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Stomatin: A New Paradigm of Membrane Organization Emerges

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ABSTRACT

Stomatin, originally identified as a major protein of the human erythrocyte membrane, is widely expressed in various tissues. Orthologues are found in vertebrates, invertebrates, plants, and microorganisms. Related proteins exhibit a common core structure, termed the prohibitin (PHB) domain, with varying extensions. Stomatin has an unusual topology, similar to caveolin-1, with a hydrophobic domain embedded at the cytoplasmic side of the membrane. Additional anchoring is provided by palmitoylation and the membrane affinity of the PHB domain. Stomatin associates with cholesterol-rich microdomains (lipid rafts), forms oligomers, and thereby displays a scaffolding function by generating large protein-lipid complexes. It regulates the activity of various membrane proteins by reversibly recruiting them to lipid rafts. This mechanism of regulation has been shown for GLUT-1 and may also apply for ion channels. Stomatin is located at the plasma membrane, particularly in microvilli, in endocytic and exocytic vesicles, and cytoplasmic granules. Stomatin-carrying endosomes are highly dynamic and interact with lipid droplets suggesting a role in intracellular lipid transport. This subcellular distribution and the caveolin-like protein structure suggest important membrane organizing functions for stomatin. A general picture emerges now that cell membranes contain cholesterol-rich domains that are generated and regulated by scaffolding proteins like caveolins, stomatins, and flotillin/reggie proteins.

Keywords: cholesterol, lipid raft, membrane microdomain, membrane protein, oligomeric complex, SPFH domain
Abbreviations: AChE, acetylcholinesterase; **CRAC**, cholesterol recognition/interaction amino acid consensus; **DRM**, detergent-resistant membrane; **EA**, glutamate-alanine; **FRAP**, fluorescence recovery after photobleaching; **GARP**, antibody produced by G. Adolf and R. Prohaska; **GFP**, green fluorescent protein; **GPI**, glycosyl phosphatidylinositol; **IM**, intramembrane; **LD**, lipid droplet; **MVB**, multi-vesicular body; **PDZ**, PSD-95, Dlg, ZO-1; **PHB**, prohibitin; **PLAP**, placental alkaline phosphatase; **SPFH**, stomatin, prohibitin, flotillin, HflC/K

CONTENTS

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Invited Review

INTRODUCTION

Stomatin, the human erythrocyte band 7.2b membrane protein

Interest in stomatin started when red cells of patients with the haemolytic anaemia Hereditary Stomatocytosis were found to be lacking a major membrane protein in the band 7 region (Lande *et al*. 1982; Eber *et al*. 1989; Morle *et al*. 1989; Stewart *et al*. 1992b). This protein, termed protein 7.2b or band 7 integral membrane protein, was isolated and characterized (Hiebl-Dirschmied *et al*. 1991a, 1991b; Wang *et al*. 1991; Stewart *et al*. 1992b) and, with reference to the disease, termed stomatin (Stewart *et al*. 1992b). However, it was found that not all patients with Hereditary Stomatocytosis were lacking stomatin in the red cells (Kanzaki *et al*. 1992) and that stomatin is only missing in the red cells of Overhydrated Hereditary Stomatocytosis (OHSt) patients but is normal in Dehydrated Hereditary Stomatocytosis (Stewart *et al*. 1993). Because OHSt red cells have a high permeability for monovalent cations, a "cation leak", it was suggested that stomatin may function as a regulator of an ion channel (Stewart *et al*. 1993). However, OHSt patients have normal amounts of stomatin mRNA in the erythroid precursors (Stewart *et al*. 1992b) and there were no mutations found in the derived cDNA (Stewart *et al*. 1992a; Wang 1992; Fricke *et al*. 2003a). Stomatin knock-out mice have a normal red cell morphology (Zhu *et al*. 1999) suggesting that stomatin is not directly linked to OHSt and may not be involved in ion channel regulation. OHSt erythroid precursors were shown to synthesize the protein but loose it during red cell maturation (Fricke *et al*. 2003a, 2005). The real defect causing OHSt is still unknown; possibly there is a fundamental trafficking abnormality (Fricke *et al*. 2005).

An interaction of erythrocyte stomatin with lipids was first shown in a labelling study using a photoreactive phosphatidylethanolamine and a lipophilic cysteine-reactive reagent, respectively (Desneves *et al*. 1996). Because of the preferential incorporation of these lipids into stomatin, it was hypothesized that stomatin may play an important role in controlling lipid organization in the erythrocyte membrane, possibly as a scramblase.

Stomatin gene organization and expression

Because the stomatin cDNA sequence was found to be normal in OHSt, it was interesting to study the gene structure and organization (Gallagher *et al*. 1995a; Unfried *et al*. 1995). The gene *EPB72/STOM* contains 7 exons; the first encodes the N-terminal region (there is no signal sequence), the second the hydrophobic domain, and 5 exons encode the C-terminal region. The 7th exon is the largest and contains more than 2 kb 3′-UTR. The gene is located on the human chromosome 9q34.1 (Westberg *et al*. 1993) / 9q33-34 (Gallagher *et al*. 1993), centromeric to the *ABL* gene that is involved in the 9;22 translocation. However, no chromosomal translocations have been reported concerning the stomatin gene. Several splice variants have been identified in cancer patients and abnormal splicing was found in a family with recessively inherited multisystem pathology (Argent *et al*. 2004); in the absence of more detailed information it is, however, difficult to judge whether this aberrant splicing is the cause or the result of these diseases. The promoter region of the stomatin gene is characteristic of a household gene suggesting a wide range of expression in various tissues. This was also shown by tissue Northern blot analyses and can be seen accordingly in microarray databases like the Gene Expression Atlas (http://symatlas.gnf.org/SymAtlas/). Whereas stomatin transcripts were readily identified in most organs and tissues, expression in the brain was comparatively low (Unfried *et al*. 1995). The mRNA comes in two sizes, differing by 1 kb in the 3′-UTR, but the biological relevance of these two transcripts has not been determined. There are several binding sites for GATA-1 and other haematopoietic transcription factors within the distal 1 kb region and therefore it may play a role by enhancing stomatin expression in haematopoietic cells (Unfried *et al*. 1995). To produce a knock-out mouse, the mouse gene and transcripts were analysed and found to be very similar to the human gene (Gallagher *et al*. 1995b, 1996); the wide tissue distribution was also seen in the mouse (Gallagher *et al*. 1995b, 1996; Schlegel *et al*. 1996). The knock-out mouse did not show a major phenotype, it was normal in appearance, activity, fertility, and red cell morphology (Zhu *et al*. 1999), however, recently it was found to react more strongly than the wild-type mouse when exposed to the volatile anaesthetic diethyl ether (Sedensky *et al*. 2006).

Stomatin orthologues and homologues

Stomatin orthologues and related proteins are widely distributed in the animal and plant kingdoms and are even found in bacteria and archaea, as revealed by genome and transcriptome analyses. In man and mouse, stomatin is widely expressed in various tissues, as described above, whereas in the nematode *Caenorhabditis elegans*, all stomatins are restricted to neurons (Zhang *et al*. 2004) indicating a broader functional spectrum in vertebrates.

In plants, two stomatin-like proteins have been described, one in *Zea mays* (Nadimpalli *et al*. 2000) and one in *Arabidopsis thaliana* (Borner *et al.* 2005). Both proteins are members of the large PID (proliferation, ion, death) superfamily that also contains plant defense response proteins like the HIR (hypersensitive induced reaction) proteins. The plant stomatins are not orthologues but similar to the human stomatin-like protein 2 (SLP-2) that is described below. More distantly related proteins, flotillin-1 and prohibitins were also identified in plants (Nadimpalli *et al*. 2000; Borner *et al.* 2005). The genome of the baker's yeast *Saccharomyces cerevisiae* does not contain any stomatin homologue whereas *Schizosaccharomyces pombe* contains an orthologue of SLP-2. A stomatin-like protein (slp) of the soil bacterium *Rhizobium etli* was described to be required for nodulation competitiveness on the common bean (You *et al.* 1998). In the hyperthermophilic archeon *Pyrococcus horikoshii*, a novel thermostable protease, nfed (nodulation formation efficiency D), was found to form an operon with a stomatin homologue that appears to be a prokaryotic orthologue (Yokoyama *et al*. 2005). It is hypothesized that the cleavage of the C-terminal region of stomatin by the protease leads to the opening of an ion channel. This hypothesis is based on a previous study by G.W. Stewart (Green *et al.* 2004) who described the occurrence of the nfed-stomatin operon in 19 prokaryotic organisms.

The *Caenorhabditis elegans* genome contains 10 stomatin-related genes (Bargmann 1998). Some gene products are highly similar to human stomatin and show little variation between them. Therefore, it is not clear which might represent "the" orthologue. Three stomatin-like genes have been identified within a group of selected genes, in studies originally started by Sydney Brenner (Brenner 1974; Park *et al*. 1986), which are responsible for neurological disorders like mechanosensory defects (MEC-2) and uncoordinated movement (UNC-1 and UNC-24). Whereas UNC-1 could be denoted as a stomatin orthologue, MEC-2 and UNC-24 are markedly different by extending their structure at one or both termini. Within these terminal regions there are specific domains that enable these proteins to interact with microtubules and ion channels (MEC-2), or lipids (UNC-24). The stomatin orthologue UNC-1 plays a role by controlling the worm's sensitivity to volatile anaesthetics (Rajaram *et al*. 1998, 1999) suggesting an association with an ion channel. UNC-1 was shown to associate with UNC-24 (Sedensky *et al*. 2001) and the degenerin channel UNC-8 (Sedensky *et al*. 2004). A mechanosensory complex in *C. elegans* touch receptor neurons has been elucidated containing MEC-2, MEC-6, UNC-24, and components of the degenerin (DEG)/ epithelial Na^+ channel (ENaC) family, MEC-4 and MEC-10 (Huang *et al*. 1995; Goodman *et al*. 2002; Zhang *et al*. 2004; O'Hagan *et al*. 2005). Moreover,

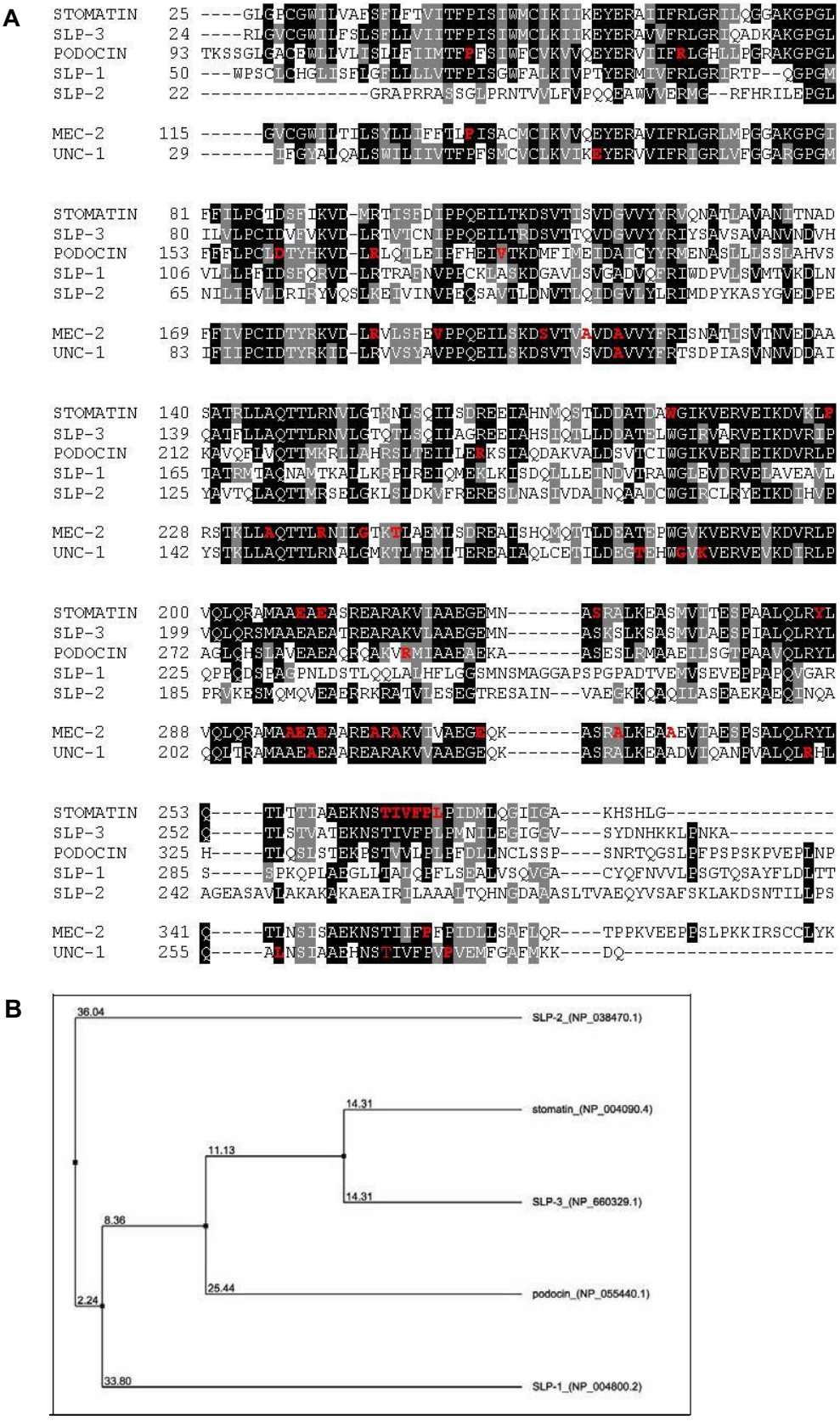

Fig. 1 Stomatin-like proteins. (**A**) Multiple sequence alignment of the human family members stomatin, SLP-1, SLP-2, SLP-3, and podocin, compared with *C. elegans* members MEC-2 and UNC-1. Amino acid exchanges that were reported to cause a phenotype *in vivo* and *in vitro* are marked in red. Note that most mutations involve highly conserved residues. These mutants are summarized in **Table 1**. Data base accession numbers for the sequences are shown in (**B**), except for MEC-2: Q27433 (SwissProt) and UNC-1: Q21190 (SwissProt). (**B**) Neighbour joining tree of the human stomatin family members. The high similarity of stomatin to SLP-3 is evident. A larger evolutionary distance is seen between stomatin and podocin, SLP-1, and SLP-2.

it was recently shown that MEC-2 binds and requires cholesterol to associate with and activate the DEG/ENaC channel in a large supercomplex (Huber *et al*. 2006).

The stomatin family and SPFH/PHB superfamily

The core region of stomatin was found to be conserved not only within the stomatin family but also in the prohibitins, flotillins/reggies, the bacterial HflC/K proteins (Tavernarakis *et al*. 1999), and most recently the erlins (Browman *et al*. 2006). The common domain was termed SPFH domain (Tavernarakis *et al*. 1999; Rivera-Milla *et al*. 2006) and, more recently, prohibitin homology (PHB) domain (http://smart.embl-heidelberg.de) (Morrow *et al*. 2005). The mammalian stomatin family comprises five proteins: stomatin, the stomatin-like proteins (SLPs) SLP-1, SLP-2, SLP-3, and podocin (**Fig. 1**). SLP-1, the orthologue of UNC-24, carries a non-specific lipid transfer protein (nsLTP)/sterol carrier protein-2 (SCP2) domain at the C-terminus suggesting a role in intracellular cholesterol/lipid transfer and transport (Seidel *et al*. 1998; Wang *et al*. 2000). SLP-2 is the only stomatin-like protein that lacks a hydrophobic do-

main for membrane anchoring and is therefore a peripheral membrane protein (Wang *et al*. 2000). Apparently, it has an essential biological function, because SLP-2 knock-out mice are not viable (Seifert *et al*. 2006). SLP-3, also known as stomatin-related olfactory protein (SRO), is the SLP with the highest similarity to stomatin (Kobayakawa *et al*. 2002). It was recently shown that SLP-3 knock-out mice have a strongly reduced touch sensation, in accordance with the worm's mec null phenotypes (Wetzel *et al*. 2007). Podocin is specifically expressed in kidney podocytes where it is part of a multiprotein complex associated with the slit-diaphragm of the glomerular filtration barrier (Benzing 2004). Mutations in podocin (*NPHS2* gene) lead to a congenital nephrotic syndrome (Huber *et al*. 2003).

The flotillin/reggie family (Langhorst *et al*. 2005; Morrow *et al*. 2005; Stuermer *et al*. 2005) is distantly related to the stomatins and has a common N-terminal SPFH/PHB domain containing two short hydrophobic regions and several palmitoylation sites that anchor these proteins to the cytoplasmic side of the membrane (Morrow *et al*. 2002; Liu *et al*. 2005). Additional anchoring for flotillin-2/reggie-1 is provided by N-terminal myristoylation (Neumann-Giesen *et al*. 2004). The distal C-terminal half of the flotillins contains the Flotillin domain that is responsible for oligomeric complex formation (Neumann-Giesen *et al*. 2004; Solis *et al*. 2007).

The prohibitins PHB1 and PHB2 (BAP37) are localized to the inner mitochondrial membrane where they form large hetero-oligomeric complexes that act as a novel type of membrane-bound chaperone (Nijtmans *et al*. 2002). Similarly, the recently discovered erlins (Browman *et al*. 2006) that are homologous to the prohibitins appear to be oligomeric and may have a related function at the cytoplasmic side of the endoplasmic reticulum. The bacterial HflC and HflK proteins form a hetero-dimer (HflKC) that is associated with the ATP-dependent and membrane-attached protease FtsH forming a complex that is now termed FtsH holo-enzyme. It is postulated that 6 FtsH holo-enzyme subunits form a hexameric complex (Saikawa *et al*. 2004).

STOMATIN STRUCTURE

Monotopic membrane protein structure

When the topology of band 7 membrane protein/stomatin of the red cell membrane was studied by proteolytic digestion or radiolabelling, it became clear that the major part of this protein is oriented toward the cytoplasm (**Fig. 2A**) (Hiebl-Dirschmied *et al*. 1991a). The primary structure deduced from the cDNA sequence revealed a highly charged N-terminal region (24 residues) followed by a 29-residue hydrophobic region, that is the putative membrane anchoring domain, and the C-terminal bulk of the protein (Hiebl-Dirschmied *et al*. 1991b). According to this structure, the cAMP-dependent phosphorylation of stomatin (Hiebl-Dirschmied *et al*. 1991a; Wang *et al*. 1991) was thought to affect the cytoplasmic C-terminal region. Surprisingly, we identified Ser-9 as the phosphorylation site (Salzer *et al*. 1993), a residue thought to face the extracellular side. Thus, it became clear that both the N- and C-terminus are oriented toward the cytoplasm and that stomatin has a monotopic membrane protein structure (Salzer *et al*. 1993). Further evidence for this structure came later by immunofluorescence microscopy studies showing that the monoclonal antibody against the N-terminus of stomatin, GARP-50 (Hiebl-Dirschmied *et al*. 1991a), is only binding after permeabilization of the cells (Snyers *et al*. 1997). Moreover, the identification of the palmitoylation sites, Cys-29 and Cys-86 that provide additional anchoring, is also in accordance with the monotopic structure (Snyers *et al*. 1999b). The monotopic structure is also a hallmark of caveolin-1 (Dupree *et al*. 1993; Monier *et al*. 1995) and the stomatin-like proteins podocin (Boute *et al*. 2000) and MEC-2 (Huang *et al*. 1995). Flotillin/reggie proteins are also monotopic integral membrane proteins, as shown in **Fig. 2A**, however, because of their shorter hydrophobic segments they are thought to rely more on the fatty acyl residues for membrane anchoring (Morrow *et al*. 2002; Neumann-Giesen *et al*. 2004).

In contrast to stomatins and flotillins, the prohibitins and related bacterial HflKC proteins are transmembrane proteins with apparently different structures (Nijtmans *et al*. 2002; Saikawa *et al*. 2004).

Oligomeric structure

When stomatin is isolated from cell membranes by solubilization and density gradient centrifugation, high-order homo-oligomers can be identified (Snyers *et al*. 1998). These oligomers have molecular masses of about 300 kDa, comprising 9-12 monomers when isolated from epithelial cell membranes and up to about 600 kDa (about 20 monomers) from red cell membranes (Salzer *et al*. 2001). The stomatin-like protein podocin was estimated to form oligomers as large as 20 to 50 monomers (Huber *et al*. 2006). Immunoprecipitation of stomatin oligomeric complexes did not reveal any additional proteins that might be specifically bound

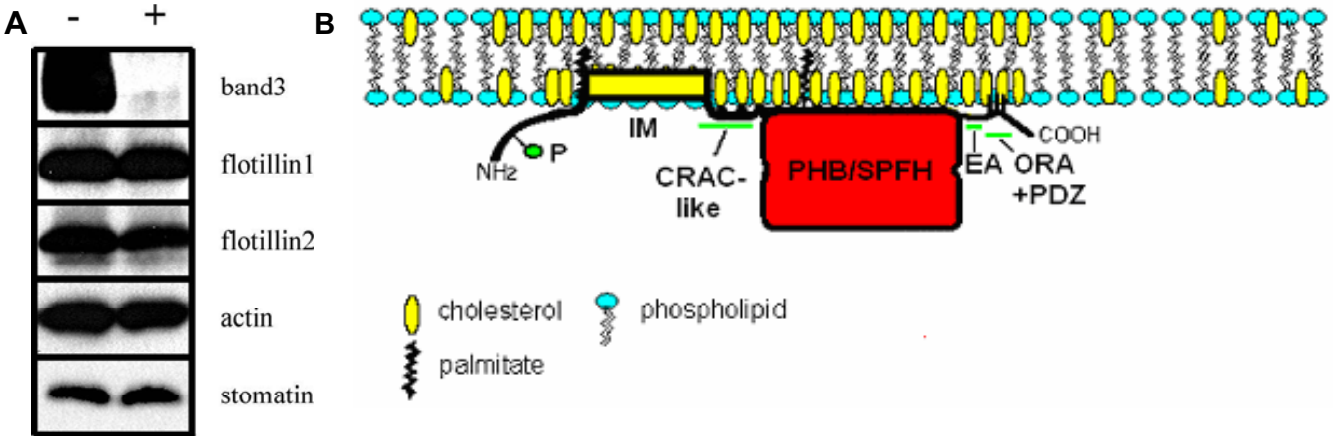

Fig. 2 Stomatin monotopic structure. (**A**) Proteolytic assessment of stomatin and flotillin topology. Human erythrocytes were digested with the highly active, unspecific proteinase K (+), washed, and analysed by Western blotting as indicated. A control, incubated without protease (-), is shown for comparison. Neither stomatin nor the flotillins were digested indicating their cytoplasmic orientation and monotopic structure. In contrast, band 3 protein, a transmembrane protein, was digested. (**B**) Schematic model of the human stomatin structure. Characteristic features are shown, the cAMP-dependent phosphorylation site (P), green ball; "IM", the 29-residue putative intramembrane domain; the juxtamembrane CRAC (cholesterol recognition/interaction amino acid consensus)-like motif, underlined in green, proposed to bind cholesterol; palmitoylation at Cys-29 and Cys-86 is indicated by zigzags. The characteristic PHB/SPFH-domain is shown in red. There are three C-terminal motifs/domains: an EA-repeat (EA) possibly involved in coiled-coil formation; a characterized domain necessary for oligomerization and lipid raft-association (ORA), and an overlapping PDZ-binding motif (PDZ). These motifs are indicated by green lines.

to these homo-oligomers (our unpublished data). The mechanism of oligomer formation is still not understood, neither for stomatins, nor caveolins or flotillins. It is clear, however, that the stomatin chains are not covalently linked, because they are readily identified by SDS-PAGE (Snyers *et al*. 1998; Salzer *et al*. 2001). We have recently identified a C-terminal hydrophobic region of stomatin (residues 264-272) that is essential for oligomerization (Umlauf *et al*. 2006) but not sufficient. The N-terminus is not involved, because an N-terminal truncation mutant readily forms oligomers. Clearly, an intact membrane anchoring domain is also necessary for oligomerization by providing the proper orientation and conformation for the lateral association at the membrane. In addition, a conserved C-terminal EA-repeat coiled-coil region, which is necessary for flotillin/reggie oligomerization (Langhorst *et al*. 2005), may also be involved in stomatin oligomerization. Flotillins form stable tetramers as basic units via coiled-coil domains that are resistant to 8 M urea (Solis *et al*. 2007). Caveolin-1 oligomers are partially resistant to cold SDS (Monier *et al*. 1995). SDS-resistant stomatin dimers are occasionally observed (our unpublished data) and chemical cross-linking predominantly yields stable dimers but also small amounts of trimers and higher oligomers (Hiebl-Dirschmied *et al*. 1991a; Snyers *et al*. 1998; Suchanek *et al*. 2005). Therefore, the dimer may represent the basic unit of stomatin oligomers. In general, the minimal requirements for oligomerization are two interacting sites to create a simple linear structure. This can be envisaged for podocin, because homophilic interactions of the N- and C-termini have been described (Huber *et al*. 2003). For caveolin-1, several interaction domains have been identified and a model of laterally associated oligomers forming a network has been proposed (Song *et al*. 1997; Schlegel *et al*. 2000). An alternative model favours the formation of filaments consisting of heptameric caveolin-1 subunits (Fernandez *et al*. 2002). These filaments are proposed to form the striated coat of caveolae. It may be speculated that similar coats might also be generated by the flotillin/reggie proteins and stomatin at non-caveolar membrane domains.

Oligomeric complexes have also been described for the prohibitins (Nijtmans *et al*. 2002), erlins (Browman *et al*. 2006), and HflKC (Saikawa *et al*. 2004).

Association with lipid rafts

Lipid rafts are dynamic cholesterol- and sphingolipid-rich membrane microdomains that are preferentially associated with GPI-anchored proteins, palmitoylated proteins like caveolin-1, and other lipid-modified proteins like src-family kinases (Simons *et al*. 1997, 2004). For biochemical studies of lipid rafts, often the chemical characteristics of cholesterol- and sphingolipid containing membrane domains are utilized, which are the low solubility in the non-ionic detergent Triton X-100 at 0-4°C and the flotation to low density upon density gradient centrifugation (Brown *et al*. 1992). Although it is clear that the detergent-resistant membranes (DRMs) are different from native membrane microdomains (Brown 2006), the analysis of DRMs may allow some relevant insight (Foster *et al*. 2003). Given the topological and biochemical similarity of stomatin with the raft marker caveolin-1 (Simons *et al*. 1997; Kurzchalia *et al*. 1999), we tested whether stomatin might also associate with DRMs or rafts. When cell membranes are solubilized with Triton X-100 at a low temperature and centrifuged, a large part of stomatin is found in the pellet fraction containing the DRMs and cytoskeleton. On density step gradient centrifugation of the pellet, stomatin floats to the low density region along with typical raft constituents, cholesterol, sphingolipids, and GPI-anchored proteins (Snyers *et al*. 1999a; Salzer *et al*. 2001). Stomatin was also found to be a representative component of lipid rafts by a different approach utilizing the cholesterol-dependency of lipid rafts (Foster *et al*. 2003). In a cell biological approach, using cell culture and immunofluorescence microscopy, we studied the co-patching of the lipid raft-associated GPI-anchored PLAP and stomatin (Snyers *et al*. 1999a). When PLAP is cross-linked on the cell surface by anti-PLAP antibody, it forms aggregates that are seen as patches. Interestingly, although stomatin is located at the cytoplasmic side of the membrane it co-localizes with PLAP in these patches. This co-patching of exo- and endocytic proteins is thought to be mediated by rafts and is just one strong argument in favour of the lipid raft hypothesis.

The characteristic association of stomatin with rafts apparently applies for all stomatin-like proteins, except SLP-2 (Foster *et al*. 2003), which lacks a hydrophobic intramembrane domain (Wang *et al*. 2000). The *C. elegans* stomatin homologues UNC-1 and UNC-24 have been identified in worm DRMs (Sedensky *et al*. 2004) and SLP-1, the human orthologue of UNC-24, is also DRM-associated (M. Mairhofer, manuscript submitted). SLP-3 is found in nerve cell DRMs (Kobayakawa *et al*. 2002) and podocin forms complexes in podocyte DRMs (Schwarz *et al*. 2001). Other SPFH/PHB family members are also associated with DRMs, particularly the flotillin/reggie proteins (Bickel *et al*. 1997; Lang *et al*. 1998), which are often used as lipid raft markers, but also the newly discovered erlins (Browman *et al*. 2006).

Interaction domains and motifs

Phosphorylation

Human stomatin is phosphorylated at Ser-9 (mature protein numbering) by a cAMP-dependent protein kinase (Salzer *et al*. 1993). This result led us to conclude that stomatin is a monotopic membrane protein (**Fig. 2B**) and not bitopic as predicted. The relevance of this previously highly emphasized phosphorylation reaction is not clear. Because Ser-9 is only found in human stomatin but not in mouse (Schlegel *et al*. 1996) or other mammalian stomatins, it is probably not of general importance.

Palmitoylation

Palmitoylation of cysteine residues has an important effect on the structure, localization, and function of a protein. Stomatin contains 3 cysteine residues at position 29, 52, and 86. For metabolic labelling studies, we mutated each of these cysteines to serine, individually and in combination, and expressed them in stable cell lines except for the triple mutant, which was instable (Snyers *et al*. 1999b). Cys-29, which is located in front of the intramembrane (IM) domain, is the major palmitoylation site, with Cys-86 being partially palmitoylated. Cys-52, which is located immediately after the IM domain, apparently is not palmitoylated; however, mass spectrometric data of peptides from endogenous stomatin indicate that Cys-52 is also palmitoylated (our unpublished data). When expressed, all the mutants are correctly targeted to the plasma membrane (Snyers *et al*. 1999b) but probably due to oligomeric complex formation with endogenous stomatin.

The intramembrane domain

This long, hydrophobic domain near the N-terminus (**Fig. 2B**) comprises 29 residues, 6 of them aromatic. Two lysine residues, Lys-24 and Lys-54 mark the domain's boundaries and 2 cysteines are flanking this domain: Cys-29, which is palmitoylated, and Cys-52, which may also be palmitoylated (see above). Two flanking tryptophans, Trp-31 and Trp-50, may provide additional anchoring at the membrane/aqueous interphase, as often found in transmembrane domains. A conserved proline residue, Pro-46, is located in the C-terminal part of the IM domain and is necessary for cholesterol binding, as shown for MEC-2 and podocin (Huber *et al*. 2006). Stomatin and stomatin-like proteins are thought to be anchored to the cytoplasmic membrane with a hairpin-like structure, like the caveolins (Monier *et al*. 1995; Parton *et al*. 2006). Hydrophobic, helical hairpins can be formed

from a minimum of 31 residues with two 14-residue helices and an intervening 3-residue turn preferentially containing a proline residue (Monne *et al*. 1999). Regarding stomatin, such a structure could only be formed, if Lys-54 was neutralized by modification or ion-pair/complex formation. Alternatively, a partially embedded helix would be conceivable, as revealed by the structure of prostaglandin H_2 synthase-1 (Picot *et al*. 1994). Within the IM domain of stomatin there are 4 Ser/Thr residues, located on one side of the putative α-helix, thus forming an amphipathic helix that may be partially inserted in the membrane. Because stomatin has been identified on the surface of lipid droplets (Umlauf *et al*. 2004), which are surrounded by a phospholipid monolayer, we postulate that this IM domain is inserted only into the cytoplasmic face of bilayer membranes. Although the insertion of the IM domain into the droplet oil phase is conceivable, we think that a model with one, possibly kinked, α-helix embedded horizontally in the cytoplasmic leaflet of the membrane (**Fig. 2B**) is more likely.

The CRAC-like motif

A juxtamembrane segment at the C-terminal side of the IM domain (**Fig. 1**, **Fig. 2B**) with the sequence IKEYER is similar to a cholesterol recognition/interaction amino acid consensus (CRAC) motif, which has the consensus L/V-X_{1-5}-Y-X_{1-5}-R/K (Epand *et al*. 2006). Although in the sequence there is an isoleucine instead of leucine/valine, there is a good chance that this motif actually binds cholesterol, because preliminary results from labelling experiments using several stomatin truncation mutants and activatable cholesterol are in good agreement with this location (E. Umlauf, C. Thiele *et al*. unpublished data). This CRAC-like motif and putative cholesterol-binding segment may be partly responsible for stomatin's partitioning into cholesterol-rich domains. An identical motif is found in SLP-3 (**Fig. 1**), and true CRAC motifs are present in MEC-2, SLP-1, and podocin.

The SPFH/PHB domain

The SPFH domain has been recognized as a common domain of several protein families, namely the stomatins, prohibitins, flotillins, and HflKC proteins (Tavernarakis *et al*. 1999), and has been implicated in regulating targeted protein turnover in stomatins and other membrane-associated proteins, because of the respective functions of prohibitins and the bacterial HflKC proteins. Such a function, regarding stomatins and flotillins, has not been verified since. Recently, the SPFH domain has become more popular by the name prohibitin homology (PHB) domain (http://smart.embl-heidelberg.de) and has been reviewed extensively, particularly in the context of flotillin/reggie proteins (Langhorst *et al*. 2005; Morrow *et al*. 2005; Stuermer *et al*. 2005; Rivera-Milla *et al*. 2006). This domain is now recognized as a scaffolding domain with an affinity for cholesterol and membrane lipids in general, often combined with a palmitoylated cysteine residue (Morrow *et al*. 2002). SPFH/PHB proteins are thus prone to oligomerize and associate with lipid microdomains leading to the formation of large protein-lipid complexes. The structure of a shortened SPFH/PHB domain of mouse flotillin-2 has been studied by NMR (Protein Data Bank accession number 1WIN) and revealed a compact, ellipsoid-globular shape that is formed by β-sheet and α-helical structures.

The C-terminal interaction domain

The C-terminus of stomatin is necessary for homo-oligomerization and lipid raft-association, as determined by density gradient centrifugation methods (Snyers *et al*. 1998, 1999a). Systematic truncation and amino acid exchange within the C-terminal region has shown that a stretch of 9 amino acids (residues 264-272) is required for oligomerization (Umlauf *et al*. 2006), with partial requirement of the flanking residues (residues 262-275). Within this region, we identified 3 amino acids (266-268), Ile-Val-Phe, to be necessary for the association with DRMs. Possibly, the aromatic ring of Phe may laterally interact with cholesterol in the membrane. The adjacent residue Pro-269 is not essential for DRM-association but is clearly required for oligomer formation. This result suggests that oligomerization and DRM association may be independent processes (Umlauf *et al*. 2006). The partial requirement of downstream residues 272-275 for oligomerization may also suggest that a PDZ3 binding motif, Ile-Asp-Met-Leu, may participate in oligomerization. Multi-PDZ proteins may thus bind to several stomatin molecules and create large complexes. Upstream of the oligomerization and raft-association (ORA) domain there is an EA-repeat region predicted to form a coiled-coil structure that may interact to form dimers and possibly trimers. EA-repeats are particularly prominent and extended in the flotillin domain of flotillin/reggie proteins (Langhorst *et al*. 2005; Rivera-Milla *et al*. 2006) and are essential for flotillin oligomerization (Neumann-Giesen *et al*. 2004; Solis *et al*. 2007).

Loss of the C-terminal interaction domain, as shown by deletion of residues 263-287, leads to the loss of oligomerization and lipid raft association (Umlauf *et al*. 2006). When we studied the lateral mobility of stomatin-GFP in the plasma membrane by FRAP analysis and compared it with the truncation mutant stomatin(1-262)-GFP, the mutant showed a significantly higher mobility (Umlauf *et al*. 2006). This indicates that the C-terminal interaction domain binds to large structures at the plasma membrane like the cytoskeleton and/or large cholesterol-rich complexes.

STOMATIN FUNCTION

Stomatin localization and dynamics

Apart from being a major erythrocyte membrane protein, it was immediately recognized that this protein is widely expressed in many tissues and cell types (Hiebl-Dirschmied *et al*. 1991a; Wang *et al*. 1991; Stewart *et al*. 1992b). In nucleated cells, stomatin is found in two pools, the plasma membrane and the juxtanuclear vesicle region (Snyers *et al*. 1997). This distribution is found in epithelial cells (UAC, MDCK, HepG2), fibroblasts (HeLa), and endothelial cells (HUVEC). At the plasma membrane, stomatin is concentrated in protrusions or microvilli and associated with actin microfilaments (Snyers *et al*. 1997), probably not directly but via adaptor proteins. The juxtanuclear stomatin-positive vesicles co-localize substantially with markers of the late endosomal/lysosomal compartment, particularly LAMP-2, in the epithelial cell lines UAC and MDCK (Snyers *et al*. 1999a) but also in HUVEC and HeLa cells (our unpublished data). When GPI-anchored proteins like PLAP and the membrane folate receptor MFRα are cross-linked on the surface of UAC cells by respective antibodies, they are internalised and partially co-localize with stomatin (Snyers *et al*. 1999a). Although the GPI-anchored proteins and stomatin are located at opposite sides of the membrane, they are co-endocytosed and traffic together via early endosomes to the late endosomal compartment. Thus, stomatin can be seen as a coat protein of these endosomes. The association of stomatin with PLAP in lipid microdomains is also demonstrated by co-patching, as already discussed in the topic "Association with lipid rafts".

When MDCK cells are grown on filter and polarize, the majority of stomatin is localized to the apical plasma membrane, in contrast to the major juxtanuclear localization in unpolarized cells (Snyers *et al*. 1999a). This is not unexpected, because the apical membrane is characterized by a high proportion of ordered lipid phase or lipid rafts and stomatin associates with rafts. The trafficking pathways leading to the concentration of stomatin at the apical membrane are currently unknown; however, it is conceivable that stomatin-containing late or recycling endosomes are targeted to the apical membrane.

Overexpression of stomatin in several cell types led to

the targeting of this protein to lipid droplets (LDs), similar to the caveolins (van Meer 2001), particularly in the presence of oleic acid (Umlauf *et al*. 2004). LDs are thought to be generated in the bilayer of the endoplasmic reticulum membrane by incorporation of triglycerides or cholesteryl esters and budding at the cytoplasmic side (Brown 2001; Martin *et al*. 2006). The role of stomatin in this process is unknown, however, it is interesting that stomatin-positive vesicles are vividly interacting with these LDs, as seen by live microscopy (Umlauf *et al*. 2004), suggesting that stomatin is involved in intracellular lipid transport. The dynamic vesicles are most probably late endosomes or lysosomes, because stomatin-positive acidic vesicles are attached to LDs (Umlauf *et al*. 2004). This interaction suggests that late endosomes are involved in the turnover of LDs, a general topic that has not yet been investigated thoroughly.

Stomatin is strongly expressed in haematopoietic cells of the myeloid lineage. Originally found in mature erythrocytes, it is also expressed in erythroid precursor cells like K562 and reticulocytes. In reticulocytes, stomatin is located at the plasma membrane and in the exosomes of multivesicular bodies (MVBs) (de Gassart *et al*. 2003), which belong to the late endocytic compartment. These exosomes are rich in transferrin receptor but also in typical lipid raft-associated components like stomatin, flotillin, src-family kinases, and GM1 that can be floated to low buoyant density (de Gassart *et al*. 2003). This is in accordance with the finding that most of the endocytic cholesterol is contained in internal vesicles of MVBs (Mobius *et al*. 2003). In thrombocytes, stomatin is highly expressed and mainly found in α-granules but not at the plasma membrane (Mairhofer *et al*. 2002). After activation, when the α-granules fuse with the plasma membrane and release the contents, stomatin is also found at the plasma membrane (**Fig. 3**). Therefore, stomatin can be seen as an α-granular coat protein that may be involved in targeting to and fusion with the plasma membrane. Similarly, neutrophils contain large amounts of stomatin on the membranes of diverse granules but not at the plasma membrane (Feuk-Lagerstedt *et al*. 2002), whereas activation leads to the transport of these granules to the periphery and fusion with the plasma membrane (**Fig. 3**). The high concentration of stomatin on the granular membranes may provide an extra stability and tightness to these membranes, similar to the tight shielding of the apical membrane, that may be needed to securely store cytotoxic enzymes and chemicals.

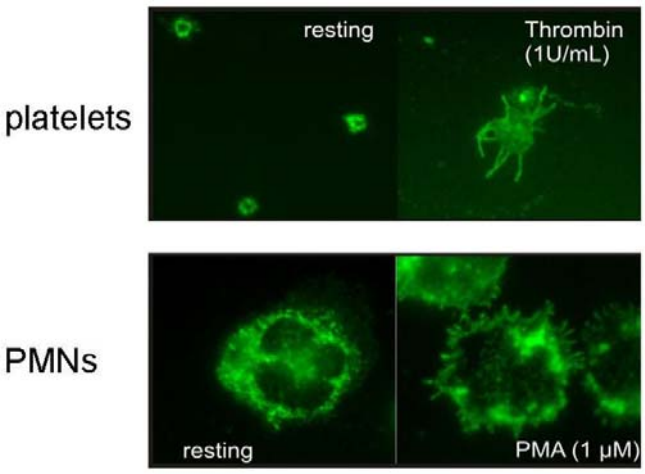

Fig. 3 Stomatin in granules of platelets and neutrophils. Platelets and neutrophilic granulocytes (PMNs) were isolated from whole blood and seeded onto coverslips coated with poly-L-lysine (platelets) or fibronectin (neutrophils). The cells were either fixed immediately with 4% formaldehyde or activated with the indicated agonists for 5 minutes at 37°C before fixation. Cells were permeabilized with 0.1% Triton X-100 and indirect immunofluorescence was performed with the monoclonal anti-stomatin antibody GARP-50 and a secondary, AlexaFluor488-conjugated anti-mouse antibody (Molecular Probes). Note the major staining of cytoplasmic granules in resting cells in contrast to the plasma membrane staining, particularly in filopodia, after activation. PMA, phorbol 12-myristate 13-acetate.

Similar to the *C. elegans* homologues, mammalian stomatin and SLP-3 have been identified in neurons. All sensory neurons of the mouse dorsal root ganglia express stomatin that is located in spots on somatic and axonal membranes (Mannsfeldt *et al*. 1999). This result suggests that stomatin may play a role in a putative vertebrate mechanotransduction complex. Similarly, stomatin mRNA was found to be co-expressed with α-, β-, and γ-ENaC in rat trigeminal mechanosensory neurons, consistent with a possible role in mechanotransduction (Fricke 2000). SLP-3, also known as stomatin-related olfactory protein (SRO), is expressed in olfactory sensory neurons (Kobayakawa *et al*. 2002). Moreover, the SLP-3 knockout mouse shows an impaired response to mechanical stimuli, in accordance with the worm's mec-2 null phenotype, supporting the idea that SLP-3 is an essential subunit of a mammalian mechano-transducer (Wetzel *et al*. 2007). Mammalian stomatin may play a role in controlling the sensitivity to volatile anaesthetics, because the stomatin knock-out mice showed an increase in sensitivity to diethyl ether, as revealed by tail clamp responses (Sedensky *et al*. 2006). Human stomatin may also interact with ENaC channels in the airway epithelium. It was identified in the ciliated cells, with the strongest signal along the cilia and the membrane of microvilli. Stomatin is suggested to play a role as a membrane-associated mechanotransducer in the control of ciliary motility (Fricke *et al*. 2003b).

The kidney-specific podocin is DRM-associated and oligomeric and interacts with the proteins CD2AP and nephrin to form a complex at the glomerular slit diaphragm of podocytes (Schwarz *et al*. 2001). It binds cholesterol and interacts with the transient receptor potential C channel protein TRPC6 in a large cholesterol-rich supercomplex (Huber *et al*. 2006). Interestingly, it requires cholesterol to regulate the activity of TRPC channels. Similarly, the *C. elegans* homologue MEC-2 binds cholesterol and interacts with DEG/ENaC channels in large supercomplexes and needs cholesterol for the activation of mechanosensation (Huber *et al*. 2006). In contrast, the peripheral membrane protein SLP-2 is a protein of the inner mitochondrial membrane, similar to the prohibitins, forming an oligomeric complex with the mitochondrial fusion mediator mitofusin 2 (Hajek *et al*. 2007). Interestingly, SLP-2 up-regulation has been described in various forms of cancer and in premalignant lesions thereby possibly serving as a marker for early detection of specific cancer types (Zhang *et al*. 2006). *In vivo* and *in vitro* transfection experiments with sense and antisense SLP-2 indicated that this protein is involved in cell proliferation and endometrial tumorigenesis (Zhang *et al*. 2006; Cui *et al.* 2007). Moreover, these studies also implicate a role of SLP-2 in cell adhesion. SLP-2 was partly found at the plasma membrane co-localizing with fibronectin 1 and it was suggested to serve as a transmembrane linker between the extracellular matrix and the cytoskeleton. In erythrocytes, SLP-2 was initially found as a peripheral plasma membrane protein associated with the cytoskeleton (Wang *et al.* 2000).

Dynamics of stomatin-specific lipid rafts

Segregation of rafts in the red cell membrane

Erythrocytes have often served as the cellular model system to address new questions in membrane biology, because the protein and lipid composition of the erythrocyte membrane is relatively simple and well characterized. The membrane cytoskeleton consists of a hexagonal lattice of spectrin tetramers that are interconnected by short actin filaments. Due to the lack of an active actin polymerisation machinery in the mature erythrocyte, the cytoskeletal activity is restricted to reversible binding to integral membrane proteins or lipids. Therefore, dynamic membrane processes have to be explained by the inherent properties of the membrane bilayer

and the integral protein and lipid components. The bilayer couple hypothesis provides a good model for erythrocyte cell shape transformations due to the surface expansion of one of the membrane leaflets (Sheetz *et al*. 1974). On the other hand, segregation processes of membrane components must be considered to be mainly driven by the physical properties of the lipid components. Both, membrane component segregation and echinocytic cell shape transformation take place upon rise of cytosolic calcium, for instance by treatment with the ionophore A23187. Stomatin is enriched at the tips of the echinocytic membrane protrusions (Hagerstrand *et al*. 2006) and does no longer co-localize with actin filaments (**Fig. 4**). In accordance, stomatin is also enriched in microvesicles (about 180 nm in diameter) generated at the tips, whereas cytoskeletal components are virtually absent (Salzer *et al*. 2002). The membrane protein composition of microvesicles indicates a segregation of different types of lipid rafts during the vesiculation process, because the GPI-anchored proteins AChE and CD55 are even more enriched than stomatin, whereas the flotillins are largely depleted (Salzer *et al*. 2002; Minetti *et al*. 2003). Interestingly, stomatin is depleted from nanovesicles (about 80 nm in diameter), which represent a small but distinct fraction of the calcium-induced, shed exovesicles. These vesicles are highly enriched in GPI-anchored proteins, the cytosolic proteins synexin and sorcin, which associate with membrane rafts upon calcium binding (Salzer *et al*. 2002), and the Alix protein (U. Salzer, unpublished observation), which is known to be involved in the formation and regulation of multivesicular bodies (Matsuo *et al*. 2004; van der Goot *et al*. 2006).

How can the segregation phenomena and vesicle formation be explained? We suggest that (i) cytoskeletal association, (ii) membrane curvature, and (iii) lipid raft aggregation are determining factors of these processes. (i) Cytoskeletal association of integral membrane proteins may be responsible for the depletion of these proteins from microvesicles by preventing the diffusion into the cytoskeleton-free membrane protrusions. For example, the ratio between cytoskeleton-associated to mobile band 3 protein correlates with the degree of band 3 depletion from microvesicles (Hagelberg *et al*. 1990; Minetti *et al*. 2003). (ii) Sterical reasons might account for the concentration of specific components like GPI-anchored proteins in the curved regions at the tip of the membrane protrusion, which might allow a tighter packing of these extrafacial monotopic raft components. Similarly, curvature-dependent segregation could be the reason for the high concentration of synexin with its convex membrane binding side at the tips of echinocytic spikes (Hagerstrand *et al*. 2006) and in the highly curved nanovesicles (Salzer *et al*. 2002). (iii) The spontaneous tendency of small rafts to aggregate and form larger liquid ordered (L_o) phase domains is thought to be associated with or to initiate membrane budding and endo- or exovesiculation processes (Turner *et al*. 2005; Hancock 2006). Theoretical considerations and experimental data from model membranes suggest that the driving force behind these processes is the minimization of the line tension at the border between the membrane domain (L_o phase or raft) and the surrounding matrix (liquid disordered L_d phase) (Lipowsky 1992; Julicher *et al*. 1993; Seifert 1993; Julicher *et al*. 1996; Baumgart *et al*. 2003).

The finding that stomatin is enriched at the tips of membrane spikes (**Fig. 4**) and in microvesicles, whereas the flotillins are depleted, indicates a differential degree of association with the cytoskeleton in calcium/ionophore treated erythrocytes. Taking into account that the ratio of stomatin to the GPI-anchored AChE is three times higher in calcium-induced microvesicles than in amphiphile-induced vesicles (Hagerstrand *et al*. 2006), one may conclude that the fraction of cytoskeleton-associated stomatin decreases upon calcium treatment. In DRMs prepared from untreated erythrocytes we usually find a large fraction of stomatin and flotillins associated with cytoskeletal components (Salzer *et al*. 2001; Salzer 2007) suggesting a tight linkage of both stomatin and flotillin rafts to the cytoskeleton. Similar results were obtained by Ciana *et al*. (2005). Stomatin rafts might selectively be released from their cytoskeletal anchorage upon calcium entry, diffuse into the cytoskeleton-free echinocytic spikes and aggregate at the tips of the protrusions to form larger L_o phase aggregates. In line with this model is our finding that DRMs can be isolated from microvesicular membranes with stomatin being the most abundant protein component (Salzer *et al*. 2002). Sterical incompatibilities may largely prevent the access of stomatin to the highly curved nanovesicular buds, because stomatin is strongly depleted from nanovesicles.

Calcium-induced vesiculation *in vitro* turns out to be a good model for the physiologically important vesiculation process that erythrocytes use as a defence mechanism against complement-mediated cell lysis. Erythrocytes can get rid of the membrane attack complex via exovesiculation and this process is dependent on calcium in the medium (Iida *et al*. 1991). Conceivably, the formation of a pore by the membrane attack complex leads to a local calcium influx that may trigger a sequence of events including the proteolytic destruction of the cytoskeleton and the aggregation of (stomatin) rafts in proximity of the membrane pore. This may cause a local budding of the respective membrane area and result in the final shedding of an exovesicle containing the membrane attack complex.

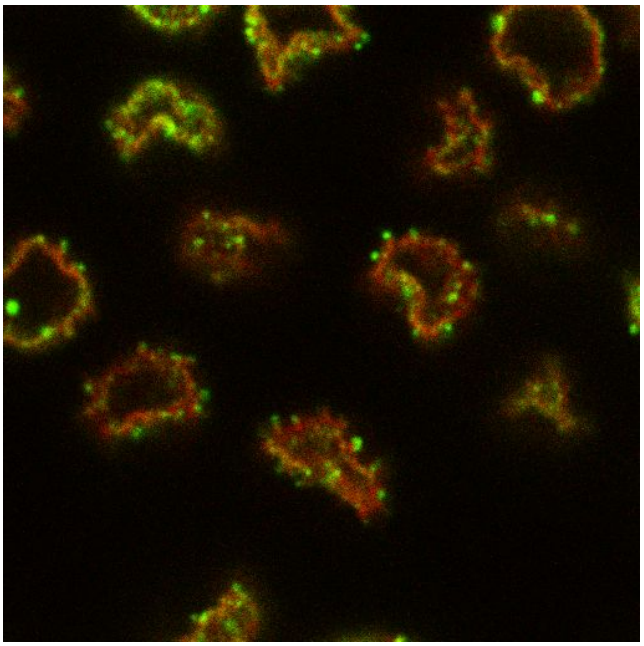

Fig. 4 Segregation of stomatin rafts on Ca^{2+} induced vesiculation of erythrocytes. Erythrocytes were treated with Ca^{2+} and ionophore A23187, fixed on a coverslip and stained for stomatin (monoclonal GARP-50 and FITC-labelled anti-mouse antibody, green) and actin (TRIC-phalloidin, red). Accumulation of stomatin at the tips of membrane protrusions devoid of actin is seen.

Segregation of rafts in the membranes of malaria infected red cells

Differential behaviour of stomatin- and flotillin-specific rafts has been observed also during the infection of erythrocytes by the malaria parasite *Plasmodium falciparum*. The parasite attaches with its apical end to the erythrocyte membrane and forms a junctional complex close to the attachment site. This complex seems to specifically "select" membrane proteins of the host cell membrane to enter the expanding parasitophorous vacuolar membrane (PVM) during invasion. The cytoskeletal and major integral proteins of the host membrane are excluded from the PVM, whereas host DRM proteins are present (Lauer *et al*. 2000). However, immunofluorescence studies show that one major DRM marker, stomatin, is a prominent exception from this "rule"

being specifically absent from the PVM (Murphy *et al*. 2004). The importance of raft-integrity is highlighted by the finding that cholesterol depletion blocks the infection process (Samuel *et al*. 2001) and induces the release of intracellular parasite probably by destabilisation of the PVM (Lauer *et al*. 2000). Depletion of cytoskeletal proteins and the involvement of lipid rafts are common features of the exocytotic calcium-induced vesicle and the "endocytotic" parasite-driven vacuole formation with the major raft-markers stomatin and flotillins showing a complementary behaviour in these processes. As signalling via the erythrocyte β_2-adrenergic receptor has been shown to be involved in malarial infection (Harrison *et al*. 2003), it is tempting to speculate that different signalling pathways may regulate the mobility of rafts by differentially modulating their association with the cytoskeleton.

A parasite homologue of stomatin, closest related to human SLP-2, is located in the invasion-associated rhoptry organelles of the extracellular parasite and inserted into the newly formed PVM during host cell invasion (Hiller *et al*. 2003). Further progress in the understanding of the functions of the stomatin family will be necessary to evaluate this finding. Three possibilities can be envisaged: (i) The *Plasmodium* stomatin homologue has a function like erythrocyte stomatin and compensates for the absence of host cell stomatin from the PVM. (ii) The *Plasmodium* stomatin has some "antagonistic stomatin function" and the depletion of erythrocyte stomatin from the PVM is specifically required. (iii) It is also possible that there is no functional interdependence between these stomatins and that the absence of host stomatin is a mere bystander effect of the invasion process.

Segregation of rafts in the membranes of platelets and neutrophils

In resting platelets and neutrophils, immunofluorescence experiments revealed a mainly cytosolic, punctuate staining of stomatin (**Fig. 3**) that is due to its predominant association with internal membranes. In platelets, stomatin is present at the membrane of α granules (Mairhofer *et al*. 2002) and in neutrophils it is localized to azurophil granules, specific granules, and probably secretory vesicles (Feuk-Lagerstedt *et al*. 2002). Both studies indicate that stomatin is associated with lipid rafts at these internal membranes. Upon activation of these cells and mobilisation of the internal granule pool, there is a shift of stomatin distribution to the plasma membrane. In activated platelets, stomatin is concentrated in long membrane protrusions; similarly, in activated neutrophils, stomatin is enriched in membrane filopodia (**Fig. 3**). Interestingly, platelet-derived microvesicles, which are shed upon thrombin or calcium/ionophore activation and are thought to exert procoagulant activity at distance from the site of activation (Heijnen *et al*. 1999), are enriched in stomatin but are depleted of flotillins (Mairhofer *et al*. 2002). This is reminiscent of the calcium-induced vesiculation of erythrocytes and suggests that a similar mechanism might account for the exovesiculation process in platelets. When platelets are activated, the α-granules fuse with the plasma membrane, a process that drastically increases the amount of stomatin rafts in the membrane. It is conceivable that mobile stomatin rafts coalesce in the membrane protrusion tips of activated platelets thereby contributing to the formation of larger L_o phase domains, which are a driving force for exovesicle fission. However, in contrast to erythrocytes, platelets do have an active actin cytoskeleton and the enrichment of stomatin in platelet exovesicles could also be due to a cytoskeleton-driven sorting process.

Interaction of stomatin with membrane proteins

A functional role of stomatin in the regulation of an ion channel has been suggested since the early studies of OHSt erythrocytes. The first direct evidence for a respective role was provided by Goodman *et al*. when they noticed that co-expression of human stomatin with the *C. elegans* DEG/ENaC channel protein MEC-4d produced a small but distinct amiloride-sensitive current in *Xenopus laevis* oocytes (Goodman *et al*. 2002). The effect of stomatin was comparable to the MEC-2 deletion containing the stomatin-core region but lacking both the MEC-2-specific N- and C-termini. Genetic screens of touch-insensitive mutants revealed that more than half of the MEC-2 missense mutations are located within this stomatin-domain thus suggesting in important role of this domain in the proper functioning of the channel (**Table 1**). It was shown that the stomatin-domain is essential for the association of MEC-2 with MEC-4 (Zhang *et al*. 2004). Co-expression of full length MEC-2 and MEC-4 produced a much larger amiloride-sensitive current in oocytes. However, interestingly, this current was strongly decreased when either the MEC-2 deletion mutant or human stomatin were expressed together with these proteins. This type of dominant-negative interference is consistent with the formation of multimers by the central stomatin-domain. Therefore, a scaffolding function for the interaction with the DEG/ENaC channel and/or with the lipids surrounding the channel has been suggested for the stomatin-domain of MEC-2 (Goodman *et al*. 2002).

Other members of the DEG/ENaC family of ion channels, the acid-sensing ion channels (ASICs), have been shown to interact with and to be modulated by stomatin (Price *et al*. 2004). ASICs 1a, 2a and 3 form homo- or heteromultimers in the membrane generating H^+-gated sodium channel activity. Upon co-expression of stomatin with each of these subunits in heterologous cells, both co-localization and co-immunoprecipitation of the respective proteins was observed in each case. However, interestingly, there was a differential effect of stomatin on the respective homomeric channel activities. Stomatin decreased the current amplitudes of ASIC 3 homomers but left the current desensitizing kinetics unchanged. The opposite effect was observed for ASIC 2a homomers, whereas ASIC 1a homomers did not show any modulation upon stomatin co-expression. ASICs 1a-3 or 2a-3 heteromers did not show altered current amplitudes but displayed increased desensitization rates upon stomatin co-expression. It is not clear yet whether stomatin modulates the gating properties of the respective ASIC channels directly or indirectly. Considering stomatin as a lipid raft-associated protein, it can be envisaged that the ASICs are recruited to these specialized membrane domains and that the altered gating behaviour is due to the different lipid environment or the interaction with raft-based signalling components. ASICs are expressed in the central nervous system and in peripheral sensory neurons where they function as sensors of acidosis (Price *et al*. 2001) but also of mechanical stimuli (Chen *et al*. 2002). Thus, Price *et al*. suggest that stomatin might regulate nociception by modulating the gating properties of various ASIC homo- and heteromers (Price *et al*. 2004).

In a related context, stomatin has been shown to be involved in the regulation of glucose transport through the plasma membrane. Co-immunoprecipitation studies of erythrocyte and rat liver cell lysates indicate an association of stomatin with the glucose transporter GLUT1 (Zhang *et al*. 1999) with the 42-amino acid cytoplasmic domain of this multiple membrane-spanning protein probably being the interaction site (Zhang *et al*. 2001). Moreover, it was shown that the amount of exogenously expressed stomatin inversely correlates with GLUT-1 activity. Since stomatin overexpression did neither affect the amount nor the plasma membrane localization of GLUT-1 but resulted in an enhanced association between stomatin and GLUT-1, it was suggested that stomatin has a "masking" effect on GLUT-1 (Zhang *et al*. 2001). Interestingly, GLUT-1 was shown to partially associate with DRMs and that this pool significantly decreases upon treatment with azide, a well-known inhibitor of oxidative phosphorylation and activator of GLUT-1 (Rubin *et al*. 2003). These data suggest that the reversible raft-association of GLUT-1 contributes to the regulation of

Table 1 Point mutations in the PHB domain of stomatin family proteins.

	Mutation	Commentary	Origin	Reference
STOMATIN	W184→A	defective oligomerization	*in vitro* mutation and expression in cell lines	E Umlauf, unpublished data
	P199→G			
	E209→K	defective PM targeting		M Mairhofer, unpublished data
	E211→K			
	S230→A	defective targeting		
	Y251→A	defective oligomerization		E Umlauf, unpublished data
	T265→A			Umlauf *et al.* 2006
	I266→A	defective oligomerization and lipid raft association		
	V267→A			
	F268→A			
	P269→A	defective oligomerization		
	L270→A			
PODOCIN	P118→L	defective targeting	steroid-resistant nephrotic syndrome	Roselli *et. al.* 2004
	R138→Q			
	D160→G			
	R168→H			
	V180→M			
	R238→S			
	R291→W	defective targeting		
MEC-2	P134→S	abolishes cholesterol binding	*C. elegans* mec-allele u274	Huber *et. al.* 2006
	R184→C	touch sensitivity	mec-allele u64	Zhang *et al.* 2004
	V190→M	touch sensitivity	mec-allele u224	
	S200→F	touch sensitivity	mec-allele u306	
	A204→T		mec-alleles e75 + u284	Gu *et al.* 1996
	A207→T	touch sensitivity	mec-allele u28	Zhang *et al.* 2004
	A234→V	touch sensitivity	mec-allele u227	Huang *et al.* 1995
	R239→H		mec-allele e1608	
	G243→E		mec-allele u7	
	T246→I		mec-allele u750	
	A296→V	touch sensitivity	mec-allele u144	Zhang *et al.* 2004
	E297→K		mec-allele u318	
	E299→K	touch sensitivity	mec-allele e1514	Huang *et al.* 1995
	A304→T		mec-allele u243	Zhang *et al.* 2004
	A306→T		mec-allele u24	
	E314→K		mec-allele u217	
	A320→V		mec-allele u311	
	A325→T		mec-allele u43	
	P357→L	touch sensitivity	mec-allele u130	
	P357→S	touch sensitivity	mec-allele u132	
UNC-1	E60→K	loss of function	*C. elegans* unc-allele n774	Rajaram *et al.* 1998
	A121→T		unc-allele e114	
	T183→R	dominant negative allele	unc-allele n494	Park *et al.* 1986; Rajaram *et al.* 1998
	G187→E		unc-allele e580	Rajaram *et al.* 1998
	K189→R+A212→V	intragenic revertant of the dominant e1598 allele	unc-allele e1598n1201	Park *et al.* 1986; Rajaram *et al.* 1998
	R252→C	cold-sensitive alleles	unc-alleles hs1+hs4	Hecht *et al.* 1996; Rajaram *et al.* 1998
	L257→I		unc-allele hs5	
	T267→I		unc-allele hs2	
	P273→S		unc-allele hs3	

glucose transport activity at the plasma membrane and that stomatin plays a crucial role in this process.

Genetic studies of *C. elegans* revealed that mutations in the *unc-1* gene, which encodes an orthologue of mammalian stomatin, cause uncoordinated locomotion (Park *et al*. 1986), increase the sensitivity to volatile anaesthetics (Rajaram *et al*. 1998), and modulate the hypersensitivity to anaesthetics induced by mutations in other genes (Morgan *et al*. 1990; Rajaram *et al*. 1999) (**Table 1**). UNC-1 is ubiquitously expressed in the nervous system of the worm and this might explain the variety of observed phenotypes. Overlapping expression patterns and genetic interactions with regard to anaesthetic sensitivity suggest a direct interaction of the proteins UNC-1 and UNC-8, which is a member of the DEG/ENaC family of ion channels (Rajaram *et al*. 1999). It was shown that UNC-1 and UNC-8 co-immunoprecipitate and that the partial presence of UNC-8 in DRMs is dependent on UNC-1 (Sedensky *et al*. 2004). Moreover, Sedensky *et al*. showed that the *unc*-24 gene, coding for another member of the stomatin protein family, was epistatic to *unc*-1 indicating a functional interdependence of the two stomatin proteins. Subcellular localization studies revealed that in worms carrying an *unc*-24 loss-of-function mutation, UNC-1 did not show its normal punctuate pattern along the major nerve tracts but was rather localized close to the cell nuclei indicating that UNC-24 affects the trafficking of UNC-1 to the cell periphery (Sedensky *et al*. 2001). Interestingly, recent data from our laboratory show that the mammalian orthologues of UNC-1 and UNC-24, i.e. stomatin and SLP-1, interact with each other and that SLP-1 affects the subcellular localization of stomatin (M. Mairhofer, manuscript submitted).

Emerging functions of stomatin

A role in lipid raft-dependent processes

In a recent review on lipid rafts, John Hancock suggests to replace the simplistic view of lipid rafts as stable, liquid ordered (L_o) domains/platforms, which float in the plane of

the membrane, by a dynamic model that proposes the spontaneous and transient formation of small, laterally mobile L_o domains (Hancock 2006). These short-lived domains can, however, gain stability by interaction with lipid-anchored or transmembrane proteins and this leads to the reduction of the line tension at the liquid ordered/disordered (L_o-L_d) domain boundary. According to this model, stomatin would be a typical raft-stabilizing protein. The dual palmitic acid modification and the cholesterol-binding property would confer this stabilizing effect, which is further enhanced by the tendency of stomatin to form oligomers. The dynamic model of lipid rafts includes any form of interdependent influence between lipids and proteins in raft formation. It is therefore conceivable that oligomerization of a raft-stabilizing protein can also induce the formation of larger L_o domains. The association of stomatin with lipid rafts is well supported, however, in light of this novel view on rafts one might speculate that stomatin plays an active role in the formation and stabilisation of membrane rafts. Reversible palmitoylation and oligomerization may contribute to this hypothetical function of stomatin as a regulated raft scaffolding protein. Caveolin is the prototypical example for a raft scaffolding protein forming caveolae, the stable, flask-shaped membrane invaginations, which are internalised in a stimulus-dependent manner (Hommelgaard *et al*. 2005). A different type of scaffolding structure, namely large, preassembled platforms of yet unclear function, is formed by flotillins/reggies in haematopoietic cells (Rajendran *et al*. 2003). In HeLa cells, a large immobile fraction of stomatin exists in the plasma membrane (Umlauf *et al*. 2006) and a large overlapping distribution of stomatin and cytoskeletal components is found in density gradients of erythrocyte DRMs (Salzer *et al*. 2001; Ciana *et al*. 2005; Salzer 2007). These data suggest an involvement of the cytoskeleton in the organisation of stomatin-specific rafts. Cytoskeletal breakdown or regulated release from the cytoskeleton would increase the mobile pool of stomatin-specific rafts. The dynamics of this mobile raft pool is significantly governed by the physical forces that are acting on mixtures of different lipid phase domains and lead to large-scale phase separation and membrane budding in order to minimize the line tension (Lipowsky 1992; Baumgart *et al*. 2003). Calcium-induced vesicle release from erythrocytes has already been suggested to be an example of such a process. The concerted endocytosis of cross-linked exoplasmic PLAP and endoplasmic stomatin (Snyers *et al*. 1999a) may rely on a similar mechanism. Mobile stomatin-specific rafts at the endoplasmic side of the membrane may be attracted by the raft aggregation on the exoplasmic surface and thereby lead to membrane budding and eventual vesicle internalisation. Flotillin-1 was recently shown to reside in punctate structures within the plasma membrane and in endocytic intermediates that contained GPI-anchored proteins and GM1 but not caveolin or clathrin. These raft-like regions seemed to bud into the cell by a clathrin- and caveolin-independent endocytic pathway (Glebov *et al*. 2006). Given the topological and biochemical similarity of stomatin to caveolin and the flotillins, it is tempting to speculate that raft-associated stomatin may also be involved in a specific, clathrin-independent, endocytic pathway. Caveolar coat formation has been suggested to be induced by polymerization of caveolin oligomers upon dephosphorylation, thereby increasing the affinity for cholesterol and imposing positive membrane curvature at the respective membrane area. A similar coat forming mechanism has been proposed for reticulons and PHB-domain proteins with respect to the presence at peripheral ER tubules and endocytic vesicles (Bauer *et al*. 2006). It may be speculated that stomatin polymerization could also take place at sites of raft aggregation thereby forming a coat for an endocytic vesicle. Conversely, polymerization should not occur at sites of negatively curved membranes (exocytic microvesicles) where stomatin rafts are also enriched (**Fig. 4**).

Regulation of channel activities

The studies of *C. elegans* stomatin homologues have clearly shown that these proteins are involved in the regulation of ion channels of the DEG/ENaC family. Although mammalian stomatin lacks the specific N- and C-terminal interactiton domains of MEC-2, it may directly bind to ion channels, as shown by the co-immunoprecipitation with ASIC channels (Price *et al*. 2001). Another example is the UNC-1 interaction with the DEG/ENaC family member UNC-8 in lipid rafts (Sedensky *et al*. 2004). The stomatin-like UNC-24 protein is also part of the mechanosensory complex comprising MEC-2, MEC-6, and the DEG/ENaC channel components MEC-4 and MEC-10 (Zhang *et al*. 2004). Ion channels at the plasma membrane are preferentially found in lipid microdomains and therefore it is conceivable that the raft-associated stomatins interact with channel proteins similar to caveolin-1 interacting with the transient receptor potential channel TRPC1 (Remillard *et al*. 2006). Recently, it was shown that the stomatin-like proteins podocin and MEC-2 bind cholesterol and associate with ion channel complexes to form large protein-cholesterol supercomplexes in the plasma membrane (Huber *et al*. 2006). Whereas MEC-2 is co-immunoprecipitated with a heterologous α-ENaC, podocin is co-precipitated with the TRPC6 channel. Interestingly, cholesterol plays an important role in regulating the channel activities within the supercomplexes. The stomatin-like proteins must contain bound cholesterol in order to function as channel modulators. A MEC-2 mutant (P134S) that contains a serine instead of the conserved proline residue in the hydrophobic IM domain (**Fig. 1**) has lost its ability to bind cholesterol and is completely touch insensitive. Multimerization and α-ENaC interaction was not affected by the mutation. This finding therefore suggests that cholesterol binding to MEC-2 is essential for touch sensitivity (Huber *et al*. 2006). Likewise, the equivalent mutation in podocin (P120S) also led to the loss of cholesterol binding and loss of TRPC6 channel activity although the podocin-TRPC6 interaction was unaffected. In this context, it is interesting that stomatin itself is able to bind cholesterol (E. Umlauf, C. Thiele *et al*. unpublished data) and thereby may modulate the activity of ion channels like ASICs in large lipid-protein supercomplexes. Moreover, the reversible recruitment of GLUT-1 to stomatin-containing rafts has been suggested as a novel mechanism of GLUT-1 regulation (Rubin *et al*. 2003). Thus, it is conceivable that stomatin is involved in the regulation of membrane processes by recruiting various membrane proteins to rafts and modulating their activities.

CONCLUDING REMARKS

Stomatin and stomatin-like proteins are monotopic, oligomeric, cholesterol-binding, lipid raft-associated proteins that are involved in membrane organization and cholesterol-dependent regulatory processes. Due to its oligomeric structure, stomatin may provide a scaffolding function by assembling small membrane microdomains to form larger lipid-protein complexes. These complexes interact with the actin cytoskeleton and may function as signalling platforms. Additionally, the lipid transfer domain of the stomatin-like protein SLP-1, known to interact with stomatin, may regulate the lipid composition of the lipid-protein complexes. Large stomatin aggregates can be formed by external lipid raft cross-linking at the plasma membrane, a process that leads to endocytosis. It is conceivable that this internalisation event is induced by stomatin polymerization similar to caveolar coat formation.

It has long been hypothesized but only recently shown that stomatin functions as a regulator of ion channels. Moreover, stomatin was found to depress GLUT-1 activity. A common view emerges that this regulatory function of stomatin is based on the reversible recruitment of these interacting membrane proteins to the lipid raft domains, with stomatin displaying a binding and modulating property.

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