Univerzita Karlova v Praze
Přírodovědecká fakulta

Studijní program: Imunologie

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Úloha membránových mikrodomén a transmembránových adaptorových proteinů PRR7 a SCIMP v regulaci imunoreceptorové signalizace

The role of membrane microdomains and transmembrane adaptor proteins PRR7 and SCIMP in the regulation of immunoreceptor signaling

Dizertační práce

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Praha, 2011

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V Praze, 26. 10. 2011

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RNDr. Matouš Hrdinka

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Acknowledgements

On this special page, I would like to express my gratitude to those people that helped me on the way of becoming a scientist. In the first place, my thanks belong to my supervisors. Prof. Václav Hořejší always showed me scientific problems in a wider perspective, encouraged me, and provided me with a plentiful support. Dr. Karel Drbal and Dr. Tomáš Brdička repeatedly brought novel ideas to my research and helped me to solve numerous technical problems. I am also thankful to all my colleagues and friends. Lastly, I thank my family and my loved ones for their help and patience with my (sometimes) long working hours. Without contribution of all these people, this dissertation would not exist. Thus, this is the most important page! Thank you!

Matouš Hrdinka

Summary

How do the plasma membrane microdomains and transmembrane adaptor proteins (TRAPs) influence the outcome of immunoreceptor signaling? These have been the important questions of molecular immunology. In spite of the years of intensive research, these problems remain incompletely understood. The plasma membrane is a highly dynamic heterogeneous bilayer spontaneously organized into microdomains of various size, composition, and lifetime. The lipid rafts are one example of such microdomains and have been implicated in many biological processes, including immunoreceptor signaling. Because rafts are enriched in many signaling proteins, they are believed to function as platforms for signal initiation and propagation. The TRAPs are important organizers and regulators of immunoreceptor signaling. For example, LAT is indispensable in T cell receptor (TCR) signaling and T cell development, PAG for the regulation of Src family tyrosine kinases (SFKs), and NTAL is a multifunctional negative and positive regulator. The presence of these TRAPs in lipid rafts seems to be crucial for their functions, however, is still a matter of debate. Moreover, other so far unidentified TRAPs could exist and play important roles in signal transduction pathways.

Here, we studied how the membrane environment influences the signaling capacity of LAT. According to our results, LAT targeted outside lipid rafts is less functional than wild-type LAT. Surprisingly, we found that LAX, a TRAP which was originally described as a typical non-raft TRAP, is targeted to a novel biochemically distinct type of membrane microdomains that can be isolated as heavy detergent resistant membranes (DRMs). We also discovered two novel leukocyte TRAPs, PRR7 (proline rich 7) and SCIMP (SLP65/SLP76 and Csk interacting membrane protein). PRR7 is up-regulated in activated T cells. When overexpressed in Jurkat T cells, the majority of cells undergo a programmed cell death. During this process, PRR7 is removed from the plasma membrane and accumulates in perinuclear vesicular compartments. A so far unidentified WW domain-containing protein probably mediates these effects. PRR7 interact with Src and selectively stimulates the transcription factor c-Jun, while all other TCR signaling pathways are suppressed in the presence of PRR7. In contrast, SCIMP expression is restricted to professional antigen presenting cells (APCs) where it associates with tetraspanin-enriched domains (TEMs). SCIMP is recruited to immunological synapse, and regulates signaling elicited from engaged MHCII molecules. In B cells tyrosine phosphorylated SCIMP organizes a protein complex composed of Lyn, Csk, BLNK, and Grb2. Overexpression and knock-down experiment revealed that SCIMP positively regulates the Erk MAP kinase pathway. Collectively, our results highlight the importance of membrane microdomains and TRAPs in many aspects of immunoreceptor signaling.

Souhrn (Summary in Czech)

Jak ovlivňují imunoreceptorovou signalizaci membránové mikrodomény a transmembránové adaptorové proteiny (TRAP)? Tyto otázky patří mezi důležité problémy současné molekulární imunologie a navzdory mnoha letům intenzivního výzkumu stále nejsou plně zodpovězeny. Buněčná membrána je velmi dynamická různorodá dvouvrstva přirozeně uspořádána do mikrodomén rozmanité velikosti, složení a délky života. Jedním z příkladů takových mikrodomén jsou lipidové rafty, které se účastní řady biologických procesů včetně imunoreceptorové signalizace. Jelikož obsahují mnoho proteinů signálních drah, jsou dokonce považovány za jakésy základny pro vznik a šíření buněčných signálů. Proteiny TRAP jsou důležité pro správné uspořádání a usměrňování imunoreceptorové signalizace. Například LAT je nepostradatelný pro signalizaci přes T buněčný receptor (TCR) a vývoj T lymfocytů, PAG pro regulaci proteinů z rodiny tyrosinových kináz Src (SFK) a NTAL je víceúčelový negativní i pozitivní regulátor. Ačkoliv se o tom neustále vedou diskuse, přítomnost TRAP v membránových mikrodoménách je zřejmě nezbytná pro jejich správné fungování. Je možné, že důležitou úlohu v přenosu signálů mohou hrát i další, ještě doposud neidentifikované TRAP.

V této studii jsme se snažili objasnit, jak jsou signalizační schpnosti proteinu LAT ovlivněny membránovým mikroprostředím. Naše výsledky ukazují, že, pokud je LAT uměle cílený mimo lipidové rafty, funguje hůře než nepozměněný LAT. Překvapivě jsme také zjistili, že typický neraftový protein LAX, se ve skutečnosti nachází v novém, doposud nepopsaném typu mikrodomén, které lze izolovat jako fragmenty membrán (DRM) odolávající působení detergentů. Zabývali jsme se také dvěma novými proteiny TRAP, PRR7 a SCIMP. Protein PRR7 je přítomen v aktivovaných T lymfocytech. Jeho umělá nadprodukce v T buňkách Jurkat vede k programové buněčné smrti. Během nadprodukce je PRR7 odstraňován z plazmatické membrány a hromadí se ve vesikulárních organelách poblíž buněčného jádra. Tyto jevy pravděpodobně způsobuje dosud neznámý protein, který se váže na PRR7 pomocí své WW části. PRR7 také váže kinázu Src a způsobuje nadprodukci transkripčního faktoru c-Jun. Všechny ostatní dráhy TCR signalizace jsou v přítomnosti PRR7 potlačeny. Naopak druhý námi popsaný protein SCIMP je přítomen pouze v buňkách které předkládají antigeny T lymfocytům v takzvaných membránových mikrodoménách obohacených o tetraspany (TEM). Protein SCIMP putuje do oblasti mezi dvěma buňkami zvané imunologická synapse a reguluje signalizaci vyvolanou MHCII proteiny. V B lymfocytech umožňuje protein SCIMP fosforylovaný na tyrosinech vznik komplexu proteinů Lyn, Csk, BLNK a Grb2. Zkoumání důsledků nadprodukce a odstranění proteinu SCIMP z buněk ukázalo, že SCIMP positivně reguluje signalizační dráhu vedoucí přes MAP kinázu Erk. Naše výsledky v souhrnu poukazují na důležitost membránových mikrodomén a proteinů TRAP v imunoreceptorové signalizaci.

Abbreviations

ADAP	Adhesion- and degranulation-promoting adaptor protein
AP1	Activator protein 1
APC	Antigen presenting cell
BCAP	B cell adaptor molecule for PI3K
Bcl10	B cell lymphoma 10
BCR	B cell antigen receptor
BLNK	B cell linker
Btk	Bruton's tyrosine kinase
CARMA	Caspase recruitment domain-containing membrane-associated guanylate kinase protein
Cbl	Casitas B-lineage lymphoma
CD	Cluster of differentiation
Cdc42	Cell division cycle 42
Csk	C-terminal Src kinase
DAG	Diacylglycerol
DAP12	DNAX-activation protein 12
DRM	Detergent-resistant membrane
Erk	Extracellular signal-regulated kinase
FcR	Fc receptor
Fos	FBJ osteosarcoma oncogene
Gab2	GRB2-associated binding protein
Gads	Grb2-related adaptor downstream of Shc
GAPT	Grb2-binding adaptor protein, transmembrane
GEF	Guanine nucleotide-exchange factor
GEM	Glycosphingolipid enriched microdomains
GPI	Glycosylphosphatidylinositol
Grb2	Growth factor receptor-bound protein 2
GSK3	Glycogen synthase kinase 3
Ig	Immunoglobulin
IKK	IκB kinase
INPP5	Inositol polyphosphate-5-phosphatase
IP3	Inositol trisphosphate
ITAM	Immunoreceptor tyrosine-based signaling motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JNK	Jun kinase
Jun	Jun proto-oncogene
KO	Knock-out (mouse deficient in a gene)
LAT	Linker for activation of T cells
LAX	Linker for activation of X cells
Lck	Leukocyte-specific tyrosine kinase
LIME	Lck-interacting molecule
MALT1	Mucosa-associated lymphoid tissue lymphoma translocation gene 1
MAPK	Mitogen-activated protein kinase

MEKK1	MEK kinase 1
MHC	Major histocompatibility complex
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-κB
NK	Natural killer
NMDAR	N-methyl-D-aspartate receptor
NTAL	Non-T cell linker
p38	Protein of 38 kDa
PAG	Phosphoprotein associated with glycosphingolipid microdomains
PBL	Peripheral blood lymphocytes
PDZ	Postsynaptic density 95, PSD-85; discs large, Dlg; zonula occludens-1, ZO-1
PH	Pleckstrin homology
PI3K	Phosphatidylinositol 3-kinase
$PIP_{2/3}$	Phosphatidylinositol bisphosphate/trisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
PRR7	Proline rich 7
PTP	Protein tyrosine phosphatase
Rac	Ras-related C3 botulinum toxin substrate
Raf	V-raf-leukemia viral oncogene
Ras	Rat sarcoma
RasGRP	Ras guanylnucleotide-releasing protein
SCIMP	SLP65/SLP76 and Csk interacting membrane protein
SFK	Src family kinase
SH2/SH3	Src homology 2/3
Shp-1	SH2-domain containing phosphatase 1
SIT	SHP2 interacting transmembrane adaptor
SLP-76	SH2-domain-containing leukocyte protein of 76 kDa
Sos	Son of sevenless
Syk	Spleen tyrosine kinase
TCR	T cell antigen receptor
TEM	Tetraspanin microdomain
Thy1	Thymus cell antigen 1
TRAP	Transmembrane adaptor protein
TREM	Triggering receptor expressed on myeloid cells
TRIM	TCR interacting molecule
Vav	Vav oncogene
Zap-70	Zeta-activated protein 70 kDa

Preface

The immune system is an inevitable adaptation of multicellular organisms to the hostile environment. It enables the organism to defend against virtually all kinds of ever evolving pathogens. However, the multicellularity itself represents a challenge, because a single malfunctioned cell could be potentially disastrous for the whole organism. By permanent controlling the health state of all cells in the body, the immune system helps to maintain tissue integrity, tissue renewal and cellular homeostasis.

The long-lived vertebrates, among all other species, developed the most complex immune system composed of two complementary units: an ancient innate and an evolutionarily modern adaptive immune system. In either case, highly specialized cells, leukocytes, and their molecular products permanently recognize and eliminate an unlimited variety of invading pathogens and compromised cells. Like all other cells in the body, leukocytes sense their environment via a number of receptors embedded in the plasma membrane or hidden inside the cell. Membrane receptors dedicated to recognition of dangerous entities or antigens are termed immunoreceptors. Once the antigen is recognized, it triggers immediate responses like cytoskeletal rearrangements and release of preformed mediators. Subsequently, the signals are transmitted to the nucleus to alter the expression of target genes and to induce long-term changes in cell behavior.

In spite of the years of intensive research, the complexity of signal processing machinery is still far beyond full understanding, and we are just uncovering some general principles and basic concepts. In the last fifteen years, the laboratory of Prof. Hořejší made a significant contribution to the field of molecular immunology and research of transmembrane adaptor proteins. During my PhD years, I continued these studies and tried to push the knowledge a little further. I touched two of the important questions in the field of molecular immunology. First: How does the membrane environment influence the outcome of signal transduction events? Second: How do the transmembrane adaptor proteins modulate the signal transduction?

Introduction

Cells of the immune system

Functionality of the immune system depends on the effective cooperation of different cell types. Unlike other cells in the body, leukocytes are not permanent residents of a tissue; instead, they enjoy a lot of mobility. Leukocytes originate from hematopoietic stem cells in the bone marrow. The cells of the innate immune system include mast cells, phagocytes (neutrophils, macrophages, and dendritic cells), basophils, eosinophils, and natural killer (NK) cells. When challenged, these cells respond quickly but rather non-specifically. On the other hand, the adaptive immune system, based on two types of lymphocytes, T cells and B cells, is prepared in advance for all future challenges. However, the main difference between innate and adaptive immune responses lies in the ability of T cells and B cells to remember the first encounter with antigen and confer a long-lasting protection.

Dendritic cells play a prominent role in antigen recognition and elimination. They are specialized in antigen recognition, processing and effective presentation to T cells via their major histocompatibility complex (MHC) molecules, thus serve as a link between innate and adaptive immune systems. Moreover, dendritic cells provide T cells with additional co-stimulatory signals for optimal activation. Most conventional T cells (i.e. those expressing TCR composed of α and β chains) recognize with unique specificity peptide-loaded MHC complexes. Surprisingly, the enormous variability of TCR binding specificities is generated by recombination events from a relatively small number of genes. During the development in thymus, $\alpha\beta$T cells differentiate into two main subsets: helper $CD4^+$ T cells recognizing antigens of extracellular origin bound to the MHCII molecules and cytotoxic $CD8^+$ T cells specific for intracellular antigens bound to MHCI molecules. The expression of MHCII molecules is restricted to professional antigen presenting cells, while MHCI molecules are found on the surface of almost all cells in the body. Thus, the main function of cytotoxic T cells is to recognize and eliminate virally infected cells or tumor cells. Helper T cells, on the other hand, assist B cells, cytotoxic T cells, and macrophages in maturation and activation processes. Activated T cells divide rapidly to create clones of the same antigen specificity. Upon activation, helper T cells further differentiate into specialized subsets.

Like dendritic cells, B cells are able to recognize antigens directly and can function as professional antigen presenting cells (APC). They express specialized immunoglobulin molecules called B cell receptors (BCR). The unique specificities of these receptors are generated analogously to TCR by recombination processes in the bone marrow. Full activation of most B cells requires help from T cells. Activated B cells can divide and differentiate into antibody producing plasma cells, thus they play the key role in the humoral immune responses. A unique adaptive mechanism is the ability

of B cells to produce antibodies with increased affinity during the course of an immune response in a process called affinity maturation.

Plasma membrane and membrane microdomains

Interior of the cell, the cytoplasm, is separated from the external environment by the plasma membrane. In addition to its obvious role in maintaining cell integrity and homeostasis, the cell membrane is also involved in many essential processes such as cell signaling, trafficking, division and adhesion. The plasma membrane is composed of lipids (phospholipids, sphingolipids, and cholesterol) and proteins. The unique chemical properties of phospholipids allow their organization into a phospholipid bilayer. Two other lipids, cholesterol and sphingolipids fine-tune membrane structure and function. It has been predicted that about 30% of all proteins in our genome code for transmembrane proteins [1], which occupy around 20% of total membrane area [2]. Membrane proteins include not only proteins spanning the membrane, but also proteins anchored to the outer leaflet of the membrane (glycosylphosphatidylinositol, GPI-anchored proteins), acylated proteins in the inner leaflet, and other membrane-associated proteins.

The oldest membrane model from the early 1930s simply assumed that phospholipids, cholesterol and glycolipids constitute the bilayer covered with membrane proteins. Forty years later, Singer and Nicholson described the dynamic nature of cell membranes. They came up with a concept of two-dimensional fluidity. It means that mobile lipid and integral protein molecules are evenly distributed and move freely within the plane of the membrane [3]. Further research in this field led to a better understanding of membrane behavior, and fluid mosaic model evolved into the "dynamically structured mosaic model" or membrane compartmentalization model [4]. This model assumes that specific membrane lipids and proteins are distributed unevenly creating heterogeneity and formation of compartments or domains. Apparently, such compartmentalization could originate from several sources. In the presence of sterols, some membrane lipids exist in two distinct liquid phases. This observation later resulted in the formulation of the so-called "raft hypothesis" [5, 6]. Furthermore, the capacity of some proteins to aggregate or oligomerize is sufficient to generate nanodomains in the plasma membrane [7]. In yet another concept, cytoskeletal or other cytosolic interactions (so called "molecular fences") restrict the free movement of individual assemblies within the membrane [8]. Functionally, microdomains specifically enhance protein-protein interactions acting as reaction platforms for enzymes, substrates and scaffold proteins.

The research of membrane microdomains has begun with the observations that some membrane components [9, 10], namely GPI-anchored proteins [10-12], and microdomains enriched in sphingolipids and cholesterol (lipid rafts) [5] resist to solubilization by some detergents. Since that time, scientists have been deliberately using the terms glycosphingolipid-enriched microdomains

(GEMs), lipid rafts and detergent resistant microdomains (DRMs) as synonyms. Obviously, it would be more correct to distinguish the membrane components present in living cells under the physiological conditions (membrane microdomains or rafts) and membrane material recovered biochemically in vitro using various detergents (DRMs) [13]. The membrane compartmentalization model implied the coexistence of regions or domains characterized by unique lipid and protein composition. So far, at least three biochemically and physiologically distinct types of membrane microdomains have been described: lipid rafts [14], caveolae [15] and tetraspanin enriched microdomains (TEMs) [16].

Lipid rafts

The first identified microdomains were related to membrane lipids and thus termed lipid rafts [5]. The underlying concept of the lipid raft hypothesis is based on unique properties of sphingolipids, cholesterol and phospholipids with saturated acyl chains. These lipid molecules can assemble into microdomains due to their ability to form the liquid ordered phase, as observed in artificial model membranes [17] and isolated plasma membrane vesicles [18] or spheres [19]. In liquid ordered phase, the saturated fatty acyl chains of lipids are in extended conformation, and thus more tightly packed. The rigid cholesterol molecules facilitate this process by intercalating among flexible phospholipids [20, 21]. Importantly, the ability of lipids to diffuse freely in the plane of membrane (the lateral mobility) remains largely unchanged [6]. Moreover, protein-protein and protein-lipid interactions further stabilize lipid-based rafts [22]. Such liquid ordered phase microdomains are sensitive to cholesterol depletion and resistant to solubilization in some mild detergents. Resulting DRMs, having a high lipid-to-protein mass ratio, remain in low-density fractions after density centrifugation [12]. Indeed, these properties have served as the main criterion for raft association. If a protein appeared in the DRM fraction after membrane solubilization in non-ionic detergents and became detergent soluble after cholesterol depletion (for example, by methyl-β-cyclodextrin treatment), it was considered as a raft constituent. Over the years, this approach led to many controversies regarding the nature (or even the existence) of lipid rafts [23]. Great variability of protocols for DRM isolation among laboratories (buffer composition, type of detergent [24], detergent concentrations, temperature and duration of cell lysis) inevitably led to incomparable results and artifacts. For example, the most common non-ionic detergent Triton X-100 may promote the formation of DRMs [25], and its activity depends heavily on buffer composition [26], temperature, and duration of lysis [27]. In addition, prolonged methyl-β-cyclodextrin treatment has major side effects on the cell physiology [28]. Collectively, these observations argue against an identification of DRMs with functional lipid rafts. Nevertheless, DRMs remain very useful in membrane microdomain studies when prepared with right detergents and interpreted carefully [29-31].

Recently, a number of sophisticated high-resolution microscopic and spectroscopic techniques enabled direct observation of lipid raft structures (briefly reviewed in [32, 33]). Using such technical advances, scientists proved that elemental rafts are nanoscale, short-lived, dynamic, and selective membrane assemblies. Under certain conditions (e.g. immunoreceptor clustering), elemental rafts coalesce to form larger and more stable functional microdomains. Many proteins prefer the lipid raft environment. For example, the lipid moiety of GPI-bound and some acylated proteins could serve as raft-targeting signals. Transmembrane segments of proteins also interact with specific (raft or non-raft) membrane lipids. In fact, it has been proposed that each single protein in the membrane naturally nucleates a tiny assembly (a "lipid shell") composed of one protein and several lipid molecules, which can be transient or stabilized by additional interactions to create larger microdomains [34].

Functionally, lipid rafts play roles in several aspects of cell biology, because they concentrate many important molecules. Among processes dependent on lipid rafts belong, for example, immunoreceptor signaling [35-39], membrane trafficking [40, 41], entry of pathogens into the cell [42], and human disease [43].

Caveolae

In contrast to lipid rafts, the first notion of caveolae comes from microscopic observations [44]. In the electron microscope, caveolae appear as 50–100-nm pits or invaginations, which could comprise up to 35% of the plasma membrane surface. Several cell types, like adipocytes, endothelia and muscle, are especially abundant in these structures. Biochemical analyses revealed that caveolae have properties quite similarly to lipid rafts and are composed of sphingolipids, cholesterol and proteins caveolins [45] and cavins [46]. Due to their raft-like properties (lipid composition, sensitivity to cholesterol extraction, recovery in DRMs), some experts in the field consider caveolae as a special subclass of lipid rafts [47, 48]. Conversely, the dynamic behavior of GFP-labeled caveolins is quite different from that of raft proteins [49].

Caveolins are palmitoylated transmembrane proteins, which bind fatty acids, cholesterol and each other to form high molecular weight oligomers. Their function is to stabilize caveolar invaginations in the membrane and prevent caveolae endocytosis [47]. Genetic studies have shown that caveolins are sufficient to induce formation of caveolae in cells naturally lacking caveolins [45] and that mice deficient in caveolins are also devoid of caveolae [50]. Cavins are members of cavin protein family containing four members. Each member of this family fulfills specific caveolae-independent roles in the cells. Moreover, all cavins constitute a multimeric complex, which is recruited to caveolae: Cavin-1 is involved in caveolae formation, Cavin-2 in caveolae elongation and Cavin-3 in regulation of vesicle budding and trafficking [51].

Caveolae have been implicated in several cellular processes like signaling and endocytosis [48]. Caveolae mediate one special sub-type of clathrin-independent (but dynamin-dependent) endocytosis leading to the formation of endocytic vesicles termed caveosomes. Several pathogens (for example, *SV40* or *Vibrio cholerae*), exploited this endocytic system to enter the cells [52].

Tetraspanin enriched microdomains

Tetraspanins are small (25-50 kDa) membrane proteins with four transmembrane segments and two extracellular loops. They belong to a large superfamily of proteins (33 members in humans) with conserved secondary and tertiary structures [53]. Some tetraspanins (CD9, CD81) are widely expressed, while the expression of others is cell specific (for example, CD37, CD53, and Tspan37 in leukocytes). Nevertheless, in each cell, several tetraspanin family members are present together, usually in high copy numbers. All tetraspanins have the ability to oligomerize and associate with other membrane proteins, including integrins, growth factor receptors and cytoplasmic signaling proteins [54]. Therefore, the resulting supra-molecular membrane complex is termed tetraspanin web [55] or tetraspanin microdomain (TEM) [16, 56]. Although primarily protein based, TEMs are not completely devoid of lipid components because tetraspanins interact with cholesterol [57] and gangliosides [58]. Palmitoylation, which modifies tetraspanins at multiple sites, seems to be crucial for tetraspanin oligomerization [59] and TEM assembly [60].

Using mild detergents, TEMs can be isolated as DRMs. Three levels of organization have been described in TEMs using different detergents and immunoprecipitation techniques [61]. The detergents digitonin and Triton X-100 maintain the most robust interactions, referred to as primary complexes, between tetraspanin and non-tetraspanin proteins (for example, CD151/α6β1 integrin) [62]. The hydrophobic interactions between transmembrane domains mediate tetraspanin-tetraspanin associations. These weaker secondary interactions are typically disrupted by more hydrophobic detergents (NP-40, Triton X-100), but preserved in less hydrophobic ones, such as Brij96 and Brij97. Very mild detergents (Brij99 and CHAPS) maintain the weakest, presumably indirect, tertiary interactions between tetraspanins and MHC proteins, proteoglycans, and various signaling molecules [63].

Several lines of evidence support the idea that TEMs are more likely discrete microdomains rather than web-like structures. Microscopic observation of labeled tetraspanins revealed that membrane contains hundreds of spatially separated TEMs (of average size 200 nm), each composed of two or more different tetraspanins [64]. Single labeled CD9 molecules enter and leave stable microdomains distinct from lipid rafts [65]. Finally, upon stimulation, the leukocyte adhesion molecules VCAM-1 and ICAM-1 localize to preformed nanoscale TEMs, which act as “endothelial adhesion platforms” [66].

Many studies have addressed the functional importance of TEMs in cell biology under physiological and pathological conditions. For example, TEMs participate in protein trafficking, cell proliferation, migration, adhesion and invasion, fertilization, antigen presentation, several aspects of viral infection, angiogenesis, kidney failure, and cancer, as reviewed in [67-70].

Immunoreceptor signaling

The BCRs, TCRs and majority of Fc receptors (FcRs) are members of the family of multichain immune-recognition receptors that share components and mechanisms for the initiation of receptor signaling. These transmembrane antigen receptors associate with common signaling subunits containing immunoreceptor tyrosine-based signaling motifs (ITAMs) within their cytoplasmic tails [71]. The engagement of antigen induces formation of receptor microclusters [72, 73]. Each receptor microcluster coalesces with other microclusters (lipid rafts) that contain important molecules of signal transduction pathways [74]. Phosphorylation of ITAMs by lipid raft associated Src family kinases (SFKs) is the first cellular event following the receptor triggering [75]. Ultimately, the signals generated thereafter involve a plethora of signaling proteins (e.g. kinases, phosphatases, lipases, ubiquitin ligases, ion channels, adapters, and scaffold-forming molecules), which become activated and induce events such as production of second messengers (inositol trisphosphate, IP3; diacylglycerol, DAG; and an increase in calcium levels), cytoskeleton rearrangements, exocytosis or endocytosis, and gene transcription.

T cell receptor signaling

T cells (namely the αβ-ones) recognize antigens by means of a multisubunit TCR:CD3 receptor complex. Heterodimeric TCRs (composed of polymorphic α and β subunits) recognize the antigen-MHC complex on the antigen presenting cells. The associated multisubunit CD3 complex (γ, δ, ε, ζ subunits) regulates the surface expression of the TCR [76] and by induced conformational changes ensures proper signal transduction across the plasma membrane [77]. The intracellular parts of CD3 chains contain in total ten ITAMs, which play a prominent role in the initiation of the TCR signaling. Upon the TCR ligation, the ITAMs are phosphorylated by the SFKs (mainly Lck, leukocyte-specific tyrosine kinase). Majority of Lck in unstimulated T cells is spatially isolated from ITAMs by association with co-receptors CD4 or CD8 residing in lipid rafts. The CD45 receptor tyrosine phosphatase modulates the phosphorylation and activation of Lck. Active Lck is brought to close proximity of TCR:CD3 only after the co-receptor binding upon the MHC molecules. Phosphorylated CD3 ITAMs create binding sites for dual SH2 (Src homology 2) domains of the Syk (spleen tyrosine kinase) family kinase Zap-70 (ζ-activated protein 70 kDa) which in turn phosphorylates multiple tyrosine residues within the linker for activation of T cells (LAT), a TRAP resident in lipid rafts [78].

Phosphorylated LAT then organizes a large multiprotein cooperative signalosome and initiates three major TCR signaling pathways [79] (Figure 1).

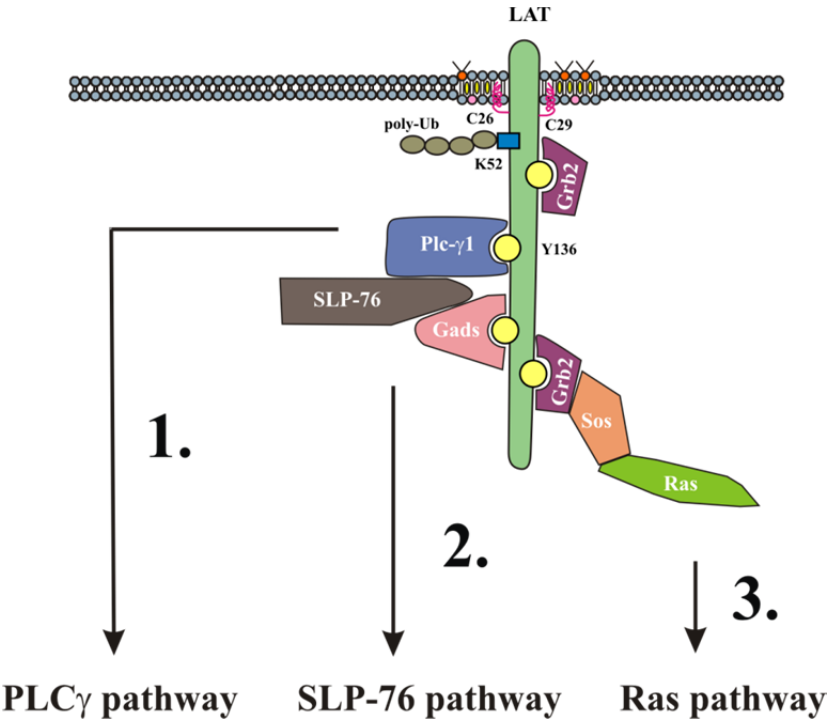

Figure 1: Three major LAT signaling pathways. LAT localizes to lipid rafts via palmitoylation of sub-membrane cysteine residues (C26/C29). Phosphorylation of LAT (yellow circles) results in recruitment of several cytoplasmic proteins and signal transduction. Post-translationally, polyubiquitination (poly-UB) regulates LAT turnover.

PLCγ pathway

First, LAT phosphorylated on tyrosine 132 (or Tyr-136 in mice) binds phospholipase C-γ1 (PLCγ1) [80], a protein indispensable for the activation of two major branches of TCR signaling, NF-κB (nuclear factor-κB) and NFAT (nuclear factor of activated T cells) pathways. Plcγ1 hydrolyzes phosphatidylinositol bisphosphate (PIP_2), creating two signaling molecules, a membrane lipid DAG and a cytoplasmic second messenger IP3 [81]. DAG recruits several cytoplasmic proteins to the plasma membrane. One of such proteins, the protein kinase C-θ (PKCθ), allows the assembly of a protein complex comprising CARMA1 (caspase recruitment domain-containing membrane-associated guanylate kinase protein 1), Bcl10 (B cell lymphoma 10) and MALT1 (mucosa-associated lymphoid tissue lymphoma translocation gene 1). This complex together with MEKK1 (MEK kinase 1) activates IKK (IκB kinase) which when activated marks IκB (inhibitor of κB) for ubiquitination and degradation, thus releasing NF-κB from inhibition and allowing it to translocate to the nucleus [82]. IP3 amplifies TCR generated signals by stimulating the opening of Ca^{2+}-permeable ion channels (IP3 receptors) in the endoplasmic reticulum. The calcium released from

the endoplasmic reticulum is monitored by STIM (stromal interaction molecule) which trigger opening of plasma membrane Ca^{2+}-permeable channels CRAC (calcium release-activated calcium channel protein). This leads to an increased and sustained influx of Ca^{2+} into the cytoplasm. The intracellular concentration of Ca^{2+} regulates the activity of the transcription factor NFAT. Under the low or resting Ca^{2+} concentrations the GSK3 (glycogen synthase kinase 3), phosphorylated NFAT is actively removed from the nucleus. Massive Ca^{2+} influx, on the other hand, activates Ca^{2+} binding protein calmodulin which then associates with the phosphatase calcineurin mediating NFAT dephosphorylation and nuclear import [83].

SLP-76 pathway

Second, phospho-LAT indirectly recruits another important adaptor, SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa) [84], via a small adaptor protein Gads (Grb2-related adaptor downstream of Shc). Recruited SLP-76 is then phosphorylated by Zap-70 and creates several binding sites for other signaling effectors. In cooperation with LAT, SLP-76 interacts with PLCγ1 discussed above. Phosphorylated SLP-76 binds Itk (interleukin-2-inducible T-cell kinase), a Tec family kinase responsible for the PLCγ1 phosphorylation and activation. Recently, SLP-76 has been found to associate with a regulatory subunit of PI3K (phosphatidylinositol 3-kinase), p85 [85]. Membrane associated PI3K generates PIP_3 phospholipids that recruit PH (pleckstrin homology) domain containing proteins to the plasma membrane. One good example of such proteins is the multifunctional pro-survival kinase Akt (v-akt murine thymoma viral oncogene homolog) [86]. Among other roles, activated Akt prevents NFAT phosphorylation and nuclear export via GSK3 phosphorylation. In addition, SLP-76 recruits two other adaptor proteins Nck (non-catalytic region of tyrosine kinase) and ADAP (adhesion- and degranulation-promoting adaptor protein) and guanine nucleotide-exchange factor (GEF) Vav (vav oncogene), the activator of small GTPases Rac (ras-related C3 botulinum toxin substrate) and Cdc42 (cell division cycle 42). Both GTPases activate Arp2/3 (actin-related protein 2/3) complex, which plays the central role in TCR-stimulated actin polymerization at the immunological synapse. Vav-dependent actin polymerization results also in an increased cell adhesion mediated by integrins, primarily by the LFA1 integrin (lymphocyte function-associated antigen 1, αLβ2 integrin). This so called “inside-out” signaling mechanism is mediated by the integrin linker talin and small GTPase Rap (Ras-related protein), which is present in ADAP protein complex associated with LAT/SLP-76 signalosome [87]. Enhanced cell adhesion facilitates the establishment of a long-lived T cell/APC contact. Lastly, SLP-76 binds the serine kinase Hpk (hematopoietic progenitor kinase), a functionally important member of the germinal center kinase family [88, 89].

Ras pathway

Third, five conserved tyrosine residues of LAT fall within Grb2 (growth factor receptor-bound protein 2) binding motifs (pYxNx) [80]. Grb2 is a small modular (one SH2 and two SH3 domains) adaptor protein involved in important signal transduction events from triggered membrane receptors to the activator protein 1 (AP1), a heterodimeric transcription factor composed of proteins Fos (FBJ osteosarcoma oncogene) and Jun (jun proto-oncogene). In TCR signaling, Grb2 recruits GEF Sos (Son of sevenless) to activate the small GTPase Ras (rat sarcoma), a crucial activator of mitogen-activated protein kinases (MAPK) pathways. In addition and maybe more importantly, Ras is also activated by GEF RasGRP (Ras guanylnucleotide-releasing protein), which is recruited to the membrane by DAG (generated by PLCγ1). Activated Ras associates with the upstream members of MAPK signal transduction cascades, a MAPK kinase kinase Raf (v-raf-leukemia viral oncogene) or MEKK1 [90]. Signal propagation in the MAPK pathways via subsequent phosphorylation events finally leads to an effector MAPK Erk (extracellular signal-regulated kinase), JNK (Jun kinase), or p38 (protein of 38 kDa). These effector MAPKs stimulate AP1 activity directly by phosphorylation of the Fos and Jun proteins and indirectly via the up-regulation of Fos and Jun transcription [91]. In addition to Ras action, JNK and p38 pathways partially respond also to SLP-76 bound GTPase Rac.

Several complementary mechanisms regulate the membrane proximal kinase mediated signaling. For example, active kinases Lck and Zap-70 are switched-off by the tyrosine phosphatase Shp-1 (SH2-domain containing phosphatase 1). Alternatively, the E3 ubiquitin ligase Cbl (Casitas B-lineage lymphoma) targets Zap-70, Lck and Vav proteins for proteasomal degradation [92]. Finally, an inhibitory mechanism based on the tyrosine kinase Csk (C-terminal Src kinase) is involved. Csk recruited to the plasma membrane by other proteins phosphorylates the inhibitory tyrosine within the C-terminal parts of SFKs. This phosphorylation induces a major conformational change and leaves SFKs in inactive conformation [93]. Additionally, proper costimulatory signals from other surface receptors such as CD2, LFA1 and CD28, which strengthen the TCR signaling, ultimately determine cell fate through regulating effector functions, cell survival, proliferation, and differentiation [94].

B cell receptor signaling

Analogously to the TCR signaling, the signal propagation after the BCR triggering follows similar general principles and signaling pathways. The B cell antigen receptor (BCR) complex is composed of antigen-binding transmembrane immunoglobulin (Ig) and associated disulfide-linked heterodimeric signaling subunits CD79α and CD79β (Igα/Igβ). Soluble antigen recognition leads to the formation of BCR microclusters and activation of SFKs (especially Lyn), which phosphorylate the ITAMs within the CD79 subunits creating binding sites for the tandem SH2 domains of tyrosine kinase Syk. This event initiates the formation of the signalosome similar to that formed in T cells after TCR

engagement [95]. The core of this protein complex is organized by cytosolic adaptor proteins such as BLNK (B cell linker, SLP-65), BCAP (B cell adaptor molecule for PI3K) and Grb2. Surprisingly, no transmembrane adaptor molecule equivalent to LAT in T cells has been identified. It suggests that BCR signaling relies on other mechanisms of protein recruitment to membrane proximity (direct binding on phosphorylated CD79 molecules or direct membrane binding via a leucine zipper motif [96]).

Two main signaling cascades originate from the BCR are the PLCγ2 and PI3K pathways [97]. The PLCγ2 pathway is initiated when Syk phosphorylates BLNK, a modification necessary for PLCγ2 and Btk (Bruton's tyrosine kinase) binding. Syk and Btk then phosphorylate PLCγ2, which in turn hydrolyses PIP_2 to produce second messengers IP3 and DAG. As discussed in the TCR signaling section, IP3 initiates signaling events leading to NFAT translocation to the nucleus and transcription of NFAT-regulated genes. The other product, DAG, activates PKCβ, an upstream factor of canonical NF-κB pathway. Activated PKCβ phosphorylates CARMA1, which is essential for CARMA1/Bcl10/MALT1 association, IKK activation, IκB phosphorylation, degradation, and NF-κB nuclear import. Deletion of PKCβ results in impaired B cell activation and maturation [98]. In addition, the coupling of BCR to the Ras/Erk pathway is mediated by PLCγ2/DAG actions. Similarly to T cells, GEFs RasGRP play a more important role than Sos in coupling the BCR to Ras activation [99]. Once activated, Ras binds Raf and initiates a phosphorylation cascade leading to MAPKs Erk1/2, which translocate to the nucleus in dimeric form and phosphorylate transcription factors of Fos, Jun, and Ets families.

The PI3K pathway depends on the recruitment of the p85 regulatory subunit to the plasma membrane by adaptor functions of CD19 and BCAP proteins. On BCR ligation, the tyrosine residues in the intracellular part of CD19 (YxxM motifs) become phosphorylated by Lyn providing binding sites for PI3K. In addition to this mechanism, PI3K is recruited to the membrane lipid rafts by the adaptor molecule BCAP phosphorylated by Syk and Btk. Once recruited to the membrane, PI3K produces PIP_3, which allows Akt to enter the PI3K pathway. Deletion of both PI3K adaptors in B cells leads to almost complete block of PI3K activation and impaired Akt response, B cell proliferation and survival [100].

The magnitude and duration of BCR signaling are limited by negative-feedback loops, including the Lyn/CD22/Shp-1 pathway, the PAG/Csk pathway, INPP5 (inositol polyphosphate-5-phosphatase, SHIP), Cbl, Dok (docking) proteins, FcγRIIb, and internalization of the BCR [97].

Fc receptor signaling

The transmembrane FcRs belonging to the Ig superfamily differ from TCRs and BCRs in several respects [101]. First, FcRs recognize Fc portions of antibodies instead of antigens proper. Antibodies, when bound to FcRs, provide antigen specificity to a variety of cells. Second, the FcRs exist as single α-chain receptors (FcγRIIa and FcγRIIb) or associate with signaling subunits (FcRγ or FcRβ) to form multisubunit receptor complexes. Third, as FcγRIIb bears immunoreceptor tyrosine-based inhibition motifs (ITIMs) instead of ITAMs in the intracellular part, it initiates an inhibitory (or regulatory) rather than activating signaling.

Activating FcγRs are important phagocytic receptors and thus serve as a link between innate and adaptive immunity [102, 103]. After cross-linking by immune complexes, the FcγR signaling pathways begin with the phosphorylation of ITAMs in receptor-associated γ-chains by SFKs. This leads to the recruitment of Syk and activation of downstream molecules, including LAT, Ras, and PI3K. Ras stimulates the Ras-Raf-MAPK pathway. PI3K creates membrane-docking sites for Btk and PLCγ. Activation of PLCγ leads to an increased intracellular calcium level and triggering of calcium-dependent pathways. The results of productive FcγR signaling are cytoskeletal reorganizations, phagocytosis, and release of chemokines and cytokines.

The inhibitory FcγRIIb limits the signaling from activation receptors. Simultaneous cross-linking of BCR and FcγRIIb leads to phosphorylation of the ITIM in the cytoplasmic tail of FcγRIIb by Lyn. This results in the recruitment of INPP5 and hydrolysis of PIP_3, which ultimately inhibits recruitment of PH-domain-containing proteins such as Btk and PLCγ. Isolated triggering of FcγRIIb, on the other hand, leads to B-cell apoptosis through ITIM- and INPP5-independent signaling pathways that involve Abl (Abelson murine leukemia) kinases, Btk, and JNK.

The activating FcεRI is crucial for the activation of mast cells and basophils and many physiological and pathological processes like inflammation and allergy [104-107]. The FcεRIs recognize antigens by means of constitutively associated IgE antibodies. Antigen recognition leads to receptor translocation into lipid rafts and SFKs Fyn and Lyn phosphorylate the ITAMs in β-chain of the receptor complex. This modification attracts Syk to phosphorylate LAT, which recruits proteins such as SLP-76, Gads, and PLCγ to the membrane. Simultaneously, Fyn phosphorylates Gab2 (GRB2-associated binding protein), which then associates with and activates PI3K. Both elevated calcium levels and activated PKC eventually induce secretion of granule-stored mediators. Activation of small GTPases such as Rac, Cdc42, and Ras leads to activation of the Erk, JNK, and p38 MAPKs that induce synthesis and secretion of cytokines, and to the release of arachidonic acid metabolites.

Other relevant receptors of immune cells

Besides the classical immunoreceptors, the cells of the immune system bear a number of other membrane receptors activating or modulating the same signaling pathways. These receptors also associate with common signaling chains (e.g. CD3ζ, FcRγ, DAP12, DNAX-activation protein 12) or contain ITAMs or ITIMs in their intracellular parts. Their expression is usually limited to specific cell types. For example, many functions of myeloid cells depend on receptors like Dectin-1 [108], FcRγ associated OSCAR (osteoclast associated, immunoglobulin-like receptor) [109], and DAP12 associated Siglec-H [110], and TREM1 (triggering receptor expressed on myeloid cells) [111]. The activation of NK cells is finely tuned by many diverse receptors associated with DAP12 (KIR2DS, NKG2C) or CD3ζ (NKp30, NKp46) [112]. B cells in addition to BCRs and FcRs need NFAM1 (NFAT activating protein with ITAM motif) during their development [113]. Proteins CLEC2 (C-type lectin domain family) and Glycoprotein VI (associated with FcRγ) regulate the activation of platelets and blood clotting [114, 115]. Widely expressed integrins associate with DAP12 or FcRγ signaling subunits and generate signals required for cell adhesion and migration [116]. Of particular interest, MHCII molecules fall into this category of receptors and deliver various signals into the antigen presenting cells after synapse formation. MHCII molecules can associate with signaling subunits of the BCR (Igα, Igβ) [117] and activate several signaling pathways involving SFKs, Syk kinase, calcium elevation and MAPK activation [118]. One of the proteins described in this thesis, SCIMP, seems to be a novel regulator of MHCII signaling pathways.

Transmembrane adaptor proteins (TRAPs)

Generally, all enzymatic signaling events depend on spatiotemporal interactions. To be effective, the enzymes must recognize specific substrates in particular cell compartments. The principles of protein modularity, which allows the cell to use one protein or enzyme in several contexts, usually assure the required specificity [119]. For example, a protein kinase can bind and phosphorylate many unrelated substrates, or a transcription factor regulates several promoter complexes. The most common protein modules used by immunoreceptor signaling pathways are SH2, SH3, PH, PTB (phosphotyrosine binding), WW, and PDZ (postsynaptic density 95, PSD-85; discs large, Dlg; zonula occludens-1, ZO-1) domains and their respective ligands [120].

A special group of proteins adds another level of complexity to cellular signaling. These proteins, ambiguously referred to as scaffolds, adaptors/adapters, linkers or docking proteins, have the ability to connect two or more proteins with otherwise incompatible interaction domains into novel functional complexes. While the most general term “scaffold function” usually describes the simultaneous binding of two or more other proteins, the specific term “adaptor function” describes the binding of two proteins via modular protein-protein interactions. The word “linker” has a functional meaning,

describing the property of joining one cellular process to another [121]. Finally, the term "docking protein" was used for membrane anchored cytosolic proteins with multiple sites for SH2 domain binding [122]. The scaffolds functionally fall into seven categories: "coordinating; kinetic thresholding; anchoring; targeting; catalytic; insulating; and concatenating" [121]. However, precise categories of scaffold proteins are hard to define, as the adaptor functions of many proteins are merely secondary to their other roles (e.g. structural or enzymatic).

As the knowledge of human genome and proteome increased, a special category of scaffold proteins emerged - the transmembrane adaptor proteins (TRAPs). These proteins share several similarities in their protein sequence, namely: a short N-terminal extracellular part, a single transmembrane segment and a rather unstructured cytoplasmic part containing amino acid motifs for binding of modular domains present in other proteins. They lack any intrinsic enzymatic or transcriptional activity. Since the cloning of LAT in 1998 [123], the TRAP family has grown to more than ten members (Figure 2).

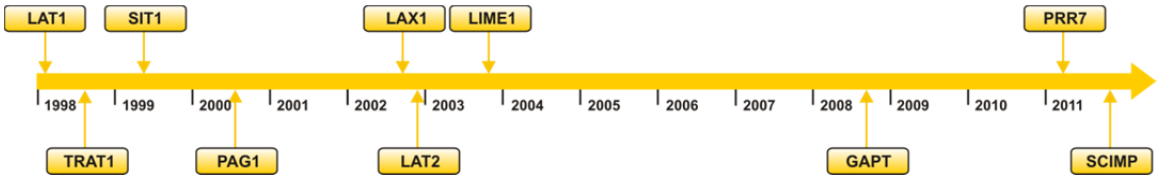

Figure 2: The timeline illustrating the discovery of TRAPs.

In addition to these "classical" TRAPs, there is a group of proteins with the same overall structure but more specialized functions. These proteins associate intimately with immunoreceptors as common signaling subunits. They include CD3 subunits of the TCR, Igα and Igβ of the BCR, FcRγ, DAP12 and several other proteins. As discussed in the "Immunoreceptor signaling" chapter, these proteins contain ITAMs which are phosphorylated as a first signaling event upon receptor triggering.

The expression of TRAPs is quite diverse. Some TRAPs are expressed ubiquitously while others show expression restricted to specific cell types (usually leukocytes) or are expressed inducibly. Post-transnationally, TRAPs may be modified by acylation, glycosylation, phosphorylation and ubiquitination. Acylation or palmitoylation are very common posttranslational modifications determining the membrane localization of many proteins [124]. The palmitoylated TRAPs apparently prefer a lipid raft or similar membrane microdomain environment as they co-purify with DRMs. Some TRAPs (namely SIT, TRIM) can form homodimers connected by disulfide bonds among extracellular cysteine residues. SIT is also heavily glycosylated. Importantly, all so far described TRAPs contain several so-called tyrosine based signaling motifs, which can be modified by transient phosphorylation and bind SH2 domain containing proteins [125] or serve as internalization motifs [126]. Trafficking or

lifetime of TRAPs is regulated by ubiquitination, which labels proteins for internalization or degradation [127].

Studies of genetically modified mice in the last two decades probed the functional importance of many genes, including all so far described TRAPs. In sharp contrast to LAT (and NTAL) knock-out (KO) mice, deletion of other TRAPs seems to be well tolerated. It is tempting to speculate that in developing mice other proteins can compensate for deficient TRAPs. However, there is little experimental evidence for such a hypothesis. In the following sections, I will point out the most important aspects of all individual TRAPs.

LAT (LAT1)

LAT is not only the first cloned TRAP, but also the archetypal one. LAT has been by far the most intensively studied and the most functionally important TRAP. As discussed in TCR signaling chapter of this dissertation and shown in Figure 1, the discovery of LAT solved the question that puzzled immunologists for a long time: How is the signal from the TCR linked to the cytoplasmic signaling cascades? Without LAT, many aspects of TCR signaling are disrupted. In T cell development, there is a block at the $CD4^-CD8^-$ stage, thus LAT-deficient mice lack mature T cells [128]. Surprisingly, the conditional deletion of LAT in mouse $CD4^+$ T cells revealed an unexpected negative regulatory role of LAT in TCR signaling. Such mice developed severe lymphoproliferative disorder and T cells could support TCR signaling [129]. Besides the prominent role in TCR signaling and T cell development, LAT is indispensable for proper signal propagation in other cell types (mast cells, platelets, NK cells, pre-B cells, etc.)[130].

Originally, LAT has been proposed to reside in lipid raft microdomains that fuse with TCRs upon stimulation [131]. However, recent research on initial steps of TCR signaling provided novel insights into LAT trafficking. Non-phosphorylated LAT exists in a monomeric as well as in a pre-clustered form (“protein islands”) in the membrane. After activation, the LAT clusters concatenate with TCR clusters [74]. Recently, super resolution microscopic techniques revealed that, in fact, LAT resides also in tiny (110 nm in diameter) sub-synaptic vesicles, which are rapidly recruited to the triggered TCRs. It seems that this (and not the pre-formed membrane-resident LAT clusters) is the main source of LAT for TCR signal propagation [132, 133].

Because a number of excellent reviews on LAT were published recently [79, 130, 134, 135], in the following sections, I will pay more attention to other (not so well documented) TRAPs. Moreover, on the example of NTAL and PAG, I would like to illustrate the diverse regulatory functions that TRAPs can play in specific cell types.

NTAL (LAB, LAT2)

NTAL (Non-T cell linker, or LAB, WBSCR5, LAT2) is structurally and functionally similar to LAT, but is absent in unstimulated T cells. Like LAT, NTAL is also palmitoylated and contains multiple conserved tyrosine phosphorylation motifs. Thus, immunologists hoped that it could be a non-T cell LAT homologue [136, 137]. Following BCR, FcγRI, or FcεRI engagement, Lyn and Syk phosphorylates NTAL, which allows the assembly of a protein complex containing NTAL, Grb2, Sos, Gab1, and Cbl. Three minutes after activation, NTAL is also strongly ubiquitinated probably via the interacting Cbl. To test whether NTAL is a functional homologue of LAT in T cells, NTAL was overexpressed in J.Cam2.5 cells (a Jurkat T cell variant lacking LAT). As a result, NTAL became phosphorylated (by Lck and Zap-70) after CD3 cross-linking and partially compensated for LAT deficiency in TCR signaling (small calcium influx and Erk phosphorylation). The absence of PLCγ and SLP-76 in the NTAL complex prevented NTAL to rescue the TCR signaling fully. Nevertheless, introduction of NTAL into LAT-deficient bone marrow cells allowed T cell development in the reconstituted mice. On the other hand, T cells from the mice reconstituted with NTAL spontaneously up-regulated activation markers and splenocytes failed to produce IL-2 after TCR stimulation. These results indicated that NTAL mimics only some aspect of LAT-mediated signaling and support the pre-TCR signaling pathways. Indeed, NTAL signaling capabilities resemble those of mutant LAT with an ablated PLCγ1 interaction [138, 139]. A final proof of this hypothesis came with a stable NTAL transgene on a LAT-deficient background. NTAL partially sufficed to restore T cell development. However, $CD4^+CD8^+$ thymocytes expressed very low levels of CD5, indicating sub-optimal signaling from NTAL. The replacement of LAT by NTAL in T cells resulted in defective calcium signaling and Erk activation, T cell development skewed towards the CD4 lineage, spontaneous activated Th2 phenotype of $CD4^+$ T cells, rapid proliferation and lymphocytic infiltration accompanied by severe organomegaly (a Th2 autoimmune disease) [140]. The partially rescued T cell development as well as minimal calcium signaling capacity of NTAL depends on three distal Grb2-binding tyrosine residues in the NTAL protein sequence [139].

In contrast to resting T cells, T cells activated via TCR express a substantial amount of NTAL. Surprisingly, aged NTAL-deficient mice developed an autoimmune syndrome accompanied by a splenomegaly, production of autoantibodies, and immune complex deposition in glomeruli due to hyper-activated T cells. Phenotypically, these cells displayed an enhanced proliferation and cytokine production after TCR stimulation. On the molecular level, it seems that NTAL negatively regulates LAT in activated T cells by at least two distinct mechanisms. First, LAT phosphorylation was markedly decreased in NTAL-deficient $CD8^+$ T cells. Second, NTAL deficiency depleted LAT from lipid rafts isolated from $CD8^+$ T cells [141].

If not an exact functional homologue of LAT, what roles does NTAL play in B cells? In agreement with originally proposed LAT-like functions, reduction of NTAL expression in B cell line A20 by specific siRNA, led to a decreased Erk phosphorylation and a diminished calcium response [137]. In chicken immature B cell line DT40, raft-localized NTAL appears to promote calcium mobilization via Grb2 binding. In these cells, Grb2 prevents sustained calcium influx from extracellular medium but Grb2 targeting to lipid rafts by phosphorylated NTAL evidently overcame this inhibition [142]. In contrast, NTAL-deficient mice had no overt defects in development of T cells, NK cells, dendritic cells and B cells [143]. Only detailed analysis of B cells from NTAL-deficient mice led to an identification of minor differences in B cell development (reduced numbers of marginal zone B cells) and signaling capacity (stronger calcium response) [144]. One study also addressed the pre-B cell development in NTAL-deficient mice. Calcium mobilization and down-regulation of pre-BCR are important steps in B cell differentiation and both BLNK and LAT, unlike NTAL, are required for these processes. To address the possibility that the lack of PLCγ1/2 binding site in NTAL prevents calcium mobilization and pre-BCR down-regulation, Herzog and Jumaa [145] engineered NTAL to contain the PLCγ-binding motif from LAT. Surprisingly, such protein supported calcium signaling and pre-BCR down-regulation but simultaneously, N-terminal part of NTAL (1-104) prevented B cell differentiation. According to recent studies, NTAL can regulate also activation-induced BCR internalization, as NTAL-deficient B cells internalize less BCR than wild-type B cells after BCR stimulation. Following the BCR engagement, NTAL is co-internalized and co-localized with BCR and ends up in the endocytic compartment. This process depends exclusively on the intracellular part of NTAL. The role of ubiquitination in this process is unclear [146]. BCR internalization begins with NTAL phosphorylation, which leads to the formation of a protein complex composed of Grb2, dynamin and Vav. Phosphorylated Vav activates Rac1/2 GTPases, important cytoskeletal regulators [147].

As indicated by the initial studies, NTAL is expressed in mast cells and is involved in FcεRI signaling [136, 137]. In these cells, Lyn and Syk phosphorylate NTAL following FcεRI engagement and this phosphorylation is potentiated in LAT [143] as well as in vimentin-deficient mice [148]. Furthermore, binding of SCF (stem cell factor) to its receptor Kit specifically leads to NTAL (but not LAT) phosphorylation independent of Lyn [149]. Kit, Lyn and Syk create different patterns of NTAL phosphorylation. For example, Syk preferentially phosphorylates two terminal residues, which could be potentially involved in PLCγ1 binding [150]. Partial functionality of LAT-deficient mast cells indicated that another signaling pathway independent of LAT might exist in mast cells [151]. Indeed, bone marrow mast cells (BMMCs) derived from NTAL-deficient mice, in comparison with wild-type mice, were hyper-reactive to FcεRI triggering [143, 152]. They exhibited an increased phosphorylation of LAT, PLCγ1/2 and Erk, enhanced PI3K activity, elevated calcium response, increased mast cell degranulation and cytokine production. However, the LAT/NTAL double deficiency led to even more

pronounced inhibition of calcium signaling indicating that NTAL plays not only negative but also positive regulatory roles in FcεRI signaling. In addition, electron microscopy revealed that small patches of NTAL or LAT occupy different membrane regions, which do not mix even after FcεRI triggering [152]. Even in the absence of antigen binding, the FcεRI delivers weak signals for sustained Erk signaling and IL-3-dependent mast cell survival. These processes are greatly impaired in LAT/NTAL double KO mice, as both adaptors are, to some extent, required for membrane targeting of Grb2-Sos-Ras complex and proper Erk activation [153]. The observation that both, NTAL overexpression and knock-down, inhibited cell degranulation after FcεRI engagement raised additional questions. The model mast cells, RBL, overexpressing NTAL have specific morphology, diminished phosphorylation of FcR, LAT, Syk and Erk, inhibited PI3K and impaired TNF-α secretion. Moreover, NTAL overexpression increased calcium uptake across plasma membrane by a not fully understood (Grb2-dependent but NTAL phosphorylation-independent) mechanism [154]. NTAL also links FcεRI signaling to cytoskeleton. BMMC derived from NTAL-deficient mice exhibited increased adhesion and reduced spreading after antigen triggering. On the other hand, NTAL-deficient BMMC migrated faster when challenged with antigen. The defects were on the level of RhoA and ROCK mediated actin polymerization. Thus, NTAL serves as a positive regulator of actin polymerization and cell spreading and a negative regulator of chemotaxis [155]. It seems that, depending on the context, NTAL negatively regulates mast cell activation and positively regulates mast cell survival via a mechanism highly dependent on LAT functions. Under specific conditions, phosphorylated LAT also binds phosphatase INPP5, which hydrolyzes IP3. NTAL counterbalances LAT functions, by competing for binding of activating molecules as well as INPP5. When NTAL is deleted, LAT binds more proteins involved in positive signaling (i.e. FcεRI signaling is enhanced), but also more INPP5, which leads to decreased cell survival (less Akt is activated). When LAT is deleted, NTAL alone is unable to support cell activation and survival. However, if both adaptors are missing, mast cell survival is restored [156]. In the absence of LAT, NTAL recruits SLP-76 to membrane, mediates its phosphorylation, and allows partial PLCγ activation with initial upstroke of calcium flux. Nevertheless, simple recruitment of SLP-76 to membrane is not sufficient for full mast cell activation [157].

In monocytes and other myeloid cells, NTAL negatively regulates pro-inflamatory TREM1/DAP12 signaling. The TREM1/DAP12 cross-linking stimulates NTAL phosphorylation and its association with Grb2. Reduction of NTAL expression in monocytic cell line U937 by shRNA, led to a marked impairment of TRAM1/DAP12-induced calcium mobilization, Erk phosphorylation and production of pro-inflammatory cytokines TNF-α and IL-8 [158]. During differentiation of monocytes into macrophages, the expression of NTAL increases and LAT expression falls. NTAL is also involved in TREM2/DAP12 pathway, which limits inflammatory responses. NTAL phosphorylated by Syk

organizes protein complex composed of Grb2, Cbl, and PI3K. In the absence of NTAL, phosphorylation of Cbl and PI3K is enhanced and IL-10 production increased, but activation of Erk pathway is abrogated and IL-12 production decreased [159].

NTAL and LAT are expressed also in NK cells. The analysis of effector functions of IL-2 stimulated NK cells (LAK cells) derived from LAT/NTAL double KO mice revealed impaired ability to kill target cells [160]. Moreover, the total NK cell numbers and NK1.1-induced IFN-γ production were lower in LAT/NTAL double KO mice. Furthermore, the Ly49 receptor repertoire was changed in the absence of either LAT or NTAL. The interplay of LAT and NTAL in NK cells is very complicated and hard to dissect. NK cells use two intertwined pathways consisting of ITAM-Syk-NTAL/LAT and ITAM-Zap-70-LAT. Under some conditions, NTAL can fully compensate for the LAT deficiency and cells without NTAL are hyper-reactive [161].

Finally, NTAL could be also potentially relevant to human pathological conditions. Upon infection of epithelial cells with *Helicobacter pylori*, NTAL is phosphorylated, associates with Grb2, and could play positive regulatory roles in Erk and phospholipase A signaling [162]. Genetic analysis of a region that is deleted in the human autosomal dominant disorder Williams–Beuren syndrome revealed that NTAL (WBSCR5, Williams–Beuren syndrome critical region 5) is one of the deleted genes [163]. Additionally, the chimeric transcription factor AML1/ETO created by the (8;21) chromosomal translocation in human acute myeloid leukemia (AML) strongly suppresses NTAL expression [164]. NTAL is highly expressed in human monocytes and macrophages but not in granulocytes, suggesting an association of NTAL with myelomonocytic features. In fact, *in vitro* differentiation of HL-60 and NB4 cells towards granulocytes by all-trans retinoic acid decreased and monocytic differentiation by phorbol 12-myristate 13-acetate (PMA) or vitamin D3 increased NTAL expression levels. Moreover, ectopic expression of NTAL in AML1/ETO-positive, NTAL-negative Kasumi-1 cells blocked their differentiation [165]. The NTAL transcriptional repression in Kasumi-1 cells could be treated either by knock-down of AML1/ETO with siRNAs or by the class I-specific HDAC inhibitors entinostat and mocetinostat [166].

PAG (Cbp, PAG1)

Three groups co-discovered PAG (Phosphoprotein associated with glycosphingolipid microdomains) using different approaches. The identification of the major Fyn-associated phosphoprotein (pp80) present in leukocyte lipid rafts led to the discovery of TRAP termed PAG. In contrast to other TRAPs, the analysis of PAG tissue expression revealed an almost ubiquitous expression pattern. PAG phosphorylated by Lck and Fyn in Jurkat cells is able to bind recombinant SH2 domains of Lck, Fyn, Lyn, Csk, Shc, Vav, GAP, PI3K, Zap-70, and Syk. *In vivo*, Fyn associates with constitutively phosphorylated PAG via SH3 domain (although the SH2 domains of Fyn are also involved), while the association with Csk, a negative regulator of SFKs, requires PAG phosphorylation on tyrosine 317. Overexpression of PAG in Jurkat cells led to decreased activity of NFAT promoter after TCR stimulation. TCR engagement decreased PAG phosphorylation and the association with Csk [167]. The Japanese group, on the other hand, used an immunoprecipitation approach to identify novel Csk binding proteins in rat brain. They succeeded and identified a protein strongly associated with Csk and termed it Cbp. Based on the observed inducible Cbp/Csk association, they explicitly proposed a negative-feedback loop in SFK signaling [168]. Finally, a third group later discovered a pp85-90 in a detergent resistant protein complex composed of Thy1 (thymus cell antigen 1), Lck, and Fyn [169]. On the SDS-PAGE, PAG migrates around 80kDa thanks to abundant acidic residues. The calculated protein mass of PAG (including all possible modifications) is 47.8 kDa. However, the recombinant human protein has a mass of 50.3 kDa. What account to the difference is currently unknown. The association of PAG with Csk is very strong and preserved even in 1.5M NaCl [170]. SFKs bind PAG via SH3 and SH2 domains. Fyn SH3 domain prefers the first proline-rich motif (RxLPxxP). This interaction allows weak binding (4-7μM) to PAG. Fyn SH2 domains then bind phosphorylated PAG tyrosine residues 163 or 181. This dual-domain docking renders Fyn insensitive to Csk regulation [171, 172]. Lyn SH3 domain, on the other hand, prefers second PAG proline-rich motif (246–266) and Lyn SH2 domain prefers Tyr-381 or 409 [173].

In addition to SH2 and SH3 binding motifs, PAG contains a C-terminal PDZ binding motif. Via this motif, PAG can interact with EBP50/Ezrin complex, thus anchoring lipid rafts to the cortical cytoskeleton [174]. This association and the presence of EBP50 in DRM fraction were lost upon T cell stimulation. Indeed, overexpressed PAG markedly decreased surface mobility of lipid-raft marker GM1, prevented the formation of a functional synapse between Jurkat and Raji cells, and inhibited IL-2 production. All these effects were dependent on PAG/EBP50 association. Thus, it seems that PAG controls T cell activation by two different mechanisms: First, by preventing raft aggregation and immune synapse formation, and second by inactivating SFKs via Csk [175]. In B cells, Ezrin associates with lipid rafts and regulates lipid-raft dynamics. Gupta et al. [176] showed that Ezrin is recruited to lipid rafts via phosphorylated PAG, and this interaction is transiently lost after BCR

stimulation. Ezrin also interacts with protein kinase A type I (PKAI), which upon the cAMP binding negatively regulates TCR signaling by Csk phosphorylatin at serine 364. Obviously, PAG assists this function by bringing Csk and ezrin-bound PKAI to close proximity [177]. When analyzed by single molecule tracking techniques, membrane trajectories of GPI-bound proteins seemed to stop for milisecond (transient anchorage). How could be the lipid moieties of GPI-bound proteins linked to cytoskeleton? Previously, PAG has been shown to interact with Thy1 in the membrane [169]. The phosphorylation of PAG and interaction of PAG with EBP50 and actin cytoskeleton is important for transient anchorage of crosslinked GPI-bound Thy1 proteins [178].

The attractive hypothesis of PAG-mediated feedback inhibition of SFKs provoked investigators to explore more details. The analysis of T cells from Fyn KO mouse pointed out the importance of raft-localized Fyn in PAG phosphorylation. Presumably, the impaired PAG function as a negative regulator, leads to decreased numbers of resting T cells in Fyn-deficient mice [179]. Functional importance of PAG in signaling processes was further illustrated in *Theleria* transformed bovine B cells. *Theleria* infection deregulates SFK Hck and permanently stimulates AP1 signaling pathway. Interestingly, drug treated cells revert to resting state. Baumgartner et al. showed that *Theleria* down-regulates the expression of PAG and thus displaces Csk from Hck-rich lipid rafts [180]. Davidson et al. [181], who studied T cells in transgenic mice overexpressing PAG and PAG mutants, made three important contributions. First, they identified the protein tyrosine phosphatase (PTP) CD45 as a main phosphatase of PAG. Second, in agreement with previous studies, the increased amount of PAG effectively inhibited T cell responses to TCR stimulation and the mutant of PAG unable to bind Csk had a dominant-negative effect. As demonstrated by experiments in which PAG was co-expressed with constitutively active Fyn, PAG inhibitory effects were solely due to the down-regulation of SFK activity in lipid rafts. Third, the overexpression PAG or dominant-negative PAG had no effects on T cell development, which is in strong contrast with the phenotype of Csk-deficient mice, suggesting that other mechanisms of Csk targeting must exist. Additionally, the receptor PTP-α regulates PAG phosphorylation, but indirectly via the raft-resident Fyn. In PTP-α-deficient thymocytes, Fyn is more active and hyper-phosphorylates PAG which in turn recruits more Csk. As a result, the long-term proliferative response of PTP-α-deficient thymocytes (but not the lymph node T cells) was reduced [182]. Where does the PAG-bound Csk go when PAG is dephosphorylated following TCR stimulation? This question was answered by Rahmouni et al. [183] who showed that protein G3BP sequesters Csk in cytosol away from the immunological synapse. Functionally, G3BP overexpression in T cells augmented IL-2 production while G3BP knock-down inhibited it.

The most prominent biochemical property of PAG-localization of PAG to lipid rafts-was studied by Posevitz-Fejfar et al. [184]. The palmitoylation defective (raft displaced) PAG mutant was localized to plasma membrane, phosphorylated, and able to recruit Fyn, Csk, and EBP50. However, such mutant

was unable to inhibit proximal TCR signaling. Remarkably, displaced PAG augmented SDF-1 induced migration of T cells probably by removing Csk from lipid rafts. Accordingly, loss of PAG expression also enhanced T cell migration to SDF-1.

The involvement of PAG in T cell anergy was first proposed by Davidson et al. [185]. In their study, they showed a decreased binding of Fyn to PAG after TCR stimulation and an augmented interaction of PAG with Fyn in anergic T cells. Forced expression of PAG tyrosine mutant binding Fyn and unable to bind Csk led to a strengthening of calcium response and anergy induction. More insights into PAG involvement in T cell anergy and Ras activation was provided by Smida et al. [186] who noticed hyper-phosphorylation of PAG in anergic T cells accompanied by increased Fyn activity. Additionally, in activated T cells, they reported formation of the anergy-promoting protein complex composed of PAG, Fyn, Sam68, and RasGAP. This model was further supported by the observation that overexpression of PAG and Fyn in Jurkat T Cells suppressed Ras activation and knock-down of PAG augmented Ras activation. Thus, PAG negatively regulates not only the SFKs but also Ras. Could be PAG involved in the aging of the immune system? Inomata et al. [187] studied T cell lipid rafts of old mice and found an age-dependent reduction of negative regulators. Specifically, PAG mRNA expression was reduced by 70% and so less PAG and Csk were recruited to lipid rafts. Whether this contributes to decreased proliferative capacity of older T cells remains to be clarified.

PAG influences erythropoietin (Epo) induced differentiation of erythroid cells by a two-step regulation of SFK Lyn. In mouse erythroblasts, Epo stimulation induces PAG phosphorylation via the pre-associated Lyn. PAG phosphorylation results in recruitment of Ctk, the hemopoietic homologue of Csk, and feedback inhibition of Lyn kinase activity. Moreover, another negative regulator of Lyn expressed after Epo stimulation, SOCS1, later competes with Ctk for PAG binding. SOCS1 is involved in ubiquitination and proteasomal degradation of many signaling molecules, including Lyn. J2E cells overexpressing PAG had significantly reduced colony forming ability, Epo-induced hemoglobin synthesis (cell differentiation), and phosphorylation of several proteins [173, 188].

In mast cells, PAG regulates immunoreceptor signaling by a slightly different mechanism. Instead of setting an activation threshold like in Jurkat T cells, in mast cell PAG probably acts as a genuine feedback-loop inhibitor. Shortly after FcεRI triggering SFKs hyper-phosphorylate PAG, which results in recruitment of Csk to lipid rafts and inactivation of SFKs. PAG overexpression in RBL effectively impaired FcεRI induced activation and degranulation [189].

High Src activity is necessary for osteoclastic bone resorption as mice deficient in Src develop severe osteopetrosis. Because PAG interferes with Src activity, the osteoclasts down-regulate PAG expression by RANKL signaling. PAG overexpression in osteoclasts inhibited Src activation and bone resorption [190].

Originally, PAG was discovered as a Csk-binding protein expressed in rat brains [168]. However, the functional role of PAG in the nervous system remained unasked until 2009. Relucio et al. [191] studied differentiation of oligodendrocytes in LAMA2 (laminin)-deficient mice. They found that oligodendrogenesis is significantly delayed due to suppressed Fyn activity. Interestingly, LAMA2 KO mice expressed twice more PAG and Csk, the negative regulators of Fyn. This suggested that laminin can regulate PAG and Csk expression and Fyn activity. In rat neurons PAG is also involved in ephrinB2 signaling by keeping Src inactive. Upon EphB2 receptor binding, ephrinB2 ligand is cleaved by γ-secretase. Georgakopoulos et al. [192] showed that the released fragment (ephrinB2/CTF2) associates with PAG and promotes PAG dephosphorylation, Csk release and Src activation. Active Src phosphorylates ephrinB2 ligand. Moreover, PAG down-modulation by siRNA resulted in impairment of EphB2-induced ephrinB2 ligand phosphorylation. This PAG function could be potentially important in human neurodegenerative disorders.

In fibroblasts, PAG remains non-phosphorylated until the cells adhere to the extracellular matrix (e.g. fibronectin). In Csk-deficient fibroblasts, PAG becomes hyper-phosphorylated. Expression of Csk reverts this phenotype. Also spreading and migration is reduced in Csk-deficient cells. Down-regulation of Cbp could induce impaired cell spreading. [193]. Furthermore, EGF stimulation of Cos-1 cells leads to PAG phosphorylation and Csk recruitment in membrane ruffles as detected by FRET microscopy. It seems that PAG senses the activatory status of SFKs specifically in lipid rafts, and could serve as a useful indicator in living cells [194]. In growth factor-evoked signaling, PTP Shp-2 opposes action of SFKs by binding and dephosphorylation of PAG. In Shp-2-deficient fibroblasts, PAG is hyper-phosphorylated and activity of SFKs up-regulated due to defective recruitment of Csk to the membrane [195]. In addition to the EGF stimulation, PAG phosphorylation and Csk recruitment in A431 or HEK293 cells was induced also after TrkA and PDGFR stimulation. Expression of PAG mutant that was unable to bind Csk or PAG knock-down led to impaired recruitment of Csk to lipid rafts. Overexpression of PAG suppressed EGF induced Erk and Akt phosphorylation, cell transformation, and colony formation in soft agar [196]. In mouse embryonic fibroblasts, PAG overexpression strongly inhibited PDGFR mitogenic signaling independently of Csk. The N-terminal part of PAG somehow (probably via sialidase Neu3 and ganglioside GM1) excludes PDGFR from caveolae, which are indispensable for PDGFR signal propagation [197].

PAG could be potentially involved in human malignant diseases. Because PAG has been proposed in SFK regulation in various models, Oneyama et al. [198] addressed PAG involvement in Src induced oncogenesis. As a model they chose Csk-deficient fibroblasts in which Src directs transformation and tumorigenesis (changed cell morphology and an anchorage-independent growth in soft agar). In these cells, PAG expression was strongly down-modulated and PAG re-expression, even in the absence of Csk, prevented Src-induced transformation. The authors proposed a mechanism according to which

phosphorylated PAG sequesters active Src in lipid rafts, thus effectively preventing phosphorylation of several Src substrates. This observation could be of a functional importance as PAG is down-regulated in many other cancer cell lines (e.g. some colon tumor cells). Interestingly, in these pathological conditions, lipid-raft targeted SFK remain inactive [199]. The oncogenic potential of SFK depends mainly on the membrane microenvironment. Fyn and Yes, the SFKs recovered in DRMs, cannot transform Csk-deficient fibroblasts, while other SFKs, distributed also (Src and Blk exclusively) in non-DRM fractions, can. The DRM-localized PAG associates with all active SFKs and thus generally serve as a suppressor for SFK-mediated cell transformation [200]. Based on the results of Oneyama et al. [198, 200], the role of PAG in regulation of Src oncogenic signaling seems to be solely lipid raft-dependent and Csk-independent. However, the Csk membrane localization is decreased in colorectal cancer cells probably due to decreased expression of PAG. Overexpression of a chimeric lipid-raft targeted Csk or overexpression of PAG reduced Src activity and cellular invasiveness [201]. The same mechanism probably operates also in human non-small cell lung cancer (NSCLC) cells. In lung cancer cells and tissues, PAG expression is down-regulated and Src activity up-regulated. Forced expression of PAG in lung cancer cells recruited Csk to lipid-rafts, suppressed Src kinase activity, and ability of these cells to invade *in vitro* and metastasize *in vivo* [202]. The molecular mechanism of PAG down-regulation in cancer cells has been explained by Suzuki et al. [203]. Oncogenic EGFR, Src and Ras signaling exceedingly stimulates PI3K-Akt and Erk/p38 MAPK pathways. This eventually leads to histone modifications (deacetylation and trimethylation) in PAG promoter and suppression of PAG mRNA production, creating a positive-feedback loop in oncogenic signaling. In addition to PAG, another Csk-binding protein, caveolin-1, regulates the oncogenic potential of Src. In caveolin-deficient cells, PAG is up-regulated and compensates for the partial loss in Csk membrane recruitment. Accordingly, PAG depletion led to increased caveolin phosphorylation and Csk recruitment. Deletion of PAG in caveolins-deficient cells markedly up-regulated Src activity [204].

In contrast to colorectal and lung cancers, various human lymphomas rather overexpress than down-modulate PAG. Svec et al. [205] found an interesting correlation between PAG expression in germinal centers of secondary lymphatic follicles and follicular malignant lymphomas. Later, they performed an extensive study to assess the expression of PAG in 155 cases of malignant lymphomas. Apparently, PAG can be used as a reliable marker of germinal center differentiation in diffuse large B cell lymphoma [206]. Moreover, Tauzin et al. [207] showed that in B-non-Hodgkin lymphoma (B-NHL) cells PAG forms raft oncogenic signalosome composed of constitutively active Lyn and STAT3, but not Csk. Inhibition of Lyn activity or PAG knock-down results in death of B-NHL cells. PAG is also overexpressed in human renal cell carcinomas (RCC, more than 70% of cases) and human RCC cell lines. Down-modulation of PAG in RCC cell lines suppressed proliferation, cell motility and invasiveness *in vitro* and in model nude mice. PAG depletion in RCC cells led to a reduction of RhoA activation and changes in actin cytoskeleton organization. These effects were dependent on the intact

PDZ binding motif in PAG [208]. Furthermore, they showed that different lymphoma cell lines contain distinct PAG-based lipid-raft signaling complexes variably containing Syk and PI3K [209]. On the other hand, ALK positive lymphomas contain PAG and Lyn in lipid rafts, but the complex is not formed, and Lyn is less active than in B-NHL cells [210].

At least three groups independently generated PAG-deficient mice. At first, Xu et al. [211] found that mice with abolished PAG, in strong contrast to Csk-deficient mice, are viable and without any developmental defects. Except the slightly increased numbers of thymocytes, the T and B cell development, T cell responses (TCR-proximal signaling, proliferation, cytokine production), humoral immune responses, and B or T cell tolerance were normal in PAG-deficient mice. Interestingly, they show that Csk is excluded from lipid raft fractions in resting thymocytes from PAG-deficient mice. The other group [212] came basically to the same conclusions regarding the lack of phenotype in PAG-deficient mice. However, in contrast to Xu et al., they did not find any difference in Csk compartmentalization to lipid rafts in thymocytes. The analysis of a neonatal nervous system in third PAG-deficient mice strain demonstrated decreased Csk lipid-raft recruitment and increased Fyn and Src activities, but the brain developed normally [213]. Remarkably, no such differences were found in adult brains indicating that other mechanisms of Src regulation may compensate for PAG deficiency.

TRIM (TRAT1)

TRIM (TCR interacting molecule) was identified as a dimeric non-raft phosphoprotein (pp29/30) associated with TCR complexes in T cells, γδT cells and NKT cells [214-217]. *In vitro*, SFK Fyn and Lck phosphorylated TRIM, which then interacted with SH2 domains of SFKs and p85 PI3K. In Jurkat T cells, TRIM associated with CD3ζ (probably via a transmembrane dimerization motif) and co-capped with CD3ε after receptor cross-linking. Moreover, TRIM overexpression significantly increased the CD3ζ and TCR surface expression and prevented TCR internalization. These effects were, however, independent of TRIM tyrosine phosphorylation [218]. Another study showed that TRIM specifically associated with the costimulatory molecule CTLA-4 [219]. Overexpression of TRIM in T cells promoted CTLA-4 transport and surface expression, while the reduction of TRIM expression had the opposite consequences accompanied by changes in co-receptor-mediated effects on cytokine production and proliferation. Possibly, these chaperon-like properties of TRIM could be of a functional importance in $CD8^+$ regulatory T cells, which express very high levels of CTLA-4 as well as TRIM following activation [220].

TRIM KO mice were normal, although they were slightly smaller than wild-type mice and had decreased cellularity in lymphoid organs [221]. However, thymus development, positive and negative selection and peripheral development of T cells were unaffected in the absence of TRIM. Analyses also indicated that TRIM was dispensable for the TCR expression, internalization and recycling.

On the molecular level, TRIM deficiency only slightly augmented Akt signaling in CD4 T cells. Thus, the functional importance of TRIM remains elusive.

SIT (SIT1)

Soon after the discovery of TRIM, the same research group described a novel TRIM-like TRAP called SIT (SHP2 interacting transmembrane adaptor) [222]. SIT is a dimeric, non-raft and heavily glycosylated protein expressed exclusively in the immune system (predominantly in thymocytes). SIT co-immunoprecipitated with CD3 molecules and was phosphorylated by SFK Lck and Fyn in a co-transfection assay. Overexpression of SIT in Jurkat T cells inhibited TCR-induced NFAT activity. In contrast to TRIM, SIT contains an inhibitory (ITIM-like) tyrosine motif that binds SH2 domain of phosphatase Shp-2. Surprisingly, this association did not mediate the negative regulatory effect of SIT. Additional experiments done with transfected cells revealed that the Csk binding motif (YASV) in the C- terminal part of SIT conferred the inhibitory function of SIT [223]. In the absence of Csk binding site, SIT provided positive rather than negative regulatory signals for T cell activation. Apparently, other proteins associated with SIT (e.g. Grb2) mediated these positive effects. However, in primary cells under more physiological conditions, the SIT-Csk interaction was not found and the functional significance of this interaction was not proven.

SIT-deficient mice exhibited a mild but interesting phenotype [224, 225]. The thymi of these mice contained increased numbers of immature ($CD4^+CD8^+$) thymocytes with up-regulated CD5 and CD69. CD5 is a negative-feedback regulator of TCR signaling required during thymocyte selection, and CD69 is a marker of ongoing TCR signaling. Indeed, on the background of the transgenic low-affinity H-Y TCR, SIT deficiency not only enhanced positive selection but also partially converted positive selection to negative selection, suggesting that SIT lowers the T cell activation threshold for positive selection. SIT deficiency also altered the composition of peripheral T cell pools (decreased total numbers of T cells, enhanced expansion of γδT cells, and accumulation of memory like $CD44^{high}CD8^+$ T cells). Moreover, T cells isolated from SIT-deficient mice proliferated better and produced more Th1-specific cytokines than T cells isolated from wild-type mice. Lastly, SIT-deficient mice were more susceptible to an induced autoimmune disease (experimental autoimmune encephalomyelitis) than wild-type mice and spontaneously developed antinuclear Abs and glomerulonephritis. In conclusion, these results demonstrated that SIT is a negative regulator of TCR-mediated signaling and finely tunes the signals required for thymic selection and peripheral T cell activation and homeostasis.

The relatively mild phenotypes of TRIM and SIT (as well as several other TRAPs) KO mice could be explained by the existence of compensatory mechanisms and functional redundancies. This conclusion

is supported by the more severe phenotype (enhanced Erk activation and positive selection of T cells in thymus) observed in TRIM/SIT double KO mice [226].

LIME (LIME1)

In 2003, two research groups independently described a lipid raft associated TRAP, LIME (Lck-interacting molecule) [227, 228]. The expression of LIME is restricted to peripheral blood lymphocytes (PBL), spleen, lymph nodes, lungs and liver. Moreover, the expression of LIME is influenced by the activation status of T cells. After cross-linking of co-receptors CD4 or CD8 with antibodies, SFK Lck strongly phosphorylates LIME and associate with it. In addition, two phosphorylated tyrosine motifs in LIME can bind Csk. Csk could in turn negative regulate the associated Lck by a mechanism very similar to that proposed for PAG. Besides Lck and Csk, phosphorylated LIME co-precipitated also with p85 PI3K, Shp-2, Grb2 and Gads. Formation of artificial synapse between T cell and B cell caused translocation of LIME to the contact site. Overexpression of LIME in Jurkat cells augmented the calcium response after TCR triggering and cross-linking of chimeric CD8-LIME proteins led to activation of MAPK Erk and JNK. Constitutive increase in IL-2 promoter activity in Jurkat transfectants was dependent on Lck kinase activity.

LIME is expressed also in B cells [229]. In B cell line A20 transfected with LIME, LIME was phosphorylated by SFK Lyn after BCR triggering and organized a protein complex composed of Lyn, Grb2, PLCγ and PI3K. BLNK was not part of this complex. Reduction of LIME expression by siRNA in A20 cells led to a decreased calcium response, decreased phosphorylation of Akt and Erk, and decreased nuclear translocation of NF-κB after BCR cross-linking. However, these LIME knock-down experiments suffered from insufficient negative controls, thus must be taken with caution.

Finally, detailed analysis of the mouse model failed to find any real functional consequences of LIME deficiency. Development and functions of T cells and B cells remained intact in LIME or LIME/NTAL double KO mice. Only on the transgenic H-Y TCR background, LIME deficiency had a minor enhancing effect on the positive selection of T cells [230].

LAX (LAX1)

The search for adaptors with multiple Grb2 binding sites, led to discovery and characterization of protein termed LAX (linker for activation of X cells) [231]. LAX is a non-raft TRAP mainly expressed in leukocytes. Interestingly, the expression of LAX increases several hours after T cell and B cell activation. LAX negatively regulates immunoreceptor signaling. After TCR or BCR cross-linking, Src and Syk family kinases phosphorylate LAX on four tyrosine residues, which in turn recruit Grb2, Gads and the regulatory subunit of PI3K. Overexpression of LAX in Jurkat T cell line resulted in a strong suppression of p38 MAPK and inhibited NFAT/AP1 transcription. Moreover,

the activity of LAX depends on constitutive association with another adaptor ALX, which brings the SFK Lck to LAX proximity [232].

Mice deficient in LAX were viable and showed no apparent developmental defects [233]. However, the LAX deficiency slightly enhanced T cell responses (e.g. global tyrosine phosphorylation, calcium signaling, IL-2 production, and proliferation) and survival. Similarly, the proliferation and survival of B cells were augmented in these mice, which correlated with increased levels of serum IgG and IgE antibodies. On the molecular level, LAX deficiency pronounced and prolonged Akt, p38 and Erk signaling. In bone derived mast cells, LAX deficiency promoted IgE-induced degranulation and viability after cytokine withdrawal [234]. Moreover, LAX was indispensable for proper expression of adaptor protein NTAL in mast cells. Based on these and other observations, the authors proposed a model explaining mast cell hyper-responsiveness, in which positive regulator LAT and negative regulator NTAL compete for a specific lipid-raft membrane environment [143].

GAPT

The hunt for LAT-like Grb2-binding TRAPs in B cells resulted in identification of small non-raft protein GAPT [235]. This protein contains four potential Grb2 binding tyrosine motifs. However, GAPT is not phosphorylated upon BCR cross-linking, but still constitutively associates with Grb2, presumably via SH3 binding proline-rich motifs. GAPT-deficient mice were viable and developed normal immune system. However, B cells isolated from GAPT-deficient mouse proliferated better after BCR cross-linking than control B cells. In old mice, GAPT deficiency also leads to two-fold enrichment of marginal zone B cells in spleens and increased concentrations of IgM and IgG antibodies in sera.

Aims of the study

During my PhD studies, I was involved in three main scientific projects:

1. In the first project, we wanted to elucidate some aspects of membrane microdomains in immunoreceptor signaling. According to the raft hypothesis, the presence of key signaling proteins (SFKs, LAT, PLC, etc.) in lipid rafts is crucial for effective signal propagation from the TCR. However, several papers questioned this requirement; at least in the case of LAT. Thus, we set to find out answers to these questions:
   - Could we explain the observations made by Zhu et al. [236] that lipid raft localization of LAT is not important for its function?
   - To what extent is a specific membrane microdomain environment important for the signaling capacity of two classical TRAPs, raft-localized LAT and non-raft LAX?
   - Could we biochemically analyze what types of membrane microdomains are involved?

2. In the second project, we studied novel TRAP PRR7, identified by a bioinformatics approach. The features of protein sequence suggested that PRR7 could be potentially involved in regulation of signal transduction events. Moreover, at the time of initial protein characterization, one study identified PRR7 in the NMDAR (N-methyl-D-aspartate receptor) protein complex in brain. In this project, we asked three basic questions:
   - Do the cells of the immune system express PRR7 as well?
   - What proteins can interact with PRR7 in the immune system?
   - Could we identify the signaling processes in which PRR7 is involved?

3. In the third project, we discovered and analyzed a novel Csk binding TRAP, which we termed SCIMP. Csk is an important negative regulator of SFKs and plasma membrane recruitment is critical for its function. Previously, several other TRAPs have been shown to provide such an anchorage. In the case of SCIMP, we wanted to investigate:
   - What is the expression profile of SCIMP?
   - Could we prove the interaction of SCIMP with Csk and other predicted interactions?
   - What is the functional relevance of such interactions?
   - In what biological processes SCIMP is involved?

Results and discussion

A New Type of Membrane Raft-Like Microdomains and Their Possible Involvement in TCR Signaling

According to numerous reports, membrane raft microdomains are indispensable for immunoreceptor signaling [36]. Lipid rafts influence many aspects of TCR signaling from the very beginning until the later steps, including intracellular signal propagation and synapse formation [37]. Lipid raft resident TRAP LAT is a master regulator of all main signaling pathways emanating from the TCR [79]. Several studies, however, cast doubts on the functional importance of lipid rafts in TCR signaling [236, 237]. In one of these studies, Zhu et al. [236] prepared a chimeric LAX-LAT protein to target LAT into the membrane but apparently outside the lipid rafts (as judged by standard biochemical criteria). When they overexpressed the LAX-LAT protein in LAT-deficient Jurkat cells, it fully restored TCR signaling. Moreover, T cells from transgenic mouse expressing LAX-LAT developed normally and were perfectly functional. These results suggested that lipid rafts are maybe not so important in LAT signaling as has been thought.

We tried to explain these observations by the existence of a novel (so far overlooked) type of membrane microdomains. We noticed that after solubilization of cell membranes in Brij98, which possess many properties of an ideal "raft detergent", we can distinguish three fractions based on size (as determined by gel filtration) and density (as determined by density gradient ultracentrifugation): (1) Fully solubilized proteins are small and evenly distributed in density gradients. (2) Large and light or classical DRMs (corresponding to lipid rafts, caveolae, and TEMs) float in density gradients. (3) Large and dense ('heavy') DRMs remain in bottom fractions during ultracentrifugation. Surprisingly, LAX, LAX-LAT, and some other proteins were isolated specifically in heavy DRMs. Similarly to classical DRMs, heavy DRMs are sensitive to the lipid raft disrupting detergent laurylmaltoside and to cholesterol depletion. Unlike classical DRMs, heavy DRMs are sensitive to the weak chaotropic agent KI (0.6M) that disrupts protein-protein interactions.

Thus, we wanted to elucidate the physiological relevance of these biochemical findings. We hypothesized that LAT targeted to these microdomains by LAX (LAX-LAT) supports TCR signaling, and that LAT targeted to non-microdomain membrane by CD25 (CD25-LAT) does not. Indeed, when we overexpressed these chimeric proteins in LAT-deficient Jurkat T cells, their signaling capacity was highly dependent on the expression level. At high expression levels, all three proteins (LAT, LAX-LAT, CD25-LAT) functioned equally well. At moderate expression levels, LAT was the best one, followed by LAX-LAT and CD25-LAT was least effective. At low expression levels, only LAT and to some extent LAX-LAT molecules were functional.

Our results clearly demonstrate that to be fully functional, LAT requires a raft microdomain environment. We described a novel type of DRMs in which LAX, originally described as a non-raft protein, is present. Our recent unpublished data indicate that LAX is targeted to these 'heavy' DRMs by its transmembrane part. Nevertheless, overexpression of LAT even in non-lipid raft membrane could lead to productive signaling [206].

PRR7 Is a Transmembrane Adaptor Protein Expressed in Activated T Cells Involved in Regulation of T Cell Receptor Signaling and Apoptosis

Transmembrane adaptor proteins (TRAPs) are important regulators of immunoreceptor signaling. Comparison of protein sequences of known TRAPs revealed common characteristics, which permitted a bioinformatics search of other similar proteins. Using this approach, we identified PRR7. It has a very short extracellular part, a single transmembrane segment and an intracellular part containing several protein binging motifs (proline rich motifs, tyrosine-based motifs, WW domain binding motif, and PDZ binding motif). Moreover, PRR7 was strikingly conserved during vertebrate evolution, suggesting its functional importance. At the time of PRR7 initial characterization, Murata et al. [238] isolated PRR7 from postsynaptic membranes of rat brain and described its direct association with PSD-95 (postsynaptic density protein of 95 kDa) and co-immunoprecipitation with NMDAR, two key proteins of neuronal synapses.

First, we performed an expression analysis. On the RNA level, we detected PRR7 in brain and weakly in other tissues including thymus and lymph nodes. Specific analysis of immune cells revealed that PRR7 expression increased in PBL after activation. Because the expression of PRR7 was rather low even in activated T cells, we used PRR7 transfected Jurkat cells for further studies (Jurkat is a T cell line suitable for TCR signaling studies). Surprisingly, we were unable to obtain a satisfactory expression of PRR7 even in transfected cells, because shortly after transfection, PRR7 expressing cells died by apoptosis. To overcome this obstacle, we established an inducible expression system. Jurkat cells induced to express PRR7 started to die around 20 h after induction. These pro-apoptotic effects were mediated mainly by the C terminus of PRR7 containing the WW domain binding motif. Interestingly, PRR7 lacking the transmembrane segment were more pro-apoptotic than wild-type PRR7. Moreover, these pro-apoptotic properties partially correlated with subcellular localization of PRR7 in perinuclear vesicular compartments. It seems likely that cells actively remove PRR7 from the plasma membrane and try to get rid of it. Furthermore, PRR7 lacking the transmembrane segment was localized in the same perinuclear compartment.

Nevertheless, the PRR7 inducible system allowed us to analyze biochemical and functional properties of PRR7 before cells started to die. Biochemically, PRR7 belongs to acylated (palmitoylated) proteins and is present in DRMs resistant even to laurylmaltoside solubilization (a detergent that solubilizes

classical rafts). In Jurkat cells, PRR7 is constitutively phosphorylated mainly by Src kinase, which also associates with PRR7. Jurkat cells overexpressing PRR7 have partially activated phenotype, spontaneous expression of CD69 and increased IL-2 production after stimulation with drugs PMA and ionomycin. Up-regulated and hyper-phosphorylated transcription factor c-Jun is probably responsible for this phenotype. On the other hand, all membrane proximal signaling events we analyzed were suppressed in the presence of PRR7: decreased surface expression of TCR (unpublished observation), decreased expression and tyrosine phosphorylation of Lck, decreased overall tyrosine phosphorylation and calcium response after TCR cross-linking by antibodies.

Although our results indicated potentially important functional roles of PRR7, many questions remain to be answered. The analysis of the overexpression phenotype could be just one side of the coin. It is very likely that a protein containing so many conserved interaction motifs (as PRR7 does) would somehow interfere with cellular processes. It would be crucial to find out if decreased expression of PRR7 will have the opposite effects. Due to higher expression of PRR7 in brain, it would be of great interest to perform such experiments in neurons. The results of Murata et al. [238] opened the possibility that PRR7, present in the core of the postsynaptic density, could regulate important neuronal functions. It would be also interesting to study PRR7 in the context of immunological synapses, which share many similarities and components with neuronal synapses (including NMDAR receptors).

Except for Src, we failed to identify more proteins interacting with PRR7. The knowledge of proteins interacting with PDZ and WW domain binding motifs could help to explain the PRR7 phenotype. Currently, the most plausible explanation of PRR7 phenotype is the association with the members of Nedd4 family of ubiquitin ligases thus deregulating their functions. These proteins have been shown to interact with exactly the same PPPY motif, which is present in PRR7 and usually rely on adaptor proteins to reach their substrates. The transcription factor c-Jun is post-translationally regulated by Itch, a Nedd4 family ubiquitin ligase [239]. Although we failed to co-immunoprecipitate PRR7 and Itch, it is still possible that such interaction may exist in living cells or that PRR7 interacts with other Nedd4 family members. Indeed, our recent unpublished data support this possibility.

The strong pro-apoptotic phenotype of PRR7 in Jurkat cells raised the questions about its relevance to primary T cell functions. Apoptosis is an important physiological process. The majority of T cells die by apoptosis during their development in thymus [240]. Apoptosis also limits the immune responses in natural processes referred to as activation-induced cell death and activated T cell autonomous death [241]. It is possible that PRR7 is involved in these processes. Indeed, the tight regulation of PRR7 expression and its pro-apoptotic functions support such assumptions. Unfortunately, in Jurkat T cells expressing PRR7 we were not able to find up-regulation of mediators of apoptosis such as FasL or Bim. Thus, the exact mechanism of PRR7-induced apoptosis remains to be solved in the future.

SCIMP: transmembrane adaptor protein involved in MHCII signaling

The question, how is Csk recruited to the plasma membrane, seems to be central to immunoreceptor signaling. When proximal to SFKs, Csk can negatively regulate their activity by phosphorylating inhibitory tyrosine residues. Many Csk binding proteins, including several TRAPs have been discovered [242]. While Csk deficiency is indispensable for T cell maturation, deletion of Csk binding TRAPs had little or no effect on T cell development. These results suggested that other Csk binding protein may exist.

The bioinformatics search for proteins containing Csk binding motif revealed a potential gene product C17orf87, which we termed SCIMP. This protein resembled other TRAPs as it is composed of single transmembrane segment, short extracellular and larger intracellular part. In addition to Csk binding motif, it contained other evolutionary conserved motifs predicted to interact with SH3 domain of Lyn, and SH2 domains of Grb2, and BLNK/SLP-76, indicating possible involvement of SCIMP in signal transduction pathways.

First, we wanted to know if SCIMP is present in the cells of the immune system. We discovered that on both RNA and protein level the expression of SCIMP is restricted to immune tissues (spleen, lymph nodes) and more specifically to professional antigen presenting cells (B cells, macrophages, dendritic cells). The expression pattern of SCIMP well correlated with the expression of MHCII. To investigate more this connection, we biochemically analyzed the composition of SCIMP membrane protein complexes. We found that SCIMP is an acylated (palmitoylated) protein interacting with tetraspanins in tetraspanin enriched microdomains (TEMs). Interestingly, the MHCII molecules are also present in TEMs [243]. Another line of evidence came from microscopic observation of GFP labeled SCIMP. It not only partitioned to uropods of migrating cells, but SCIMP was also recruited to the immunological synapse formed between T and B cell lines. Collectively, these results, rather restricted expression profile of SCIMP and presence in TEMs and IS, suggested that SCIMP could possibly regulate signaling processes during antigen presentation.

The analysis of cellular signaling provided more direct evidence of SCIMP involvement in MHCII signaling. In B cells, SCIMP was phosphorylated upon MHCII cross-linking with antibodies by Lyn and assembled a protein complex composed of Grb2, BLNK, Csk and Lyn. The simultaneous binding of negative regulator Csk and positive regulators Grb2 and BLNK was novel and counterintuitive. To dissect the signaling pathways specifically, we constructed chimeric SCIMP (CD25-SCIMP), which could be cross-linked by antibodies and its mutated versions unable to bind BLNK or Csk. Cross-linking of CD25-SCIMP in B cell line K46 led to a strong calcium response and Erk activation. When a mutation prevented BLNK binging to SCIMP, the cellular signaling response was lost. On

the other hand, the removal of Csk binding motif from SCIMP augmented the signaling response. These analyses confirmed that SCIMP could exert both positive and negative signaling functions.

Finally, we studied if there is a functional consequence of SCIMP deficiency in B cell line K46. The cells were transfected with specific shRNA vectors to silence the expression of SCIMP. In these cells, SCIMP down-modulation led to the impaired Erk activation after MHCII cross-linking. This impairment was rescued by the expression of SCIMP, but not by SCIMP with mutated BLNK binding site. Collectively, we provided the evidence that SCIMP is a potent regulator of MHCII signaling in B cells. Further research should focus on *in vivo* studies of SCIMP-deficient mice. We predict that in these mice, the adaptive immune responses will be impaired.

Conclusions

During my PhD studies, I was involved in three scientific projects. The common theme of these projects was the role of plasma membrane microenvironment and adaptor proteins in immunoreceptor signaling in leukocytes.

In the first project, we studied how membrane microdomains influence the signaling capacity of TRAP LAT, a key protein in TCR signaling pathways. Using informative mutants of LAT we showed that targeting LAT to the raft microdomain environment is a prerequisite for the full signaling capacity of LAT. Moreover, we discovered a novel type of membrane microdomains biochemically distinct from lipid rafts and TEMs. We called it heavy rafts, because they produced DRMs present during ultracentrifugation in density gradients in bottom, high-density fractions, probably due to higher protein content. These heavy DRMs containing LAX and other proteins also elegantly explained the results of Zhu et al. [236] who questioned the importance of lipid rafts in LAT signaling.

In the second project, we dealt with TRAP PRR7. We were first to recognize that PRR7 is a TRAP expressed in activated T cells, and we performed a basic biochemical and functional analysis. The most striking observation was the pro-apoptotic function of PRR7 in T cells. Although we have not identified the exact apoptotic mechanism, we provide the evidence that it is mediated by the WW domain-binding motif, which also targets PRR7 to peculiar intracellular vesicular compartments. We also investigated PRR7 functions in T cell signaling. PRR7 constitutively associates with and is phosphorylated by Src. All TCR proximal signaling events were suppressed in the presence of PRR7. Moreover, the expression level of Lck was decreased as well. On the other hand, transcription factor c-Jun was strongly up-regulated in the presence of PRR7. This led to a partially activated phenotype of Jurkat cells in the absence of TCR stimulation (up-regulated CD69) and increased expression of IL-2 in the presence of PMA and ionomycin. Collectively, our data pointed out that PRR7 is a potent regulator of T cell physiology and should be further studied, best in PRR7-deficient mice.

In the third project, we investigated the functional role of a novel TRAP SCIMP. Based on our results, SCIMP appears to be an important regulator of MHCII signaling. It is present in microdomains (TEMs) in the membrane of all professional antigen-presenting cells and translocates to the site of immunological synapse. In B cells, the kinase Lyn binds and phosphorylates SCIMP, which leads to the assembly of protein complex composed of BLNK, Csk, and Grb2. Such protein complex is able to transmit the signals emanating from the engaged MHCII receptors and provide positive (BLNK dependent) as well as negative (Csk-dependent) signaling.

In conclusion, this study highlighted the importance of membrane microdomains in immunoreceptor signaling and elucidated so far unexplored signaling mechanisms in lymphocytes mediated by two novel TRAPs PRR7 and SCIMP.

Contributions

A New Type of Membrane Raft-Like Microdomains and Their Possible Involvement in TCR Signaling

I joined this project at the very beginning and prepared several constructs for targeting LAT to various membrane microdomains. I also transfected J.Cam2.5 cells for biochemical analyses. Now, I am continuing these studies trying to find out what targets LAX to heavy DRMs.

PRR7 Is a Transmembrane Adaptor Protein Expressed in Activated T Cells Involved in Regulation of T cell Receptor Signaling and Apoptosis

This was my main PhD project. I designed, performed and analyzed the majority of experiments and wrote substantial parts of the manuscript.

SCIMP: transmembrane adaptor protein involved in MHCII signaling

In this project, I performed the real-time PCR expression analysis of SCIMP in human tissues (Figure 1C).

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Reprints of publications

1. Otahal P, Angelisová P, Hrdinka M, Brdicka T, Novak P, Drbal K, Horejsi V. 2010. **A new type of membrane raft-like microdomains and their possible involvement in TCR signaling.** J Immunol. 2010 Apr 1;184(7):3689-96. Epub 2010 Mar 5.

2. Hrdinka M, Draber P, Stepanek O, Ormsby T, Otahal P, Angelisova P, Brdicka T, Paces J, Horejsi V, Drbal K. 2011. **PRR7 is a transmembrane adaptor protein expressed in activated T cells involved in regulation of T cell receptor signaling and apoptosis.** *J Biol Chem* 286: 19617-29

3. Draber P, Vonkova I, Stepanek O, Hrdinka M, Kucova M, Skopcova T, Otahal P, Angelisova P, Horejsi V, Yeung M, Weiss A, Brdicka T. 2011. **SCIMP: transmembrane adaptor protein involved in MHCII signaling.** *Moll Cell Biol* [published ahead of print on 19 September 2011, doi:10.1128/MCB.05817-11]

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A New Type of Membrane Raft-Like Microdomains and Their Possible Involvement in TCR Signaling

Pavel Otáhal,*[,1] Pavla Angelisová,*[,1] Matouš Hrdinka,* Tomáš Brdička,* Petr Novák,[†] Karel Drbal,* and Václav Hořejší*

Membrane rafts and signaling molecules associated with them are thought to play important roles in immunoreceptor signaling. Rafts differ in their lipid and protein compositions from the rest of the membrane and are relatively resistant to solubilization by Triton X-100 or similar detergents, producing buoyant, detergent-resistant membranes (DRMs) that can be isolated by density gradient ultracentrifugation. One of the key signaling molecules present in T cell DRMs is the transmembrane adaptor protein LAT (linker for activation of T cells). In contrast to previous results, a recent study demonstrated that a LAT construct not present in the buoyant DRMs is fully able to support TCR signaling and development of T cells in vivo. This finding caused doubts about the real physiological role of rafts in TCR signaling. In this study, we demonstrate that these results can be explained by the existence of a novel type of membrane raft-like microdomains, producing upon detergent solubilization "heavy DRMs" containing a number of membrane molecules. At a moderate level of expression, LAT supported TCR signaling more efficiently than constructs targeted to the microdomains producing heavy DRMs or to nonraft membrane. We suggest that different types of membrane microdomains provide environments regulating the functional efficiencies of signaling molecules present therein. ***The Journal of Immunology*****, 2010, 184: 3689–3696.**

Membrane rafts (also less correctly called lipid rafts), specific microdomains of the plasma membrane, are distinguished from the rest of the membrane by their relative resistance to solubilization by Triton X-100 or similar detergents (1, 2). Compared to the rest of the plasma membrane, rafts appear to be enriched in cholesterol, sphingomyelins, and glycosphingolipids, containing long saturated acyl residues. These sphingolipids form a liquid-ordered phase further stabilized by cholesterol (3). Detergent-solubilized rafts (also called detergent-resistant membranes [DRMs] or detergent-insoluble glycosphingolipid complexes) can be conveniently isolated by density gradient ultracentrifugation, because they float to the low-density fractions of the gradient and therefore can be separated from completely solubilized membrane proteins and their complexes present in the bottom high-density fractions (4). Membrane rafts apparently participate in a number of biologically important phenomena, such as in immunoreceptor (TCR, BCR, and FcR) signaling, because they concentrate several critical signaling molecules and after initiation of immunoreceptor signaling they associate with the ligated immunoreceptors (5–7). In TCR-mediated signaling pathways, one such raft-associated protein is a transmembrane adaptor protein, linker for activation of T cells (LAT) (8, 9), which after TCR-induced phosphorylation serves as a scaffold for organizing membrane-proximal multicomponent complexes of cytoplasmic signaling proteins. An early report indicated that palmitoylation of LAT is critical for its raft association and for its functionality (10).

However, despite multiple sources of evidence indicating important roles for rafts in immunoreceptor signaling, not only the details of the raft-based mechanisms but even their very existence remain a topic of debate (11, 12). The main problem is that membrane rafts are difficult to visualize as distinct morphological structures. Therefore, most of the structural and functional data are based on (or inferred from) results of biochemical experiments based on detergent extraction of membranes. Such an approach may however almost inevitably induce various artifacts, especially due to selective extraction of some raft components. Other methods often used to demonstrate involvement of membrane rafts in receptor signaling and other phenomena are based on depletion of cholesterol; however, this can also produce artifacts due to generalized alterations in the physical properties the membrane (13). Also, low temperature used during membrane solubilization and production of DRMs may induce artifacts due to phase separations in the mixtures of membrane lipids; however, DRMs can be readily obtained also at 37°C (14), and membrane fragments resembling DRMs can be obtained even by detergent-free membrane disintegration (15, 16).

One of the strongest arguments for the real functional involvement of membrane rafts in TCR signaling has been based on the correlation between loss of (biochemically defined) raft association of palmitoylation-deficient mutants of Lck (17) and LAT (10) and loss of their signaling functionality. Furthermore, inhibition of Lck, Fyn, and LAT palmitoylation led to their raft displacement and inhibition of TCR signaling (18). Therefore, it came as a surprise when a mutant LAT construct was described that did not contain any palmitoylation motif and was not associated with buoyant DRMs but was fully functional in TCR signaling (19). This construct was composed of the extracellular and transmembrane domains of another transmembrane adaptor protein,

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Received for publication June 29, 2009. Accepted for publication January 31, 2010.

This work was supported in part by project no. AV0Z50520514 awarded by the Academy of Sciences of the Czech Republic, Czech Science Foundation (project MEM/09/E011), and the Center of Molecular and Cellular Immunology (project 1M0506, Ministry of Education, Youth and Sports of the Czech Republic).

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Abbreviations used in this paper: DRM, detergent-resistant membrane; LAT, linker for activation of T cells; LAX, linker for activation of X; LM, laurylmaltoside; LNGFR, a truncated form of human nerve growth factor receptor; MbCD, methyl β-cyclodextrin; OFP, orange fluorescent protein; RV, retrovirus.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0902075

linker for activation of X (LAX) (which is not present in conventionally defined membrane rafts), and the cytoplasmic domain of LAT. Moreover, recently it was shown that the palmitoylation-deficient cysteine mutant of LAT is actually not effectively incorporated into plasma membrane (20). A simple interpretation was that membrane rafts may not be, after all, important in the signaling process.

In this study, we set out to clarify this issue. We hypothesized that the LAX–LAT construct (as well as LAX) may be actually present in a novel type of raft-like microdomains that upon detergent solubilization produce nonbuoyant DRMs but are able to support, at least partially, the TCR signaling. Our results indeed support such a hypothesis, and we describe such DRMs resembling in some respects those corresponding to "conventional rafts." Furthermore, we found that targeting of LAT intracellular domain into different membrane microenvironments (putative conventional rafts, novel raft-like microdomains producing heavy DRMs, and nonraft membrane) affects signaling performance of the molecule, especially at low levels of expression.

Materials and Methods

Plasmids, cells, and Abs

The CD25–LAT construct was created by fusion of sequences encoding the extracellular and transmembrane parts of human CD25 (aa 1–259), the submembrane peptide of human CD3ε (aa 153–160), and the intracellular part of human LAT (aa 35–233); this construct was cloned into the pcDNA3 vector. Constructs encoding fluorescently tagged proteins were made by in-frame fusion of sequences encoding LAT, LAX–LAT, and CD25–LAT with orange fluorescent protein (OFP) (21) using Myc tag as a spacer. All of these constructs were subsequently cloned into the pMXsIN retroviral vector (kindly provided by Dr. A. Cerwenka) (22), which expresses a truncated form of human nerve growth factor receptor (LNGFR) from an internal ribosomal entry site sequence.

Jurkat cells were from the American Type Culture Collection (Manassas, VA), LAT-negative J.CaM2 cells were kindly provided by Dr. A. Weiss, and J.CaM2 cells expressing the LAX–LAT fusion protein (19) were kindly provided by Dr. W. Zhang (LAX–LAT fusion protein comprises the extracellular and transmembrane domains of human LAX [aa 1–68] and the intracellular part of human LAT [aa 34–233]).

J.CaM2 cells expressing the CD25–LAT fusion protein were created by electroporation with plasmid DNA and subsequent selection in the presence of 1 mg/ml G418.

Rabbit antiserum to human LAT (8) was kindly provided by Dr. L. Samelson (National Institutes of Health, Bethesda, MD), rabbit antiserum to human Lck was kindly provided by Dr. A. Veillette (Clinical Research Institute of Montreal, Montreal, QC, Canada), mAb HC10 to HLA class I was kindly provided by Dr. H. Ploegh (Whitehead Institute for Biomedical Research, Cambridge, MA), mAb βF1 to TCRβ was kindly provided by Dr. M. Brenner (Harvard Medical School, Boston, MA), mAb C305 to Jurkat cell TCR was kindly provided by Dr. A. Weiss (University of California, San Francisco, CA), mAb to LAX was kindly provided by Dr. W. Zhang (Duke University Medical Center, Durham, NC), and mAb C33 to CD82 was kindly provided by Dr. O. Yoshie (Kinki University School of Medicine, Osaka, Japan).

Abs to the following molecules were obtained from the indicated commercial sources: LAT (Dy647-labeled; Exbio, Prague, Czech Republic), LNGFR (FITC-labeled; Miltenyi Biotec, Bergisch Gladbach, Germany), CD69 (FITC-labeled; BD Biosciences, San Jose, CA), Myc tag (Cell Signaling Technology, Danvers, MA), CD28 (eBioscience, San Diego, CA), CD71 (Zymed, San Francisco, CA), TCR-ζ (Santa Cruz Biotechnology, Santa Cruz, CA), CD3ε (Becton Dickinson), actin (Sigma-Aldrich, St. Louis, MO). mAbs to CD5 (MEM-32), CD45 (MEM-28), TRIM (TRIM-4), H-Ras (RAS-01), and SIT (SIT-02) are commercially available from Exbio.

Cell and membrane solubilization

A total of 5×10^7 Jurkat cells or various Jurkat transfectant cells, or corresponding amounts of membrane preparations, were lysed in 1 ml lysis buffer (20 mM Tris [pH 8.2], containing 100 mM NaCl, 5 mM iodoacetamide, Protease Inhibitor Cocktail III [Calbiochem/Merck, Darmstadt, Germany], 10 mM EDTA, 50 mM NaF, and 10 mM $Na_4P_2O_7$), containing as detergents either 1% Brij-98 (polyoxyethylene 20 oleyl ether; Sigma-Aldrich) or 1% laurylmaltoside (LM) (*n*-dodecyl-β-D-maltoside; Calbiochem), for 30 min on ice. This lysate was either used directly for density gradient ultracentrifugation (without removing insoluble components) or spun at $25,000 \times g$ for 3 min to remove nuclei and other insoluble materials. Where indicated, the cells were pretreated with 10 mM methyl-β-cyclodextrin (MbCD; 20 min, 37°C), washed with PBS, and solubilized as described above.

Plasma membrane preparation

Cells (2×10^8) were resuspended in 1 ml ice-cold hypotonic buffer (10 mM HEPES [pH 7.4], 42 mM KCl, 5 mM $MgCl_2$, and protease inhibitor mixture), incubated on ice for 15 min, and then passed 10× through the 30-gauge needle (23). The suspension was centrifuged 5 min at $580 \times g$ and 0°C to remove nuclei. The ice-cold postnuclear supernatant was centrifuged 10 min at $25,000 \times g$ and 2°C to pellet the membranes.

Density gradient ultracentrifugation

Cells (5×10^7) were solubilized in 0.5 ml lysis buffer containing 1% Brij-98 (30 min on ice), then 0.5 ml ice-cold 80% (w/v) sucrose in lysis buffer was added, and the mixture was placed at the bottom of a 5.2 ml polyallomer centrifuge tube (Beckman Coulter, Brea, CA), then carefully overlaid with 1.8 ml 30%, 0.8 ml 20%, 0.8 ml 10%, and 0.7 ml 5% sucrose in lysis buffer (with the detergent) and finally with 0.1 ml lysis buffer. Centrifugation was performed at 2°C in Beckman Optima MAX-E ultracentrifuge, using the MLS50 swing-out rotor (18 h, 50,000 rpm). Nine 0.57 ml fractions (and sediment as 10th fraction) were collected gradually from the top of the gradient; proteins were separated by SDS–PAGE and analyzed by immunoblotting.

In some experiments, selected fractions were diluted with six volumes of sucrose-free buffer (20 mM Tris [pH 8.2] and 100 mM NaCl) and centrifuged at 2°C and $50,000 \times g$ for 2 h to recover any large complexes. The sediment was analyzed by SDS–PAGE and immunoblotting.

Gel filtration

A total of 0.1 ml of the cell lysate (after nuclei removal by 3 min centrifugation at $25,000 \times g$) was applied at the top of a 1 ml Sepharose 4B column (in the lysis buffer with detergent) and washed with the lysis buffer; 0.1 ml fractions were collected (all performed at 4°C) and analyzed by SDS–PAGE and immunoblotting. In some experiments, the detergent lysates were supplemented with 0.6 M KI (24) before gel filtration. The void volume fractions of this highly porous gel (fraction 4) contain very large complexes or particles (approximately $>10^7$ Da); maxima of m.w. standards IgM and IgG elute in fractions 7 and 9, respectively (25).

Other biochemical methods (SDS–PAGE and Western blotting) were performed essentially as described previously (26).

Retroviral infection and MACS sorting

Retroviruses (RVs) were prepared by transfection of Phoenix-Ampho cells (Origene, Rockville, MD) with plasmid DNA using Lipofectamine (Invitrogen, Carlsbad, CA) in six-well plates. RV-containing supernatant was centrifuged to remove debris and then used to spin-infect ($1200 \times g$ for 90 min at room temperature) J.CaM2 cells in the presence of Polybrene (Sigma-Aldrich; 10 μg/ml). Cells were allowed to expand in culture for 3 d, and then the infected cells were enriched by magnetic bead sorting because all RVs express the surface marker LNGFR from an internal ribosomal entry site. Infected J.CaM2 cells were stained with FITC-labeled Ab to LNGFR on ice, cells were then washed and incubated with anti-FITC beads (Miltenyi Biotec), and labeled cells were then sorted on AutoMACS cell sorter (Miltenyi Biotec).

Microscopy

J.CaM2 cell transfectants were washed with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 for 5 min on ice. Cells were then incubated with anti-Myc Ab followed with Alexa Fluor 488-labeled goat anti-mouse IgG secondary Ab (Invitrogen). Nuclei were visualized with Hoechst 33258 dye. Images were acquired on Leica SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany).

Cell stimulation and cytofluorometry

To measure upregulation of CD69, J.CaM2 cells were stimulated overnight with C305 Ab (purified, 1 μg/ml) or left unstimulated, and cells were then washed with FACS buffer (PBS containing 2% FCS and 0.1% sodium azide) and stained on ice with FITC-labeled anti-CD69 Ab. The level of CD69 expression was analyzed by gating on Hoechst 33258-negative (live)

cells. To measure calcium response to anti-TCR activation, cells expressing LAT chimeras fused with OFP were first loaded with 5 μM Fura-Red (Invitrogen) in loading buffer (1× HBSS, 2% FCS, without Ca^{2+} or Mg^{2+}) for 30 min at 37°C. After being washed, cells were resuspended in loading buffer supplemented with Ca^{2+} and Mg^{2+} and kept on ice. Before the calcium response was imaged, cells were warmed for 10 min at 37°C and then stimulated with C305 mAb (50 μg/ml). For MbCD treatment, cells were incubated for 30 min at 37°C in loading buffer containing 5 μM Fura-Red with or without 1 mM MbCD. After being washed, cells were then resususpended in calcium-free loading buffer containing 1 mM EGTA, stimulated with C305 mAb (50 μg/ml), and analyzed for 2 min until the intracellular calcium stores were depleted. Then, calcium was added to a final concentration of 2 mM, and cells were again analyzed for 2 min to determine the magnitude of the extracellular calcium flux. Cytofluorometry was carried out on a LSRII instrument (Becton Dickinson) in a ratiometric setup using 405/480 nm excitation. Analysis of the data was performed using the FlowJo software (Tree Star, Ashland, OR). To determine the levels of expression of LAT by cytofluorometry, cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized with cold methanol on ice for 30 min, and nonspecific staining was blocked with 1% BSA in PBS containing 0.01% Triton X-100. Subsequently, cells were incubated with Dy647-labeled anti-LAT Ab, washed, and analyzed on a LSRII flow cytofluorometer.

Results

To explain the results of Zhu et al. (19), we first hypothesized that (a fraction of) the LAX–LAT construct might be actually raft-associated but more sensitive to detergent extraction. Thus, we tested a milder detergent Brij-98 instead of the standard Triton X-100. However, even in the presence of this milder detergent, a large majority of the LAX–LAT protein was absent from the buoyant fractions of the sucrose gradient corresponding to DRMs, whereas almost all LAT was present in the buoyant fractions under the same conditions (Fig. 1*A*).

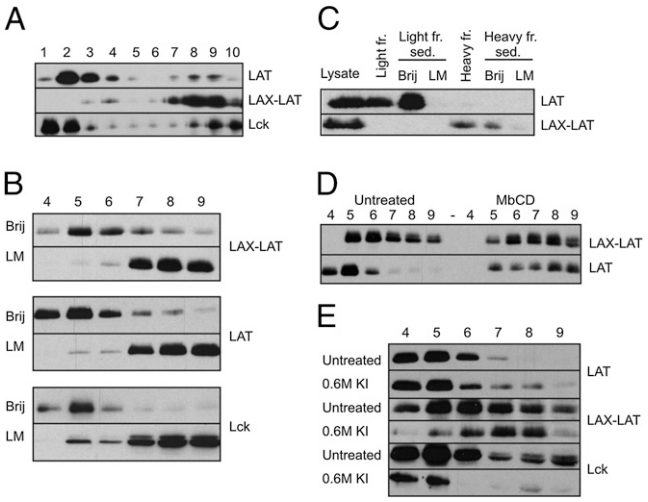

FIGURE 1. Properties of the detergent-resistant membrane complexes containing the LAX–LAT protein. *A*, Brij-98 lysate of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT, Lck) were subjected to density gradient ultracentrifugation, and the separated fractions were analyzed by SDS–PAGE and immunoblotting. Polyclonal Ab to LAT was used to detect both LAT and LAX–LAT. The fractions are numbered from the top. No. 10 represents the sediment. *B*, Brij-98 or LM lysates of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT, Lck) were subjected to gel filtration on Sepharose 4B, and the separated fractions were analyzed by SDS–PAGE and immunoblotting. *C*, Brij-98 or LM lysates of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT) were subjected to density gradient ultracentrifugation as in *A*, and combined fractions 2 and 3 (light) and 8 and 9 (heavy) were diluted and centrifuged to recover large complexes as described in *Materials and Methods*. Samples were analyzed by SDS–PAGE and immunoblotting. *D*, Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT) were pretreated with MbCD or left untreated and solubilized by Brij-98, and the lysates were subjected to gel filtration as in *B*. *E*, Brij-98 lysates of Jurkat (LAT) or LAX–LAT J.CaM2 transfectants (LAX–LAT, Lck) were treated with KI or left untreated and subjected to gel filtration as in *B*.

Next, we tested the hypothesis that the LAX–LAT chimera might be present in a sort of atypical heavy DRM originating from microdomains different from conventional rafts (i.e., large, nonbuoyant complexes) of a similar detergent sensitivity and resistance as the classical DRMs (resistant to Brij-98 but sensitive to LM).

Thus, we performed gel filtration of 1% Brij-98 or LM detergent lysates of the LAX–LAT transfectants on minicolumns of the highly porous gel Sepharose 4B. As shown in Fig. 1*B*, a substantial fraction of LAX–LAT was indeed found in large complexes in the presence of the raft-preserving detergent Brij-98 but not in the presence of the raft-breaking detergent LM. As expected, typical raft molecules LAT and Lck exhibited similar behaviors. Similar results were obtained using Brij-58. When using 1% Triton X-100, only ~30–50% of typical raft molecules (LAT, Lck, and CD55) floated to the low-density fractions of the gradient, and these, as well as LAX–LAT, were found not in the void volume fractions of the Sepharose 4B gel filtration columns but were spread in retarded fractions 6–8, corresponding to lower-m.w. complexes (data not shown).

Alternatively, we isolated the heavy DRMs also from the high-density fractions of the sucrose gradient by simple dilution followed by high-speed centrifugation when Brij-98 lysate (but not LM) was used (Fig. 1*C*).

Next, we compared the sensitivities of the heavy and classical (light) DRMs to cholesterol depletion by MbCD. As shown in Fig. 1*D*, both types of DRMs exhibited similar sensitivities to the pretreatment of the cells by MbCD before Brij-98 solubilization, because both LAT and LAX–LAT largely shifted to fractions corresponding to smaller complexes after the treatment.

Thus, we concluded that the majority of the LAX–LAT construct is apparently present in previously undetected membrane structures resembling in their detergent sensitivity and sensitivity to cholesterol extraction classical membrane rafts but differing from them in the higher buoyant density of the corresponding DRMs. The difference in the densities of the heavy and classical (light) DRMs could be obviously due to a higher protein-to-lipid ratio in the former, and therefore the integrity of the heavy DRMs could be more dependent on protein–protein interactions. Thus, we tested the resistance of both types of DRMs to the chaotropic agent 0.6 M KI (24), which is known to be a mild disruptor of protein–protein interactions (27). As shown in Fig. 1*E*, heavy but not light DRMs were disrupted under these conditions, indicating potentially a more important role for protein–protein interactions in the former.

This result might indicate potential involvement of F-actin in maintaining the heavy DRMs. However, distribution of actin in the gel filtration fractions of the Brij-98 cell lysate corresponded to fully solubilized molecules and was very different from the distribution of LAX–LAT (Fig. 2); the same result was obtained also when using Brij-98–solubilized membranes (data not shown).

Next, we tested which other molecules might be present in the membrane microdomains yielding heavy DRMs. Although several well-known classical raft resident molecules (LAT, Lck, Fyn, and CD55) almost quantitatively floated up in the sucrose gradients containing 1% Brij-98 (Fig. 1*A* and data not shown), a number of others (e.g., CD3ε, CD5, CD28, CD45, CD71(TfR), CD82, TRIM, SIT, LAX, H-Ras, HLA class I, ζ-chain, TCRβ-chain, PLCγ, ZAP70, SLP76, Csk, and tubulin) were found almost exclusively in the bottom fractions of the gradient (data not shown). Of these, under the conditions of gel filtration, LAX, CD5, CD45, H-Ras, and CD28 behaved similarly to LAX–LAT (i.e., were present mostly in large complexes resistant to Brij-98 and sensitive to LM). CD71 (transferrin receptor), TRIM, HLA class I, and TCRζ-chain were distributed almost homogeneously in all of the fractions obtained by gel filtration in the

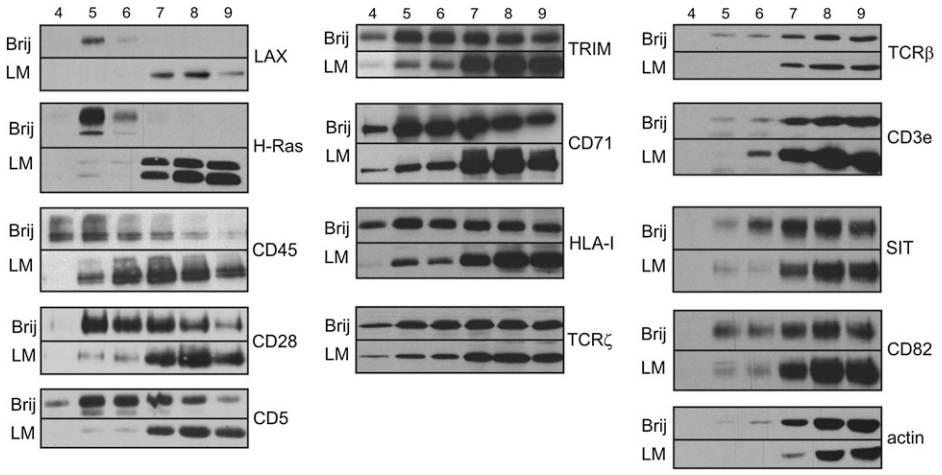

FIGURE 2. Gel filtration of specific membrane proteins solubilized in the presence of Brij-98 or LM. Brij-98 or LM lysates of LAX–LAT J.CaM2 transfectants were subjected to gel filtration on Sepharose 4B as in Fig. 1*B*, and the indicated proteins were visualized by immunoblotting.

presence of Brij-98 and largely shifted to fractions corresponding to small complexes or molecules in the presence of LM. However, TCR/CD3, CD82, SIT, actin, and cytoplasmic molecules, such as PLCγ, Csk, ZAP70, SLP76, and tubulin, were mostly or exclusively present in low-m.w. fractions in the presence of both Brij-98 and LM detergents (Fig. 2 and data not shown). As expected, the molecules known to be present in classical buoyant DRMs (e.g., LAT, Lck, Fyn, and CD55) were also detected almost exclusively in void volume fractions of the Sepharose 4B gel filtration columns in the presence of Brij-98 and shifted markedly to low-m.w. fractions in the presence of LM (data not shown).

To exclude the possibility that almost all of the membrane proteins are present in some sort of Brij-98–resistant complexes simply because the detergent poorly solubilizes membranes, detergent (Brij-98 or LM) lysates of partially purified plasma membranes were subjected to gel filtration on Sepharose 4B, and the protein profiles of the fractions were compared for both of these detergents. As shown in Fig. 3, a large majority of the plasma membrane proteins are present in low-m.w. fractions even after Brij-98 solubilization.

Next, we tried to prepare a LAT construct that would not be targeted to heavy DRMs to be able to compare signaling capacities of the LAT versions targeted to rafts, the newly described microdomains, and nonraft membrane. To this end, we prepared a construct containing the extracellular and transmembrane domains of CD25,

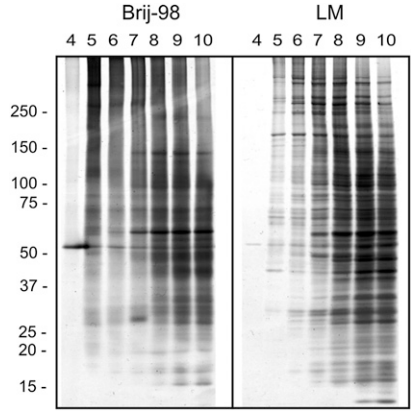

FIGURE 3. Distribution of detergent-solubilized total membrane proteins in gel filtration fractions. Brij-98 or LM lysates of LAX–LAT J.CaM2 transfectant plasma membranes were subjected to gel filtration on Sepharose 4B as in Fig. 1*B*, separated fractions were subjected to 5–15% gradient gel SDS–PAGE, and proteins were visualized by silver staining. Positions of m.w. standards are indicated.

a short submembrane segment coming from CD3ε, and the major cytoplasmic part of LAT (*Materials and Methods* and Fig. 4*A*). This construct was transfected into LAT-deficient J.CaM2 cells, and the stable transfectants were subjected to Brij-98 solubilization followed by gel filtration on Sepharose 4B. As shown in Fig. 4*B*, in contrast to LAT and LAX–LAT, the CD25–LAT chimeric protein was found in the fractions corresponding to solubilized molecules (or small complexes) and therefore was apparently targeted to nonraft membrane sensitive to Brij-98 solubilization.

Finally, we wished to compare LAT, LAX–LAT, and CD25–LAT chimeras as to their abilities to support TCR signaling when expressed in LAT-deficient J.CaM2 cells. The results could be obviously affected by the level of expression of these molecules, and therefore we prepared all of these constructs with C-terminal OFP tags to be able to compare signaling events in cell populations with comparable expression of these constructs. This approach was considered safer than selection of multiple clones with similar expression, because individual clones might exhibit various poorly defined idiosyncratic anomalies. Therefore, for measurement of proximal (calcium flux) and distal (CD69 expression) effects of TCR activation, we compared the cell populations of comparable levels of expression of the constructs, as determined by the OFP fluorescence.

As can be seen in Fig. 5*A*, all three constructs were properly expressed in the plasma membrane and supported TCR signaling equally well if the cells expressed high amounts of the constructs. However, at moderate levels of expression (corresponding to the expression level of endogenous LAT; Fig. 5*B*), LAT (present mostly in classical rafts) supported signaling better than LAX–LAT (present in the novel type of microdomains), whereas CD25–LAT was the least effective one (Fig. 5*C–E*). Therefore, the differences in signaling potencies of these LAT chimaeras become significant only when the amount of LAT available to initiate the TCR signaling cascade is limiting. The functional differences among the three constructs become even more pronounced when calcium release from intracellular stores and from extracellular medium is measured separately (Fig. 5*D*, −MbCD). These data also demonstrate that the major functional difference between LAT and LAX–LAT constructs is in how they support the extracellular calcium influx rather than calcium release from intracellular stores.

Signaling potency of LAT (present in conventional rafts) is obviously more sensitive to partial cholesterol depletion by MbCD as compared with that of LAX–LAT (Fig. 5*D*). This is in agreement with the observed higher resistance of the LAX–LAT-containing microdomains to cholesterol depletion (Fig. 1*D*).

We also tried to compare the LAT constructs as to their redistribution to the immunological synapse formed between Jurkat

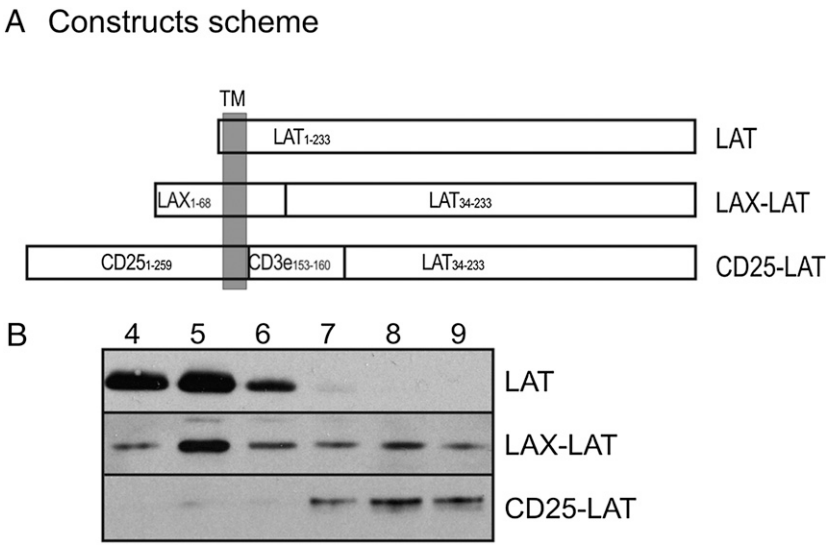

FIGURE 4. Sensitivity of CD25–LAT chimeric protein to Brij-98 solubilization. *A*, Scheme of the LAT constructs used. *B*, Brij-98 lysates of Jurkat (LAT), LAX–LAT J.CaM2, or CD25–LAT transfectants were subjected to gel filtration on Sepharose 4B as in Fig. 1*B*.

cell transfectants and Raji B cells treated with staphylococcal enterotoxin E superantigen. However, even the control wild-type LAT was not significantly redistributed (data not shown).

Discussion

The finding that an apparently nonraft form of LAT (the LAX–LAT chimera) was able to support TCR signaling both ex vivo and in vivo (19) was a serious blow to the widely accepted hypothesis on the importance of membrane rafts in immunoreceptor signaling. In the present paper, we demonstrate that this result can be plausibly explained by the existence of a so far rather overlooked type of membrane microdomain. These appear to be similar to the classical rafts in yielding DRMs resistant to solubilization by polyoxyethylene type detergents, such as Brij-98, but sensitive to LM and to cholesterol extraction (Fig. 1).

These heavy DRMs (and also the classical, buoyant DRMs) can be conveniently separated from fully soluble proteins by gel chromatography on highly porous gel Sepharose 4B. However, this method is essentially not able to distinguish between classical and heavy DRMs, because both these types of large (and probably heterogeneous) complexes are eluted in or near the void volume fractions of the Sepharose 4B column. Nevertheless, it is possible to separate them conveniently by ultracentrifugation in a sucrose density gradient followed by simple dilution of the respective fractions (to decrease density) and sedimentation of the large complexes by ultracentrifugation (Fig. 1*C*).

The heavy DRMs are apparently more dependent on protein–protein interactions because they are, in contrast to the light DRMs, sensitive to treatment with the chaotropic agent 0.6 M KI (Fig. 1*E*). This is in agreement with their higher density, probably due to a higher protein-to-lipid ratio. Interestingly, these structures are apparently not dependent on F-actin, because the distribution of actin in gel filtration corresponds to small molecules (Fig. 2). We have not yet examined the lipid composition of these assemblies to compare it with that of the classical, light DRMs. Nevertheless, it seems likely that lipid-based interactions (in addition to protein–protein interactions) also play an important role in maintaining the integrity of the heavy DRMs, because the detergent LM (which is known to generally preserve protein–protein interactions) as well as cholesterol extraction effectively disrupted them (Fig. 1*C*, 1*D*). The heavy DRMs were less sensitive to cholesterol depletion by MbCD treatment (Fig. 1*D*), and also the signaling functionality of the corresponding microdomains was less sensitive to cholesterol depletion (Fig. 5*D*), whereas signaling capacity of nonraft CD25–LAT was essentially unchanged. The heavy DRMs obviously did not contain a characteristic marker of classical DRMs, GM1, because this glycolipid was detected in many published studies exclusively in the buoyant fractions of the density gradient.

It should be noted that under the conditions used there is not a perfect separation of the components of the heavy and light DRMs (e.g., minor amounts of LAX–LAT can be detected in the floating fractions and minor amounts of LAT or Lck can be detected in the bottom fractions of the gradient [Fig. 1*A*]). It is difficult to determine to what extent it reflects a real situation in the corresponding membrane microdomains before the detergent solubilization. However, the presence of small amounts of LAX–LAT in the classical rafts could obviously contribute to the functional effects of this construct, especially at high levels of expression (see also further discussion).

As stated above, instead of the more commonly used Triton X-100 detergent, we used Brij-98 because the results were more clear-cut and more reproducible; when using Triton X-100, large fractions of typical raft molecules (Lck, LAT, and GPI-anchored proteins) were fully solubilized and found outside the large buoyant detergent-resistant complexes. In our hands, Brij-98 appears to be an optimal detergent for reproducible preparation of DRMs. We are of course aware that the use of any detergent is connected with a certain degree of artefactuality; however, it seems reasonable to assume that the milder detergents, such as Brij-98, preserve the structures corresponding to native membrane rafts better than more stringent ones (e.g., Triton X-100).

We demonstrated that the newly observed type of DRMs (and presumably the membrane microdomains from which they originate) contains not only the artificial construct LAX–LAT but also (as could be expected) endogenous LAX and a number of other membrane proteins (Fig. 2). These membrane proteins include a number of functionally important ones, and therefore this new type of membrane microdomains may be of special functional importance. At the moment, it is not clear how heterogeneous these DRMs (and the presumed corresponding native microdomains) are (i.e., whether one type is rich in LAX only, another in CD28, etc. or whether various mixed DRMs [and microdomains] exist, containing various combinations of these molecules).

It is also unclear what structural motifs are responsible for targeting membrane proteins into this type of membrane environment. Our preliminary unpublished results indicate that the submembrane basic motif present in LAX is of importance, but this question has yet to be studied in more detail. It should be noted that even in the case of

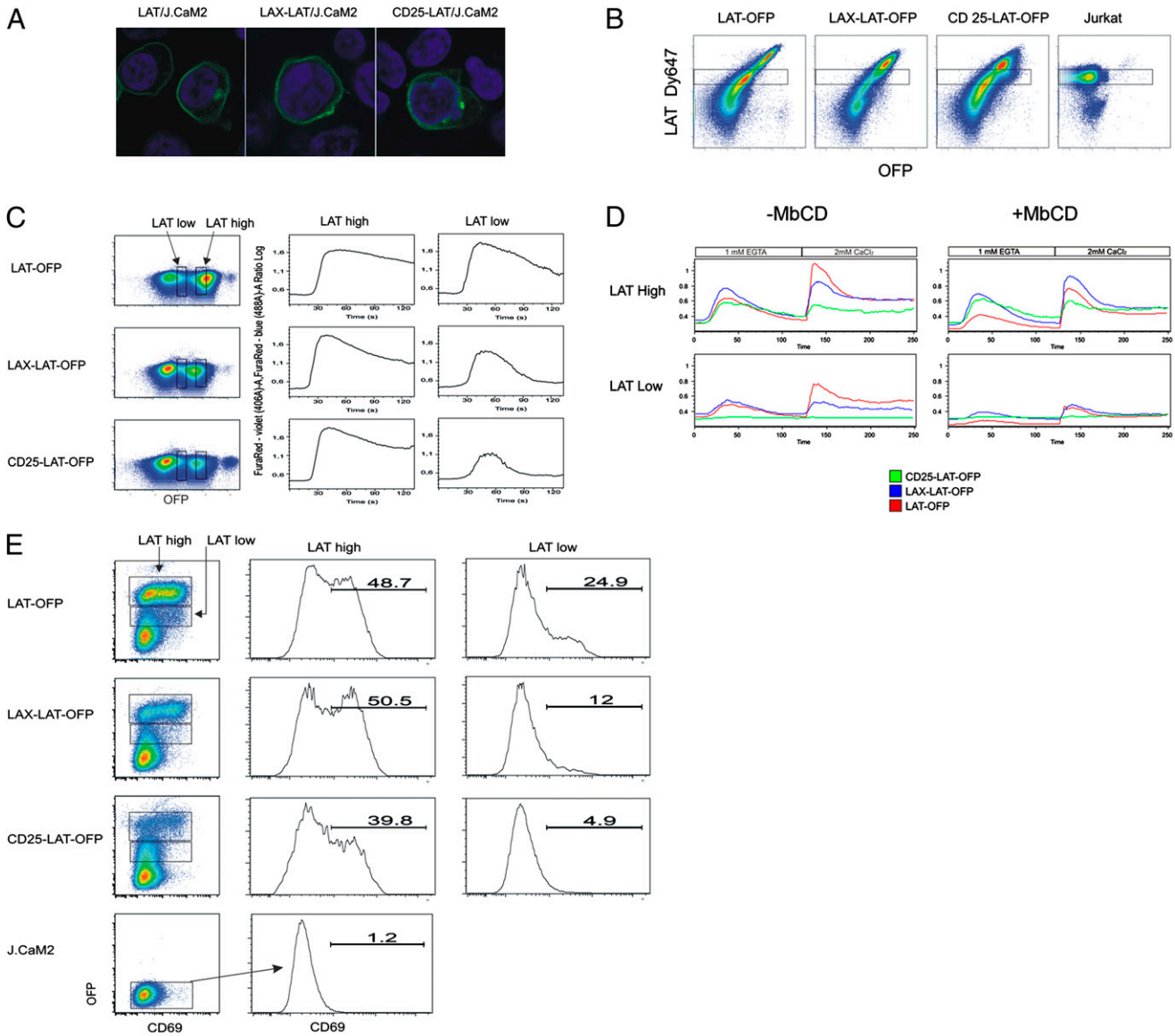

FIGURE 5. Activation responses of J.CaM2 transfectants expressing various LAT constructs containing Myc and OFP tags. *A*, The indicated fixed and permeabilized J.CaM2 cell transfectants were stained with anti-Myc Ab followed by Alexa Fluor 488-labeled goat anti-mouse IgG secondary Ab, nuclei were stained with Hoechst 33258 dye, and images were acquired on a Leica SP5 confocal microscope (original magnification ×800). *B*, The indicated fixed and methanol-permeabilized cells were stained with Dy647-labeled anti-LAT Ab and analyzed by flow cytometry. *C*, The indicated transfectants of J.CaM2 were stimulated with C305 Ab, and dynamics of their cytoplasmic calcium response were measured in populations gated for high or low LAT construct expression, respectively, as described in *Materials and Methods*. *D*, To determine the effect of MbCD (1 mM) treatment on TCR signaling, cells were treated with MbCD as described in *Materials and Methods*, and the magnitude of intracellular and extracellular calcium response was determined following stimulation with C305 Ab. *E*, The indicated transfectants of J.CaM2 were stimulated overnight with C305 Ab, and surface expression of the activation marker CD69 was measured by cytofluorometry in populations gated for high or low LAT construct expression, respectively.

conventional rafts (or classical DRMs) the motifs targeting proteins into them are not entirely clear; palmitoylation appears to be important, but there are cases of raft-associated nonpalmitoylated proteins as well as palmitoylated nonraft molecules (28).

It is also obvious that some molecules are present in these complexes almost completely (e.g., LAX, TRIM, CD5, CD45, H-Ras, and CD28), whereas others (CD71, HLA class I, and TCR) only partially (Fig. 2). Importantly, most of the membrane proteins are fully solubilized even by Brij-98, thus excluding the possible objection that the mild detergent used simply did not solubilize the membrane (Fig. 3).

It should be noted that similar DRMs corresponding to some sort of raft-like membrane microdomains (called heavy rafts by the authors) were already described previously in other cell types (29–33). These heavy DRMs did, somewhat in contrast to those described in our present study, clearly float in the density gradient but less than the conventional ones (i.e., into intermediate density fractions).

We are of course aware of the fact that the biochemical results based on detergent solubilization must be judged with caution because of possible artifacts caused by detergents (selective extraction of some components from the microdomains originally present in the native membrane, possible detergent-induced fusion of native microdomains, etc.).

Therefore, probably the most significant finding of this study is that at a moderate level of expression, similar to the physiological level of the endogenous protein, LAT (present mostly in conventional rafts) supported signaling better than LAX–LAT (present in the newly described raft-like microdomains), whereas CD25–LAT was the least effective one (Fig. 5); it should be noted that the lack of functionality of the nonpalmitoylated cysteine mutant of LAT as described in previous

papers (10, 34) is actually mostly due to its defective plasma membrane targeting (20). In contrast, all types of LAT constructs, targeted either to the conventional rafts (wild-type LAT), novel raft-like microdomains (LAX–LAT), or nonraft membrane (CD25–LAT) supported the TCR signaling almost equally at high levels of expression (Fig. 5). Interestingly, targeting of LAT outside conventional lipid rafts most profoundly impaired calcium influx from extracellular medium. In B cells, it has been shown that functionality of CRAC channels is dependent on the presence of Src and Syk family kinases (35). Because LAT is a substrate of Syk family kinases, it may be somehow involved in the regulation of CRAC current; however, the detailed mechanism still remains to be elucidated.

Our results indicate that different microdomains do exist in the native membrane and that the environment corresponding to conventional rafts may be under certain conditions (moderate level of expression) optimal for signaling functions of LAT (and perhaps also some other molecules). We therefore propose that conventional rafts (producing upon detergent solubilization buoyant DRMs) might provide an optimal environment for sensitive signaling, which may be important (e.g., in the case of weak stimuli). Nevertheless, the putative novel type of raft-like microdomains (detected here as heavy DRMs) are apparently also able to communicate with TCR complex and participate in the signaling processes.

In conclusion, we believe that our results plausibly explain the startling results by Zhang and colleagues (19). We suggest that their failure to detect any differences in TCR signaling outcome between LAT and LAX–LAT transfectants of LAT-negative T cell lines was due to a relatively high level of expression in these transient transfectants. Their in vivo results demonstrating rescue of LAT-negative thymocyte development by stable transfection with the LAX–LAT construct may simply indicate that even a suboptimal form of LAT (the LAX–LAT construct) can be sufficient [especially if its level of expression may be higher than that in the WT cells, as seems to be the case in Fig. 2*B* of their paper (19)]. It may be speculated that the use of a suboptimal LAX–LAT (and even more CD25–LAT) construct instead of the optimal LAT could lead to (subtle) changes in TCR repertoire (selection for T cells expressing higher-affinity TCRs during development in thymus).

A recent study (36) addressed also a similar problem (i.e., whether a nonraft mutant of LAT is able to support TCR signaling). The authors used another chimeric, nonpalmitoylated LAT construct consisting of the plasma membrane-targeting N-terminal sequence of Src kinase and the LAT cytoplasmic domain (Src–LAT) localized as a peripheral membrane protein in the plasma membrane but outside lipid rafts. This Src–LAT construct restored T cell development and activation, just as the LAX–LAT construct in the earlier study (19). Using the Src–LAT construct kindly provided by M. Hundt and A. Altman, we confirmed its non-DRM localization (similar to our CD25–LAT) even under our mild detergent conditions (1% Brij-98) (data not shown). We suggest that even the suboptimal signaling capacity of the Src–LAT construct, if expressed at its proper level, is sufficient to support TCR signaling and T cell development, similarly to the previously described LAX–LAT construct. Nevertheless, our data suggest that such constructs are less effective than wild-type LAT; the optimal efficiency of wild-type LAT can be best observed at low levels of expression. In fact, some of the data presented in the study by Hundt at al. (36) also suggested that compared with wild-type LAT higher expression levels of Src–LAT were required to complete thymic development, which is in good agreement with our conclusions.

Our findings not only explain the results of Zhu et al. (19) but also point to yet another level of plasma membrane heterogeneity. It will be important to determine the mechanisms responsible for targeting various proteins into the different types of membrane microdomains and clarify basic properties and biological roles of the putative raft-like microdomains described in this study.

Acknowledgments

We thank all colleagues who provided us with the cells, vectors, and Abs, as indicated in *Materials and Methods*.

Disclosures

The authors have no financial conflicts of interest.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 22, pp. 19617–19629, June 3, 2011

PRR7 Is a Transmembrane Adaptor Protein Expressed in Activated T Cells Involved in Regulation of T Cell Receptor Signaling and Apoptosis*

Received for publication, August 13, 2010, and in revised form, March 20, 2011 Published, JBC Papers in Press, April 1, 2011, DOI 10.1074/jbc.M110.175117

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Transmembrane adaptor proteins (TRAPs) are important organizers and regulators of immunoreceptor-mediated signaling. A bioinformatic search revealed several potential novel TRAPs, including a highly conserved protein, proline rich 7 (PRR7), previously described as a component of the PSD-95/*N*-methyl-D-aspartate receptor protein complex in postsynaptic densities (PSD) of rat neurons. Our data demonstrate that PRR7 is weakly expressed in other tissues but is readily up-regulated in activated human peripheral blood lymphocytes. Transient overexpression of PRR7 in Jurkat T cell line led to gradual apoptotic death dependent on the WW domain binding motif surrounding Tyr-166 in the intracellular part of PRR7. To circumvent the pro-apoptotic effect of PRR7, we generated Jurkat clones with inducible expression of PRR7 (J-iPRR7). In these cells acute induction of PRR7 expression had a dual effect. It resulted in up-regulation of the transcription factor c-Jun and the activation marker CD69 as well as enhanced production of IL-2 after phorbol 12-myristate 13-acetate (PMA) and ionomycin treatment. On the other hand, expression of PRR7 inhibited general tyrosine phosphorylation and calcium influx after T cell receptor cross-linking by antibodies. Moreover, we found PRR7 constitutively tyrosine-phosphorylated and associated with Src. Collectively, these data indicate that PRR7 is a potential regulator of signaling and apoptosis in activated T cells.

Activation of leukocytes through multichain immunoreceptors (TCR,[3] BCR, FcRs) involves a complex array of membrane-linked and cytoplasmic proteins, including transmembrane and cytoplasmic adaptor proteins (1). Several of the transmembrane adaptors (TRAPs) are associated with lipid rafts, namely LAT (2, 3), PAG (4, 5), NTAL (6, 7), and LIME (8, 9), whereas others (LAX, SIT, TRIM, GAPT) are present in non-raft membrane (10–13). All of these proteins are composed of a short N-terminal extracellular peptide, a single transmembrane segment, and a cytoplasmic part containing multiple tyrosine and other protein interaction motifs. In the case of the raft-associated proteins, a palmitoylation motif (*CXXC* or *CXC*) lies between the transmembrane segment and the intracellular part (14). TRAPs play positive and negative regulatory roles in immunoreceptor signaling. The most prominent role is played by LAT, which is essential for several aspects of TCR signaling and plays an important role in the immunoreceptor signaling in other leukocyte subsets (15). In addition, experiments on genetic models have assigned important regulatory functions to other members of TRAP family, including NTAL, LAX, TRIM, and SIT (16, 17).

In an effort to discover additional TRAPs of functional importance, we performed an *in silico* search for proteins possessing the features characteristic of known TRAPs. Among 149 candidate genes, one of the best hits was PRR7. Previously, PRR7 was identified in the PSD fraction of rat forebrain tissue by a proteomic approach (18). It was shown to associate with PSD-95 (via a PDZ domain binding motif) and was found in a protein complex containing the *N*-methyl-D-aspartate receptor subunits NR1 and NR2B. These biochemical data suggested that PRR7 could be potentially involved in modulation of neural activities.

In the present study we found that PRR7 is weakly expressed in many other tissues, including immune cells, and is up-regulated during T cell activation. Moreover, we show that PRR7 overexpression in Jurkat cells substantially affected TCR signaling and cell survival.

EXPERIMENTAL PROCEDURES

Bioinformatic Search—The *in silico* search in human genome (USCS build hg16) for proteins possessing the features characteristic of known TRAPs was done with following parameters: a short extracellular N-terminal sequence, a single hydrophobic sequence starting at amino acids (aa) 5–50 (prediction using TMHMM Version 2.0) (19), a palmitoylation motif at aa 20–60 (*CXC* or *CXXC*), tyrosine-based phosphorylation motifs (*YXX*(I/L/V/A)) and/or C-terminal Group I PDZ binding motifs ((S/T)*X*(L/V)).

* This work was supported in part by Academy of Sciences of the Czech Republic Project AV0Z50520514, GACR (Project MEM/09/E011), and by the Center of Molecular and Cellular Immunology (Project 1M0506, Ministry of Education, Youth, and Sports of the Czech Republic).

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[3] The abbreviations used are: TCR, T cell receptor; aa, amino acid; Brij-98, polyoxyethylene 20 oleyl ether; J-iPRR7, Jurkat clones with PRR7-inducible expression; LM, lauryl maltoside (*n*-dodecyl-β-D-maltoside); PBL, peripheral blood lymphocytes; PSD, postsynaptic density; RSL1, RheoSwitch Ligand 1, diacylhydrazine [(*N*-(2-ethyl-3-methoxybenzoyl)-*N*′-(3,5-dimethylbenzoyl)-*N*′-*tert*-butylhydrazine]; SFK, Src family kinase; TRAP, transmembrane adaptor protein; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin; qPCR, quantitative PCR; Z-VAD-FMK, carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone; PLC, phospholipase C.

RNA and RT-qPCR—RNA was isolated using a Mini RNA purification kit (Zymo Research, Orange, CA) or TRI Reagent RT (MRC, Cincinnati, OH). Contaminating DNA was removed using a DNAfree kit (Ambion, Austin, TX). A human normal tissue FirstChoice RNA Survey Panel and a lymph node FirstChoice Total RNA were purchased from Ambion. Human hippocampal RNA was purchased from BioChain Institute (Hayward, CA). Total RNA (1 μg) was transcribed using SuperScript III RT (Invitrogen) with a combination of random pentadecamer and anchored oligo$(dT)_{20}$ primers. RT-qPCR was performed using a LightCycler 480 SYBR Green I Master chemistry (Roche Applied Science) and an amount of cDNA equivalent to 20 ng of total RNA. Primers specific for human PRR7 were 5′-tgtccagtgaagcgtctgag-3′ (forward) and 5′-aagcacgtgaggaacgtgta-3′ (reverse). Raw RT-qPCR data (Ct values) were preprocessed (normalization to optimal reference genes, GAPDH or β-microglobulin, relative expression calculation) and analyzed using GenEx Software (MultiD, Göteborg, Sweden).

Northern Blotting—Total RNA was isolated from 1×10^7 cells using TRI Reagent RT. RNA was resolved (15 μg/sample) by formaldehyde-agarose gel electrophoresis, transferred to positively charged nylon membrane, and hybridized to radioactively ([α-^{32}P]dCTP)-labeled probe in Super Hyb solution (MRC). The probe was prepared from cloned PRR7 cDNA using DecaLabel DNA labeling kit (Fermentas, Ontario, Canada).

Cells and Media—Cell line MEG-01 was provided by S. Watson (University of Birmingham, Birmingham, UK), MOLT-4 and HPB-ALL were from the IMG cell line collection (Institute of Molecular Genetics, Prague, Czech Republic), and Caco-2, U937, and THP-1 were from DSMZ (Braunschweig, Germany). Jurkat, Ramos, and COS-7 cell lines were from ATCC (Manassas, VA), and HEK293FT cells were obtained from Invitrogen. Caco-2, HEK293FT, and COS-7 cells were cultured in DMEM supplemented with 10% FCS (Biochrom AG, Berlin, Germany) and antibiotics at 37 °C in 5% CO_2. All other cells were cultured in RPMI 1640 supplemented with 10% FCS and antibiotics at 37 °C in 5% CO_2. Human peripheral blood lymphocytes (PBL) were isolated from whole blood of healthy donors by Ficoll-Paque Plus (GE Healthcare) gradient centrifugation. For depletion of adherent cells, PBL were subjected to plastic adherence for 2 h at 37 °C. PBL were cultured as stated above for Jurkat cells.

DNA Constructs—The coding region of human PRR7 was amplified from human T cell line MOLT-4 cDNA and inserted into mammalian expression plasmid pEFIRES-P, provided by Dr. S. Hobbs (Institute of Cancer Research, London, UK) (20). For inducible expression, PRR7 was subcloned into the pZX-LR vector in-frame with the hemagglutinin (HA) tag. The pZX-LR vector was a kind gift of Dr. Claude Labrie (CHUL Research Center, Quebec, Canada) (21). Deletion mutants of PRR7 (Δ2–39, Δ44–274, Δ114–274, Δ151–274, Δ159–274, Δ171–274, Δ207–274, ΔTAV) were generated by PCR and fused to enhanced green fluorescent protein (EGFP) in the pEGFP vector (Clontech, Palo Alto, CA). A tyrosine mutant of PRR7 (all Tyr residues mutated to Phe) was generated by sequential PCR oligonucleotide-directed site-specific mutagenesis and subcloned into the pEGFP vector (Clontech). For co-transfection experiments in COS-7 cells, the following cDNA constructs were used: Src in the pSM vector provided by Dr. A. Weiss (University of California, San Francisco, CA), FLAG-tagged Lck inserted into the pcDNA3 vector provided by Dr. R. Abraham (Mayo Clinic, Rochester, MN), FynT and FynF (constitutively active Fyn) in the pEF-BOS vector provided by Dr. B. Schraven (Otto-von-Guericke-University, Magdeburg, Germany), Yes in the pSG5 vector provided by Dr. C. Benistant (CRBM, CNRS, Universities of Montpellier I and II, France), Myc-tagged Lyn in the pcDNA3.1 vector provided by Dr. S. Watson (University of Birmingham, Birmingham, UK), FLAG-tagged Hck in the pcDNA1 vector provided by Dr. G. Langsley (Institut Pasteur, Paris, France).

Antibodies—Hybridomas producing monoclonal antibodies (mAbs, TRAP-3/01–13) to human PRR7 were prepared by standard hybridoma techniques using mice immunized with the recombinant protein corresponding to aa 147–274. Using the deletion mutants, the epitope recognized by the mAbs was mapped to the stretch of aa 147–170. The mAbs were cross-reactive with murine and rat PRR7 in immunoblots (not shown). The phosphotyrosine antibody (P-TYR-02) used for detection of phospho-LAT in Jurkat whole cell lysates was developed in our laboratory as well. For cell stimulation, the following non-commercial antibodies were used: C305 IgM mAb to Jurkat cell TCR provided by Dr. A. Weiss and 248.23.2 IgM mAb to CD28 (22). Antibodies to the following antigens were obtained from the indicated commercial sources: LAT (LAT-01), PAG (MEM-255), CD3 (MEM-57), CD5 (MEM-32), Lck (LCK-01), ZAP-70 (ZAP-03), CD69 (FN50, Alexa Fluor 647), tubulin (TU-01), and LAMP-1 (CD107a, B-T47) from Exbio (Vestec, Czech Republic); GAPDH from Sigma; Src (N-16), c-Jun (N), ERK2 (C-14), TCRζ (6B10.2) from Santa Cruz Biotechnology (Santa Cruz, CA); IL-2 (MQ1–17H12, APC) from eBioscience (San Diego, CA); phosphotyrosine (4G10) from Upstate Biotechnology (Lake Placid, NY); HA tag (6E2), pZAP-70 Tyr-319, pPLCγ1 Tyr-783, PLCγ1, pp42/44 ERK1/2 (Thr-202/Tyr-204), pp38 MAPK (T180/Y182), p38 MAPK, pJNK (T183/Y185), JNK, pc-Jun (Ser-63/Ser-73), pSFK (Y416), and pLck (Y505) from Cell Signaling Technology (Beverly, MA).

Inhibitors and Inhibitor Assay—The following inhibitors were used: the inhibitor of the Src family kinases, PP2 (Calbiochem), final concentration 10 μM and the pan-caspase inhibitor, Z-VAD-FMK (Alexis Biochemicals), final concentration 10 μM. Z-VAD-FMK inhibitor assay was performed using 2.5×10^5 cells in 96-well tissue culture plates in triplicates. Viability was assessed by flow cytometry (propidium iodide exclusion and side plus forward scatter gating).

Transfections and Inducible Expression—Jurkat cells (10^7) were transfected with 15 μg of the corresponding plasmid DNA in 300 μl of RPMI medium supplemented with 10% FCS by electroporation (250 V, 950 microfarads) using a GenePulser electroporator (Bio-Rad). For the pZRD-inducible system (21), based on the RheoSwitch Mammalian Inducible Expression System (New England Biolabs, Ipswich, MA), transfectants were selected in the culture medium containing Zeocin (200 μg/ml, Invitrogen). Stably transfected GFP-positive cells were

sorted and further subcloned by limiting dilution. The clones (denoted J-iPRR7) that expressed PRR7 only after induction with RSL1 (500 nM for maximal induction, RheoSwitch Ligand 1, New England Biolabs) were used in further experiments. COS-7 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Cell Stimulation—To analyze PRR7 up-regulation, freshly isolated PBL were stimulated using 1) anti-CD3 IgG (MEM-57) immobilized on tissue culture plastic (10 μg/ml) and soluble anti-CD28 IgM (100× diluted hybridoma supernatant), 2) PHA-L (2.5 μg/ml, Sigma), and 3) a combination of PMA (10 ng/ml, Sigma) and ionomycin (500 ng/ml, Sigma).

For short term activation (Ca^{2+} flux, phospho-Tyr blots), Jurkat cells were stimulated with anti-TCR IgM (C305) (23) (10 μg/ml) at 37 °C for 2 min. For activation of Jurkat cells in an IL-2 production assay, the combination of PMA and ionomycin was used as described above.

Preparation of Cell Lysates, Immunoprecipitations, Biochemical Methods—Generally, 5×10^7 cells were lysed in 1 ml of lysis buffer (20 mM Tris pH 7.5, 100 mM NaCl, 5 mM iodoacetamide, 10 mM EDTA, 50 mM NaF, 10 mM $Na_4P_2O_7$, 10% v/v glycerol, and Protease Inhibitor Mixture III, Calbiochem) containing 1% w/v detergent LM (Calbiochem) or Brij-98 (Sigma) for 30 min on ice. To remove nuclei and other insoluble materials, the lysate was spun down at 16,000 × *g* for 10 min at 4 °C. Density gradient ultracentrifugation and gel filtration on Sepharose 4B were performed as previously described (24). For immunoprecipitation experiments, we coupled the anti-PRR7 IgG (TRAP3-03) to CNBr-Sepharose 4B (Amersham Biosciences) or used soluble antibodies and Protein A/G PLUS-agarose IP Reagent (Santa Cruz). Palmitoylation of PRR7 was examined using the acyl-biotinyl exchange chemistry-based method (25). Briefly, plasma membranes from 5×10^7 cells were isolated, and palmitate protein modifications were removed by hydroxylamine and replaced with biotin. Biotinylated proteins were then immunoprecipitated on streptavidin-agarose beads. Other biochemical methods (SDS-PAGE, immunoblotting) were performed essentially as described before (4).

Microscopy—Jurkat cells were allowed to adhere to polylysine-coated coverslips for 30 min, then fixed with 4% w/v formaldehyde for 15 min at room temperature and permeabilized with 0.1% Triton X-100 (Sigma) for 5 min. Blocking was performed in 2.5% BSA and 10% goat serum (Sigma) in PBS for 30 min. Cells were then incubated with 100×-diluted LAMP-1 antibody followed by 750×-diluted Alexa 647-labeled goat anti-mouse IgG secondary antibody (Molecular Probes, Invitrogen). The DNA dye, Hoechst 33258 (1 μg/ml, Invitrogen), was used to visualize nuclei. Images were captured with a Leica SP5 confocal microscope and a 63× objective lens (Leica Microsystems, Mannheim, Germany).

Flow Cytometry—CD69 surface staining and intracellular IL-2 staining were done according to the standard protocols. To measure apoptosis in cells expressing PRR7, we performed annexin V/Dy-647 (Apronex Biotechnologies, Vestec, Czech Republic) staining in combination with the DNA dye, Hoechst 33258, according to the manufacturer's instructions. To measure calcium response to anti-TCR activation, cells were first loaded with 5 μM Fura Red (Molecular Probes, Invitrogen) in loading buffer (1 × Hanks' balanced salt solution, 2% FCS, without Ca^{2+} or Mg^{2+}) for 30 min at 37 °C. After washing, cells were resuspended in loading buffer supplemented with Ca^{2+} and Mg^{2+} and kept on ice. Cells were warmed up for 10 min at 37 °C and then stimulated with an irrelevant control antibody or the anti-TCR C305 mAb (10 μg/ml). Calcium flux was monitored for 240 s. Flow cytometry was carried out on an LSRII instrument (BD Biosciences), and cells were sorted on FACSVantage (BD Biosciences). Analysis of the data was performed using FlowJo software (Tree Star, Ashland, OR) as specified in the figure legends (Figs. 6 and 8).

RESULTS

PRR7 Sequence Analysis—Human PRR7 (18) is a 274-aa protein with a sequence typical for TRAPs. It has a short N-terminal extracellular peptide, a single transmembrane segment, and a cytoplasmic part containing several conserved binding motifs (Fig. 1*A*). These motifs include multiple SH2 domain binding and/or endocytic tyrosine-based motifs (Y*XX*(I/L/V/A)), multiple proline-rich SH3 binding motifs (P*XX*P), group I WW domain binding motifs (PP*X*Y), and a C-terminal class I PDZ domain binding motif (TTAV). In addition, PRR7 contains a potential submembrane palmitoylation motif (CC*X*C, Fig. 1*A*).

PRR7 is a highly conserved protein. The percentage of aa identity is more than 94% among available sequences from placental mammals, and a substantial level of aa identity is observed even if lower vertebrate species (amphibian, fish) are included (Table 1). The highest degree of conservation of PRR7 protein sequence was observed in the extracellular, transmembrane, and submembrane parts, including the palmitoylation motif. In addition, the region containing three tyrosine residues, Tyr-153, Tyr-166, and Tyr-177, and the C terminus with the PDZ domain binding motif also displayed a very high degree of homology (Fig. 1*B*). Interestingly, N-terminal part of PRR7 as well as sequences surrounding tyrosines 153 and 166 form a putative domain identified in Pfam data base (26) as the WBP-1 domain (PF11669). Among other proteins, this domain is present in WW domain-binding protein 1 (WBP-1) where it mediates the interaction with WW domains of Yes-associated protein (YAP) via tandem PP*X*Y motifs in its C terminus (27). Similar motifs encompass tyrosines 153 and 166 in PRR7, suggesting possible involvement of this region in an interaction with a WW domain containing protein (Fig. 1*C*).

PRR7 Expression Profile—To determine the PRR7 expression pattern in human tissues, we performed RT-qPCR on a panel of multiple tissue RNAs. As shown in Fig. 2*A*, PRR7 mRNA is expressed most strongly in brain tissue and moderately in several other tissues: esophagus, trachea, lung, ovary, cervix, prostate, testes, thyroid, including the immune organs thymus and lymph nodes. We also found low levels of PRR7 mRNA in a number of human cell lines (Jurkat, U937, THP-1, MEG-01, CaCo2, HEK293FT); the only cell line exhibiting relatively high expression was T cell line MOLT-4 (Fig. 2*B*). We next analyzed whether the expression level of PRR7 mRNA in PBL changes after activation. Interestingly, we observed rapid up-regulation of PRR7 mRNA in PHA-stimulated PBL. It was detectable at 1 h and peaked at 8 h after stimulation (Fig. 2*C*).

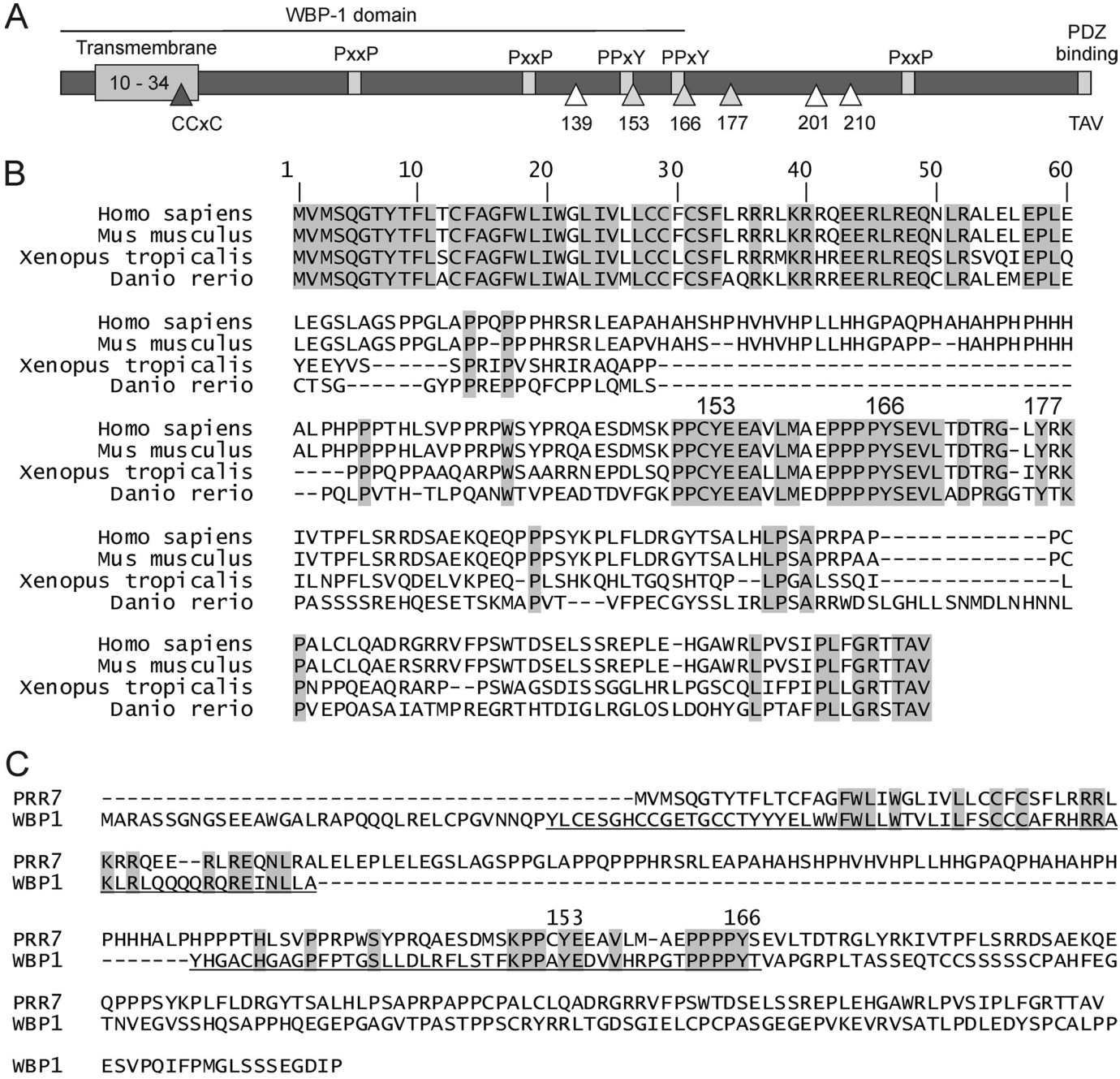

FIGURE 1. **Human PRR7 protein scheme and conservation.** *A*, PRR7 features a short extracellular part (predicted aa 1–9) followed by a single transmembrane segment (predicted aa 10–34), a submembrane palmitoylation motif CC*X*C (aa 28–31), six intracellular tyrosine residues (*gray triangles* for fully conserved residues, *white triangles* for less conserved residues), multiple SH3 domain binding proline-rich motifs (P*XX*P, only conserved motifs are shown, aa 74–77, 123–126, 222–225), group I WW domain binding motifs (PP*XY*, only conserved motifs shown, aa 150–153, 163–166), and a C-terminal PDZ-binding motif (TTAV, aa 271–274). The N-terminal part of PRR7 (aa 1–167) corresponds to the predicted WBP-1 domain as indicated. This scheme is based on GenBank™ protein sequence NP_085044. *B*, shown is alignment of PRR7 protein sequences from four vertebrate species: *Homo sapiens* (human), *Mus musculus* (mouse), *Xenopus tropicalis* (frog), and *Danio rerio* (zebrafish). The sequences were aligned using ClustalW program from the EMBL-EBI website. Absolutely conserved residues are shaded in *gray*. Tyrosine residues conserved in all vertebrates (Tyr-153, Tyr-166, Tyr-177 in humans) are numbered *above the alignment*. *C*, a comparison of human PRR7 and WBP-1 protein sequences is shown. The sequence corresponding to the predicted WBP-1 domain is *underlined*. Identical residues are *shaded in gray*. Two conserved tyrosine residues (Tyr-153 and Tyr-166 in human PRR7) in the predicted WW domain binding motifs are indicated *above the sequences*.

To examine the expression of PRR7 at the protein level, we generated anti-human PRR7 monoclonal antibodies. Unfortunately, none of the generated antibodies could clearly detect PRR7 after immunoblotting of whole primary cell lysates (except for mouse or rat brain tissues; not shown). Moreover, native PRR7 could not be quantitatively immunoprecipitated from cell lysates using these antibodies, suggesting partial blocking of the epitope (not shown). Nevertheless, it was still possible to use immunoprecipitation to concentrate PRR7 protein to the degree sufficient for detection by immunoblotting also in leukocytes. We then used this approach to verify our results from previous experiments. As shown in Fig. 2*D*, PRR7 protein was detected in PBL activated by various stimuli, including activation with anti-CD3 and anti-CD28 antibodies,

TABLE 1

Conservation of PRR7 protein in vertebrates

Orthologous protein sequences were aligned and compared to human PRR7 using ClustalW program. Species in this table are sorted according to the calculated protein identity with human PRR7.

Species	Length	Identity
	aa	*%*
Pongo pygmaeus (*orangutan*)	273	99
Pan troglodytes (*chimpanzee*)	259	98
Macaca mulatta (*macaque*)	276	98
Rattus norvegicus (*rat*)	269	98
M. musculus (*mouse*)	269	97
Spermophilus tridecemlineatus (*squirrel*)	269	97
Canis familiaris (*dog*)	272	97
Bos taurus (*cattle*)	269	97
Echinops telfairi (*tenrec*)	270	95
Sus scrofa (*pig*)	269	94
Monodelphis domestica (*opossum*)	280	81
X. tropicalis (*frog*)	227	56
D. rerio (*fish*)	246	48
Gasterosteus aculeatus (*fish*)	279	44

PHA, or PMA and ionomycin. We could also detect PRR7 protein in several lymphoid cell lines, including Jurkat, Ramos, and MOLT-4 (Fig. 2*E*). The highest protein levels were observed in MOLT-4, which correlated well with the higher PRR7 mRNA level in this cell line (Fig. 2, *B* and *E*).

Overexpression of PRR7 Results in Apoptosis in the Jurkat T Cell Line—Because the level of PRR7 expression in leukocytes (cell lines and primary PBL) was low and its direct immunoprecipitation was inefficient, we generated multiple Jurkat cell lines transfected with PRR7 constructs containing a C-terminal GFP tag. Surprisingly, we were not able to establish a Jurkat cell line with a sufficiently high expression of PRR7. Direct observation of the PRR7-GFP-transfected Jurkat cells excluded a rapid necrotic cell death of the transfectants. Instead, we consistently observed a gradual loss of the PRR7 transfectants from the culture within a few days (not shown). PRR7^{high} cells were stained with the DNA dye, propidium iodide, and analysis of the staining suggested that overexpression of PRR7 protein caused apoptosis (not shown). To exclude the possibility that the apoptosis induction is at least in part the result of the stress caused by the transfection procedure (electroporation), we generated a stable Jurkat clone (J-iPRR7) inducibly expressing full-length HA-tagged PRR7. In this case a modified RheoSwitch Mammalian Inducible Expression System (21) was utilized. It allowed us to tightly regulate the expression of PRR7 using the synthetic compound RSL1. Without RSL1, PRR7 expression was not detectable; maximal expression was achieved in the presence of RSL1 12 h after induction (Fig. 3*A*). Strikingly, we observed that in J-iPRR7 cells, apoptosis could be clearly detected within 24 h of induction by RSL1 and gradually increased with time (Fig. 3, *B* and *C*), suggesting that apoptosis induction was a direct consequence of PRR7 expression. This process could be to a substantial degree inhibited by caspase inhibitor Z-VAD, demonstrating the involvement of caspase-dependent apoptotic pathways (Fig. 3*D*).

Region Surrounding Tyr-166 Is Critical for Apoptosis Induction—To identify the regions of the PRR7 molecule involved in the apoptosis induction, we generated a set of Jurkat cell lines transiently expressing either full-length PRR7-GFP or its mutants gradually truncated from the C terminus (Fig. 3*E*). We also generated a mutant where all the tyrosines in the PRR7 sequence were mutated to phenylalanines (all Y to F) and a mutant lacking the N terminus, including extracellular peptide, transmembrane segment, and the palmitoylation motif (Δ2–39-PRR7-GFP). As expected, expression of the full-length PRR7 resulted in a high degree of apoptosis in Jurkat cells (Fig. 3*F*). Similar results were obtained when aa downstream of Thr-171 were removed, suggesting that the C-terminal region, including the highly conserved Tyr-177 and the PDZ binding motif, are dispensable for apoptosis induction. In contrast, when 12 more aa were removed (Δ159–274-PRR7-GFP), a significant drop in the percentage of apoptotic cells was observed, and only a very mild additional reduction was noticed after further shortening of PRR7 protein by 8 or more aa (Δ151–274-PRR7-GFP) (Fig. 3*F* and not shown). These data suggested that the region critical for the apoptosis induction is located between aa 159 and 171 of PRR7. Importantly, the tyrosine mutant of PRR7 (all Y to F) also induced only a mild level of apoptosis under the same conditions, indicating that one or more tyrosine residues are involved. The only tyrosine in the critical region identified by the deletion mutants is the highly conserved Tyr-166. Thus, our observations suggest that a tyrosine-based motif surrounding this Tyr-166 is essential for the induction of apoptosis by PRR7, although the involvement of other tyrosine residues cannot be completely excluded by this type of analysis.

Finally, the mutant lacking extracellular and transmembrane parts induced the highest level of apoptosis in Jurkat transfectants (Fig. 3*F*). This finding was surprising as we rather expected a loss of function when removing the membrane localization sequences.

Intracellular Localization of PRR7 Mutants Partially Correlates with Apoptosis Induction—Strong pro-apoptotic effects of the construct lacking putative membrane anchoring sequences prompted us to analyze subcellular localization of PRR7 and its mutants. Full-length PRR7 was localized at the plasma membrane and to large vesicular perinuclear structures, which did not co-localize with an early endosomal marker transferrin (not shown) nor with lysosomal marker LAMP-1 (CD107a, Fig. 4, *A* and *B*). The C-terminal tag modifications had no effect on this localization pattern as tagged versions of PRR7 (PRR7-GFP, PRR7-HA) were indistinguishable from non-tagged PRR7 (Fig. 4*A*), suggesting that the C-terminal PDZ binding motif requiring a free carboxyl group at the C terminus for binding (28) is not involved in this process.

Time course analysis of PRR7 in the inducible system revealed that shortly after expression induction by RSL1, PRR7 was found mainly at the plasma membrane and later gradually accumulated in the perinuclear compartment. After RSL1 withdrawal, the plasma membrane staining was lost, and PRR7 could be detected only in the perinuclear cytoplasmic vesicles (Fig. 4*C*). These data suggest a continuous active process of PRR7 removal from the plasma membrane by endocytosis.

We next analyzed PRR7 deletion mutants. C-terminal deletions up to aa 171 (Δ207–274, Δ171–274-PRR7-GFP) did not have any effect on PRR7 localization. However, further shortening of the cytoplasmic part of PRR7 up to aa 159 and 151 (Δ159–274, Δ151–274-PRR7-GFP) resulted in gradual loss of vesicular accumulation and a shift to an almost exclusive sur-

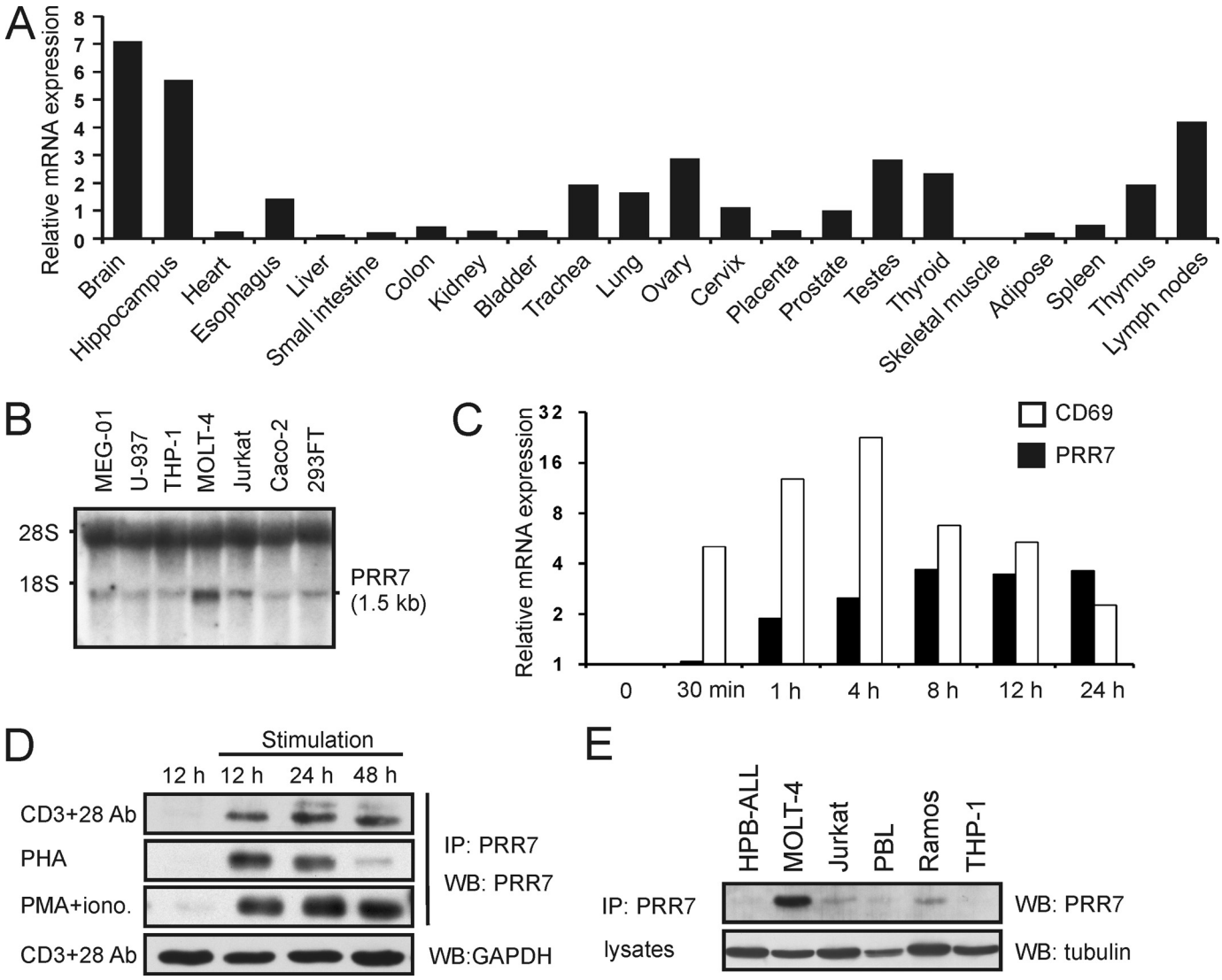

FIGURE 2. **Expression of PRR7 in primary human cells and tissues.** *A*, shown is RT-qPCR analysis of PRR7 mRNA expression in normal human tissues. PCR reactions were done in triplicate, and mean Ct values were used for further data processing in GenEx software. First, data were normalized to GAPDH mRNA expression, and then relative expression values were calculated. An average expression in all tissues corresponds to value 1 on the vertical axis. *B*, Northern blot analysis of PRR7 mRNA in human cell lines is shown. Total RNA (15 μg) was resolved on 1% agarose gel and, after transfer to nylon membrane, probed for PRR7 mRNA. The positions of ribosomal RNA bands 18 S and 28 S are indicated. *C*, shown is an RT-qPCR analysis of kinetics of PRR7 mRNA up-regulation in human PBL after PHA treatment. PCR reactions were done in triplicate, and mean Ct values were used for further data processing in GenEx software. Data were normalized to the optimal reference gene, β2-microglobulin, and relative expression values were calculated. Value 1 on the *vertical axis* corresponds to basal PRR7 mRNA expression in unstimulated PBL. Expression of the activation marker CD69 was used as a positive control. *D*, human PBL were stimulated with plate-bound anti-CD3 IgG and soluble anti-CD28 IgM, PHA, or PMA and ionomycin. Cell lysates were concentrated by immunoprecipitation (*IP*) with the anti-PRR7 mAb (TRAP3-03). Expression of PRR7 was analyzed by immunoblotting (*WB*) using the anti-PRR7 mAb (TRAP3-10). In addition, equivalence of sample sizes was verified via staining of lysates for GAPDH. A representative result for each type of stimulation is provided. *E*, PRR7 was immunoprecipitated from lysates of human cell lines with the anti-PRR7-Sepharose. Expression of PRR7 was analyzed by immunoblotting using the anti-PRR7 mAb. The equivalence of sample sizes was verified via staining of lysates with the anti-tubulin mAb.

face localization (Fig. 4*B*). Additional shortening of PRR7 sequence did not have any further effect on the subcellular localization of this protein (not shown). These results suggest that the region critical for internalization of PRR7 is positioned between aa 151 and 171. Strikingly, this region partially overlaps with the sequence responsible for the apoptosis induction (159–171), suggesting a link between PRR7 internalization and apoptosis. In agreement with this, the PRR7 N-terminal deletion mutant, which caused the highest level of apoptosis (Δ2–39-PRR7-GFP), was localized exclusively in the intracellular vesicular compartment (Fig. 4*B*). The region found to be critical for PRR7 internalization contains two highly conserved tyrosine motifs, suggesting that they may serve as tyrosine-based internalization motifs. However, the all Y to F mutant of PRR7 showed only a mild reduction in the size of intracellular PRR7 compartment (not shown), displaying at the same time strong defects in the apoptosis induction. These results indicated that tyrosines within the critical conserved region are not a part of the tyrosine-based internalization sequences, but rather, they are involved in pro-apoptotic signaling mediated by PRR7. Importantly, these tyrosines form the putative WW domain binding motif of the PRR7 WBP-1 domain, suggesting possible involvement of a WW-containing protein in this process.

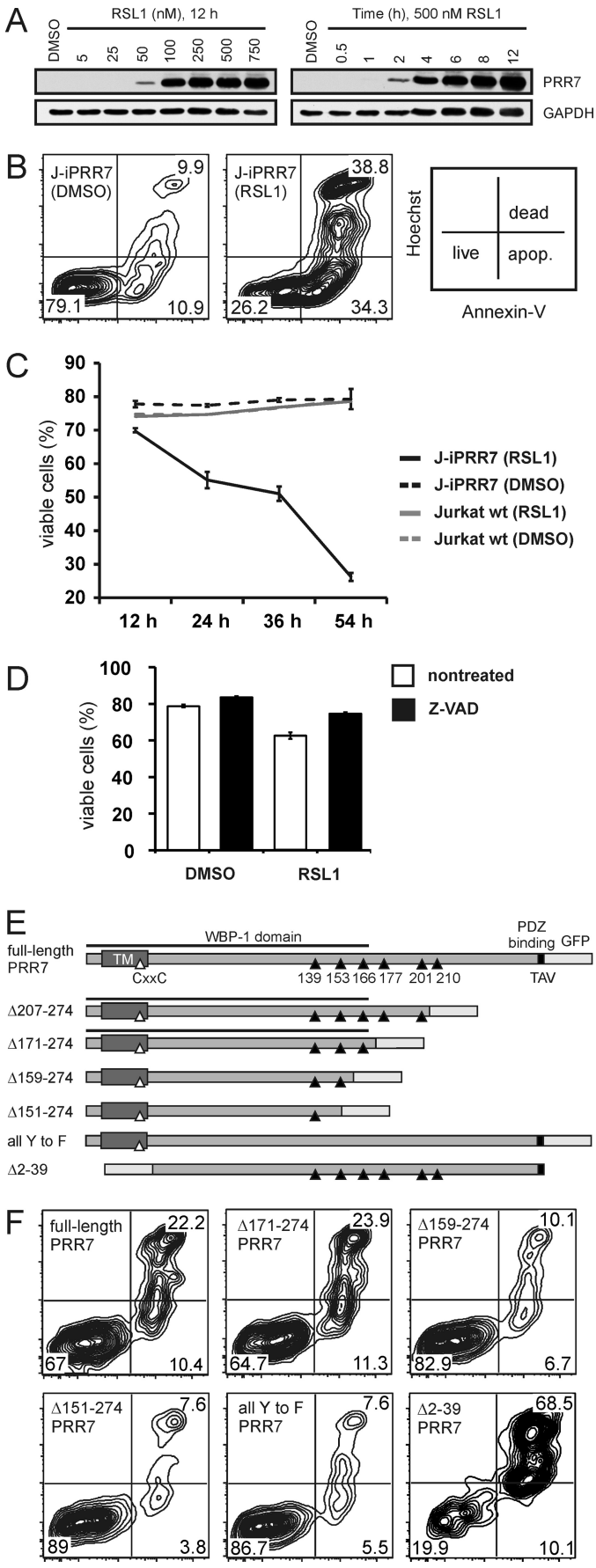

FIGURE 3. **PRR7-inducible system and induction of apoptosis by overexpression of PRR7 in Jurkat cells.** *A*, to determine the optimal concentration of RSL1 for PRR7 induction, J-iPRR7 cells were treated with an increasing amount of RSL1 for 12 h (*left panel*). J-iPRR7 cells were treated with 500 nM RSL1, and expression of PRR7 in several time intervals was analyzed by immunoblotting with the anti-PRR7 mAb (TRAP3-10, *right panel*). Staining for GAPDH was used as a sample loading control. *B*, J-iPRR7 cells were induced to express PRR7 or left uninduced (DMSO control). After 54 h, apoptosis in both cultures was determined by annexin V and Hoechst 33258 staining. *C*, J-iPRR7 and Jurkat wt cells were treated with 500 nM RSL1 to induce PRR7 expression or left untreated (DMSO control). Cell viability was determined by flow cytometry after 12, 24, 36, and 54 h. Data are presented as the mean of three experiments (±S.D.). *D*, J-iPRR7 cells were induced to express PRR7 or left uninduced (DMSO control) for 24 h in the presence of the pan-caspase inhibitor, Z-VAD-FMK (10 μM). The percentage of viable cells was determined by propidium iodide (*PI*) staining and flow cytometry. The experiment was done in triplicate cultures, and data are represented as the mean values ± S.D. *E*, shown is a scheme of PRR7-GFP mutants used in this study. Tyrosine residues are represented by *black triangles*. *F*, Jurkat cells were transfected with corresponding plasmid DNA coding for wt or mutated PRR7 fused to GFP. After 48 h, cells were stained with annexin V/Dy647 and Hoechst 33258 to analyze apoptosis in the GFP-positive cells.

PRR7 Partitions to Large Detergent-resistant Complexes—Similarity to palmitoylated lipid raft-associated TRAPs suggested that PRR7 may employ lipid rafts as signaling platforms. Indeed, we found PRR7 to be palmitoylated in the J-iPRR7 cells (Fig. 5*A*). However, in contrast to these adaptors, PRR7 was found in a different type of detergent (Brij-98)-resistant complexes. These were large (as determined by gel filtration on Sepharose 4B) but almost non-buoyant (as determined by density gradient ultracentrifugation) and resistant to lipid raft-disrupting detergent laurylmaltoside (Fig. 5, *B* and *C*).

Variable Effects of PRR7 on the Activity of Signaling Pathways in J-iPRR7—To elucidate how PRR7 affects T cell signaling pathways, we compared the phenotype of induced *versus* non-induced J-iPRR7 cells. Induction of PRR7 expression led to a mild spontaneous up-regulation of the activation marker CD69 in quiescent cells (Fig. 6*A*). Moreover, treatment of induced J-iPRR7 with PMA and ionomycin resulted in a substantial increase in IL-2 production (Fig. 6*B*), suggesting that these cells are in a partially activated state. In contrast, at the same time, the induced J-iPRR7 cells exhibited an attenuated calcium response (Fig. 6*C*) and reduced global tyrosine phosphorylation (Fig. 6*D*) after TCR stimulation.

Interestingly, we detected strong constitutive tyrosine phosphorylation of PRR7, which was not altered by TCR cross-linking (Fig. 6, *D* and *E*). This observation raised questions about the mechanisms mediating PRR7 phosphorylation and the role of tyrosine phosphorylation in the diverse effects of PRR7 on T cell signaling pathways.

To elucidate the mechanisms of PRR7 phosphorylation, we analyzed lysates of J-iPRR7 cells treated with a specific inhibitor of Src-family kinases (SFK) PP2 by phosphotyrosine immunoblotting. As shown in Fig. 7*A*, PRR7 phosphorylation was almost completely inhibited in the presence of PP2, suggesting that PRR7 is constitutively phosphorylated by SFKs. To identify potential SFKs capable of PRR7 phosphorylation, constructs encoding PRR7 and various tyrosine kinases were co-transfected into COS-7 cells. The results revealed that several SFKs (most strongly Src) are able to phosphorylate PRR7 (Fig. 7*B*). The multiple predicted SH3 domain binding motifs in the intracellular part of PRR7 using iSPOT (29) indicated a possible

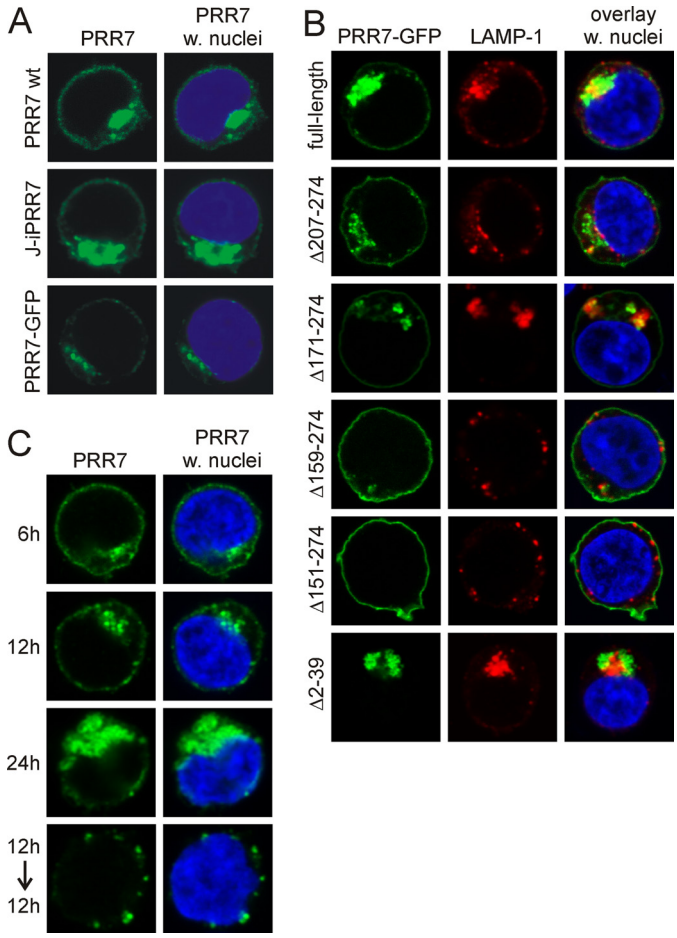

FIGURE 4. **Subcellular localization of full-length PRR7 and deletion mutants in Jurkat cells.** *A*, shown is a comparison of subcellular localization of PRR7 protein with (*w.*) and without tags. Jurkat cells expressing the PRR7 wt protein (without a tag) were fixed 18 h post-transfection and stained with PRR7 antibody. J-iPRR7 (PRR7 with HA-tag) cells were induced for 18 h to express PRR7 with 500 nM RSL1, fixed, and stained with the anti-HA-tag mAb. Jurkat PRR7-GFP transfectants were fixed 18 h post-transfection. Samples were analyzed by confocal microscopy. *B*, cells were transfected with corresponding plasmid DNA, and after 18 h cells were allowed to adhere to poly-lysine-coated coverslips, fixed, stained for lysosomal marker LAMP-1 (CD107a) and DNA dye Hoechst 33258, and analyzed by confocal microscopy. *C*, J-iPRR7 cells were induced to express PRR7 with 500 nM RSL1. Cells were fixed 6, 12, and 24 h afterward or induced for 12 h, washed, and incubated an additional 12 h in fresh medium without RSL1 before fixation. All samples were stained with the anti-HA-tag mAb and DNA dye Hoechst 33258 and analyzed by confocal microscopy.

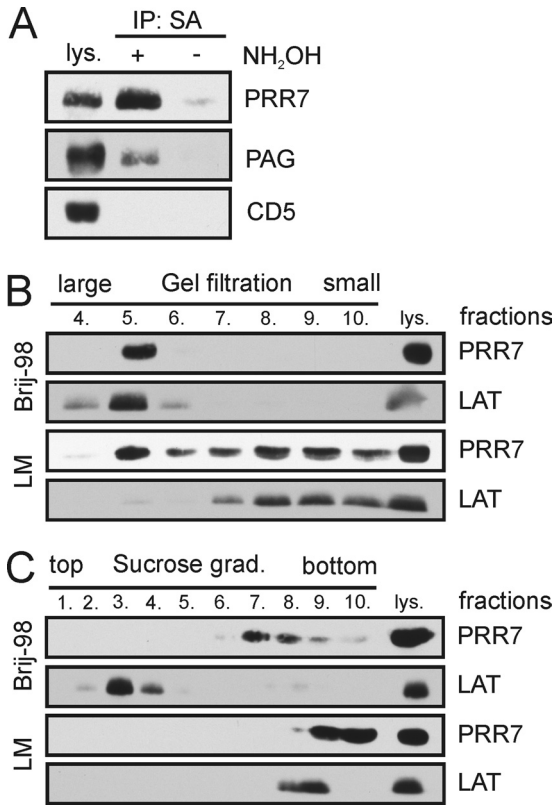

FIGURE 5. **Biochemical characterization of the PRR7 protein.** J-iPRR7 cells were induced to express PRR7 (16 h) and subjected to several biochemical assays. *A*, palmitoylation of PRR7 was examined using the acyl-biotinyl exchange chemistry-based method as described under "Experimental Procedures." A palmitoylated transmembrane adaptor protein, PAG, was used as a positive control; a non-palmitoylated transmembrane protein, CD5, and lysate that was not treated with NH_2OH (hydroxylamine) were used as negative controls. IP, immunoprecipitate. *SA*, streptavidin beads. *B*, Brij-98 or LM lysates of J-iPRR7 cells were subjected to gel filtration on Sepharose 4B, and the separated fractions were analyzed by immunoblotting. A raft-associated transmembrane adaptor protein, LAT, was used as a control. *C*, Brij-98 or LM lysates of J-iPRR7 cells were subjected to density gradient ultracentrifugation, and the separated fractions were analyzed by immunoblotting. The fractions are numbered from the *top*. *10* represents the sediment. The lipid raft protein, LAT, was used as control.

association of PRR7 with SFKs (via their SH3 domains). Thus we immunoprecipitated PRR7 from the induced J-iPRR7 cells and tested the immunoprecipitates for the presence of associated SFKs by immunoblotting. Among the tested SFKs (Src, Lck, Fyn), the only identified binding partner was Src (Fig. 7*C* and not shown).

Mechanisms of PRR7-dependent Regulation of J-iPRR7 Signaling—So far, our observations suggested that PRR7 expression had a dual activating/inhibitory effect. It resulted in selective up-regulation of c-Jun and CD69, whereas at the same time it led to the inhibition of proximal TCR signaling. Among the hypothetical explanations for the inhibitory effects of PRR7 on TCR-mediated signaling was the possibility that the cells are already in the process of apoptosis advanced enough to have negative effects on TCR signal transduction. To test this, we treated J-iPRR7 cells with caspase inhibitor Z-VAD during RSL1-mediated PRR7 induction and then followed a calcium response downstream of TCR. Z-VAD treatment had a negligible effect on calcium response in PRR7-expressing J-iPRR7 cells, suggesting an apoptosis-independent mechanism of PRR7-mediated inhibition of T cell signaling (Fig. 8*A*).

To identify the point where PRR7 interferes with TCR signaling pathways, we treated J-iPRR7 cells with RSL1 to induce PRR7 expression, stimulated the cells by TCR cross-linking, and followed the phosphorylation of individual proteins in the cell lysates. We observed reduced phosphorylation of a number of important signaling molecules, including ZAP-70, LAT, PLCγ1, ERK1/2, and JNK (Fig. 8*B*) after TCR stimulation. The only remarkable exception was the up-regulation of c-Jun protein level, which was also reflected in the increased amount of the phosphorylated form of this transcription factor (Fig. 8*B*).

The reduction in tyrosine phosphorylation of proteins in proximal TCR signaling pathways, including ZAP-70, LAT, and PLCγ1, suggested the effects of PRR7 at the level of TCR itself. To analyze this, we followed the phosphorylation of TCRζ

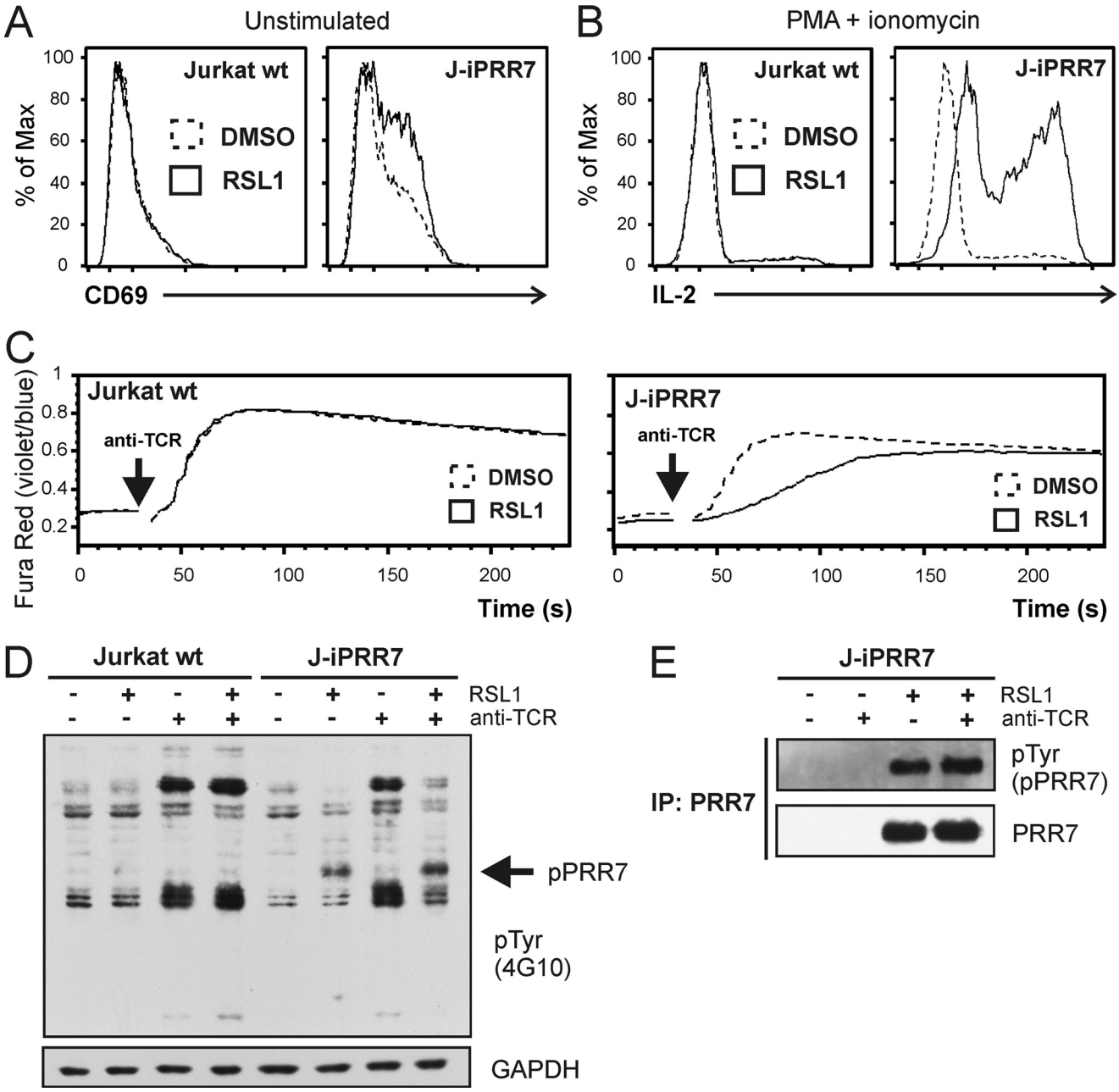

FIGURE 6. **Activated phenotype and impaired TCR signaling in J-iPRR7 cells.** *A*, J-iPRR7 cells were induced for 12 h to express PRR7, stained for the surface lymphocyte activation marker, CD69, and analyzed by flow cytometry. *B*, J-iPRR7 cells were induced to express PRR7. After 12 h, the cells were stimulated for additional 6 h with PMA and ionomycin in the presence of brefeldin A (5 μg/ml), fixed, stained for cytokine IL-2, and analyzed by flow cytometry. *C*, J-iPRR7 cells were induced for 16 h to express PRR7 and then loaded with a calcium indicator dye Fura Red. Calcium response after the anti-TCR IgM (C305, 10 μg/ml) stimulation was measured by flow cytometry. Collected data were analyzed in FlowJo software and plotted as mean fluorescence values. *D*, J-iPRR7 cells were induced to express PRR7 (16 h). Cells were stimulated with the anti-TCR IgM (C305, 10 μg/ml, 2 min) or left unstimulated. Whole cell lysates were analyzed by immunoblotting with the anti-phospho-Tyr mAb (4G10). Membrane was reprobed with GAPDH antibody as a loading control. *E*, J-iPRR7 cells were treated as in *panel D*, and lysate was subjected to immunoprecipitation (*IP*) with the anti-PRR7 mAb. Immunoprecipitated material was analyzed by immunoblotting with the anti-phospho-Tyr mAb (4G10) and the anti-PRR7 mAb.

chain after TCR cross-linking. We observed a substantial reduction in TCRζ phosphorylation both in a basal state and after stimulation in PRR7-expressing cells (Fig. 8*C*). As TCRζ is a substrate SFKs, we next analyzed the activation status of these kinases by immunoblotting with antibodies specific to their activating and inhibitory tyrosines with a focus on Lck, the major Src-family member in T cells. Surprisingly, we observed reduction in the phosphorylation of both regulatory tyrosines (SFK activation loop tyrosine and Lck inhibitory tyrosine) in PRR7-expressing cells. However, we also detected a highly reproducible reduction in total Lck staining (Fig. 8*D*). These data showed that the decreased Lck level is probably the major consequence of PRR7 expression, which is then reflected in the reduced content of both regulatory phosphotyrosines in the samples and reduced activity of downstream signaling pathways.

Finally, to assess the role of SFK activity and tyrosine phosphorylation of PRR7 in the opposing functions of PRR7, we

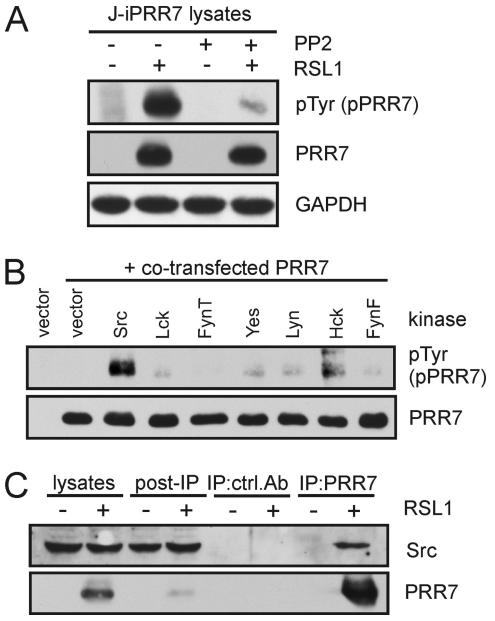

FIGURE 7. **Tyrosine phosphorylation of PRR7 and association of PRR7 with Src.** *A*, J-iPRR7 cells were induced to express PRR7 and simultaneously treated with the SFK inhibitor, PP2 (10 μM), for 18 h or left untreated. Tyrosine phosphorylation status of PRR7 was analyzed by immunoblotting with the anti-phospho-Tyr mAb (4G10). GAPDH staining served as a sample loading control. *B*, PRR7 was co-transfected with Src kinases into COS-7 cells. After 24 h, cells were lysed, and tyrosine phosphorylation of PRR7 was detected by immunoblotting with the anti-phospho-Tyr (4G10) mAb. *C*, J-iPRR7 cells were induced to express PRR7 or left uninduced for 18 h and lysed in LM buffer, and PRR7 was immunoprecipitated (*IP*) using the anti-PRR7-Sepharose or an isotype control mAb. Eluted proteins were analyzed by immunoblotting.

treated J-iPRR7 cells with SFK inhibitor PP2 during the PRR7 induction. Surprisingly, PP2 treatment had only a minor effect on c-Jun up-regulation as well as apoptosis induction (not shown), indicating that the effect of PRR7 on these pathways is independent of SFK signaling. In contrast, PP2 treatment inhibited the up-regulation of CD69 (Fig. 8*E*).

DISCUSSION

Thus far, PRR7 has been described in a single paper (18) as a component of the postsynaptic density (PSD) fraction of the rat forebrain, suggesting that it may play a role in modulating neural activities via interactions with the PSD-95/*N*-methyl-D-aspartate receptor complex or in PSD core formation. Here, we report that PRR7 is expressed in many tissues, including leukocytes, and is significantly up-regulated during T cell activation (Fig. 2, *C* and *D*). Nevertheless, even in activated T cells we had difficulties detecting PRR7 using our monoclonal antibodies on the immunoblot of whole cell lysates, which was most likely the result of both low expression level of PRR7 and suboptimal antibody reactivity. Therefore, we have used mainly Jurkat transfectants transiently or inducibly expressing recombinant PRR7. In these cells, our monoclonal antibodies could readily detect PRR7 in cell lysates and could be used in biochemical studies.

PRR7 is highly evolutionarily conserved (Table 1), suggesting that its function might be preserved across all vertebrates. The intracellular part of this protein (Fig. 1*A*) contains several motifs (proline-rich WW binding motif, tyrosine-based SH2 domain binding and/or internalization motifs, proline-based SH3 binding motifs, and a C-terminal PDZ-binding motif) indicating potential for interactions with other proteins.

The PDZ binding motif is responsible for the reported association with the MAGUK family scaffold protein PSD-95 in neurons (18). We assumed that in leukocytes it could be associated with the leukocyte counterpart of PSD-95, DLG1 (30). However, in our co-transfection experiments, we were not able to confirm such an association, whereas the association with PSD-95 was readily demonstrable under the same conditions (not shown). Src is another binding partner of PRR7, as identified in this work. PRR7 is most likely a substrate of Src, as we observed strong tyrosine phosphorylation of PRR7 in Jurkat cells, which was inhibited by the SFK inhibitor, PP2.

The most striking consequence of PRR7 overexpression in Jurkat T cell line was increased apoptosis. We used multiple deletion mutants to identify the region critical for apoptosis induction. Our analysis limited this region to the sequence between aa 159–171 in the WBP-1 domain of PRR7. Importantly, this region contains only a single tyrosine residue, Tyr-166, suggesting that phosphorylation of this site may be involved. However, our experiments with PP2 treatment of J-iPRR7 cells did not provide sufficient evidence for the role of SFK-mediated PRR7 tyrosine phosphorylation in apoptosis induction (not shown). We could also rule out the activation-induced cell death, as we did not observe any up-regulation of FasL in J-iPRR7 cells nor were we able to block PRR7-induced apoptosis by blocking antibodies to FasL (not shown). Thus, the mechanism of how the region around Tyr-166 mediates pro-apoptotic signaling remains unknown. One of the remaining hypotheses is phosphorylation-independent binding of a WW domain-containing protein to the tyrosine motif in the critical region. However, the identity of such a putative protein remains to be determined.

PRR7 inducibly expressed in the Jurkat cells was initially present at the plasma membrane, and later it was mainly detected in reversible, perinuclear, vesicular structures (Fig. 4, *B* and *C*). Time course analysis of PRR7 localization in the inducible system after RSL1 withdrawal suggests that the perinuclear accumulation is a continuous, active process involving removal from the plasma membrane by endocytosis (Fig. 4*C*).

Interestingly, the region involved in PRR7-mediated internalization partially overlaps with the section critical for PRR7-mediated apoptosis, suggesting the link between PRR7 internalization and induction of apoptosis. However, the region required for PRR7 internalization also includes the sequences outside the area critical for apoptosis. Moreover, the mutation of all PRR7 tyrosine residues that had strong inhibitory effects on apoptosis induction resulted only in a mild reduction in PRR7 internalization (not shown), suggesting that this region mediates apoptosis via other mechanisms then solely by triggering PRR7 endocytosis. On the other hand, the mutant lacking the membrane-anchoring sequences, which localized exclusively in the intracellular compartment, induced the highest degree of apoptosis. Taken together these data suggest that internalization and tyrosine phosphorylation are two independent mechanisms, which may cooperate to efficiently trigger apoptosis in J-iPRR7. An important question remains of how the region between aa 151 and 171 mediates PRR7 inter-

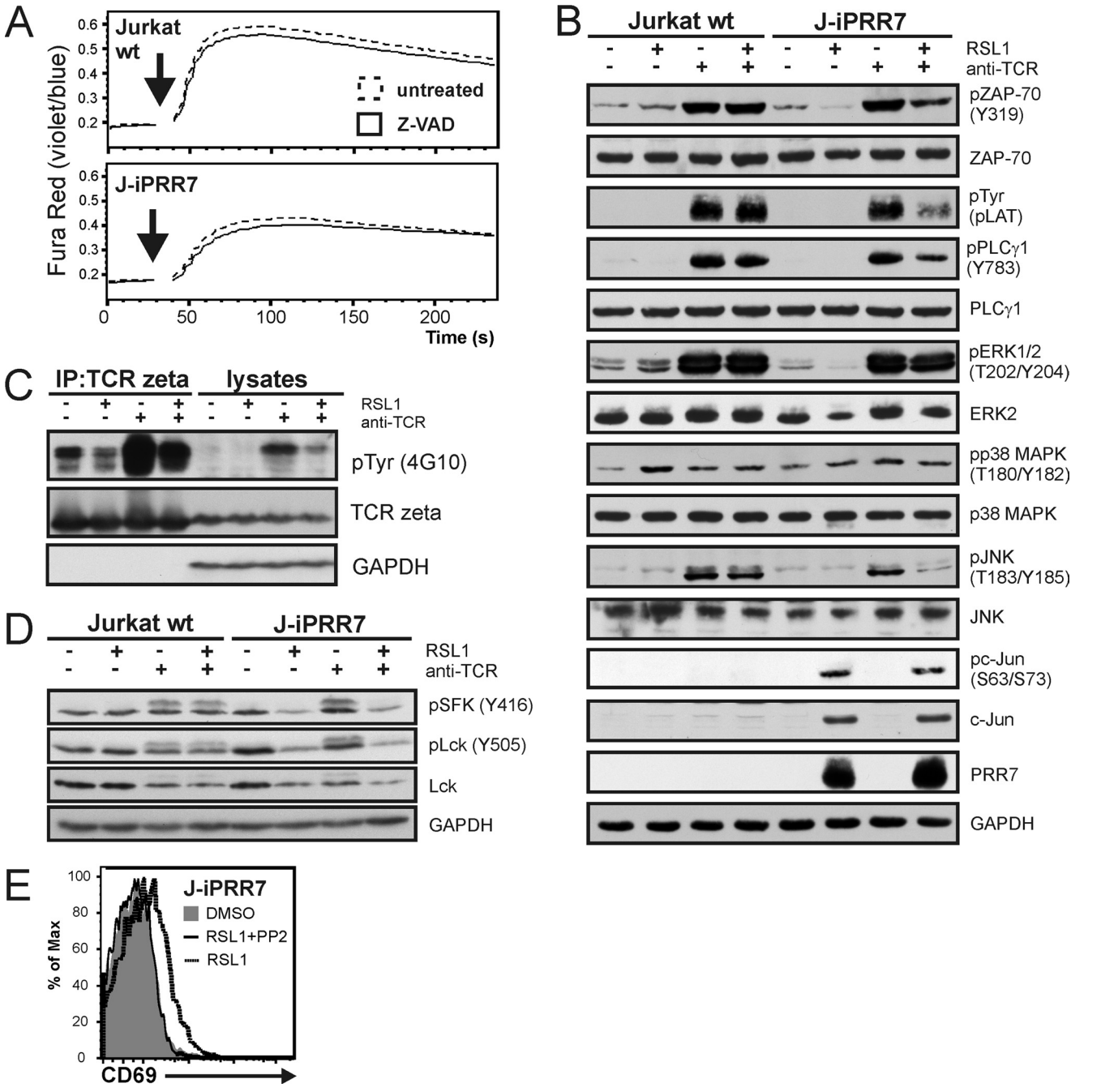

FIGURE 8. **Mechanisms of PRR7 dependent regulation of J-iPRR7 signaling.** *A*, J-iPRR7 cells were induced to express PRR7 in the presence of the pan-caspase inhibitor Z-VAD-FMK (10 μM, 16 h) and then loaded with a calcium indicator dye Fura Red. Calcium response after the anti-TCR IgM (C305, 10 μg/ml, *black arrows*) stimulation was measured by flow cytometry. Collected data were analyzed in FlowJo software and plotted as mean fluorescence values. *B*, J-iPRR7 cells induced to express PRR7 (16 h) were stimulated with the anti-TCR IgM (C305, 10 μg/ml, 2 min) or left unstimulated. Whole cell lysates were analyzed by immunoblotting with antibodies specific for proteins involved in TCR signaling. Phosphorylation status of LAT was detected using anti-Tyr(P) antibody (clone P-TYR-02) developed in our laboratory. Staining of GAPDH was used as a sample loading control. *C*, J-iPRR7 cells were treated as stated above (*panel B*), and lysates were subjected to immunoprecipitation (*IC*) with the anti-TCRζ mAb. Immunoprecipitates and whole cell lysates were immunoblotted with the anti-phospho-Tyr mAb (4G10) and Abs specific for TCRζ and GAPDH (sample loading control). *D*, J-iPRR7 cells were treated as stated above (*panel B*). Whole cell lysates were analyzed by immunoblotting with antibodies specific for phosphorylated forms of Lck, total Lck, and GAPDH (sample loading control). *E*, J-iPRR7 cells were induced to express PRR7 in the presence or absence of PP2 (10 μM, 18 h), stained for CD69, and analyzed by flow cytometry. In all the above assays, Jurkat wt cells and DMSO treatment were used as controls (not shown in *panels C* and *E*).

nalization. Tyrosines 153 and 166 within this critical region are part of potential tyrosine-based internalization sequences fitting the consensus Y*XX*Φ (31). Tyrosine to phenylalanine mutations within similar motifs can substantially reduce the rate of endocytosis (32). Mutations of PRR7 tyrosines had only a mild effect on PRR7 internalization, suggesting that these motifs are not genuine internalization sequences.

Other phenotypic features of the induced J-iPRR7 cells were also quite striking. They exhibited some traits of partially activated or "primed" T cells, including a spontaneous increase in

the expression of the surface activation marker, CD69, and strongly enhanced IL-2 production after PMA and ionomycin stimulation (Fig. 6, *A* and *B*). On the other hand, proximal elements of immunoreceptor signaling cascades (tyrosine phosphorylation of cytoplasmic substrates and cytoplasmic calcium elevation upon TCR cross-linking) were clearly diminished or down-regulated (Fig. 6, *C* and *D*, and 8, *B–D*). A likely explanation is that PRR7 by a so-far unknown mechanism down-regulates protein levels of a critical SFK Lck, which in turn results in the reduction of the activity of all the downstream pathways. On the other hand, a concomitant positive regulatory function of PRR7 was evidenced by a strong up-regulation of AP-1 transcription factor component c-Jun accompanied by a mild induction of the activation marker CD69 in the cells where PRR7 expression was induced (Figs. 6*A* and 8*B*). Intriguingly, c-Jun up-regulation was not affected by PP2 treatment (not shown), suggesting that this phenomenon is independent of Src or other SFKs. Moreover, we did not see any up-regulation of upstream pathways such as JNK or Erk that could lead to c-Jun up-regulation. c-Jun turnover is regulated via ubiquitin-dependent degradation, and thus a plausible hypothesis is that PRR7 somehow interferes with this process. This could lead to up-regulation of c-Jun protein levels independently of the activity of upstream MAP kinase pathways. Interestingly, one of the proteins involved in the regulation of c-Jun turnover is Itch, an E3 ubiquitin ligase of NEDD4 family (33) that also possesses multiple WW domains and could potentially interact with PRR7. However, we could not detect any Itch protein in PRR7 immunoprecipitates (not shown), although it remains possible that the interaction is labile or that some other member of the NEDD4 family is involved in such an interaction.

It can be speculated that increased c-Jun/AP-1 activity is responsible for the primed state of PRR7 expressing Jurkat, resulting in a high level of IL-2 production after stimulation. Although AP-1 sites are important components of the IL-2 promoter (34, 35), additional factors such as NF-AT and NF-κB are required for the full activity. If these are provided, *e.g.* after stimulation with PMA and ionomycin, a strong response can result. Regulation of CD69 expression also involves AP-1, but in contrast to c-Jun, CD69 up-regulation could be inhibited by PP2. A somewhat speculative explanation can be that tonic signaling by TCR (which is dependent on SFK) is required for CD69 expression in addition to c-Jun activity. In Jurkat cells, basal TCR signaling results in low level of CD69 expression (36). It is likely that it can be further potentiated by c-Jun overexpression while still being dependent on SFK-driven basal signaling. In line with this explanation is the observation that PRR7-mediated CD69 up-regulation could be partially blocked by inhibitors of several pathways (NF-AT, NF-κB, and p38 MAPK, SAPK/JNK) known to be initiated by TCR/SFK mediated signaling (not shown).

Although our present results show that PRR7 is able to influence T cell physiology rather dramatically, it remains to be determined whether it plays a regulatory role under more physiological conditions, *e.g.* in the natural process of T cell activation (37). Such roles may be indicated by the evidently tightly controlled expression of PRR7 in resting T cells and its marked up-regulation upon activation (Fig. 2). In contrast, cell types other than T lymphocytes, *e.g.* fibroblasts, may be much less sensitive to increased levels of PRR7 expression; in this context it should be noted that overexpression of full-length PRR7 was well tolerated by COS-7 or HEK293FT cells. Clearly, preparation of conditional *Prr7* gene knock-out mice may provide more definitive answers concerning the roles of PRR7 in various types of cells and tissues.

Acknowledgments—We thank all colleagues who provided the cells, vectors, and antibodies as indicated under "Experimental Procedures."

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MOLECULAR AND CELLULAR BIOLOGY, Nov. 2011, p. 000 Vol. 31, No. 22
0270-7306/11/$12.00 doi:10.1128/MCB.05817-11

SCIMP, a Transmembrane Adaptor Protein Involved in Major Histocompatibility Complex Class II Signaling▽†

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Received 15 June 2011/Returned for modification 14 July 2011/Accepted 2 September 2011

Formation of the immunological synapse between an antigen-presenting cell (APC) and a T cell leads to signal generation in both cells involved. In T cells, the lipid raft-associated transmembrane adaptor protein LAT plays a central role. Its phosphorylation is a crucial step in signal propagation, including the calcium response and mitogen-activated protein (MAP) kinase activation, and largely depends on its association with the SLP76 adaptor protein. Here we report the discovery of a new palmitoylated transmembrane adaptor protein, termed SCIMP. SCIMP is expressed in B cells and other professional APCs and is localized in the immunological synapse due to its association with tetraspanin-enriched microdomains. In B cells, it is constitutively associated with Lyn kinase and becomes tyrosine phosphorylated after major histocompatibility complex type II (MHC-II) stimulation. When phosphorylated, SCIMP binds to the SLP65 adaptor protein and also to the inhibitory kinase Csk. While association with SLP65 initiates the downstream signaling cascades, Csk binding functions as a negative regulatory loop. The results suggest that SCIMP is involved in signal transduction after MHC-II stimulation and therefore serves as a regulator of antigen presentation and other APC functions.

The adaptive immune response is initiated by T cell recognition of antigen peptide-loaded major histocompatibility complex (MHC) glycoproteins present on the surfaces of professional antigen-presenting cells (APCs), such as dendritic cells (DCs), macrophages, and B cells (41, 45). This leads to the formation of the immunological synapse (IS) at the cell-cell contact site. The hallmark of the IS is accumulation of T cell receptors (TCRs) paired with peptide-MHC, together with pairs of adhesion and costimulatory molecules (14).

Numerous proteins participating in the formation of IS have been described as constituents of plasma membrane microdomains, such as lipid rafts or tetraspanin-enriched microdomains (TEMs). Lipid rafts are lipid-based structures enriched with cholesterol, sphingolipids, and glycosphingolipids that contain certain glycosylphosphatidylinositol (GPI)-linked transmembrane or acylated cytoplasmic proteins (33). In contrast, TEMs are based on protein-protein interactions among different tetraspanins, such as CD9, CD37, CD53, CD81, or CD82. All tetraspanins share a similar structure, including four transmembrane domains and structurally conserved small and large extracellular domains. Tetraspanins can also interact with additional transmembrane proteins, including integrins and MHC-II, leading to the formation of membrane platforms (23, 49).

Active signal transduction takes place on both sides of the IS. This leads to cross talk between APCs and T cells, required for efficient antigen presentation. In addition, formation of IS in DCs results in apoptosis inhibition and prolonged life span (40). Antigen presentation inducing activation of naive T cells is a primary function of DCs. Although B cells too are able to activate T lymphocytes, the primary function of antigen presentation in B cells appears to be the soliciting of T cell help required for productive activation (11). An important role is played by costimulatory molecules, such as CD40, but many of the signaling events occurring at the B cell side of IS are also dependent on the presence of specific peptide–MHC-II complexes and thus presumably on direct engagement of MHC-II molecules. However, the complexity of IS, as well as the ability of MHC-II to transmit signals in both directions, makes the analysis of direct involvement of MHC-II in APC signal transduction relatively difficult. One possibility to overcome this problem is antibody-mediated cross-linking of MHC-II molecules. Interestingly, this can recapitulate a number of events observed during B cell interaction with T cells, including cytoskeleton reorganization, an increase in tyrosine phosphorylation and the calcium concentration, proliferation, differentiation, or apoptosis (1). In addition, high sensitivity of certain B cell lymphomas to cell death following MHC-II cross-linking make anti-MHC-II antibodies potential therapeutic agents (9, 37).

The mechanism by which the signaling is initiated upon MHC-II stimulation is still incompletely defined. In B cells, MHC-II-associated molecules, such as signal-transducing sub-

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† Supplemental material for this article may be found at http://mcb.asm.org/.

▽ Published ahead of print on 19 September 2011.

units of B cell receptor (BCR) Igα/Igβ (31), CD19 (5), or an innate immune signaling adaptor, MPYS/STING (26), are thought to mediate signal transmission. Partitioning to lipid rafts (2) or tetraspanin-enriched microdomains (30) may also equip MHC-II molecules with signaling capabilities. As a result, MHC-II signaling in B cells is rather complex and involves BCR signaling machinery, as well as other BCR-independent mechanisms. However, there still are large gaps in our understanding of precise pathways and molecules involved.

In contrast, signal propagation on the T cell side of IS is well defined. TCR engagement in the IS leads to subsequent activation of Src and Syk family kinases and tyrosine phosphorylation of a number of molecules, including the transmembrane adaptor protein (TRAP) LAT. LAT is a critical component of several signaling pathways, mainly due to its ability to recruit a complex of phospholipase C gamma 1 (PLC-γ1) with the adaptors Gads and SLP76 (16).

SLP76 and its related homologue SLP65 (also known as BLNK or BASH) are involved in the transduction of signals emanating from various immunoreceptors, like the TCRs, BCRs, or Fc receptors (28). Following cell activation, these adaptors are brought to the plasma membrane. Their recruitment is largely dependent upon tyrosine phosphorylation of transmembrane proteins and can be accomplished either indirectly, as in the case of the inducible association between LAT and SLP76 mediated via a cytosolic adaptor, Gads, or directly, as in the complex of SLP65 and the BCR subunit Igα (13). Once localized at the membrane, the SLP65/SLP76 proteins become phosphorylated by Syk family kinases and form complexes with various effector molecules, such as Nck and Vav, Tec family kinases, and PLC-γ1 or PLC-γ2. This leads to initiation of downstream responses, including actin cytoskeleton remodeling, calcium flux, and activation of mitogen-activated protein kinases (MAPKs).

Reversible membrane localization is a key feature of another critical regulatory circuit of immunoreceptor signaling, involving the cytoplasmic inhibitory kinase Csk. Csk inhibits SFKs by phosphorylating the inhibitory tyrosine near their C termini, an event necessary for setting a proper threshold for numerous signaling pathways (10). In order to reach its substrates, Csk must be recruited to the plasma membrane. This process is in part mediated by the lipid raft-associated TRAPs PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains) (6, 27) and LIME (7, 22).

In this study, we describe the discovery of a new TRAP which binds both the Src homology 2 (SH2) domain of the inhibitory kinase Csk and the SH2 domains of the SLP65 and SLP76 adaptors. Based on the described observations and the fact that this protein has to fulfill its function with only a comparably small number of 145 amino acids (aa) constituting its sequence, we decided to call it *S*LP65/SLP76, *C*sk *I*nteracting *M*embrane *P*rotein (SCIMP). We provide an initial characterization of SCIMP, which reveals expression restricted to APCs, association with TEMs, localization in the IS, and involvement in signaling downstream of MHC-II molecules.

MATERIALS AND METHODS

SCIMP identification. The ScanProsite tool at the ExPASy Proteomics server (http://www.expasy.org/tools/scanprosite/) (12) was employed to generate the list of proteins containing the consensus Csk SH2 domain binding motif: Y-[A/S/T]-X-[V/P]-[N/Q/C]-[K/R] (7). The resulting list was submitted to the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM-2.0/) (29, 46) to detect putative transmembrane domains. This step was then followed by manual selection of potentially interesting proteins.

Antibodies. Antibodies to the following antigens were used in this study: Myc (9B11), PLC-γ1, PLC-γ2, phosphorylated PLC-γ1 (P-PLC-γ1) (Y783), P-PLC-γ2 (Y1217), phosphorylated extracellular signal-regulated kinase 1 and 2 (P-Erk1/2) (T202/Y204), P-MEK1/2 (S217/221), and phosphorylated p90 ribosomal S6 kinase (P-p90RSK) (S380) were from Cell Signaling Technology, Erk2, Lyn, SLP65 (2B11), Grb2 (C-23), and Csk were from Santa Cruz Biotechnology, CD81 (M38) was from Exbio, P-Tyr (4G10) was from Upstate Biotechnology, Fc Block (2.4G2) was from BD Biosciences, mouse MHC-II I-A/I-E-biotin (M5/114.15.2) was from BioLegend, goat antimouse-fluorescein isothiocyanate (FITC) and Thy1.1-FITC (HIS51) were from eBioscience, mouse Igμ, human Igμ, and goat antimouse-horseradish peroxidase (HRP) light-chain-specific antibodies were from Jackson ImmunoResearch, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), goat antimouse-HRP IgG specific, rabbit antigoat-HRP, and rabbit antichicken-HRP were from Sigma-Aldrich, goat antirabbit-HRP was from Bio-Rad, goat antimouse-Alexa Fluor 488 and goat antimouse Alexa Fluor 568 were from Molecular Probes, CD19 (B-d3) was kindly provided by J. Wijdenes (Innotherapie, Besançon, France), CD37 (HD28) was kindly provided by G. Moldenhauer (DKFZ, Heidelberg, Germany), CD3ε (MEM-57), CD56 (MEM-188), CD14 (MEM-18), CD25 (MEM-145), MHC-II (SLE-01), NTAL (NAP-04), PAG (MEM-255), CD53 (MEM-53), CD8 (control for mouse IgG1, MEM-146; control for mouse IgG2a, MEM-31), CD45 (MEM-28), and SLP76 (SLP76/3) were prepared at Laboratory of Molecular Immunology, IMG ASCR (Prague, Czech Republic).

Rabbit polyclonal antibodies against human and murine SCIMP were prepared by immunization of rabbits with recombinant proteins corresponding to intracellular parts of human SCIMP (aa 43 to 145) or murine SCIMP (aa 32 to 150) produced in *Escherichia coli*. Mouse monoclonal antibodies to human SCIMP were generated by standard techniques using splenocytes of mice (F1 hybrids of BALB/c × B10) immunized with the human recombinant protein described above and Sp2/0 myeloma cells as a fusion partner.

Cell lines and primary cells. All cell lines were cultured at 37°C with 5% CO_2 in the following media, supplemented with 10% fetal calf serum and antibiotics: Ramos, Raji, Daudi, and THP1 cell lines (all from ATCC) in RPMI 1640, HEK293FT cells (Invitrogen) and Phoenix Ampho cells (Origene) in Dulbecco's modified Eagle medium (DMEM), and K46 cells (kindly provided by J. Cambier, National Jewish Medical Research Center, Denver, CO) in IMDM.

Human peripheral blood lymphocytes (PBLs) were prepared from buffy coats obtained at Thomayer University Hospital (Prague, Czech Republic) using Ficoll-Paque Plus (GE Healthcare) gradient centrifugation (900 × *g* for 30 min). To isolate different PBL subpopulations, T cells, B cells, NK cells, and monocytes were labeled with CD3, CD19, CD56, or CD14 antibodies, respectively, followed by FITC-conjugated secondary antibodies and anti-FITC magnetic beads (Miltenyi Biotec), and separated by positive selection on an AutoMACS magnetic cell sorter (Miltenyi Biotec). Purity was confirmed by fluorescence-activated cell sorter (FACS) analysis. Human Peripheral blood monocyte-derived dendritic cells (MDDCs) were obtained by differentiation of monocytes in complete RPMI 1640 medium containing interleukin 4 (IL-4) (10 ng/ml; PeproTech) and granulocyte-macrophage colony-stimulated factor (GM-CSF) (100 ng/ml, Leucomax; Novartis) for 8 days. For isolation of granulocytes, a gradient composed of Histopaque 1119 (Sigma-Aldrich), overlaid by Ficoll-Paque Plus and freshly collected blood from healthy donors, diluted with phosphate-buffered saline (PBS), was prepared and centrifuged (900 × *g* for 30 min). Granulocytes were collected from the interface between Ficoll-Paque and Histopaque 1119 layers. In addition, erythrocytes were isolated from the pellet and subjected to hypotonic lysis in 0.04% acetic acid to obtain red blood cell membranes (RBC ghosts). Platelets were prepared from the top phase of the gradient by two-step centrifugation (400 × *g* for 10 min to remove cells and large particles and 600 × *g* for 10 min to pellet platelets).

Mouse organs were isolated from C57BL/6 mice (Animal Facility of IMG ASCR, Prague, Czech Republic) sacrificed by cervical dislocation. Splenic B cells were prepared by negative selection using CD43 and CD11b magnetic beads (Miltenyi Biotec).

For SCIMP expression analysis, murine organs and tissues were snap-frozen in liquid nitrogen, homogenized in grinding mortar, and lysed in 2% SDS. As an exception, splenocytes and PBLs were first incubated with ACK buffer to remove red blood cells and then directly lysed in 2% SDS. Bone marrow cells isolated from femurs and human blood cells were directly lysed in 2% SDS. All samples were centrifuged (70,000 × *g* for 30 min) to remove nuclei and debris. Protein

concentrations were measured as absorbance at 280 nm and adjusted to obtain equal protein concentrations in all samples.

Where applicable, the procedures were performed after obtaining an informed consent from the donors and in accordance with national ethical guidelines. They were also approved by the Animal Care and Use Committee of the Institute of Molecular Genetics (Academy of Sciences of the Czech Republic).

RNA and RT-qPCR. A human normal tissue FirstChoice RNA survey panel and lymph node FirstChoice RNA were purchased from Ambion. Total RNA (1 μg) was transcribed using SuperScript III reverse transcriptase (RT) (Invitrogen) or RevertAid reverse transcriptase (Fermentas) with a combination of random pentadecamer and anchored oligo(dT)$_{20}$ primers. RT-quantitative PCR (qPCR) was carried out using LightCycler 480 SYBR green I master chemistry (Roche). Primers specific for human SCIMP cDNA were as follows: fwd-1, 5′-AGTCGC CAGTTCAATTACCG-3′; rev-1, 5′-GACTTGGGGCTTCCTGTG-3′, and fwd-2, 5′-TGCTCACATATGGATACTTTCACA-3′; rev-2, 5′-GGCCACAGCT AAGATGATCC-3′. RT-qPCR data (C_T values) were normalized to reference gene GAPDH data and analyzed using the GenEx software program (MultiD).

DNA constructs, cloning, and cell transfections. Human SCIMP cDNA was cloned from a leukocyte cDNA library (Clontech Laboratories) using the primers 5′-caGGATCCgctcacatatggatactttcacag and 5′-gtGAATTCtcaaaatgatgctttttcag tatt (restriction sites are in capital letters) and ligated into BamHI/EcoRI sites of pcDNA3. Mouse SCIMP was cloned from mouse splenic cDNA using the primers: 5′-gtGAATTCtcccacagcaatgagttggtg and 5′-gtCTCGAGctaaaacgagctgtgtaa acccacttc and ligated into EcoRI/XhoI sites of pcDNA3.

For immunoprecipitation experiments, the Myc coding sequence was added after the last codon of human SCIMP and this construct was cloned into MSCV-IThy1.1 (a retroviral vector with internal ribosome entry site (IRES)-Thy1.1 sequence for simultaneous expression of the Thy1.1 surface reporter; kindly provided by P. Marrack, National Jewish Health, Denver, CO). Y69F, Y107F, Y124F, and Y131F tyrosine mutants were prepared using PCR mutagenesis. The proline-rich sequence deletion mutant lacking aa 81 to 104 was prepared by fusion PCR. To obtain SCIMP-green fluorescent protein (GFP), SCIMP was cloned into pEGFP-N3 (Clontech Laboratories), and SCIMP-GFP sequence was subsequently ligated into MSCV-IThy1.1. CD25-SCIMP chimeras were prepared by cloning the sequence of extracellular part of human CD25 (coding aa 1 to 240), followed by sequence of SCIMP (coding aa 13 to 145, with or without a C-terminal Myc tag) into MSCV-IThy1.1. All constructs were sequenced.

The following constructs were used for cotransfection experiments in HEK293: Lyn in pcDNA3 (kindly provided by S. Watson, University of Birmingham, Birmingham, United Kingdom), c-Src in pSM (kindly provided by D. Littman, New York University School of Medicine), FynT in pEF-BOS (kindly provided by B. Schraven, Otto-von-Guericke University, Magdeburg, Germany), and Syk in pRK5 (kindly provided by W. Kolanus, University of Cologne, Cologne, Germany).

The Lipofectamine 2000 reagent (Invitrogen) was used according to the manufacturer's instructions for transfection of both HEK293FT (cotransfection studies) and Phoenix Ampho cells (production of viral particles using the MSCV-IThy1.1 vector). Retrovirus-containing supernatants were supplemented with Polybrene (10 μg/ml; Sigma-Aldrich) and added to cells. Cells were then centrifuged at 1,250 × *g* for 90 min. Infected cells were labeled with anti-Thy1.1-FITC antibody followed by anti-FITC magnetic beads and isolated on an AutoMACS cell sorter.

Cell activation. Cells (5×10^7 cells/ml) were stimulated with 1 mM pervanadate for 20 min or with the required antibody (10 μg/ml) for 2 min or as indicated in serum-free medium at 37°C. In the case of MHC-II cross-linking, Fc receptors were blocked by incubation with 2.4G2 antibody on ice. Primary biotinylated antibody (10 μg/ml) was then added for 30 min, cells were washed and resuspended in warm medium, and streptavidin (10 μg/ml; Jackson ImmunoResearch) was added after 5 min. Cells were incubated at 37°C as appropriate. Samples were solubilized in lysis buffer (for immunoprecipitation studies, see below) or in SDS-PAGE sample buffer (for analysis of signaling pathways by phospho-specific antibodies).

Biochemical procedures. Lysis with detergents was carried out by solubilization of 5×10^7 cells in 1 ml of cold lysis buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1 mM Pefabloc [Sigma-Aldrich], 5 mM iodoacetamide, 50 mM NaF, 1 mM Na_3VO_4, 10 mM EDTA, 10% [vol/vol] glycerol) supplemented with 1% (wt/vol) detergent {laurylmaltoside, Brij 98, NP-40, or 3-[(3-cholamidopropyl)-dimethylammonio]-1- propanesulfonate [CHAPS], as required} for 30 min. Nuclei and debris were removed by centrifugation (16,000 × *g* for 10 min).

For immunoprecipitation, detergent lysates were incubated for 1 h with primary antibody (500× diluted), followed by 2 h of incubation with protein A/G Plus-agarose beads (Santa Cruz Biotechnology). When preclearing was used, this procedure was performed first with the control and then repeated with specific antibodies. After washing, immunoprecipitates were eluted with 60 μl SDS-PAGE sample buffer.

For flotation in sucrose density gradient, 0.5-ml aliquots of cell lysates (without glycerol) were mixed with 0.5 ml 80% (wt/vol) sucrose in lysis buffer in an ultracentrifuge tube and overlaid with 3.5 ml 35% sucrose and finally 0.5 ml of lysis buffer. Samples were subjected to ultracentrifugation (50,000 × *g* for 20 h), and 0.6-ml fractions were collected from the top of the gradient.

For gel filtration, 0.2-ml aliquots of detergent lysate were separated on a column containing 2 ml of Sepharose 4B and continually washed with lysis buffer with the corresponding detergent, and 0.2-ml fractions were collected.

For cell fractionation, 9×10^7 cells were resuspended in 0.5 ml of cold hypotonic buffer (10 mM HEPES [pH 7.4], 42 mM KCl, 5 mM $MgCl_2$, 1 mM Pefabloc, 5 mM iodoacetamide, 50 mM NaF, 1 mM Na_3VO_4, 10 mM EDTA) and mechanically disintegrated by forcing the cell suspension 10 times through a 25-gauge injection needle. The homogenate was centrifuged at 400 × *g* for 10 min to pellet the nuclear fraction, followed by centrifugation at 25,000 × *g* for 10 min to pellet the membrane fraction. The resulting supernatant contained the cytoplasmic fraction.

An acyl-biotinyl exchange reaction was carried out as previously described (48). Isolated plasma membrane fraction proteins from 5×10^7 cells were incubated with *N*-ethylmaleimide to block free thiols. The samples were then incubated with hydroxylamine to remove palmitate residues (this step was omitted with control samples), and the resulting free thiol groups were labeled with biotin-HPDP (Pierce, Thermo Fisher Scientific). Biotinylated proteins were immunoprecipitated on streptavidin agarose beads and eluted with SDS-PAGE sample buffer.

Calcium flux measurement. Cells were loaded with 2 μg/ml calcium indicator dye Fluo-4 (Invitrogen). Samples were analyzed by using a FACSCalibur flow cytometer for 30 s at rest and then another 3 min after activation with CD25 antibody (10 μg/ml). The calcium response index was calculated as the percentage of cells with Fluo-4 fluorescence higher than the 95th percentile of resting cells during the time interval between 10 and 20 s. Data were analyzed using the FlowJo software program (TreeStar).

Microscopy. For live cell microscopy, Ramos or Daudi B cells expressing SCIMP-GFP were loaded with staphylococcal enterotoxin E (SEE) (1 μg/ml; Toxin Technology) and transferred into Lab-Tek chambered cover glass (Nunc; Thermo Fisher Scientific), and DDAO (Molecular Probes, Invitrogen)-labeled Jurkat T cells were subsequently added at a ratio of 1:1. Cells were observed in a climate chamber (37°C, 5% CO_2) under a Leica DMI6000 B fluorescence microscope using a 40× objective lens (Leica Microsystems), and images were acquired every 30 s. Eventually, the percentage of conjugates with SCIMP-GFP localized at the IS was assessed after 1 h.

For antibody staining, the spontaneously polarized Ramos cells or SEE-induced conjugates were fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 0.1% (wt/vol) Triton X-100, and blocked in PBS with 0.5% bovine serum albumin (BSA), 5% human AB serum, and 5% goat serum. After staining with primary and Alexa Fluor 568-conjugated secondary antibodies, the samples were mounted in Mowiol medium and observed with a Leica TCS SP5 confocal microscope using a 63× objective lens (Leica Microsystems). Data were analyzed using the LAS AF software program (Leica Microsystems).

Lentiviral shRNA-mediated knockdown. The vector pLKO.1, containing small-hairpin RNA (shRNA) sequences against SCIMP (shRNA 1, TRCN0000267281; shRNA 2, TRCN0000267278; shRNA 3, TRCN0000283513), and empty vector were purchased from Sigma, mixed with ViraPower packaging mix (Invitrogen) according to the manufacturer's instructions, and transfected to HEK293FT using the Lipofectamine 2000 reagent. Virus-containing supernatants were concentrated with PEG-it virus precipitation solution (System Biosciences) supplemented with Polybrene (10 μg/ml) and added to the K46 cell line. Cells were cultured in the presence of puromycin (6 μg/ml; InvivoGen). Stable clones obtained were analyzed for expression of SCIMP. For immunoblot analysis of signaling defects, the Odyssey infrared imaging system (LI-COR Biotechnology) was used.

RESULTS

SCIMP identification and primary structure. To identify new candidate proteins that could recruit Csk to the proximity of its substrates at the plasma membrane, we searched the Uniprot protein database for transmembrane proteins that could potentially interact with the Csk Src homology 2 (SH2) domain. In addition to the well-established Csk-binding partners, including

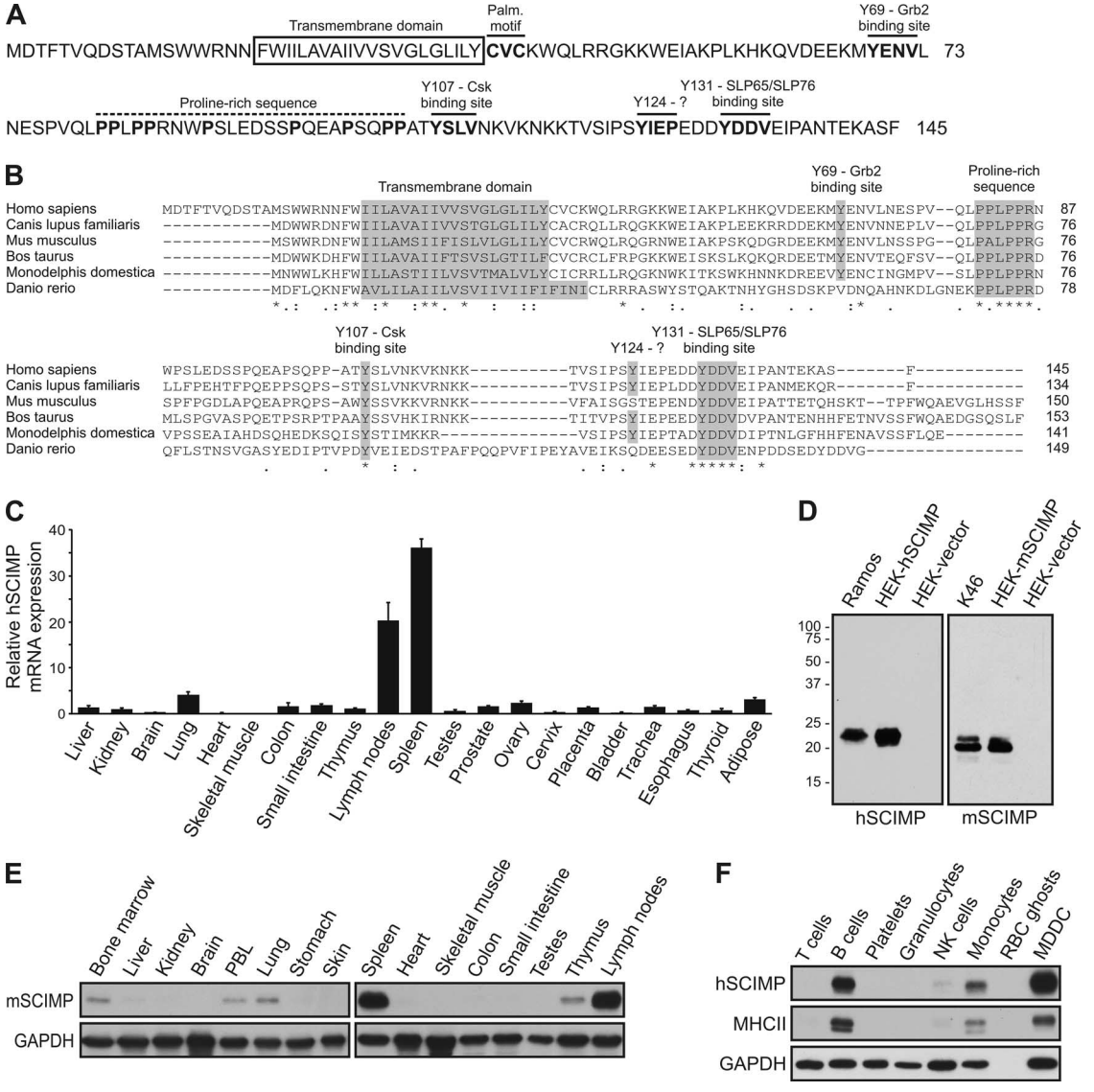

FIG. 1. SCIMP is a transmembrane adaptor protein expressed in antigen-presenting cells. (A) Sequence of human SCIMP. Positions of the transmembrane domain, palmitoylation motif, proline-rich sequence, and predicted Grb2, Csk, and SLP65/SLP76 binding sites are indicated. (B) Comparison of SCIMP sequences from human (*Homo sapiens*), dog (*Canis lupus familiaris*), mouse (*Mus musculus*), cattle (*Bos taurus*), opossum (*Monodelphis domestica*), and zebrafish (*Danio rerio*). The transmembrane domain, highly conserved part of the proline-rich sequence and SLP65/SLP76 binding motif, as well as other conserved tyrosine residues, are highlighted, and positions of corresponding tyrosines in human sequence are marked. Respective GenBank accession numbers are: NP_996986, XP_849038, NP_001038991, XP_001251000, and XP_001377777. Sequence of *Danio rerio* SCIMP was deduced from two overlapping expressed sequence tag (EST) sequences, EB765140 and EB768085 (C) RT-qPCR analysis of SCIMP mRNA expression in human tissues. Average expression in all tissues corresponds to value 1 on the vertical axis. Data are presented as the mean of four experiments (± SD). (D) Western blot reactivity of anti-SCIMP antibodies on lysates from nontransfected B cell lines Ramos (human) and K46 (murine) or from HEK293 cells transfected with human (hSCIMP) or murine SCIMP (mSCIMP) constructs or empty vector. hSCIMP was stained with mouse monoclonal antibody (NVL-07) and mSCIMP with rabbit antiserum to mSCIMP. (E) SCIMP expression in mouse tissues and peripheral blood leukocytes (PBLs), analyzed by immunoblotting. (F) SCIMP and MHC-II expression in isolated human blood leukocyte subsets and in monocyte-derived dendritic cells (MDDC), analyzed by immunoblotting. GAPDH serves as a loading control; data are representatives of two independent experiments (E and F).

PAG and LIME, we identified an unknown polypeptide (145 aa long in humans), termed c17orf87. As mentioned above, we renamed this protein SCIMP. It possesses features typical of TRAPs. A short leaderless extracellular domain is followed by a predicted transmembrane domain, a potential palmitoylation (S-acylation) motif (CxC), and a larger cytosolic tail containing four potential tyrosine phosphorylation sites that may interact with SH2 domains (Fig. 1A). A putative binding site for Csk is represented by Y_{107}SLV sequence. Y_{69}ENV sequence is a typical binding site for Grb2 family adaptor proteins (19, 35). Y_{131}DDV sequence is a predicted motif for binding to the adaptor proteins SLP65 and SLP76 (17, 44). In addition, SCIMP contains a proline-rich sequence, a likely binding site for Src homology 3 (SH3) domains (32) (Fig. 1A).

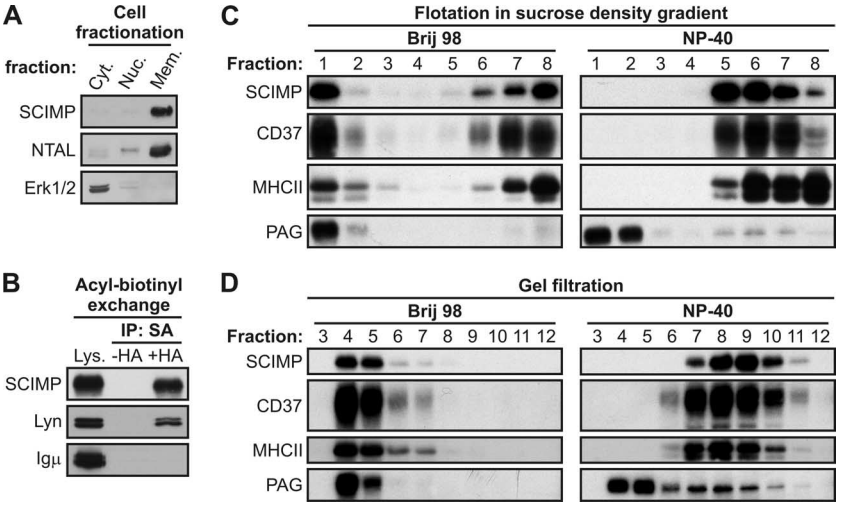

FIG. 2. SCIMP is a palmitoylated transmembrane protein present in Brij 98- but not NP-40-resistant membrane microdomains. (A) Fractionation of Ramos cells into cytoplasmic (Cyt.), nuclear (Nuc.), and membrane (Mem.) fractions, followed by SCIMP immunoblotting. NTAL and Erk2 staining served as controls for membrane and cytoplasmic fractions, respectively. (B) Acyl-biotinyl exchange reaction performed on lysates (Lys.) from membrane fractions of Ramos cells. Non-hydroxylamine-treated samples (−HA) served as a negative control, while HA treatment (+HA) allowed exchange of palmitate for biotin moiety and isolation of palmitoylated proteins on immobilized streptavidin. Lyn and Igμ staining, respectively, served as positive and negative controls for specificity of isolation. (C and D) Brij 98 or NP-40 lysates from Ramos cells were fractionated by flotation in sucrose density gradient (C) or by gel filtration on Sepharose 4B (D). Samples were analyzed for distribution of SCIMP, CD37, MHC-II, and PAG. Data are representative of two independent experiments.

To detect evolutionarily conserved regions in SCIMP, we employed the Basic Local Alignment Search Tool (BLAST) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to search for SCIMP homologues from different vertebrate species in GenBank nucleotide and protein databases, including genomic and expressed sequence tag (EST) collections. We identified SCIMP in a number of mammals and in zebrafish. The alignment of a representative selection of SCIMP sequences revealed that the SCIMP typical domain organization of a TRAP is conserved throughout evolution (Fig. 1B). Only two of the predicted protein interaction motifs were preserved in all species analyzed: the putative SLP65/SLP76 interaction motif (duplicated in zebrafish) and the proline-rich sequence.

Expression of SCIMP in antigen-presenting cells. We investigated the expression of human SCIMP in various tissues at the mRNA level and observed the strongest expression in lymph nodes and spleen (Fig. 1C), whereas only a negligible amount of SCIMP mRNA was detected in the majority of nonimmune system tissues.

Subsequently, we generated both mouse monoclonal antibodies and rabbit antisera against the cytoplasmic part of human SCIMP, as well as rabbit antisera against the cytoplasmic part of mouse SCIMP, which detected an endogenous protein with an apparent molecular mass of ~20 kDa in murine and human B cell lines (Fig. 1D and data not shown). The size is somewhat higher than that predicted from SCIMP sequence (17 kDa). However, the same mobility was also observed for SCIMP expressed from the cDNA construct in HEK293 cells, suggesting unusual electrophoretic mobility of this polypeptide (Fig. 1D). We used the antibodies to analyze expression of SCIMP at the protein level in mice. Western blot analysis of various organs and tissues revealed an expression pattern similar to that observed before at the mRNA level in human tissues. A large amount of the SCIMP protein was detected in the spleen and lymph nodes and a low level in other tissues of the immune system, including bone marrow, peripheral blood leukocytes (PBLs), and thymus; a lack of SCIMP expression was again confirmed in the majority of nonimmune tissues, with the exception of lung (Fig. 1E).

To assess SCIMP expression in individual leukocyte subpopulations, we purified different leukocyte subsets from human blood. Peripheral blood monocyte-derived dendritic cells (MDDCs) were also included. Interestingly, we observed a significant amount of SCIMP just in professional APCs, namely, B cells, monocytes and MDDCs, whereas only negligible to no amounts of SCIMP were detected in other cell types. Accordingly, the expression of SCIMP correlated with that of MHC-II in these samples (Fig. 1F).

Subcellular distribution and palmitoylation of SCIMP. The SCIMP sequence contains a predicted transmembrane domain followed by a potential palmitoylation motif. Indeed, the majority of SCIMP was recovered from the membrane fraction of Ramos B cells (Fig. 2A). Moreover, using an acyl-biotinyl exchange assay (48), a method based on the exchange of palmitoyl residues in proteins for the biotin moiety, we demonstrated that SCIMP is palmitoylated (Fig. 2B), likely at one or both of the cysteines within the potential submembrane palmitoylation motif, since these are the only cysteines in the human SCIMP sequence. It should be noted that this method cannot distinguish between palmitoylation and other, much rarer types of protein S-acylation involving different fatty acyl residues. On the other hand, it detects neither N-terminal myristoylation nor C-terminal prenylation. However, for these modifications, SCIMP does not contain consensus sequences.

Palmitoylation is one of the prerequisites for protein targeting to membrane microdomains, such as lipid rafts. One of the characteristic features of these domains is their resistance to solubilization in particular detergents (8). To verify whether

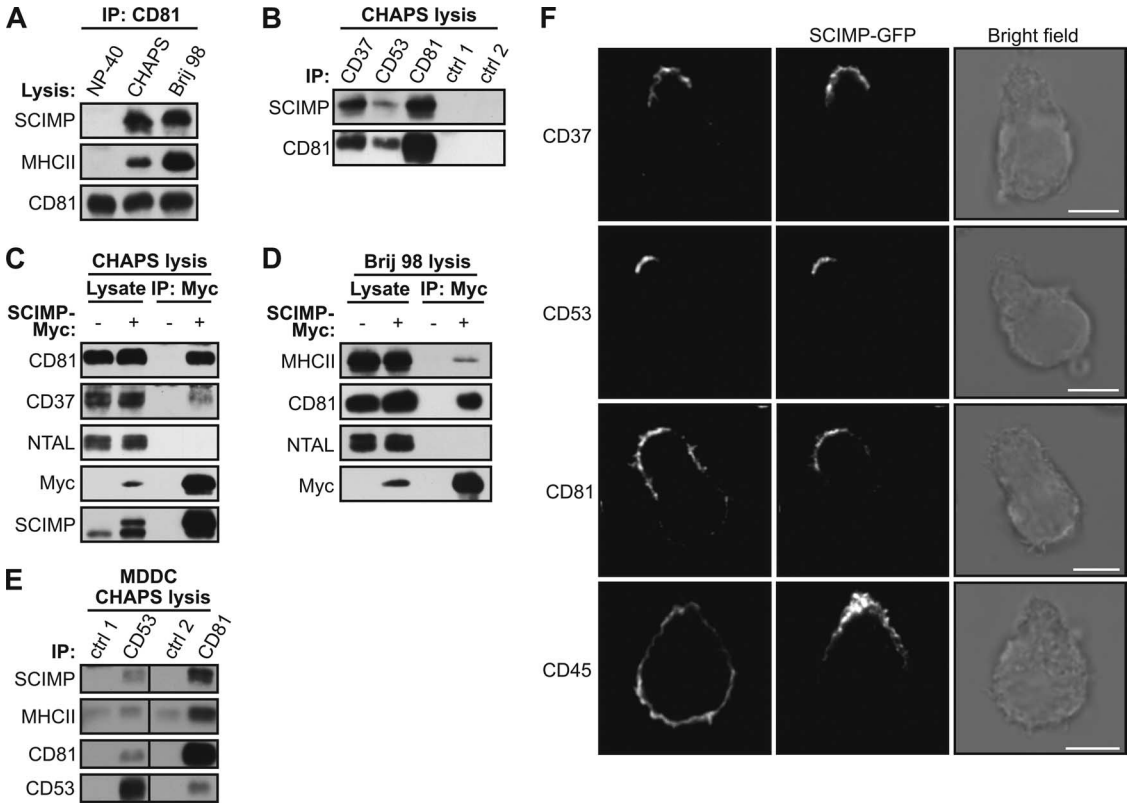

FIG. 3. Localization of SCIMP in tetraspanin-enriched microdomains. (A) NP-40, CHAPS, or Brij 98 lysates from Ramos cells were immunoprecipitated with anti-CD81 antibody, and samples were stained for SCIMP, MHC-II, and CD81. (B) CHAPS lysates of Ramos cells were immunoprecipitated with anti-CD37, anti-CD53, anti-CD81, or isotype control antibodies, followed by staining for SCIMP and CD81. (C and D) CHAPS (C) or Brij 98 (D) lysates of Ramos cells transfected with SCIMP-Myc or empty vector were immunoprecipitated with anti-Myc antibody, and samples were stained as indicated. (E) CHAPS lysates of human monocyte-derived dendritic cells (MDDC) immunoprecipitated with isotype control antibodies, followed by immunoprecipitation with anti-CD53 or anti-CD81, were stained for SCIMP, MHC-II, CD81, and CD53. (F) Spontaneously polarized Ramos cells transfected with SCIMP-GFP were stained for CD37, CD53, CD81, or control CD45 and analyzed by confocal microscopy. Scale bar = 5 μm. Data are representative of two (A and F) or three (B, C, and D) independent experiments.

SCIMP is localized in lipid rafts, we solubilized Ramos cells in Brij 98- or NP-40-containing lysis buffers and fractionated the resulting lysates using flotation in a sucrose density gradient. About half of SCIMP was present in the low-density fractions when a mild detergent, Brij 98, was used, but upon solubilization in the more stringent detergent NP-40, SCIMP was recovered only from bottom fractions of the gradient (Fig. 2C). This was in marked contrast to the raft resident protein PAG (6), which was recovered from low-density fractions in both detergents, demonstrating that SCIMP is not localized in classical lipid rafts determined to be microdomains resistant to TX-100-related detergents, such as NP-40. Interestingly, tetraspanin CD37, as well as tetraspanin-associated MHC-II (3), exhibited a behavior similar to that of SCIMP (Fig. 2C). Moreover, upon fractionation of cell lysates by gel filtration on Sepharose 4B, SCIMP was recovered in large detergent-resistant complexes upon lysis in Brij 98 but was completely solubilized in NP-40. Again, this behavior was markedly different from that of NP-40-resistant PAG containing microdomains but resembled the behavior of tetraspanin CD37 and of MHC-II (Fig. 2D).

Association of SCIMP with TEMs. Similar solubility profiles of SCIMP and tetraspanin proteins led us to hypothesize that SCIMP could be a constituent of TEMs (49). To test this idea, we performed a series of immunoprecipitation experiments. First we lysed Ramos cells in buffers containing Brij 98, CHAPS, or NP-40 detergents and immunoprecipitated tetraspanin CD81. Upon solubilization of cells in Brij 98 or CHAPS, detergents that preserve TEMs (4, 42), we detected an interaction between CD81 and both MHC-II and SCIMP (Fig. 3A). These associations were completely lost upon solubilization in the tetraspanin-disrupting detergent NP-40. Furthermore, SCIMP coimmunoprecipitated with CD37, CD53, and CD81 after solubilization of cells in CHAPS (Fig. 3B). We also detected an interaction of CD81 with CD37 and CD53, proving that the integrity of TEMs was not compromised.

Next, we prepared Ramos cells virally infected with a vector coding for Myc-tagged SCIMP (SCIMP-Myc) or an empty vector. Upon solubilization of cells in CHAPS, it was possible to specifically coimmunoprecipitate CD37 and CD81 with SCIMP-Myc (Fig. 3C); however, no interaction with a lipid raft marker, NTAL (25), was observed; this further confirms that SCIMP is not a component of lipid rafts (Fig. 3C). When the same experiment was performed with Brij 98 cell lysates, a weak interaction was detectable between SCIMP-Myc and MHC-II, but the binding of SCIMP-Myc to CD81 was much stronger (Fig. 3D). This suggests that while the interaction with

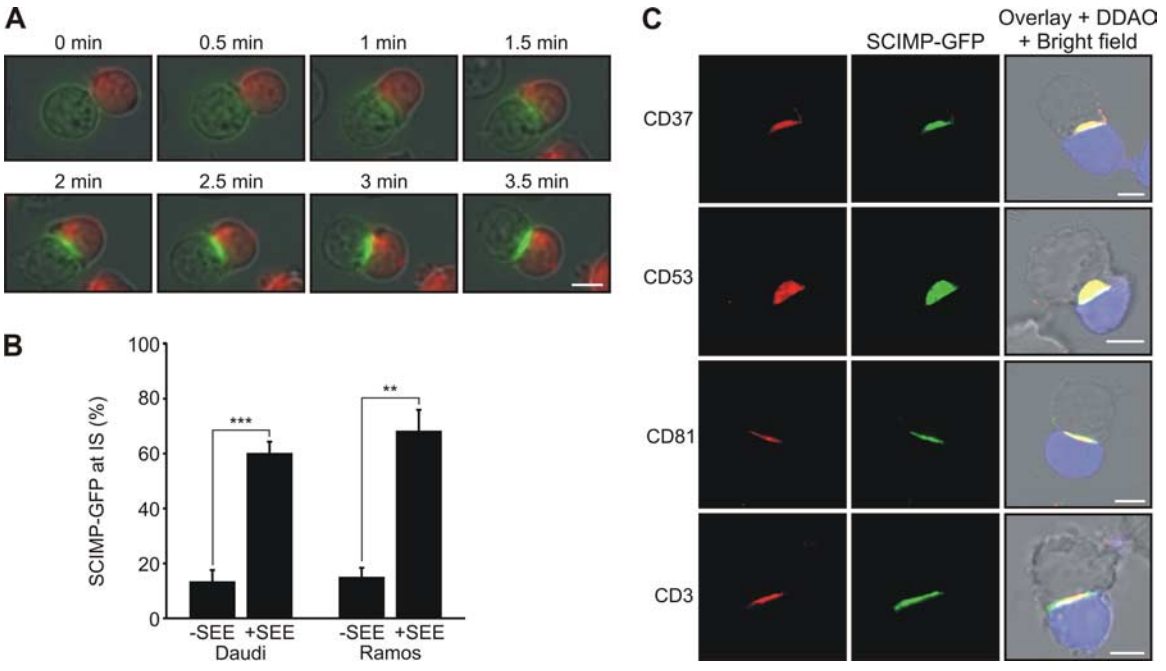

FIG. 4. Translocation of SCIMP into immunological synapse. (A) Ramos cells transfected with SCIMP-GFP (green) were loaded with superantigen, and subsequently Jurkat T cells (labeled by DDAO in red) were added. Immunological synapse formation was observed by live cell imaging. (B) Daudi or Ramos cells transfected with SCIMP-GFP were loaded (+SEE) or not (−SEE) with superantigen, and Jurkat cells were added. The percentage of cells with SCIMP-GFP translocated to the cell-cell contact is shown. Results are means ± SD from three independent experiments; at least 100 conjugates were analyzed in each experiment. **, $P < 0.01$; ***, $P < 0.001$ (two-tailed Student test with assumed nonequal variances). (C) Ramos cells transfected with SCIMP-GFP were loaded with superantigen and mixed with Jurkat T cells labeled with DDAO (in blue). After IS formation, cells were fixed and stained for CD37, CD53, CD81, or CD3 and analyzed by confocal microscopy. Scale bar = 5 μm. Data are representative of two independent experiments.

some of the tetraspanins may be direct, binding to MHC-II is likely to be indirect, perhaps mediated by members of the tetraspanin family. Finally, we also could coimmunoprecipitate both SCIMP and MHC-II with CD37 and CD81 from CHAPS lysates of human MDDC, thus proving that this interaction can also be detected in primary cells (Fig. 3E).

Tetraspanins and tetraspanin-associated proteins have been observed in the uropod of migrating cells (43). To characterize the localization of SCIMP in polarized cells, we transfected the Ramos cell line with SCIMP fused to C-terminal GFP. Interestingly, a fraction of Ramos cells displayed a migratory morphology with a well-distinguished leading edge and uropod. In these cells, a vast majority of SCIMP colocalized with tetraspanins CD37, CD53, and CD81 in the uropod (Fig. 3F). Together these results provide strong evidence at biochemical and microscopic levels for an association of SCIMP with TEMs.

SCIMP localization to IS. Thus far, we have demonstrated that SCIMP is a part of MHC-II-containing TEMs. Some of the tetraspanins have been shown to partition to the IS between the APC and the T cell (34). To find out whether SCIMP may also localize to the IS, we used Ramos and Daudi B cell lines expressing SCIMP-GFP as APCs in a superantigen staphylococcal enterotoxin E (SEE)-based assay. Live-cell imaging of superantigen-induced conjugates between SCIMP-GFP-expressing Ramos or Daudi cells and the Jurkat T cell line revealed that early after conjugate formation, SCIMP was rapidly translocated into the IS (Fig. 4A and B; see also video S1 in the supplemental material). Similar behavior was also observed for several tetraspanin molecules, including CD37, CD53, and CD81 (Fig. 4C). These studies strongly suggest a connection between SCIMP and processes originating at the APC side of the IS.

Phosphorylation of SCIMP upon MHC-II cross-linking. Expression of SCIMP in APCs and localization in TEMs together with MHC-II and clustering of SCIMP at the IS implied that SCIMP may be involved in signaling pathways resulting from engagement of MHC-II molecules during antigen recognition. To determine whether the engagement of MHC-II molecules could possibly influence SCIMP phosphorylation, we therefore isolated mouse splenic B cells, stimulated them with either anti-BCR or anti-MHC-II antibodies, and subsequently isolated SCIMP via immunoprecipitation. Compared to BCR activation, MHC-II molecule cross-linking induced only a mild increase in overall tyrosine phosphorylation (Fig. 5A). However, stimulation with anti-MHC-II led to a marked increase in SCIMP tyrosine phosphorylation, whereas BCR activation had no effect on phosphorylation of SCIMP (Fig. 5B).

After MHC-II stimulation, both Src and Syk family kinases are activated (1). To determine the kinases responsible for phosphorylation of SCIMP, we cotransfected HEK293 cells with Syk, Lyn, Src, Fyn, or empty vector together with either SCIMP or NTAL. In contrast to the established Syk substrate NTAL (25), SCIMP was most strongly phosphorylated by Lyn and Src, while Syk had no effect (Fig. 5C). Based on these findings, we conclude that Src, but not Syk, family kinases are

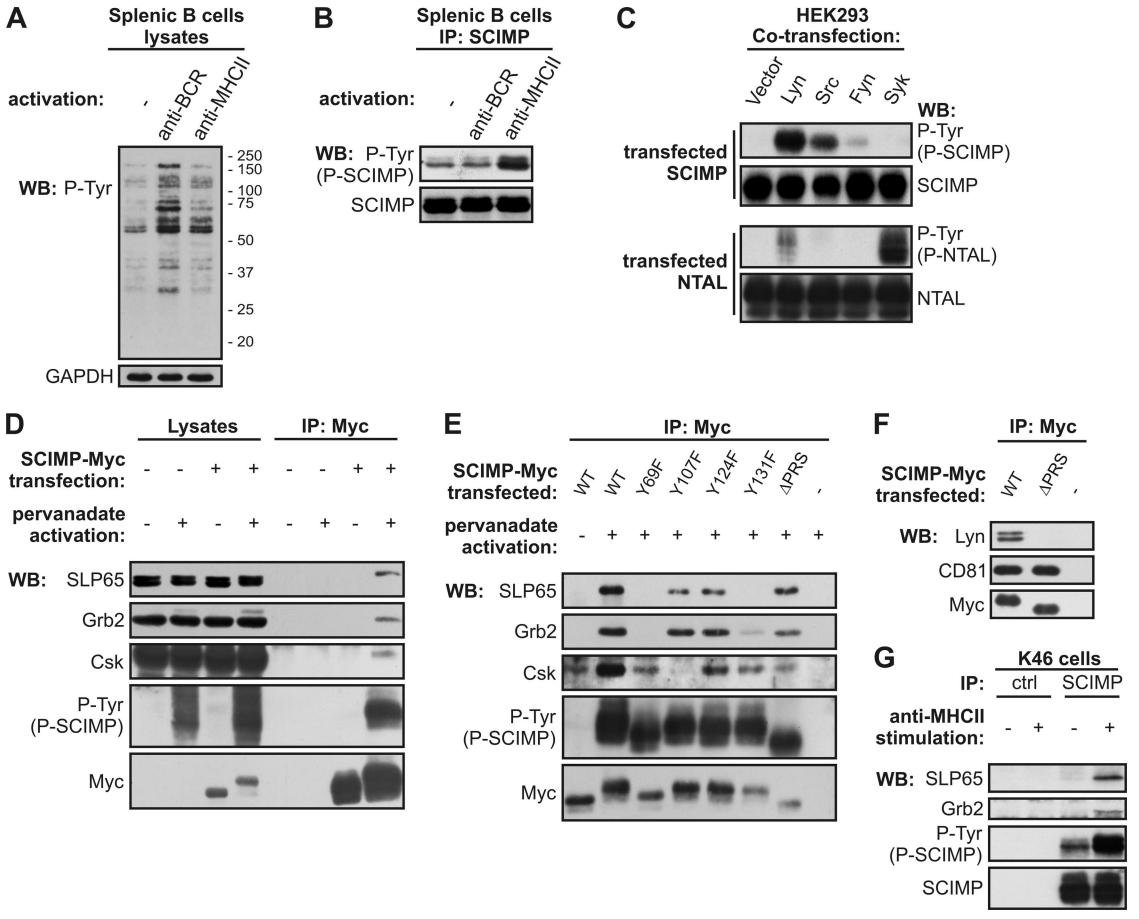

FIG. 5. SCIMP is phosphorylated upon MHC-II stimulation and binds Grb2-SLP65 complex, Csk, and Lyn. (A) Isolated splenic B cells were left untreated (−) or activated with anti-BCR or anti-MHC-II antibodies for 2 min and lysed in laurylmaltoside, and whole-cell lysates were analyzed by antiphosphotyrosine staining. GAPDH served as a loading control. (B) Isolated splenic B cells were treated as for panel A, SCIMP was immunoprecipitated, and samples were probed for phosphorylated SCIMP (P-Tyr) or for total SCIMP. (C) HEK293 cells were cotransfected with SCIMP or NTAL together with either empty vector or plasmids coding for Lyn, Src, Fyn, or Syk kinases. Phosphorylation of SCIMP or NTAL was analyzed by antiphosphotyrosine staining. (D) Ramos cells transfected with SCIMP-Myc or empty vector were stimulated or not with pervanadate and lysed in laurylmaltoside, and SCIMP-Myc was immunoprecipitated. Samples were probed with SLP65, Grb2, Csk, Myc, and phosphotyrosine (P-Tyr) antibodies. (E) Ramos cells transfected with either wild-type (WT) SCIMP-Myc, SCIMP-Myc with indicated tyrosines mutated to phenylalanines, or SCIMP-Myc containing deletion of proline-rich sequence (ΔPRS) were treated as for panel D. (F) Ramos cells transfected with WT SCIMP-Myc or ΔPRS SCIMP-Myc were lysed in Brij 98, and SCIMP-Myc was immunoprecipitated. Samples were stained for Myc, Lyn, and CD81. (G) K46 cells were left untreated or activated with anti-MHC-II antibody, lysed in laurylmaltoside, and immunoprecipitated with antisera to SCIMP or preimmune control sera. Samples were probed for phosphorylated SCIMP using antiphosphotyrosine antibody and for total SCIMP, SLP65, and Grb2. Data are representatives of two (A, B, and C) or three (D to G) independent experiments.

likely to be responsible for the phosphorylation of SCIMP upon MHC-II cross-linking.

SCIMP interaction with SLP65/SLP76, Grb2, Csk, and Lyn. Phosphorylated SCIMP may serve as a binding platform for SH2 domains of several proteins, as deduced from sequence analysis. To verify the predicted binding partners, we first induced strong protein phosphorylation by treating SCIMP-Myc-transfected Ramos cells with pervanadate (PV), a potent inhibitor of protein tyrosine phosphatases, and lysed the cells with a relatively stringent detergent, laurylmaltoside. Subsequently, SCIMP-Myc was immunoprecipitated with anti-Myc antibody. Upon PV treatment, SCIMP became strongly phosphorylated, and we could easily detect interactions of SCIMP with the SLP65, Grb2, and Csk proteins in these immunoprecipitates (Fig. 5D). Interestingly, even though SLP65 is expressed in two isoforms, SCIMP interacted only with the longer form, which contains a binding site for the SH3 domain of Grb2 (18). This implies that binding of SLP65 to SCIMP may require the formation of a trimolecular complex involving Grb2.

In order to identify the phosphorylation sites responsible for binding of these signaling molecules, we mutated all individual tyrosines in the intracellular part of SCIMP-Myc to phenylalanines. In addition, we also deleted the proline-rich sequence between aa 81 and 104. We treated transfected cells with PV and immunoprecipitated SCIMP-Myc. As expected, mutation of Y107 led to the loss of Csk binding capacity (Fig. 5E). Moreover, mutation of either of the predicted binding sites for Grb2 and SLP65, Y69 and Y131, respectively, resulted in a simultaneous loss of binding of both adaptors to SCIMP (Fig. 5E). These studies suggest a level of cooperativity in the interaction among SCIMP, SLP65, and Grb2. It was also possible to

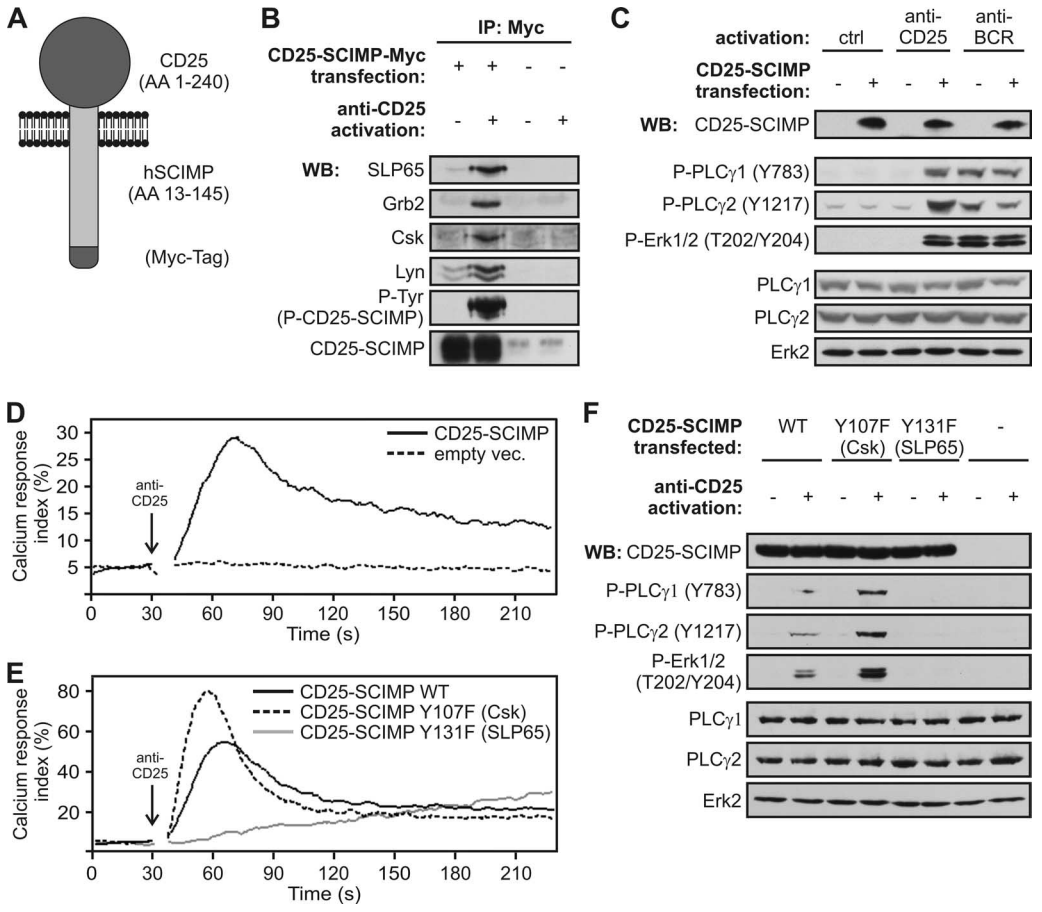

FIG. 6. Cross-linking of SCIMP initiates signaling events. (A) Schematic representation of CD25-SCIMP chimera containing or not containing a Myc tag. (B) Raji cells transfected with CD25-SCIMP-Myc or empty vector were activated (or not) with anti-CD25 antibody, lysed in laurylmaltoside, immunoprecipitated with anti-Myc antibody, and stained with SLP65, Grb2, Lyn, Csk, SCIMP, and phosphotyrosine antibodies (band corresponding to CD25-SCIMP is shown). (C) Raji cells transfected with CD25-SCIMP or empty vector were activated for 5 min or not by anti-CD25 or anti-BCR antibody, and samples were stained as indicated. (D) Calcium response of Raji cells transfected with CD25-SCIMP or empty vector after CD25 cross-linking. (E) Calcium response after CD25 cross-linking in Raji cells transfected with a CD25-SCIMP-Myc chimera containing either WT sequence of SCIMP or SCIMP with indicated tyrosine-to-phenylalanine mutations (Y107F [Csk binding site] or Y131F [SLP65 binding site]). (F) Raji cells transfected with CD25-SCIMP-Myc chimeric constructs coding for WT or tyrosine mutants of SCIMP or with empty vector were activated with anti-CD25 antibodies and stained as indicated. Data (B to F) are representative of two independent experiments.

detect an interaction of SCIMP with the Src family kinase Lyn after cell solubilization in a mild detergent, Brij 98 (Fig. 5F). The constitutive nature of this association implies that it might be based on interactions between the proline-rich sequence of SCIMP and the SH3 domain of Lyn. Indeed, deletion of the proline-rich sequence of SCIMP abolished this association (Fig. 5F). Similar interactions were also observed in the myeloid cell line THP1, except that SLP76 was the predominant member of the SLP adaptor family detected in SCIMP immunoprecipitates (not shown).

Finally, these data were confirmed in an experiment with endogenous proteins from nontransfected cells using only MHC-II stimulation. Murine B cell line K46, a well-established model for MHC-II signaling (31), was stimulated with anti-MHC-II antibody, and SCIMP was immunoprecipitated from the corresponding cell lysates. SLP65 and Grb2 coimmunoprecipitated with SCIMP (Fig. 5G), but no Csk or Lyn could be detected in these samples. Since SCIMP is a very small protein, it is possible that our anti-SCIMP antibodies competed with Csk and Lyn for binding to SCIMP.

Cross-linking of CD25-SCIMP fusion protein results in multiple signaling events. The results indicate that SCIMP may be involved in signal transduction events initiated by MHC-II/SCIMP aggregation at the IS. MHC-II glycoproteins are known to trigger multiple signaling pathways employing a range of coreceptors and adapter molecules. Thus, it was essential to find out which of these pathways can be directly regulated by SCIMP and can be attributed to its association with SLP family adaptors and other binding partners. To address this, we prepared a fusion construct composed of the extracellular portion of human CD25 followed by the human SCIMP sequence (starting at S_{13}) and a Myc tag for immunoprecipitation studies at the C terminus (Fig. 6A). Transduction of the CD25-negative B cell line Raji with this construct resulted in surface expression of the CD25-Nvl chimera at an expression level comparable to that of endogenous SCIMP in

these cells (not shown). We cross-linked the CD25-SCIMP fusion protein on the surface of these cells by CD25-specific antibody. Immunoprecipitation of CD25-SCIMP then revealed a significant increase in CD25-SCIMP phosphorylation and also induced an association with Csk, SLP65, and Grb2 and surprisingly with Lyn as well (Fig. 6B).

Since aggregation of SCIMP led to its association with potential effector molecules, we decided to find out whether it would also initiate the downstream effector pathways. After CD25-SCIMP cross-linking, we observed a strong increase in phosphorylation of PLC-γ1 and PLC-γ2 (Fig. 6C), accompanied by an intracellular calcium increase (Fig. 6D). Moreover, when looking at further downstream events, we detected a significant increase in phosphorylation of the MAPK Erk1/2, comparable to the levels of phosphorylation induced by BCR (Fig. 6C).

In order to specify which of the SCIMP-associated molecules are involved in the regulation of these events, we generated CD25-SCIMP-Myc chimeras where tyrosines Y107 (Csk binding site) and Y131 (SLP65 binding site) were mutated to phenylalanines, and we transfected these constructs into Raji cells at similar expression levels (not shown). Strikingly, mutation of the SLP65 binding site almost completely abolished the calcium response downstream of CD25-SCIMP chimeras, while mutation of the Csk binding site had a strong enhancing effect (Fig. 6E). In agreement with this result, no increase in phosphorylation of PLC-γ1, PLC-γ2, or Erk1/2 could be detected after cross-linking of the chimeras with the mutated SLP65 binding site. In contrast, hyperphosphorylation of these molecules was observed after mutating the tyrosine responsible for binding to Csk (Fig. 6F). These data strongly suggest that SCIMP can initiate and propagate signaling by recruiting SLP65 and that it is regulated via the recruitment of Csk.

SCIMP is required for sustained Erk signaling upon MHC-II stimulation. All the data obtained so far indicate that SCIMP is directly involved in propagation of signaling from MHC-II molecules, mainly by recruiting the SLP65 adaptor protein. However, this adaptor also binds to Igα/β heterodimers (38) known to be involved in MHC-II signal transduction, and there may also be other, less well defined mechanisms of its membrane recruitment dependent on its N-terminal region. In order to find out if there is any nonredundant role for SCIMP and SCIMP-associated SLP65 in MHC-II signal transduction, we infected the K46 murine B cell line with lentiviruses carrying vectors coding for mouse SCIMP-specific shRNAs. Infections with viruses coding shRNA 1 and shRNA 3 resulted in a more than 90% downregulation of SCIMP, while the vector coding nonsilencing shRNA 2 and the empty vector served as negative controls (Fig. 7A and B). Two clones were selected from each transfected cell line to avoid the risk of clonal bias. After activation of these cell lines via MHC-II molecule cross-linking, no effect of SCIMP downregulation on calcium response was observed (not shown). On the other hand, sustained activity of the Erk pathway was strongly affected by SCIMP deficiency. Only a transient Erk1/2 phosphorylation was detectable in SCIMP-deficient cell lines, with a marked reduction at the 10-min time point (Fig. 7A and B). Importantly, this effect was observed across the entire Erk pathway, since other components of this pathway, MEK1/2 (activator of Erk1/2) and p90RSK (Erk1/2 downstream target), were similarly affected (Fig. 7A and B).

To examine the effect of SCIMP-associated SLP65 and Csk on this pathway, we performed a knockdown rescue experiment in which two K46 clones expressing shRNA 3 were retrovirally infected with vectors coding for wild-type human SCIMP or human SCIMP with a mutated Csk binding site (Y107F) or SLP65 binding site (Y131F) and with a control empty vector. Note that the human SCIMP constructs would not be affected by mouse shRNA 3 sequences. Ten minutes after MHC-II molecule stimulation, cells expressing wild-type human SCIMP exhibited increased Erk1/2 phosphorylation compared to the control. An even stronger effect was observed when the Csk binding site was mutated (Fig. 7C and D). In contrast, cells transfected with the human SCIMP mutant unable to bind SLP65 were incapable of sustaining Erk1/2 phosphorylation. Again, the same applied for activation of MEK1/2 and p90RSK. Together, these data strongly suggest that SLP65 binding to SCIMP is necessary for sustained Erk1/2 signaling downstream of MHC-II molecules. Concomitant binding of Csk serves as a negative regulatory loop to modulate the SCIMP function.

DISCUSSION

TRAPs are implicated as regulators and/or organizers of signaling from various plasma membrane receptors. Expression of some of them, like PAG, is ubiquitous, indicating that they have a general role in cells of various origins (6), while others, such as LAT, are expressed in a highly tissue-restricted pattern, pointing toward a more specific role. Indeed, LAT is present mainly in T cells, NK cells, and mast cells and functions as a critical regulator of TCR and Fc receptor signaling (21). Analysis of SCIMP expression revealed its presence to be strictly limited to tissues of the immune system, most prominently in the lymph nodes and spleen. Probing this further, we observed a clear correlation between the expression of SCIMP and MHC-II molecules in APCs. This suggested that the functions of SCIMP may be coupled to the regulation of antigen presentation or APC activation.

SCIMP contains a transmembrane domain followed by palmitoylated (S-acylated) cysteines. It should be noted that even though most S-acylated proteins contain palmitate, other fatty acid residues have also been detected, such as palmitoleate, stearate, oleate, arachidonate or eicosapentanoate (36, 39). In contrast to other palmitoylated TRAPs (LAT, NTAL, LIME, and PAG), SCIMP is not found in "classical" lipid rafts. Instead, we provided biochemical evidence that SCIMP is associated with tetraspanins CD37, CD53, and CD81 and established SCIMP as a novel constituent of TEMs.

Current knowledge of TEM function in antigen presentation is rather limited. MHC-II molecules loaded with specific antigenic peptides appear to be enriched in TEMs (30, 47), and it has been suggested that TEMs might increase the chance of successful presentation of a limited pool of MHC-II molecules loaded with specific peptides. This would be in agreement with the observation that tetraspanins are constituents of IS (34). SCIMP is also rapidly translocated to IS. Since SCIMP contains only a very short extracellular part, this process is most likely mediated through interaction with TEMs.

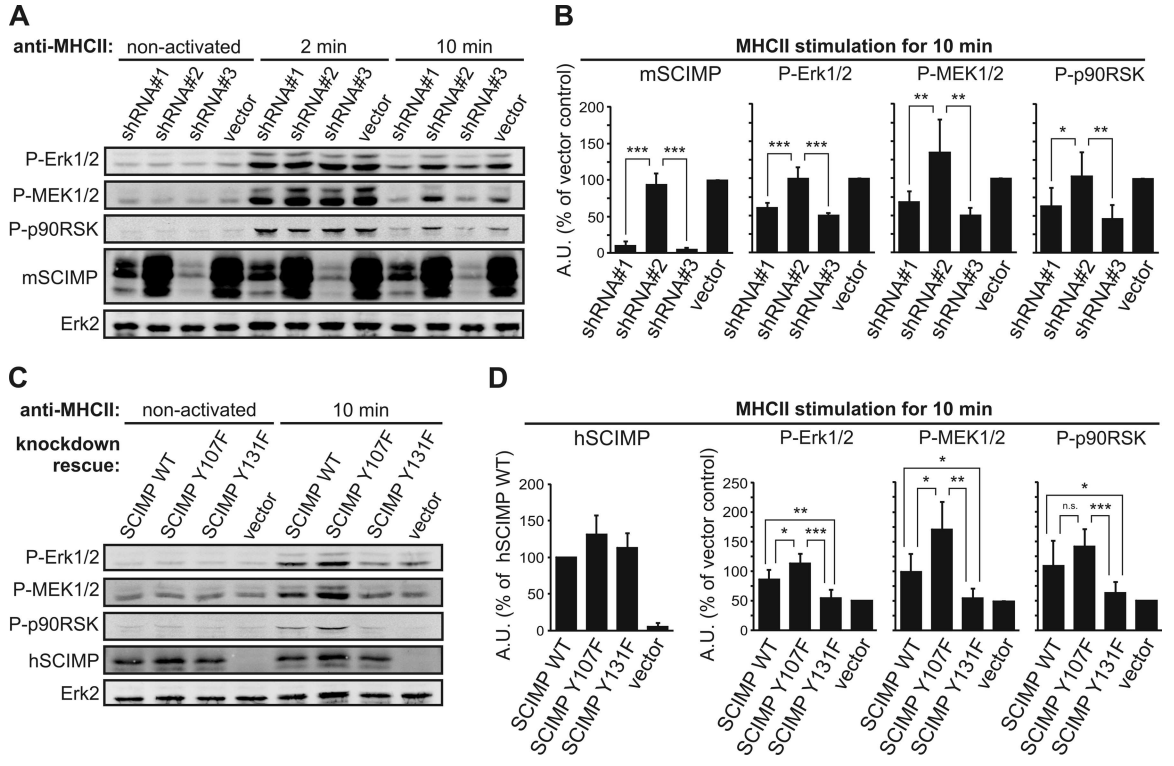

FIG. 7. SCIMP mediates sustained Erk signaling after MHC-II-mediated activation. (A) K46 murine B cells were lentivirally infected with vectors coding for shRNAs against SCIMP or with empty vector. While shRNA 1 and shRNA 3 mediated strong knockdown, shRNA 2 served as a control. Cells were activated with anti-MHC-II antibodies for indicated times and analyzed for phosphorylation of Erk1/2 (T202/Y204), MEK1/2 (S217/221), and p90RSK (S380) or for expression of mSCIMP. Total Erk2 staining served as a loading control. (B) Statistical analysis of results in panel A from six independent experiments using two clones from each cell line. Samples were normalized to the loading control (means ± SD). (C) Clones expressing shRNA 3 were reconstituted with wild-type (WT) human SCIMP or with human SCIMP with a mutated Csk binding site (Y107F) or SLP65 binding site (Y131F) or with empty vector. Cells were activated and analyzed as described for panel A, with staining for transfected human SCIMP added. (D) Statistical analysis of results in panel C from six independent experiments using two clones from each cell line. Samples were normalized to the loading control (means ± SD). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (two-tailed Student test with assumed nonequal variances). All data were collected using the Odyssey infrared imaging system.

Even though the role of signals generated by interactions with MHC-II molecules is still rather unclear, there is ample evidence that MHC-II molecules are not just passive tools to display peptides on the cell surface. Responses generated via MHC-II molecule ligation range from APC activation and differentiation to apoptosis. Engagement of MHC-II on the APC surface using specific antibodies results in activation of Src and Syk family kinases, induction of calcium influx, and activation of MAPKs, protein kinase C (PKC) family members, and the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, as well as downstream activation of transcription factors NF-AT and AP1 (1, 20). The key question is how the signaling by MHC-II molecules is initiated, since MHC-II molecules possess only short cytoplasmic parts without any apparent signaling motifs. Signaling mechanisms of MHC-II glycoproteins were studied mainly in B cells, where MHC-II interactions with Igα/β, CD19, or MPYS were suggested to be involved in mediating the signal transmission (5, 26, 31), but the precise pathways and number of key players still remain incompletely defined. Here we have identified SCIMP as one of possible mediators of MHC-II molecule signal transduction. In splenic B cells, SCIMP phosphorylation substantially increased after MHC-II cross-linking but not after BCR cross-linking. The SCIMP signaling capacity probably depends to a large extent on phosphorylation of the tyrosine motifs in its intracellular domain. Based on the current knowledge of SH2 domain binding preferences, it was possible to predict and confirm by coimmunoprecipitation the binding partners for three of these tyrosines (Grb2 for Y_{69}, Csk for Y_{107}, and SLP65/SLP76 for Y_{131}). It should be noted that the list of binding partners for phosphorylated SCIMP might be incomplete, and some other, so far unknown proteins could bind via their SH2 domains to phosphorylated SCIMP in a competitive manner.

We observed a high level of cooperativity in SLP65/SLP76 and Grb2 binding to SCIMP. It has been shown that both SLP65 and SLP76 interact via their proline-rich motifs with the SH3 domain of Grb2 (15, 24), thereby forming a complex where both proteins have SH2 domains available for binding. We suggest that this "bivalent" mode of binding would substantially increase the avidity of the interaction between Grb2, SLP65/SLP76, and SCIMP.

Binding of SCIMP to both Lyn and Csk is reminiscent of findings for LIME and PAG (6, 7). These adaptors also bind both the Src family kinases and Csk and are mainly considered negative regulators, since they anchor the inhibitory Csk in lipid rafts. However, binding of SCIMP to SLP65/SLP76 adap-

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tors also suggests an active role for SCIMP in signal transduction. To better understand the dual activatory/inhibitory nature of this adapter and its direct effect on signaling pathways, we generated the CD25-SCIMP chimeric protein. Direct cross-linking of this construct led to increased CD25-SCIMP phosphorylation and concomitant binding of the SLP65/SLP76-Grb2 complex and Csk kinase. The overall functional outcome of CD25-SCIMP cross-linking was activation of several signaling pathways, including phosphorylation of PLC-γ1 and PLC-γ2, a robust calcium response, and Erk1/2 activation. Since we did not observe a direct association between SCIMP and PLC-γ isoforms (not shown), the signal events initiated by cross-linking of CD25-SCIMP were most likely mediated by anchoring SLP65 to the plasma membrane. SLP65 could associate there with various effector proteins, such as PLC-γ isoforms and their activator, Btk (28). This hypothesis is corroborated by the observation that all signaling pathways emanating from CD25-SCIMP were abolished by mutation of the SLP65/SLP76 binding site. Notably, the BCR signaling subunit Igα also contains a SLP65 binding tyrosine, and its mutation compromises the BCR signaling pathway (13, 38).

An opposite phenotype was observed when the Csk-binding tyrosine was mutated, since all the responses generated after cross-linking of CD25-SCIMP were substantially enhanced, indicating a direct role of Csk in the regulation of signaling mediated by SCIMP. These results lead to important conclusions. First, SCIMP has an overall activating function dependent on SLP65 binding, and interaction with Csk is employed to modulate SCIMP's own activity. Second, among the many responses elicited by MHC-II stimulation, SCIMP is directly involved in generating the calcium response and enhancing Erk1/2 activity.

A complementary approach to provide evidence for the signaling capabilities of SCIMP was the generation of K46 cell lines with diminished expression of SCIMP. Surprisingly, we observed no effects on the calcium response after MHC-II molecule cross-linking. This observation does not rule out the role of SCIMP in the calcium response, but combined with our results with CD25-SCIMP chimeras it rather suggests that MHC-II-dependent calcium signaling may be under the control of multiple redundant pathways. In contrast, the Erk pathway was substantially affected. The SCIMP deficiency led to premature termination of Erk signaling pathway activity, as judged from the reduced phosphorylation of Erk1/2, its activator, MEK1/2, and its downstream target, p90RSK, clearly demonstrating the nonredundant role of SCIMP in this process. Importantly, we were able to rescue this phenotype by reconstituting these cells with wild-type SCIMP or SCIMP with a mutated Csk binding site but not with SCIMP unable to bind SLP65. This complemented our data from CD25-SCIMP chimeras and provided a strong evidence that the main function of SCIMP is to anchor SLP65/SLP76 adaptors to the membrane, while binding of Csk drives a negative feedback loop (Fig. 8). We still do not know the precise downstream effects of SCIMP-mediated signaling on APC function. One possibility is the regulation of MHC-II induced apoptosis that is largely dependent on Erk1/2 (26). However, we did not observe any changes in the rate of apoptosis in SCIMP-depleted cells (not shown). Another possibility might be the regulation of antigen presentation. We believe that further study using SCIMP-deficient mice (currently in preparation) would shed more light on the physiological effects of signaling mediated by SCIMP.

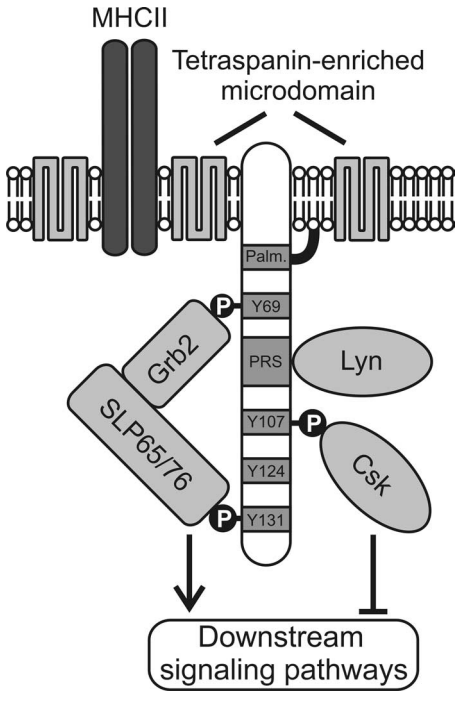

FIG. 8. A model of SCIMP-mediated signal transduction. SCIMP is palmitoylated on its submembrane palmitoylation motif (Palm.) and is localized in tetraspanin-enriched microdomains together with MHC-II. Via its proline-rich sequence (PRS), SCIMP constitutively binds the SH3 domain of Lyn kinase. Upon cross-linking of SCIMP in the immunological synapse, SCIMP becomes phosphorylated (P) at several tyrosine residues and recruits a complex of Grb2 and SLP65 or SLP76 adaptors to the plasma membrane. Subsequently, SLP65 or SLP76 initiates a signaling cascade leading to activation of downstream signaling pathways. This process is controlled by the negative regulatory kinase Csk, which is also recruited to phosphorylated SCIMP.

At present, SCIMP is the only protein in TEMs with a functional similarity to LAT, a lipid raft-associated transmembrane adaptor critically involved in signaling mediated by TCRs and other immunoreceptors. LAT interacts with the SLP family adaptor SLP76 and transduces signals of similar types to those transduced by SCIMP (16). However, the range of receptors employing SCIMP as a signal transducer will most likely be different due to its association with TEMs. We have demonstrated the importance of SCIMP in signaling via MHC-II molecules. On the other hand, many different proteins are associated with the tetraspanin web, and thus it is reasonable to speculate that more receptors dependent on SCIMP signaling will be discovered during our future efforts to understand this interesting protein.

ACKNOWLEDGMENTS

We thank all the colleagues who provided us with the cells, plasmids, and antibodies, as indicated above, and Tamiko Katsumoto for critical reading of the manuscript.

This work was supported in part by project no. AV0Z50520514, awarded by the Academy of Sciences of the Czech Republic, GACR (project MEM/09/E011), and by the Center of Molecular and Cellular Immunology (project 1M0506, Ministry of Education, Youth and Sports of the Czech Republic). P.D., O.S., and M.H. are Ph.D. stu-

dents supported in part by the Faculty of Science, Charles University, Prague, Czech Republic.

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