

Chapter 5

Membrane Rafts in the Erythrocyte Membrane: A Novel Role of MPP1p55

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Abbreviations

aZO1	A mammalian zonula occludens protein
DIgA	A Drosophila disc large tumor suppressor
DRM	Detergent resistant membrane
FLIM	Fluorescence
lifetime imaging L27	Lin-2 and Lin-7 receptor targeting proteins
ld	Liquid disordered
lo	Liquid ordered
MAGUK	Membrane associated guanylate kinase
MPP1	Membrane palmitoylated protein-1
PC	Phosphatidylcholine
PDZ PSD	Post synaptic density protein
PE	Phosphatidylethanolamine
PS	Phosphatidylserine
SH3	Src homology 3 domain
SM	Sphingomyelin

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Introduction

Eukaryotic cell membranes are organized into functional lipid and protein domains, the most widely studied being membrane rafts. They are enriched in sphingolipids and cholesterol and contain several types of membrane proteins such as stomatin and flotillins, which are their “permanent” residents, along with several others, such as receptors, including growth factor receptors. Membrane rafts organize receptors and their downstream molecules to regulate a number of intracellular signaling pathways (for review see (Grzybek et al. 2005; Pike 2006; Hancock 2006; Simons and Sampaio 2011)). Important post-translational modifications, such as palmitoylation, addition of GPI anchors or sterol molecules, regulate raft-affinity of the majority of raft proteins.

Lateral interactions of cholesterol with membrane raft lipids seem to be crucial for maintaining these microdomains in the ‘lo’ (liquid-ordered) state, which is characterized by decreased conformational (trans-gauche) freedom and, consequently, reduced “fluidity”, compared to the bulk cholesterol-poor membrane which exists in the ‘ld’ (liquid disordered) state. However, unlike the gel phase in artificial lipid systems they are characterized by similar rotational and lateral (translational) mobility to the bulk membrane (Ipsen et al. 1987; Simons and Vaz 2004). Rafts are enriched not only in cholesterol and sphingolipids, but also glycolipids and specific inner-layer phospholipid PE and PS species. Data on the lipidomics of the DRM reveal that, in addition to an abundance of sphingomyelin and cholesterol, they contain other phospholipids that mostly contain fully saturated or monounsaturated acyl chains. Predominant among these are the phosphatidylethanolamine glycerophospholipids and plasmalogens (Macdonald and Pike 2005; Pike et al. 2005; Koumanov et al. 2005; Brugger et al. 2006). Phosphatidylserine, which is a relatively minor membrane component, is three times more prevalent in the DRM than in the bulk volume of the plasma membrane, while phosphatidylinositols are rather diminished within the DRM, as are phosphatidylcholine species. PEs occur in the membrane predominantly as sn-1 saturated, sn-2 unsaturated glycerophospholipids, and recent data show that some DRM preparations are enriched in 1-stearoyl-2-linoleoyl-sn-glycero-3-phosphoethanolamine (SLPE), regardless of the method of isolation (Pike et al. 2005). Our own data indicate that this PE interacts with cholesterol comparably to SM, while dipalmitoyl-PE does not bind cholesterol (Grzybek et al. 2009). This suggests the importance of the structure of acyl chains of particular phospholipids (which, incidentally not all are saturated) and also explains the background of the mechanism by which inner-layer phospholipids participate in membrane rafts.

Membrane rafts contain several specific sets of membrane proteins (Brown and London 2000), which include membrane proteins belonging to the SPFH family (stomatin/prohibitin/flotillin/HflK), such as raft scaffold proteins flotillin-1 and -2, and stomatin or stomatin-like protein. These proteins share a common feature in that they associate with the raft domains, possibly through cholesterol-binding (Epanand et al. 2005; Epanand 2006; Browman et al. 2007) or oligomerization (Browman

et al. 2007). Apart from these proteins, and other palmitoylated and transmembrane proteins, membrane rafts also include GPI-anchored proteins and proteins involved in signal transduction, such as, by way of example, tyrosine kinases of the src family, $G\alpha$ subunits of heterotrimeric G proteins, endothelial nitric oxide synthase and hedgehog protein. Many of the raft proteins are modified with saturated acyl (palmitoyl) chains, or with cholesterol, which is thought to facilitate interactions with lo-domains (Wang et al. 2000). The mechanism(s) by which the proteins partition into membrane rafts may involve preferred solubility in the ordered domains and/or chemical affinity for raft lipids, as exemplified by the cholesterol-binding properties of some of the proteins (Fantini and Barrantes 2013). Another example includes a structural protein motif recognizing sphingolipids or specific glycolipids such as gangliosides (Hakomori 2002; Mahfoud et al. 2002).

It has long been known that rafts are engaged in cellular signal transduction pathways by hosting a number of receptors and their associate adapter proteins, and that they also facilitate signaling switches during the activation of the respective pathways. These proteins include receptor tyrosine kinases (EGF-R, IGF-1, c-kit), non-receptor kinases (e.g. src kinases: Src, Lck, Hck, Fyn, Blk, Lyn, Fgr, Yes, and Yrk (Boggon and Eck 2004)), serpentine (G-protein-linked seven transmembrane domain) receptors (Pike 2003), sigma receptors (Aydar et al. 2004) as well as heterotrimeric and monomeric G-proteins and other adaptor proteins. Also pro- or anti-apoptotic signaling elements have been found in raft domains, as recently reviewed (Hryniewicz-Jankowska et al. 2014). Of particular interest are the potential roles of membrane rafts as signaling platforms in neoplasia (Staubach and Hanisch 2011) and their possible usage as targets for anticancer drugs (Hryniewicz-Jankowska et al. 2014)

Erythrocyte Membrane Rafts

The existence of lateral heterogeneity within the erythrocyte membrane (Karnovsky et al. 1982; Schroeder et al. 1995; Rodgers and Glaser 1991) was known even before the formulation of the raft hypothesis by Simons and Ikonen (1997). As in the case with other cell types, the first attempts to characterize the lateral heterogeneity of erythrocyte membranes involved the extraction of membranes with the nonionic detergent, Triton X-100 (Salzer and Prohaska 2001). Subsequent freeze-fracture electron microscopy studies showed that the DRM fraction derived from human and ruminant erythrocytes were devoid of intramembrane particles from the inner fracture plane (Quinn et al. 2005), which is in agreement with the notion that transmembrane proteins are excluded from highly-ordered regions of the bilayer (McIntosh et al. 2003). Synchrotron X-ray diffraction studies indicated that d-spacings obtained for multilamellar stacks and vesicular suspensions of raft membranes (DRM) were, on average, more than 0.5 nm greater than corresponding arrangements of the original erythrocyte membranes from which they were obtained (Quinn et al. 2005).

Salzer and Prohaska provided the first systematic analysis of the protein composition of the DRM fraction of the erythrocyte membrane (Salzer and Prohaska 2001). Prominent components of this fraction were flotillins-1 and -2, typical components of raft-fractions in other cell types (Bickel et al. 1997; Lang et al. 1998). They also found variable amounts of membrane-skeleton and membrane-skeleton adapter proteins, such as spectrin, actin and proteins 4.1 and 4.2. The major integral protein, erythrocyte anion-exchanger, was absent from this fraction, as was glycophorin C.

Kamata et al. provided functional evidence for the presence of membrane rafts in erythrocyte membranes by showing inactivation of the Gs α -mediated signal transduction pathway by reversible disruption of rafts by lidocaine treatment (2008).

Stomatin, Flotillins and MPP1 Are the Stable Components of Erythrocyte Rafts

Among the constant protein components of erythrocyte membrane rafts, stomatin, flotillin-1 and flotillin-2 seem to be the major components playing an important structural role in their organization. They are considered fundamental marker-proteins of these structures. Although MPP1 is not commonly recognized as a membrane raft marker protein, our observations indicate that, at least in erythrocyte and erythroid cells, MPP1 is consistently found in DRM preparations of these cells, as described below.

Stomatin

Stomatin was first described as an integral protein of the erythrocyte membrane and was subsequently found to be widely expressed in various tissues and cells (Hiebl-Dirschmied et al. 1991a, b). Stomatin is absent in the erythrocyte membrane of patients suffering from a specific form of hemolytic anemia called stomatocytosis (for a review see e.g. (Boguslawska et al. 2010)). The protein consists of 287 amino acid residues, characterized by the presence of a highly charged 24-residue long N-terminus. This is followed by a 29-residue hydrophobic hairpin-domain, which is highly conserved amongst the close stomatin homologue proteins SLP-3, SLP-1, podocin and MEC-2 (Kadurin et al. 2009) and a large C-terminal region containing 234 residues. Stomatin typically has an unusual topology, with the afore-mentioned “hairpin domain” located within the lipid bilayer and the N- and C-termini facing the cytosol. There is data suggesting that a small portion of stomatin is expressed as a transmembrane protein, with the C-terminal domain being N-glycosylated and exposed to the cell surface (Kadurin et al. 2009). Palmitoylation of cysteine residues Cys-29 and Cys-86 further increases the affinity of stomatin for the membrane (Snyers et al. 1999).

Stomatin domain (SPFH, in mouse stomatin residues 86–213) has a mixed alpha/beta-fold structure. The N-terminal part forms an antiparallel curved beta-sheet (beta strands 1a, 1b, 2 and 3). Helices alpha-2 and alpha-4 extend in parallel and occupy the groove of the sheet, while the short alpha-1 and alpha-3 helices are oriented perpendicularly at both ends of the beta-sheet. The N- and C-termini are located at opposing sides of the molecule (Brand et al. 2012). Mouse stomatin forms a banana-shaped dimer via the four residues located at the C-terminal position of beta-3, in contrast to its archeal homologue from *Pyrococcus horikoshii*, which is trimeric (Yokoyama et al. 2008).

There is substantial evidence that stomatin forms higher-order oligomers; one of the crystal forms was found to form a ring-like oligomer 8 nm in diameter (Brand et al. 2012). Others had earlier found that erythrocyte stomatin forms oligomers of 9–12 mers, or even structures that were twice as large, reporting the presence of a short hydrophobic sequence (residues 264STIVFPLPI272) within the C-terminal region of stomatin that was responsible for oligomer formation (Umlauf et al. 2006).

Flotillins

Flotillins are also members of the SPFH-protein family, containing a common structural motif known as the SPFH domain (see above). Flotillin-1 is alternatively known as reggie-2, whilst flotillin-2 is known as reggie-1. Flotillin-1 has a hydrophobic sequence located within the C-terminal part of the SPFH domain (residues 134–151) in addition to the N-terminal hydrophobic stretch (residues 10–36). The N-terminal domain is considered as being important in the interaction of flotillin-1 with the membrane raft-domain and the C-terminal hydrophobic sequence is thought to be responsible for localization of flotillin-1 in the plasma membrane (Liu et al. 2005). SPFH domains in both flotillins (flotillin-1: residues 36–179, and flotillin-2: residues 41–183) are followed by another flotillin domain comprised of three coiled-coil stretches rich in EA residues (Solis et al. 2007). The C-terminal part of both molecules contains a PDZ3 domain (Chi et al. 2012). These proteins share 48 % structural identity and 71 % sequence homology. Flotillin-1 contains a palmitoylation site which is thought to be important for its membrane localization in kidney cells (Morrow et al. 2002). However, separate data indicates that palmitoylation at this site is less important in defining membrane localization than is the presence of the hydrophobic stretches located in this protein (Liu et al. 2005).

Flotillin-2 is myristoylated on the G2 residue, which is unique within the SPFH protein family members, and is palmitoylated on C4, 19 and 20. These post-translational modifications seem indispensable for membrane binding of flotillin-2 (Neumann-Giesen et al. 2004).

Cross-linking studies have shown that flotillins form rather stable homo- and hetero-tetramers of flotillin-1 and -2 in membranes and that oligomerization is dependent upon the C-terminal part of the molecules. Deletion studies have indicated that the presence of coiled-coil-2 and partially-coiled-coil-1 domains are responsible for tetramerization of flotillin-2. Moreover, the stability of flotillin-1 in the cell depends on the co-presence of flotillin-2 (Solis et al. 2007).

MPP1

MPP1 (p55) was first cloned by Ruff et al. (1991) and identified as a major palmitoylation substrate in the erythrocyte membrane. It is a member of the MAGUK (membrane associated guanylate kinase) family of proteins (te Velthuis et al. 2007; de Mendoza et al. 2010). It partially fulfills the criteria for scaffolding proteins (Pan et al. 2012), in that it contains several functional domains which are potentially responsible for simultaneous binding of regulatory and skeletal proteins and is, therefore, an important protein of the membrane skeleton ternary complex (Marfatia et al. 1995). All MAGUK family proteins share an enzymatically inactive guanylate kinase domain that mediates protein–protein interactions and intramolecular interactions with the SH3 domain and 0–1 unit of SH3 domain; 0–6 units of the PDZ domain, and 0–2 units of L27 (found in Lin-2 and Lin-7 receptor targeting proteins) domains. Different members of this family have ww, card, ZU5 or protein kinase domains. MPP1 also shares single GUK, SH3 and PDZ domains, as well as a D5/Hook/I3 domain which is responsible for protein 4.1R binding (Marfatia et al. 1997; Seo et al. 2009a). The best known role of MPP1 is its participation in the junctional complex formation of the erythrocyte membrane skeleton (for a current review see (Machnicka et al. 2012)). In this role, the PDZ-domain of MPP1 interacts with the cytoplasmic domain of glycophorin C, while the central region (D5-domain) is responsible for the interaction with protein 4.1R (Seo et al. 2009a, b). This interaction markedly strengthens protein 4.1-glycophorin C binding (Hemming et al. 1995). The functional role of MPP1 palmitoylation is currently not well understood, but one of the apparent functional aspects of this post-translational modification is discussed below.

The role of MPP1 in non-erythroid cells is understood rather poorly. Mburu et al. (2006) have shown that MPP1 forms a complex with whirlin, the protein which binds to the Usher protein network in the cochlea and the Crumbs network in the retina, by direct association with USH2A (usherin) and VLGR1 (a member of the 7-transmembrane receptor G-protein, which binds calcium and is expressed in the central nervous system). Mutations in this gene are associated with Usher syndrome 2 and familial febrile seizures. MPP1 binds also to MPP5, another member of the MPP subfamily of proteins (Gosens et al. 2007).

One of the established physiological roles of MPP1 is its engagement in the regulation of neutrophil polarity. Using a MPP1 knockout (p55^{-/-}) mouse model, Quinn et al. (2009) showed that, upon agonist-stimulation of neutrophils, MPP1 is rapidly recruited to the leading edge. Neutrophils of knockout mouse do not migrate efficiently *in vitro* and form multiple pseudopods upon chemotactic stimulation, in contrast to normal mouse neutrophils, which form a single pseudopod at the cell front required for efficient chemotaxis. Phosphorylation of Akt is decreased in these cells upon stimulation with chemoattractant, and this appears to be mediated by a PI3K γ kinase-independent mechanism.

Visualization of Erythrocyte Membrane Rafts

Several techniques have been used to visualize membrane rafts *in situ* within living cells, including such methodologies as homo- and hetero-transfer, fluorescence polarization anisotropy, FCS, total internal reflection (TIRF), single-molecule microscopy and the super-resolution microscopical techniques of stimulated emission depletion (STED), photo-activated localization microscopy (PALM), and stochastic optical reconstruction (STORM). All of these techniques (and combinations of them) support the hypothesis of lateral membrane heterogeneity and the existence of nano-domains [for reviews, see: (Jacobson et al. 2007; He and Marguet 2011)]. Among others erythrocyte membranes were also a subject of several experimental efforts.

Mikhalyov and Samsonov (2011) used the fluorescent probe N-(BODIPY®-FL-propionyl)-neuraminosyl-GM1 (BODIPY-GM1) to detect rafts in erythrocyte membranes via fluorescence video-microscopy. Their observations indicated that the probe was uniformly distributed over the plasma membrane at 23 °C. However, a partial phase-separation of the probe was observed at 4 °C, detectable as discrete bright spots whose dimensions were comparable to the limit of resolution of the technique. They also observed red-shifted fluorescence of the probe, characteristic of high local concentrations of the BODIPY fluorophore. The shift was greatest at 4 °C, and the smallest at 37 °C and was eliminated by treatment of the erythrocytes with methyl- β -cyclodextrin. They also observed that distinct GM1 patches distributed over the entire membrane at both 23 °C and at 37 °C in erythrocytes stained with Alexa FL 647 cholera toxin subunit B conjugate (CTB-A647). The authors conclude that rafts could be detected in erythrocytes based on fluorometry and fluorescence microscopy methods.

The combined FCS (scanning fluorescence correlation spectroscopy) and Laurdan GP (generalized polarization) can be used to measure the fluctuations of the membrane packing microheterogeneity. Such studies have facilitated the measurement of generalized polarization fluctuations in the plasma membrane of intact rabbit erythrocytes and Chinese hamster ovary cells, indicative of the existence of tightly packed micro-domains moving within a more fluid background phase. These structures, which are characterized by different lipid packing, have different properties: 1: fast fluctuations with a small amplitude, corresponding to the domains of ~50 nm in diameter, 2: slow fluctuations, with a large amplitude, corresponding to domains of ~150 nm in diameter and 3: slow fluctuations with a small amplitude, corresponding to the large domains of diameter within a range of 150–600 nm (Sanchez et al. 2012). According to these authors, the small size and characteristic high-lipid packing indicate that these micro-domains have properties that have been proposed for lipid rafts.

The data presented above, however, do not reach the resolution achieved in the observation of other living cells through using STED far-field fluorescence nanoscopy, where raft components were found trapped transiently (~10–20 ms) in cholesterol-mediated complexes of areas <20 nm in diameter (Eggeling et al. 2009).

Role of Erythrocyte Membrane Rafts in Signaling

As was previously mentioned, one of the most recognized physiological functions of membrane rafts in living cells is the functional organization of signal-transduction platforms. Although the erythrocyte is a specialized cell in which not many signal transduction events can be observed, there are several indications that those signaling pathways do exist and are also raft-dependent in these cells.

Murphy et al. (2006) demonstrated that, by using a peptide that inhibits Gs-receptor interaction, erythrocyte Gs is functional and that propranolol, an antagonist of G protein-coupled β -adrenergic receptors, blocked Gs activity in erythrocytes. This drug was proposed for use in malarial infection treatment (Murphy et al. 2004, 2006, 2007).

One of the best examples of a role of membrane rafts in cell signaling in erythrocytes is the above-mentioned work demonstrating that disruption of rafts in erythrocyte membranes via lidocaine treatment, which occurs without altering membrane cholesterol content, suppressed Gs α -mediated signal transduction. This signaling pathway can be triggered via the activation of the adenosine receptor by the agonist, 5'-N-ethylcarboxamido-adenosine. Furthermore, they showed that raft-mediated signal transduction resulted in elevated cAMP levels, leading subsequently to the phosphorylation of adducin, a membrane skeletal protein. Signal transduction was restored upon removal of lidocaine from the erythrocyte membrane (Kamata et al. 2008). These authors (Koshino and Takakuwa 2009) showed that disruption of erythrocyte membrane-rafts with lidocaine also prevented *Plasmodium* invasion of erythrocytes in culture.

Another signaling pathway that has been discovered in erythrocytes is an apoptotic signal transduction mechanism. Mature red cells contain Fas, FasL, FADD (Fas-associated death domain), caspase 8, and caspase 3. Circulating, aged and oxidatively-stressed erythrocytes showed co-localization of Fas with the raft marker proteins Gs α and CD59. Aged red cells had significantly lower aminophospholipid translocase activity. These events were independent of calpain, but dependent on reactive oxygen species (ROS). Upon inhibition of ROS generation by treatment of cells with *t*-butyl hydroperoxide, Fas did not translocate into rafts and neither formation of a Fas-associated signaling complex nor caspase activation was observed. This indicates that translocation of Fas into rafts was the trigger for the signal leading to caspase 3 activation (Mandal et al. 2005). It was shown that administration of *N*-acetyl cysteine in combination with atorvastatin, an inhibitor of cholesterol synthesis, proved to be more effective than either of the drugs alone towards the rectification of arsenic-mediated early erythrocyte death. Cholesterol depletion via atorvastatin treatment, along with reduced glutathione (GSH) repletion via *N*-acetyl cysteine treatment blocked Fas activation, leading to premature death of erythrocytes (Biswas et al. 2011). Similarly, mouse erythrocytes exposed to lead ions (Pb²⁺) undergo FAS-activated apoptotic death, with FAS observed to translocate to the DRM fraction in such cells (Mandal et al. 2012).

Erythrocyte Rafts and Pathology

Although numerous reports exist on the importance of rafts in many pathologies, such as neuropathological disorders and neoplasia, there is sparse data on red cell pathologies in which membrane rafts are reported to play an important role.

As one of the main protein components of erythrocyte membrane rafts is stomatin, the behavior of this protein in stomatocytosis, particularly in overhydrated stomatocytosis, was studied (Wilkinson et al. 2008). In this rare, dominantly-inherited, hemolytic anemia, erythrocytes show stomatocytic morphology, deficiency of stomatin in the membrane and a major leak of the monovalent cations Na^+ and K^+ across the membrane (Lande et al. 1982). However, the *STOM* gene encoding stomatin is almost certainly not mutated in this pathology. Rather, it appears that there is probably a trafficking defect in the developing erythroid cells, so that stomatin is obstructed within the secretory pathway and is not able to be incorporated into the plasma membrane of the mature erythrocytes (Fricke et al. 2005). When the DRM fraction was obtained from the erythrocytes of such patients, it was found to contain a reduced amount of stomatin, with the total membrane stomatin content reduced by more than 50 %. At the same time, the other raft proteins, such as flotillin-1 and -2, and Glut-1, were present in the DRM fraction in normal quantities. According to the authors, the DRM of patient erythrocytes contained decreased amounts of DRM-associated actin and tropomodulin (Wilkinson et al. 2008). The authors suggest that the raft tropomodulin, seen in normal erythrocytes, may act as a reservoir of available tropomodulin accessible for control of actin turnover. A fuller answer to this question still awaits further studies. As our own experience is concerned (see below), DRM isolated from human erythrocytes under alkaline conditions, or in the presence of latrunculin, contained normal quantities of flotillins and stomatin, but they contained only traces of actin or spectrin (Lach et al. 2012). This might be an indication of really weak raft-membrane skeleton interactions.

In some of the cases of hereditary hereditart spherocytosis, erythrocyte membranes were found to have membrane raft proteins flotillin-1 and -2 (Margetis et al. 2007) mildly reduced (75–90 %) and were observed to contain sorcin. Sorcin is a 22 kDa (band 8) protein, which is found in nanovesicles released from normal erythrocytes in the presence of high concentrations of Ca^{2+} (Allan et al. 1980; Salzer et al. 2002).

During our studies on hereditary spherocytoses, we found a family whose two male siblings (brothers) displayed symptoms of hemolytic anemia that did not fit to the traditional characteristics of the known disease. The parents of the brothers and their sister, along with the family members on both sides of the siblings' parents, were without symptoms of hemolytic anemia. As the patients showed the presence of stomatocytes, their Na^+/K^+ equilibrium was tested, along with their red blood cell membrane protein profile via SDS-PAGE. However, no changes were noticed in either of these parameters, compared to the unaffected individuals tested. Further, no protein acyl transferase (PAT) activity was detectable in their erythrocytes, which was caused by an absence of membrane DHH17 protein, while near-normal transcript levels of DHH17 gene were present in the reticulocytes of the two patients.

DHHC17 is a member of the protein acyltransferase (PAT) family of genes, of which 23 are present in the human genome (Korycka et al. 2012), but only one form is expressed in reticulocytes (Lach et al. 2012).

MPP1 and Its Palmitoylation Play a Crucial Role in Lateral Membrane Organization in Erythroid Cells

Lack of palmitoylation activity was found to lead to marked changes in lateral membrane organization, as revealed by a marked decrease in the DRM fraction of the erythrocyte membrane (see above). It was found that the mechanism underlying these changes involves MPP1, which is the major palmitoylation substrate of the erythrocyte membrane. It was confirmed that this protein was absent from the DRM fraction of erythrocytes of both of the patients (in the case of above-described hemolytic anemia) or from DRM erythrocyte fractions derived from healthy control individuals in which palmitoylation was inhibited by treatment with the potent PAT inhibitor, 2-BrP (2-bromo palmitate), first by MS/MS analysis (Grzybek and Sikorski –unpublished data) and then, by immunoblotting (Lach et al. 2012).

Separately, DHHC17-directed siRNA treatment was performed on reticulocytes isolated from human umbilical cord blood. Here, Western Blot analysis of sucrose density gradient fractions using anti-MPP1 antibodies indicated that the DHHC17 siRNA-transfected reticulocyte DRM fraction contained much less MPP1 than the same fraction obtained from reticulocytes transfected with control RNA of a scrambled sequence. A decreased amount of DRM was also observed when erythrocyte ghosts were resealed with a specific anti-MPP1 antibody.

The possibility of a mutation in the *MPP1* gene seemed very likely, as this gene locus is located in the X chromosome, which would fit the pattern of inheritance of the studied family, where only male individuals were affected. However, only a silent mutation (ACG_ACT; T85T) was identified in these patients. No mutations were found in the nucleotide sequences coding for the cytoplasmic-domain of glycoporphin C (*GYP C*), or in the coding sequence of the *EPB4.1R* gene (coding for protein 4.1). Moreover, sequencing of the stomatin transcript, which was present at the normal level, did not reveal mutations or polymorphisms.

The change in membrane solubility in 1 % Triton X-100 solution cannot be an effect of differences in lipid composition, as both the TLC and quantitative analysis of lipids in lipid extracts from erythrocyte ghosts showed no significant variations among the major lipid classes (including cholesterol content) between control healthy individuals and patients. Therefore, we concluded that (palmitoylated) MPP1 might play a crucial role in lateral membrane organization in the human erythrocyte, which is connected to the unique pathology of this cell.

Moreover, further studies performed on HEL cells (derived from erythroid precursors) showed a similar effect of palmitoylation inhibition on the DRM fraction.

When a stable HEL cell-line with a silenced *MPP1* gene was used for experiments, the same effect was observed. Moreover, the FLIM experiments using di-4 probe performed on normal and 2-BrP-treated erythrocytes, normal and 2-BrP-treated HEL cells and HEL cells with stably-silenced *MPP1* expression, demonstrated significant decrease in membrane-order upon inhibition of palmitoylation, or decrease in cellular MPP1 level (Biernatowska et al. 2013; Lach et al. 2012). Similar conclusions were drawn from studies on giant plasma membrane vesicles derived from HEL cells, performed by using several advanced biophysical techniques in parallel (Podkalicka, Grzybek et al. – to be published).

Furthermore, *MPP1*-knockdown significantly affects the activation of MAP-kinase signaling via raft-dependent tyrosine kinase receptors, indicating the importance of MPP1 for lateral membrane organization (Biernatowska et al. 2013).

In conclusion, palmitoylation of MPP1 appears to be at least one of the mechanisms controlling lateral organization of the cell membrane. Thus, these studies point to a new role for MPP1, and present a novel linkage between membrane-raft organization and protein palmitoylation.

An important question that remains is how MPP1 affects the organization of the membrane domains. Our hypothesis is that, upon palmitoylation, the affinity of MPP1 for membrane-skeleton binding decreases and MPP1 becomes freely available for binding to the pre-existing nanoclusters or ‘unstable rafts elements’ within the membrane, namely, protein-cholesterol/lipid complexes corresponding to cholesterol-depletion sensitive, short-lived (<0.1 ms) nanoclusters (<10 nm in diameter), observed previously by others (Fujita and Kinoshita 2010; Mayor and Rao 2004; Sharma et al. 2004). This suggestion is based on the observations that, in normal erythrocytes and in HEL cells, the unpalmitoylated MPP1 remains in the high-density “skeletal” fraction within the density-gradient profile of the DRM. Binding of palmitoylated MPP1 to the pre-existing nanoclusters induces their fusion into nanodomains and stabilizes them as membrane “rafts” (resting-state rafts), which are larger (~20 nm) in diameter (Eggeling et al. 2009), more stable, and detergent-resistant domains, which become functional. This is consistent with the model proposed by Hancock (2006). Since palmitoylation has been reported previously to be involved in the regulation of membrane-protein clustering, such as tetraspanin (Berditchevski et al. 2002; Hemler 2005) or GABA_A receptors (Rathenberg et al. 2004), and has also been shown to promote oligomerization of certain proteins (Charollais and Van Der Goot 2009; Feig et al. 2007), we postulate that palmitoylated MPP1 in erythroid cells plays a role in raft protein(s) oligomerization.

It should be stressed, however, that the MPP1-based mechanism of raft organization is not the only possible mechanism driving lateral membrane organization. For example, a similar function was recently suggested for the palmitoylation of Rac1 in COS-7 and MEF cells (Navarro-Lerida et al. 2012), and these authors implicate a role for the actin cytoskeleton in the mechanism of raft clustering in these cells.

The Involvement of Actin in Erythrocyte Membrane Rafts Organization?

Substantial indications are available in the literature on the relationship of domain formation and stabilization with cortical actin (Goswami et al. 2008; Hummel et al. 2011). In addition, MPP1 is a membrane-skeleton protein that participates in the formation of “vertical” linkages between the membrane-skeleton and the membrane bilayer containing integral proteins (for recent reviews, see (Machnicka et al. 2012, 2014)). It is known that the DRM fraction from erythrocyte membranes contains both spectrin and actin, in addition to MPP1 and raft marker proteins such as flotilins, stomatin and other GPI-anchored proteins. It could be speculated that the presence of MPP1 in raft domains could arise as a result of spectrin-actin complex formation. Also, direct interactions of membrane skeletal proteins with membrane-lipids have been implied by numerous works (for reviews, see e.g. (Hanus-Lorenz et al. 2001; Boguslawska et al. 2014)). Such data might contribute to establishing the link between detergent-resistant membranes and MPP1. However, the above-mentioned simple experiments on the isolation of a DRM fraction under alkaline conditions, i.e. in the presence of Na_2CO_3 , or from latrunculin-treated erythrocyte ghosts, rather exclude this possibility (Lach et al. 2012).

Conclusions

From the above discussion, several conclusions can be drawn. The most important and, probably, the most obvious is that membrane rafts in the mature erythrocyte are not the remnants from erythroid precursor cell(s), but are real and dynamic domains functioning within the membrane, helping to fulfil physiological roles, of which only a few of them are just now being recognized.

The next conclusion is the presence and role of the MPP1 protein in these domains. It has been known for some time that palmitoylation drives proteins into the raft-domain in non-erythroid cells (Levental et al. 2009; Yang et al. 2010; Barnes et al. 2006; Babina et al. 2014), but the role of MPP1 in this remained unknown until recently, although it was well known that this was a major palmitoylation target in erythrocytes (Maretzki et al. 1990; Ruff et al. 1991). This fact was noticed in early studies, explaining the property of tighter binding of MPP1 to the membrane, even after extraction at pH 11 (Ruff et al. 1991). However, the most important conclusion stems from the fact that (palmitoylated) MPP1 functions as an organizer of membrane rafts in erythroid cells. We postulate that the binding of palmitoylated MPP1 to the pre-existing nano-assemblies induces their fusion into nano-domains and stabilizes them as larger (~20 nm diameter) “resting state rafts” that are more stable and detergent-resistant domains which assume a level of functional organization.

The above conclusion is strengthened by the fact that a lack of MPP1 palmitoylation, resulting from an absence of DHHC17, the only erythroid palmitoyltransferase, leads to the observed pathology. However, the molecular mechanism by which this occurs is still to be identified.

Therefore, erythrocyte membrane understanding will increasingly focus on what components and conditions are necessary to fulfill the functional roles of membrane domains, such as raft assemblies, and the mechanisms by which these are effected. In this regard, MPP1, particularly its altered behavior in the free and in the palmitoylated states, now becomes an increasingly important component and target in the regulation, maintenance and function of the normal erythrocyte membrane.

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