc42 or TC10 (a mammalian homolog of Cdc42). Surprisingly, it was shown, that the interaction between Exo70 and TC10 is needed for fusion of vesicles, rather than their delivery to the plasma membrane (reviewed in (He and Guo, 2009)).

Last but not least, in mammalian cells the exocyst is also regulated by small GTPase Ral (Moskalenko et al., 2002). Activation of Ral is one of the results of Ras signaling pathway (Ras GTPase is connected to oncogenic transformation, endocytosis and actin cytoskeleton dynamics). Moskalenko et al. found out that Ral binds two exocyst subunits – Sec5 and Exo84 and through their connection mediates exocyst complex assembly (Moskalenko et al., 2003). It was discovered, that Ral is involved in such processes as targeting vesicles and exocytosis, cytokinesis, neurite branching and actin-based membrane protrusions. These processes may be therefore mediated by exocyst and regulated by Ral GTPases (He and Guo, 2009).

3. Structures for cell migration

Recently, several studies found the exocyst to play a crucial role in cell migration and tumor cell invasiveness. In these studies was shown exocyst importance in formation of structures required for cell migration such as lamellipodia, filopodia and invadopodia. These are all actin-based membrane protrusions and the exocyst was revealed to be involved in actin assembly. In addition, the exocyst is needed for secretion of matrix metalloproteinases (MMPs), the process critical for tumor cell invasiveness.

3.1 Lamellipodia

Lamellipodia are thin membrane protrusions formed at the leading edge of migrating cells and regarded as major structures for cell migration. Their breadth vary from 1 to 5 μm and they are usually 0,1 – 0,2 μm thick (Small et al., 2002; Ladwein and Rottner, 2008). Lamellipodia contain network of branched actin filaments, whose barbed ends are orientated towards the plasma membrane (Ladwein and Rottner, 2008). Actin filaments polymerize at the leading edge and depolymerize at the rear of the cell and this process of lamellipodia turnover is known as treadmilling (Le Clainche and Carlier, 2008). The actin polymerization at the leading edge promotes membrane protrusions (Ladwein and Rottner, 2008; Small et al., 2002) and orientated cell extension needed in process of cell migration.

In addition to actin filament network, lamellipodia contain also a variety of proteins that regulate actin cytoskeleton. The main activator of actin filaments nucleation is the Arp2/3 complex. It branches preexisting actin filaments and creates free barbed ends to be elongated. The Arp2/3 complex is under the control of WAVE proteins (also known as Scar or WANP; belonging to the WASP family proteins), that directly bind to the Arp2/3 complex and activate it. Another important actin-regulatory protein is cortactin. Cortactin promotes the Arp2/3-mediated actin nucleation (directly through activation of the Arp2/3 complex or indirectly through activation of N-WASP) and stabilizes new actin filament branches. Cofilin has a contradictory role in lamellipodia. On one hand, cofilin is responsible for disassembly and turnover of lamellipodia. On the other hand, cofilin was shown to be important for lamellipodia formation and stability by severing actin filaments and consequent actin polymerization that leads to membrane protrusions. The stability of actin network is provided by actin-crosslinking proteins filamin, α-actinin and coronin. Capping protein and gelsolin are

important for protecting free filaments away from the cell edge from unproductive actin polymerization. The role of myosins is probably carrying and targeting the cargo to the plus ends of actin filaments (reviewed in (Yamaguchi and Condeelis, 2006); (Machesky, 2008); (Ladwein and Rottner, 2008); (Small et al., 2002); Fig. 3).

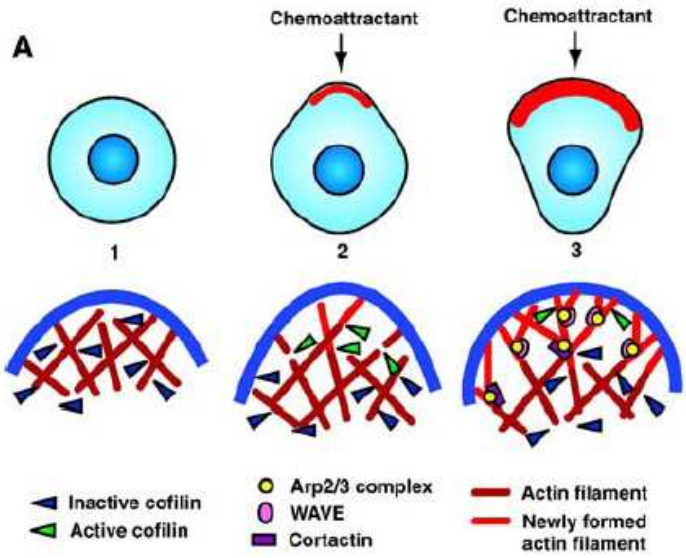

Figure 3. Formation of lamellipodia. Lamellipodia are actin-based structures, the main proteins implicated in lamellipodia formation are the Arp2/3 complex, WAVE proteins, cortactin and cofilin. (Adapted from Yamaguchi, H. and Condeelis, J. (2006). Biochimica et Biophysica Acta 1773, 642-652. Regulation of the actin cytoskeleton in cancer cell migration and invasion).

Lamellipodia formation can be induced by both growth factor and integrin receptors (Small et al., 2002). The well-known stimulator of lamellipodia is epidermal growth factor (EGF). EGF stimulate the WAVE-Arp2/3 complex and LIM kinase/cofilin pathways (Yamaguchi and Condeelis, 2006). Pathways downstream of EGF activate small GTPases of Rho family Rac1 and Cdc42 (Small et al., 2002). Rac1 stimulate WAVE indirectly, probably through interaction with IRSp53 (insulin receptor tyrosine kinase substrate of 53 kDa) or some of the members of the WAVE complex (Ladwein and Rottner, 2008). The effect of Cdc42 is probably mediated by indirect activation of Rac1 (Ladwein and Rottner, 2008). EGF also stimulates PLC-mediated activation of cofilin and LIM kinase-mediated inactivation of cofilin. This leads to localized and transient actin polymerization and this process is therefore thought to contribute to chemotactic sensing during invasion (Yamaguchi and Condeelis, 2008).

Lamellipodia are structures promoting cell migration. They form adhesions that connect the extracellular matrix to actin cytoskeleton and attach the cell to the substrate. By anchoring the cell to the substrate at the leading edge and disassembly of adhesions at the rear, the cell can retract its trailing edge by acto-myosin contractility and the cell body is therefore pulled forward (Yamaguchi and Condeelis, 2006; Le Clainche and Carlier, 2008). The role of lamellipodia as promoters of migration is important also in invasive cancer cells. The WAVE - Arp2/3 complex and the LIM kinase - cofilin pathways are upregulated in several types of cancer and are connected to increased metastatic and invasive potential both in vitro and in vivo (Yamaguchi and Condeelis, 2006; Machesky, 2008).

3.2 Filopodia

Filopodia are thin (0,1 - 0,3 μm), rod-like plasma membrane protrusions containing long parallel bundles of filamentous actin (F-actin). They often protrude from lamellipodial actin network (Matilla and Lappalainen, 2008). The fast-growing barbed ends of F-actin are orientated towards the plasma membrane and their rapid polymerization leads to the membrane protrusion and filopodia extension that promotes cell migration (Matila and Lappalainen, 2008; Faix et al., 2009).

Filopodia are filled with tight bundles of F-actin, but they contain also many other proteins that can regulate their formation and function. Necessary inductors of filopodia are formins (especially diaphanous protein (Dia)) that induce the formation of unbranched actin filaments by processive barbed ends nucleation and elongation. Ena/VASP proteins (for example Mena, Evl, VASP) protect actin filaments from capping proteins and promote their elongation. Fascin is actin-bundling protein that creates parallel filament bundles in filopodia. I-BAR proteins (such as MIM, IRSp53) are determined in direct membrane deformation and tubulation. Myosin-X is also important in filopodia formation and function, however, its function in filopodia was not described yet. The other regulators of actin cytoskeleton such as α-actinin, fimbrin, espin, filamin are also present in filopodia. The role of N-WASP and the Arp2/3 complex in filopodia formation is controversial, as they are not essential for filopodia formation and do not localize at filopodia, but their inhibition causes filopodia reduction (reviewed in (Matilla and Lappalainen, 2008); (Machesky, 2008); (Faix et al., 2000); Fig. 4).

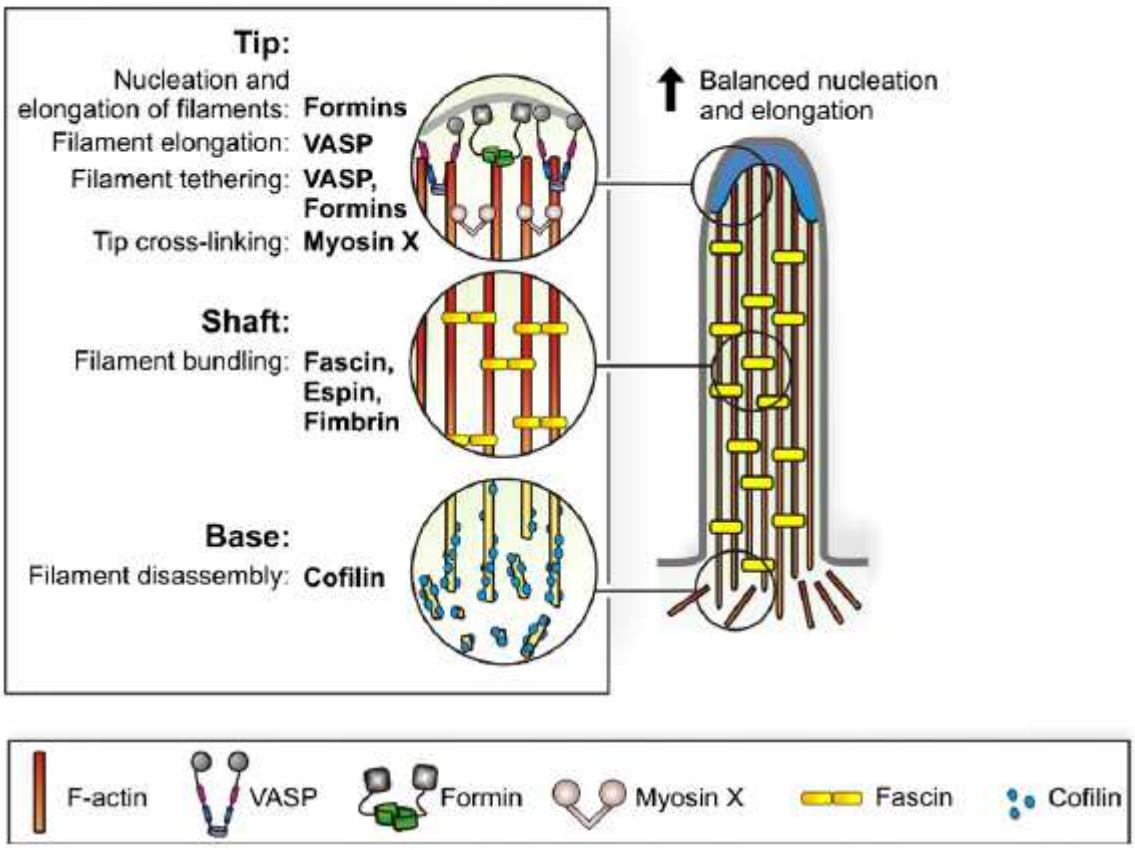

Figure 4. Formation of filopodia. Filopodia contain tight bundles of actin, nucleated by formins and elongated by VASP proteins. The main actin-bundling protein is fascin. Cofilin is implicated in filopodia turnover. (adapted from Faix, J., Breitsprecher, D., Stradal, T. E. B. and Rottner, K. (2009). The International Journal of Biochemistry and Cell Biology 41, 1656-1664. Filopodia: Complex models for simple rods).

Filopodia formation is positively regulated by small GTPases of Rho superfamily – Cdc42, Rho in filopodia (RIF), Wrch1, RhoD, RhoT, TC10 (Faix et al., 2009; Matilla and Lappalainen, 2008). There are two main models of filopodia formation. According to the convergent elongation model, filopodial actin filaments are derived from lamellipodial actin network. On the other hand, de novo filament nucleation model suggests nucleation of actin filaments in filopodial tips. Contradictory observations in this area suggest that there exist more ways of filopodia formation and they can differ in different organisms or different cell types (Matilla and Lappalainen, 2008; Faix et al., 2009).

Filopodia are essential for cell migration. They provide actin-based protrusion of plasma membrane at the leading edge and therefore directed cell extension. Afterwards, focal adhesions linked to stress fibers are formed under the leading edge and so the consequent acto-myosin contraction can push the cell body and nucleus forward (Matilla and Lappalainen

2008). Filopodia are also implicated in cancer metastasis and invasion. Filopodial proteins Ena/VASP are overexpressed in cancer cells and fascin is transiently up-regulated during invasive stages of cancer metastasis. Fascin increases invasive potential of cancer cells, probably by mediating enhanced filopodia activity that leads to enhanced cell motility (Machesky, 2008).

Because of presence of receptors for signaling molecules and extracellular matrix molecules (especially integrins and cadherins), filopodia are regarded also as sites of signal transduction, cell adhesion and sensing cell´s surroundings. In addition to migration and invasion, filopodia are important also in phagocytosis of macrophages, fusion of sheets of epithelial cells during embryonic development or wound healing and adherens junctions formation, neuronal growth cones function, development of dendritic spines and neurite formation of cortical neurons (Matilla and Lappalainen, 2008).

3.3 Invadopodia

Invadopodia are specialized structures formed by migrating cancer cells. They are able to degrade the extracellular matrix and thus mediate tumor cell invasion (Fig. 5; Fig. 6).

Invadopodia are membrane protrusions localized at the ventral surface of cells and extending into the extracellular matrix. Their size varies from 0,1μm to 0,8μm in diameter and can reach 2μm or more in length (Stylli et al., 2008). They can be organized into clusters – many smaller surface extensions protrude from a larger invagination (Buccione et al., 2004; Stylli et al., 2008). Often, they are localized in proximity to Golgi apparatus (Stylli et al., 2008; Gimona and Buccione, 2006). Their lifetime varies from minutes to hours (Yamaguchi et al., 2005).

Invadopodia are actin based structures. They contain filamentous actin (F-actin) and are enriched in actin regulatory proteins (the Arp2/3 complex, neural-Wiskott Aldrich Syndrome protein (N-WASP), cortactin), adhesion molecules (integrins and focal adhesion molecules), signaling molecules (especially Src kinase) and matrix degradation enzymes (matrix metalloproteinases) (reviewed in (Weaver, 2006)). Matrix metalloproteinases (MMPs) are critical components of invadopodia, because they mediate the extracellular matrix degradation. MMPs are a large family of Zn-binding endopeptidases (Stylli et al., 2008) which can together degrade all the components of the extracellular matrix (Buccione et al., 2004). The MMPs typical for invadopodia are membrane-associated collagenase MT1-MMP

(also known as MMP-14) and soluble gelatinases MMP-2 and MMP-9 (Stylli et al., 2008). The secretion of soluble MMPs is directed towards the leading edge of cells and is dependent on microtubules (Gimona and Buccione, 2006). However, soluble MMPs are synthetized as inactive proenzymes that require cleavage for its activation. MT1-MMP activates MMP-2 in this way at sites of invadopodia (Kelly et al., 1998).

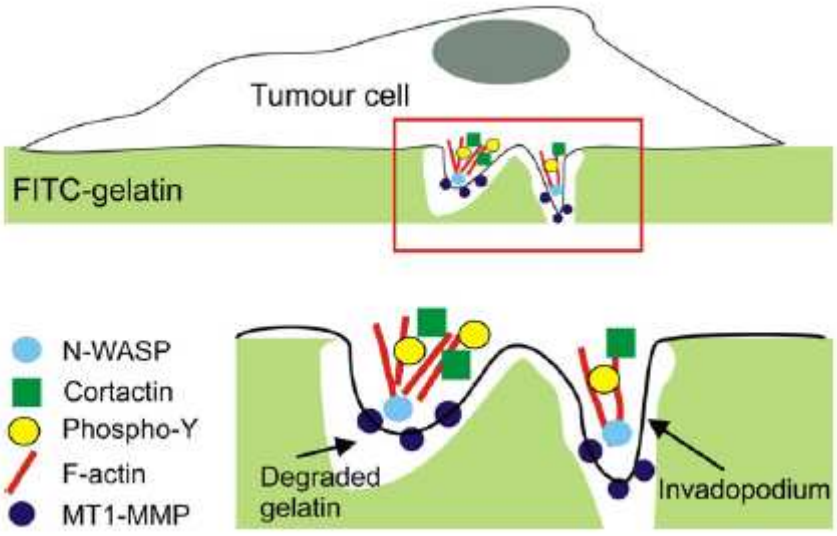

Figure 5. Invadopodia. Invadopodia are actin-based membrane protrusions. Their formation is activated by N-WASP, the Arp2/3 complex, dynamin and cortactin. Invadopodia are sites of matrix degradation mediated by secretion of MMPs. (adapted from Stylli, S. S., Kaye, A. H. and Lock, P. (2008). Journal of Clinical Neuroscience 15, 725-737. Invadopodia: At the cutting edge of tumor invasion).

Invadopodia formation can be induced by activation of actin polymerization, which results in membrane protrusions. According to Buccione et al., the machinery essential for invadopodia formation is the N-WASP-Arp2/3-cortactin-dynamin complex (Buccione et al., 2004). N-WASP stimulates actin polymerization by activating the Arp2/3 complex, which catalyzes actin nucleation. N-WASP is directly activated by Cdc42, Nck1 and WIP (WASP-interacting protein) and can be stimulated in response to extracellular stimuli such as EGF (Yamaguchi et al., 2005).

Invadopodia are essential for cancer cell invasion. Invadopodia formation is typical for highly invasive cancer cells and invadopodia markers are enriched in many human tumors (Weaver, 2006). Moreover, there was shown a correlation between the ability of cells to form invadopodia and their invasive potential both in vitro (Matrigel invasion assays) and in vivo

(metastasis in nude mouse models) (Stylli et al., 2008; Weaver, 2006). Furthermore, invasion was shown to be dependent on matrix degradation mediated by MMPs as invasive potential of cells is correlated with levels of MMPs and invadopodial ability to digest the extracellular matrix (Kelly et al., 1998; Stylli et al., 2008). The ability to degrade the extracellular matrix can be used for remodeling and migration through the extracellular matrix. Metastatic cells are able to degrade the stromal tissue surrounding primary tumors and invade through the tumor stroma towards vascular or lymphatic vessels (Yamaguchi et al., 2005). Moreover, they can also penetrate the basement membrane surrounding blood and lymphatic vessel walls and squeeze through the endothelial cell barrier into vessels (Yamaguchi et al., 2005). Subsequently, in bloodstream they can overcome long distances and after extravasation they can establish a new tumor site.

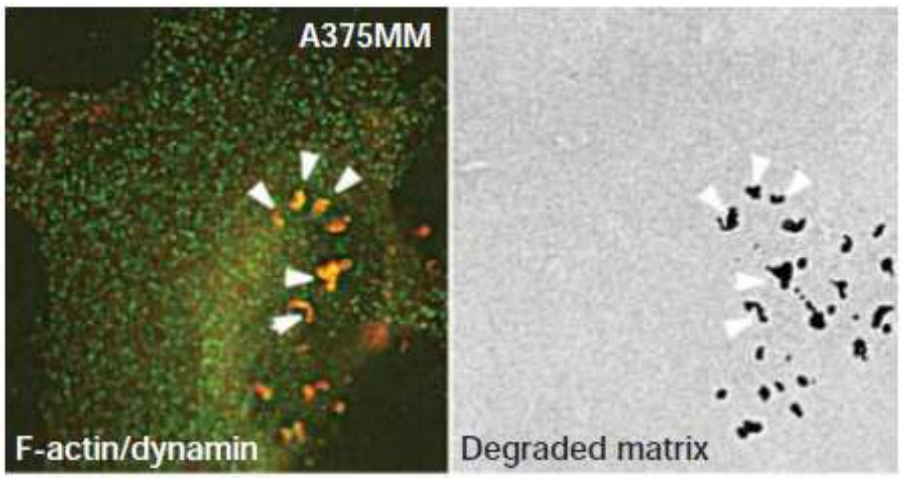

Figure 6. Invadopodia function – degradation of the extracellular matrix. The colocalization of F-actin and dynamin marks the sites of invadopodia (left panel). The extracellular matrix degradation is situated to invadopodia (right panel). (adapted from Buccione, R., Orth, J. D. and McNiven, M. A. (2004). Nature Reviews, Molecular Cell Biology 5, 647-657. Foot and mouth: podosomes, invadopodia and circular dorsal ruffles).

4. The exocyst in filopodia formation

Sugihara et al. directly showed the exocyst to participate in filopodia formation (Sugihara et al., 2002). The exocyst was also found to associate with RalA GTPase, which is known to induce filopodia formation (Ohta et al., 1999).

4.1 The interaction between the exocyst and RalA

The interaction between the exocyst and RalA was found by screen for RalA downstream effectors. This interaction was confirmed by precipitation in vitro and in vivo and was then better characterized. The exocyst was found to bind RalA GTPase directly through Sec5 subunit. This association is GTP-dependent – RalA GTPase was bound only during its activated (that means GTP-bound) state. For this interaction N-terminal 60 amino acid sequence of RalA and N-terminal 80 amino acid sequence of Sec5 are important (Sugihara et al., 2002).

4.2 The exocyst-mediated regulation of filopodia formation

As previously mentioned, RalA induces filopodia formation in Swiss 3T3 cells (Ohta et al., 1999). However, use of anti-Sec5 antibody (that specifically interacts with binding site for association with RalA) in cells microinjected with activated RalA caused reduced filopodia formation. The similar phenotype was observed in cells treated with Sec5 N-terminal RalA-binding fragment (Sugihara et al., 2002). This suggests that the interaction between Sec5 and RalA is needed for filopodia formation. The fact that in cells microinjected with activated RalA Sec5 was localized along filopodia supports this hypothesis. This study also demonstrated Sec5 colocalization with another exocyst subunit, Sec8. This indicates that the exocyst is present in filopodia as a whole complex (Sugihara et al., 2002).

Other stimulators of filopodia formation include Cdc42 (Nobes and Hall, 1995), IL-1 (Puls et al., 1999) and TNF-α (TNF-α also induces lamellipodia formation; Puls et al., 1999). Disruption of interaction between the exocyst and RalA was shown to be critical also in this case of filopodia induction. Cdc42-mediated filopodia were abolished by anti-Sec5 antibody. Similarly, microinjection of GDP-bound RalA (RalA in its inactive conformation) and anti-Sec5 antibody into TNF-α or IL-1 treated cells caused block of filopodia formation.

Interestingly, Cdc42-induced formation of lamellipodia was not influenced (Sugihara et al., 2002).

Previous studies revealed Cdc42 to be a downstream effector of TNF-α and IL-1 (Puls et al., 1999). Moreover, Ohta et al. showed that Cdc42 induces filopodia through pathway including RalA (Ohta et al., 1999). RalA was in this study implicated in actin cytoskeletal dynamics, precisely by binding filamin (actin filaments cross-linking protein that also links membrane and other intracellular proteins to actin; Ohta et al., 1999). Considering results of Sugihara et al., the exocyst also seems to be part of this signaling cascade leading to formation of filopodia (Sugihara et al., 2002). The authors themselves pronounced hypothesis, that TNF-α and IL-1 may activate Cdc42, which in turns activates RalA, and that the binding of GTP-bound RalA to Sec5 may be necessary to induce formation of filopodia (Sugihara et al., 2002). On the other hand, RalA and its interaction with exocyst were not found to be important in lamellipodia formation. It is consistent with experiments of Nobes and Hall, who implicated Rac1 GTPase in lamellipodia formation (Nobes and Hall, 1995).

4.3 The way of action of the exocyst-RalA complex

Unexpectedly, Sugihara et al. found out, that process of filopodia formation by the exocyst complex is not mediated by vesicle trafficking (Sugihara et al., 2002). Treatment of cells with brefeldinA (brefeldinA inhibits vesicle trafficking by disrupting Golgi apparatus) did not have any effect on filopodia formation. Similarly, there was no effect during treatment with SNAP-23 (SNAP-23 is known to inhibit fusion of vesicles with the plasma membrane). The authors presumed, that the exocyst could be involved in actin remodeling independently of vesicle trafficking. However, they did not exclude the possibility that vesicle transport might contribute to filopodia formation. Vesicle-associated RalA may be selectively activated and targeted to the exocyst complex at the plasma membrane to promote filopodia formation (Sugihara et al., 2002).

Later on, the hypothesis that the exocyst regulates actin cytoskeleton was proved by Zuo et al. (Zuo et al., 2006). It was found out that the exocyst interacts with the Arp2/3 complex, which is involved in actin polymerization, and therefore regulates filopodia formation (see below).

5. The exocyst in invadopodia formation

Recently, two studies implied the exocyst in invadopodia formation and activity. Sakurai-Yageta et al. showed the exocyst to be involved in Cdc42- and RhoA-activated signaling cascade leading to invadopodia formation through direct interaction of Sec3 and Sec8 with IQGAP1 (Sakurai-Yageta et al., 2008). Liu et al. confirmed that the exocyst plays a crucial role in invadopodia formation and described its contribution to matrix metalloproteases (MMPs) secretion and actin dynamics (Liu et al., 2009).

5.1 The interaction of the exocyst with IQGAP1

Recently, Sakurai-Yageta et al. described the interaction between the exocyst and IQGAP1 (IQGAP1 is Cdc42 and Rac effector, which regulates cell polarization during cell migration (Noritake et al., 2005) and was implicated also in tumorigenesis and invasion (Jadeski et al., 2008)). Using sets of pulldown assays they found out that IQGAP1 is able to directly bind Sec3 and Sec8 exocyst subunits through its conserved C-terminus. This region displays coiled-coil conformation and so do the Sec3 and Sec8 N-terminal IQGAP1-binding domains. Other exocyst subunits connected to IQGAP1 are bound indirectly through association with Sec3 and Sec8 (Sakurai-Yageta et al., 2008).

In this study it was also shown that the expression of Cdc42 or RhoA GTPases mediates the interaction between IQGAP1 and Sec3/Sec8. Although IQGAP1 directly interacts with GTP-bound Cdc42 GTPase, this interaction is not required for mediation of IQGAP1 and Sec3/Sec8 association. On the other hand, GTP-bound Cdc42 and RhoA associate with Sec3/Sec8 and this is the condition of IQGAP1 and Sec3/Sec8 interaction (Sakurai-Yageta et al., 2008).

5.2 The role of the exocyst in invadopodia formation and activity

The effect of the exocyst on invadopodia formation of human breast carcinoma cells (MDA-MB-231) was examined using fluorescent microscopy and matrix degradation assay. It was discovered, that siRNA knockdown of either Exo70 or Sec8 exocyst subunits resulted in decreased number of invadopodia per cell. These cells produced fewer matrix degradation sites and so the total matrix degradation area was smaller. However, this deficiency in

invadopodia formation was rescued by rat Exo70, which is more than 90% identical in amino acids sequence. On the other hand, overexpression of Exo70 resulted in higher number of invadopodia and greater matrix degradation area (Liu et al., 2009). These observations suggest that the exocyst is needed in process of invadopodia formation and because of greater capacity of matrix degradation also for tumor cell invasion. This is consistent with independent observations of Sakurai-Yageta et al. that knockdown of Sec6, Sec8, Sec10 in the same tumor cells caused inhibition of matrix degradation and also reduced invasiveness in transwell chamber invasion (Sakurai-Yageta et al., 2008).

Knockdown of IQGAP1 by siRNA also reduced matrix degradation. On the other hand, expression of IQGAP1 increased matrix degradation, but less than expression of constitutively active IQGAP. However, deletion of C-terminal Sec3/Sec8-binding region of IQGAP1 completely abolished this stimulating effect (Sakurai-Yageta et al., 2008). These results show that the interaction between IQGAP1 and exocyst is required for invadopodial activity. Knockdown of Cdc42 or RhoA decreased matrix degradation too, which supports the idea that interaction between the exocyst and IQGAP1 is regulated by these GTPases (Sakurai-Yageta et al., 2008).

5.3 The localization of the exocyst at invadopodia

The hypothesis that the exocyst can regulate invadopodia formation and function is also supported by localization of endogenous Exo70 and IQGAP1. Endogenous Exo70 was found to be enriched at the plasma membrane and in invadopodia. Similar was the distribution of overexpressed Exo70 and endogenous Sec8. These exocyst subunits colocalized with F-actin sites (Liu et al., 2009). Likewise, Sakurai-Yageta et al. observed colocalization of overexpressed Sec8 with F-actin, but also with endogenous IQGAP1, at sites of matrix degradation. In these sites also MT1-MMP was present (Sakurai-Yageta et al., 2008).

It is noteworthy, that Exo70 was not detected at all sites of matrix degradation. This may be caused by very dynamic character of invadopodia (F-actin and regulatory proteins also do not always localize at sites of matrix degradation). Another explanation could be that exocyst is localized in invadopodia just transiently, during its acting in invadopodia formation (Liu et al., 2009).

5.4 The mechanism of exocyst function in invadopodia

Because the role of the exocyst is tethering secretory vesicles to sites of their fusion, it was not surprising that the role of the exocyst in invadopodia function could be mediating secretion of MMPs. The levels of secreted MMPs were analyzed by gelatin zymography. In Exo70 and Sec8 knockdown cells, the secretion of MMP-2 and MMP-9 was dramatically reduced compared to control cells. Again, expression of rat Exo70 in these cells was able to restore the secretion of MMPs almost to their initial levels (Liu et al., 2009). Similarly, depletion of Sec8 or IQGAP1 caused reduction of MT1-MMP positive invadopodia (Sakurai-Yageta et al., 2008).

Previously was shown, that Exo70 interacts with phosphatidylinositol-4,5-bisphosphate through its C-terminus and that this association is required for its localization at the plasma membrane (Liu et al., 2007). Liu et al. demonstrated that overexpression of mutant Exo70, which had deleted C-terminus and therefore could not bind phosphatidylinositol-4,5-bisphosphate, resulted in striking decrease of MMP-2 and MMP-9 secretion levels (Liu et al., 2009). On the contrary, overexpression of full-length Exo70 increased MMP-2 and MMP-9 secretion (Liu et al., 2009).

These results suggest that the exocyst is essential for secretion of MMPs at invadopodia. Furthermore, few studies claimed, that MMPs secretion is regulated by endocytosis and membrane recycling, a process, in which the exocyst was lately shown to be involved (Sakurai-Yageta et al., 2008).

Exocyst role is probably targeting secretory vesicles with MMPs to sites of their fusion with the plasma membrane for secretion (Liu et al., 2009). However, except for function of MMPs as major tools of cell to degrade the extracellular matrix, Sakurai-Yageta et al. assumed that MMPs could also play important role in invadopodia formation as a “building foundation” of these structures (Sakurai-Yageta et al., 2008), as knockdown of MMPs inhibits invadopodia formation (Clark et al., 2007).

Exocyst function seems to be regulated by Cdc42 and RhoA GTPases which control the interaction of Sec3/Sec8 with IQGAP1, because this interaction probably targets the exocyst to IQGAP1-enriched invadopodia. The exocyst assembly results in tethering vesicles and their polarized exocytosis leading to accumulation of MMPs at invadopodia. Therefore the exocyst is one of the major effectors of Cdc42 and RhoA-regulated signaling cascade leading to invadopodia formation and function (Sakurai-Yageta et al., 2008).

IQGAP1 is a scaffold protein that mediates multiple interactions. Besides its interaction with the exocyst, the mechanism of its involvement in invadopodia formation could also be the activation of N-WASP – Arp2/3 pathway resulting in actin polymerization (Le Clainche et al., 2007). In addition to this, the exocyst was also directly implicated in regulation of actin filaments assembly. This aspect of exocyst function is very recent and is discussed below.

6. The exocyst and actin polymerization

Polymerization of actin is necessary for formation of membrane protrusions such as lamellipodia, filopodia and invadopodia. For that reason is actin polymerization also important in cell motility.

6.1 The interaction of the exocyst and the Arp2/3 complex

Actin polymerization is catalyzed by the Arp2/3 complex, which generates branched filamentous actin network. Lately, Zuo et al. identified Exo70 exocyst subunit as one of the binding partners of the Arp2/3 complex (Zuo et al., 2006).

In this study, Exo70 was found to interact with Arpc1 subunit of the Arp2/3 complex (also known as Arc40 or p40). GST-fusion rat Exo70 protein interacted with two isoforms of mammalian Arpc1 – Arpc1A and Arpc1B in vitro. In vivo, imunoprecipitation was performed using anti-Arp3 antibodies. Exo70 and Sec8 co-precipitated with the Arp2/3 complex. Neither of these two subunits co-imunoprecipitated with Arp2/3 in Exo70 knockdown cells, although Exo70 co-imunoprecipitated with Arp2/3 in Sec8 knockdown cells. It is therefore obvious, that the Arp2/3 complex interacts with the exocyst through Exo70 subunit. These results were confirmed by pull-down of Arp2/3 by GST-CA fusion protein (CA-domain of WASP is known to bind Arp2/3) in which Exo70 and Sec8 coprecipitated with Arp2/3.

The binding domain of Exo70 was determined by expressing different fragments of Exo70. Cell lysates were used for GST-CA pull-down of Arp2/3. It was discovered, that full-length Exo70 (amino acids 1 - 653) and C-terminus of Exo70 (amino acids 404 - 653) are able to bind the Arp2/3 complex. On the contrary, N-terminus of Exo70 (amino acids 1 - 408) binds Sec8, which was verified by co-imunoprecipitation (Zuo et al., 2006).

6.2 The function of Exo70 in complex with Arp2/3

Liu et al. examined the influence of Exo70 on actin polymerization in vitro using pyrene actin assay (Liu et al, 2009). It was discovered, that lysates from both Exo70 and Sec8 knockdown cells showed inhibited actin polymerization comparing to control cells, though Sec8 knockdown had much smaller effect than Exo70 knockdown. On the other hand, overexpression of Exo70 in cells stimulated actin polymerization. As expected,

overexpression of mutant Exo70 lacking C-terminal domain inhibited actin polymerization. Exo70 thus seems to be a positive regulator of the Arp2/3 – mediated actin polymerization (Liu et al., 2009).

It is very interesting, that the interaction between Exo70 and Arp2/3 is much stronger in cells with high invasive potential. The strength of association of Exo70 and Arp2/3 was examined by GST-CA pulldown of Arp2/3. Three cell types with different levels of invasiveness were used and it was demonstrated, that intensity of interaction depends on invasive potential of cells (Liu et al., 2009). Likewise, by GST-CA pulldown of Arp2/3 was found out, that the interaction between Exo70 and Arp2/3 complex was greatly stimulated in presence of epidermal growth factor (EGF; known regulator of cell migration that induce membrane protrusions) (Zuo et al., 2006). Therefore it seems that the strength of interaction between Exo70 and Arp2/3 is important for regulation of actin polymerization.

6.3 The localization of the exocyst and the Arp2/3 complex

Because the interaction between the exocyst and the Arp2/3 complex was demonstrated both in vitro and in vivo, it is not surprising, that Exo70 and Arp2 colocalize in migrating cells. Imunofluorescence showed that Exo70 is recruited to the plasma membrane at the leading edge of migrating NRK cells (normal rat kidney cells). In these cells, Arp2 was also localized at the leading edge and co-localized with Exo70. Nevertheless, Exo70 knockdown caused changed localization of Arp2, which was no more situated at the leading edge (Zuo et al., 2006). This observation suggests that localization of the Arp2/3 complex is dependent on localization of Exo70, which could in fact target Arp2/3 to specific sites of plasma membrane that are intended to become membrane protrusions.

Consistent with this hypothesis is observation, that epidermal growth factor (EGF; known regulator of cell migration that induce membrane protrusions) stimulation of cells mediated recruitment of Exo70 and Arp2/3 from intracellular structures to the plasma membrane. It is noteworthy, that although Exo70 knockdown cells were stimulated by EGF, Arp2/3 was not localized at plasma membrane in these cells (Zuo et al., 2006).

6.4 Actin polymerization and formation of lamellipodia

Exo70 was demonstrated to be essential in cell migration as Exo70 knockdown cells inhibited cell migration in transwell migration assay. In migration at single-cell level, Exo70 knockdown caused migration velocity decrease and cells were much less able to keep their direction of movement (Zuo et al., 2006).

In Exo70 knockdown cells, formation of lamellipodia was influenced. Migrating cells formed one or two broad lamellipodia extending in a relatively fixed direction, but Exo70 knockdown cells were either without any lamellipodia or formed multiple lamellipodia in random directions. Also, the length of lamellipodia was diminished. These observations could explain the decreased migration rate and lower directional persistence (Zuo et al., 2006).

Although there is no direct evidence, it seems that the exocyst plays a regulatory role in lamellipodia formation through Arp2/3. Reduced length of lamellipodia indicate defect in actin polymerization and random organization points to failure of specific localization of Arp2/3. Together with the previously mentioned facts, that in Exo70 knockdown cells Arp2/3 complex do not localize at plasma membrane sites of membrane protrusions and it in fact cannot be stimulated by Exo70, this indicates, that the interaction between the exocyst and the Arp2/3 complex is needed for lamellipodia formation and cell motility.

6.5 Actin polymerization and formation of filopodia and invadopodia

Actin polymerization is important also for formation of filopodia and invadopodia. It was previously shown, that overexpression of Exo70 induces actin-based membrane protrusions resembling filopodia (Wang et al., 2004). However, cells expressing mutant fragment of Exo70 lacking C-terminal Arp2/3 binding domain did not form any protrusions. By GST-CA pulldown it was verified that this Exo70 fragment was not able to bind Arp2/3, but its capacity of binding other exocyst subunits remained unchanged. As essential amino acids for membrane protrusion formation residues 571-572 and 628-630 of rat Exo70 were identified (Zuo et al., 2006).

Similarly, overexpression of Exo70 was shown to induce invadopodia formation and enhance matrix degradation under the cell. However, cells expressing mutant Exo70 defective in interacting with Arp2/3 (deletion of amino acids 628-630) were neither able to form invadopodia nor degrade matrix (Liu et al., 2009).

Membrane protrusions can be induced also by constitutively active form of Cdc42. However, in Exo70 knockdown cells this effect was suppressed. Moreover, injection of anti-Exo70 antibody (that specifically binds to Exo70 C-terminus and therefore blocks Arp2/3 binding domain) into cells with constitutively active Cdc42 also inhibited formation of filopodia (Zuo et al., 2006).

These observations directly show the association of Exo70 and Arp2/3 to be essential for formation of both filopodia and invadopodia. As in the case of lamellipodia formation, this interaction may be necessary for specific Arp2/3 localization and its activation. Arp2/3–mediated actin-polymerization subsequently mediates actin-based protrusions formation, which is required for cell migration.

7. Conclusion

The aim of this work was to show the importance of the exocyst in formation and function of cell migration structures such as lamellipodia, filopodia and invadopodia. From view of translational research, invadopodia are the most interesting, because they mediate cancer cell invasion during tumor metastasis.

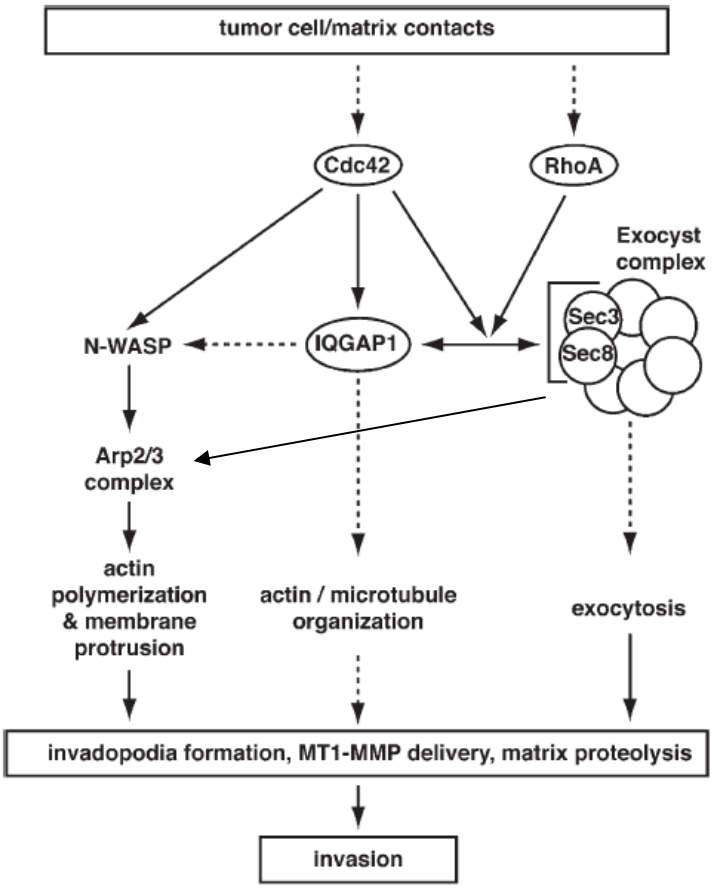

Figure 7. The role of the exocyst in cancer cell invasion. The exocyst was shown to mediate MMPs secretion and also to activate the Arp2/3 complex for actin polymerization, both processes essential for invadopodia formation and function. (adjusted and from Sakurai-Yageta, M., Recchi, C., Le Dez, G., Sibarita, J. B., Daviet, L., Camonis, J., D´Souza-Schorey, C. and Chavrier, P. (2008). The Journal of Cell Biology 181, 985-998. The interaction of IQGAP1 with the exocyst complex is required for tumor cell invasion downstream of Cdc42 and RhoA).

The exocyst is an effector of Cdc42 and RhoA-mediated pathway leading to invadopodia formation. Cdc42 and RhoA GTPases mediate the interaction between Sec3 exocyst subunit and IQGAP1 protein and this interaction is probably important for specific

localization of the exocyst at sites of future invadopodia. In these sites, Exo70 exocyst subunit interacts with the Arp2/3 complex, the activator of actin filament nucleation, and activates it. Therefore the exocyst mediates actin polymerization, which is needed for invadopodia formation. Nevertheless, the exocyst major role is thought to be mediating the secretion of MMPs that are essential for the degradation of the extracellular matrix, the process critical in tumor cell invasion (Fig. 7).

As mentioned before, invadopodia are structures essential, but also specific for invasive tumor cells and therefore they are good targets for anti-metastatic therapy. The anti-invadopodia drugs could be good complementation in metastatic cancer treatment to chemotherapy and radiation therapy. So far, there are a few candidates for inhibition in metastatic cancer treatment, including FAK, cortactin and Src kinase. The exocyst and other proteins implicated in Cdc42 and RhoA-mediated pathway of invadopodia formation could potentially be also such targets for metastatic cancer therapy. However, we are still at the beginning of understanding this pathway and more studies are needed to better characterize both upstream regulators and downstream effectors of the exocyst and also the exocyst itself.

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