MPP1 directly interacts with flotillins in erythrocyte membrane - Possible mechanism of raft domain formation

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**A R T I C L E   I N F O**

Keywords:
- Red blood cell
- MAGUK proteins
- Raft-associated proteins
- Lateral membrane organization

**A B S T R A C T**

Flotillins are prominent, oligomeric protein components of erythrocyte (RBC) membrane raft domains and are considered to play an important structural role in lateral organization of the plasma membrane. In our previous work on erythroid membranes and giant plasma membrane vesicles (GPMVs) derived from them we have shown that formation of functional domains (resting state rafts) depends on the presence of membrane palmitoylated protein 1 (MPP1/p55), pointing to its new physiological role. Exploration of the molecular mechanism of MPP1 function in organizing membrane domains described here, through searching for its molecular partners in RBC membrane by using different methods, led to the identification of the raft-marker proteins, flotillin 1 and flotillin 2, as hitherto unreported direct MPP1 binding-partners in the RBC membrane. These proteins are found in high molecular-weight complexes in native RBC membrane and, significantly, their presence was shown to be separate from the well-known protein 4.1-dependent interactions of MPP1 with membrane proteins. Furthermore, FLIM analysis revealed that loss of the endogenous MPP1-flotillins interactions resulted in significant changes in RBC membrane-fluidity, emphasizing the physiological importance of such interactions in vivo. Therefore, our data establish a new perspective on the role of MPP1 in erythroid cells and suggests that direct MPP1-flotillins interactions could be the major driving-force behind the formation of raft domains in RBC.

**1. Introduction**

The functional diversity of biological membranes is based on the presence of dynamic sterol-sphingolipid-enriched assemblies of specific proteins, whose propensity to coalesce facilitates compartmentalization of the membrane constituents [1]. A variety of specific protein-lipid, lipid-lipid and protein-protein interactions are thought to be the driving-force to activate the clustering of raft precursor nano-assemblies into functional domains (resting state rafts) [2–5]. Nevertheless, the endogenous factors regulating such a switch-like mechanism are poorly characterized. Prominent components of RBC membrane functional domains are proteins belonging to the SPFH (stomatin/prohibitin/otillin/HflK) family, such as flotillin 1 (reggie 2) and flotillin 2 (reggie 1), whose strong tendency to oligomerize is considered essential in fulfilling their structural role [6,7]. Flotillins facilitate assembly of cholesterol- and sphingolipid-rich membrane micromdomains, organizing therefore the lateral membrane environment and act as protein raft-markers of many different cells. Although coassembly of pre-existing flotillin 1 and flotillin 2 hetero-oligomers into larger complexes has been shown to be crucial for the formation of membrane rafts [8] and is important in different cellular processes, including clustering of membrane receptors, regulation of signaling pathways [9–12] and cell adhesion [13], the mechanism underlying their clustering remains unclear.

MAGUKs (membrane-associated guanylate kinase homologs) are an ubiquitously expressed class of scaffolding proteins involved in sequestering specific molecules to localized regions of the plasma membrane, playing a pivotal role in clustering multi-protein complexes in

**Abbreviations:** CRAC, cholesterol recognition amino acid consensus motif; di-4, di-4-ANEPPDHQ; DRM, detergent resistant membrane; DTBP, dimethyl 3,3′-dithiobiisproinomimidate; FLIM, fluorescence lifetime imaging; GLUT4, glucose transporter 4; GPI, glycosyl-phosphatidyl inositol; GPMVs, giant plasma membrane vesicles; GuK, guanylate kinase domain; HEL, human erythroleukemia cells; Ld, liquid disordered; Lo, liquid ordered; MAGUKs, membrane-associated guanylate kinase homologs; MPP1/p55, membrane palmitoylated protein 1; PDZ, PSD-95/Dlg/ZO-1 domain; FLA, proximity ligation assay; PSD-95, postsynaptic density protein 95; RBC, red blood cells; SH3, Src homology 3 domain; SPFH, stomatin/prohibitin/otillin/HflK domain; TBS, Tris-buffed saline; TCR, T-cell receptor

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http://dx.doi.org/10.1016/j.bbamem.2017.08.021
Received 23 March 2017; Received in revised form 21 August 2017; Accepted 27 August 2017
Available online 01 September 2017
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different cells [14]. This family of proteins is highly conserved through evolution and is characterized by the presence of a core structural module composed of a PSD-95/Dlg/ZO-1 (PDZ) domain, an Src homology 3 (SH3) domain, and a catalytically inactive guanylate kinase (GuK) domain, arranged sequentially into a PDZ-SH3-GuK tandem. Despite different cellular localization and functions [15–17] that are ascribed for MAGUKs, they share a fundamental feature in organizing molecules into complexes, thereby maintaining the structural heterogeneity of the plasma membrane. MPP1, one of the MAGUK members identified in RBC membranes [18]. Similar to other MAGUKs, MPP1 shares characteristic core domains, and an additional D5 domain responsible for 4.1 binding [19,20]. Involvement of PDZ and D5 domain of MPP1 in the binding of transmembrane glycoporphin C and 4.1 protein respectively, was shown to be crucial in the formation of ternary complex attaching spectrin-based skeleton to the RBC membrane [21], assigning therefore the major function of MPP1 in stabilizing the mechanical properties of RBC membrane. Apart from its role in strengthening the interactions between protein 4.1 and glycoporphin C, the other important role of MPP1 in RBC which is functional domains organizer has, until recently, remained poorly understood.

Significantly, we have shown previously that MPP1 acts as a crucial molecule in organizing lateral heterogeneity in native erythroid cell membranes. We came to this conclusion by studying the RBCs obtained from two patients with unusual hemolytic anemia, wherein suppression of membrane functional domains formation was a result of the lack of palmitoylated MPP1 in the membranes leading, in turn, to the loss of proper RBC structure [22]. Moreover, we have used RBC cells obtained from healthy donors as controls, where we inhibited MPP1 palmitoylation and erythrocyte precursor HEL (human erythroleukemia) cells, where MPP1 knockdown was performed and, in both cases, we observed significant changes in the physicochemical properties of the plasma membrane or GPMVs, namely, changes in fluidity and phase-separation properties. Furthermore, MPP1 has been shown to critically regulate raft-dependent insulin or c-kit receptor signaling pathways in HEL cells [23,24]. Detailed analyses revealed that the inhibition of signal transduction occurs at the level of the small GTPase, H-Ras activity pointing to the novel functional link in between MPP1-dependent membrane-domain formation and H-Ras activation (Podkalicka et al., submitted). Altogether, this principle provides a general mechanism whereby formation of functional domains in RBC is effected by MPP1. In this regard, to understand the mechanism underlying MPP1-driven domain-formation, in this study we have addressed the question concerning the new, yet unknown, binding partners of MPP1 in RBC membrane domains. Using different methods such as in situ cross-linking experiments, proximity ligation assay coupled to both, co-immunoprecipitation and pull-down assays, as well as FLIM measurements, we demonstrate the existence of a novel, physiologically significant interaction between MPP1 and the raft-marker proteins, flotillins. These interactions are most probably responsible for the clustering of precursor nano-assemblies into raft domains (which we believe are resting state membrane rafts) and seems to be independent from well-known interactions of MPP1 with glycoporphin C and protein 4.1. Our data provide therefore an interesting outlook into understanding of how domain formation might be regulated via direct MPP1-flotillins interactions in native membranes.

2. Materials and methods

2.1. Antibodies and constructs

All antibodies and constructs used in this study are listed in Supplementary Table 1.

2.2. Isolation of RBC membranes and DRM

Whole blood was obtained from healthy volunteers and native RBC membranes were isolated in ice-cold hypotonic lysis 20 mMOS sodium phosphate buffer pH 7.4, supplemented with 0.1 mM PMSF as described by Dodge et al. [25]. Skeleton-depleted RBC membranes were obtained by incubating RBC membranes with 0.1 M NaOH (1:10) for 10 min at 4 °C, pelleted (10 min at 15000 × g, 10 min, 4 °C) and washed with hypotonic lysis buffer as described previously. DRM (detergent resistant membrane) fraction was isolated from RBC membranes as described previously [22,23] by treatment of RBC membranes with an equal volume of ice-cold DRM isolation buffer (10 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 µM PMSF, protease inhibitor cocktail, Sigma), incubated on ice for 20 min, and vortexed occasionally. The samples were then mixed with an equal volume of 80% sucrose containing 0.1 M Na2CO3 in the DRM isolation buffer, overlaid with 2.7 ml of 30% sucrose and 0.9 ml of 5% sucrose and ultracentrifuged in a Beckman 60Ti SW rotor (16 h, 35,000 RPM, 4 °C). Collected DRM fractions were used for further analysis.

2.3. Co-immunoprecipitation

Native, packed RBC membranes (200 µL) or skeleton-depleted RBC membranes were solubilized in 20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% Triton-X-100, 2 mM EDTA, protease cocktail inhibitor (Sigma) for 30 min on ice and incubated with 5 µg anti-MPP1 or anti FL-2 antibodies coupled to Protein G Dynabeads (Life Technologies) for 24h at 4 °C on rotation, according to the manufacturer's protocol. IgG (5 µg) was used as a control. The beads were eluted in 5 × SDS sample buffer (20% SDS, 50% glycerol, 25 mM EDTA, 250 mM DTT, 0.25 M Tris pH 6.8) and analyzed by 10% polyacrylamide gel on SDS–PAGE and Western blot with appropriate antibody listed in Supplementary Table 1. Co-immunoprecipitation of MPP1 from the DRM fraction was conducted using the Pierce™ DirectMagneticIP/Co-IP kit (Thermo Fisher Scientific).

2.4. Cross-linking of RBC proteins in vivo

RBCs were cross-linked with 1 mM DTBP (dimethyl 3,3'-dithiobispropionimidate) (Thermo Fisher Scientific) at RT for 1 h on rotation, washed in PBS-glucose (PBS, 5 mM glucose, 0.5 mM EDTA pH 7.4), followed by RBC membrane preparation and DRM isolation. For identification of cross-linked protein complexes in DRM, eight fractions were collected from the top of a sucrose gradient (see Supplementary Fig. 1A) and aliquots from each fraction were mixed with SDS sample buffer without reducing agent, boiled for 5 min, 95 °C and subjected to SDS-PAGE and Western blotting. Nitrocellulose membrane was probed against MPP1-antibody (rabbit), stripped and re-probed with anti-flotillins 1 (mouse) and anti-stomatin (goat) antibodies, respectively. Separation and releasing of monomers from cross-linked protein complexes was performed by 2D SDS-PAGE, the lane containing cross-linked proteins from DRM fraction number 2 was excised, and incubated in running buffer with 50 mM DTT and 14 mM 2-mercaptoethanol for 1 h at 37 °C, before being applied horizontally on top of a 12% SDS-PAGE gel. For Western blot analysis, proteins from the gels were transferred to a nitrocellulose membrane and probed with polyclonal antibodies raised against MPP1, then stripped and probed again against anti flotillin 1 and anti stomatin, respectively.

2.5. Expression of recombinant proteins

Recombinant proteins: MPP1-GST, MPP1-His, FL-1-His and FL-2-His were prepared in E. coli strain BL21DE3, isolated and immobilized on glutathione-Sepharose 4B beads (GE Healthcare) or TALON resin (Clontech) respectively. His-tagged proteins were purified under denaturation conditions using 8 M urea based on TBS-buffers (20 mM Tris, 150 mM NaCl, pH 7.4) whereas MPP1-GST protein purification was performed in the native conditions based on PBS-buffers. GST-tag was cleaved off on the column with the PreScission protease (Sigma)
according to the manufacturer’s protocol. Purified untagged MPP1 as well as MPP1-His/FL-1-His/FL-2-His tagged proteins were checked by polyacrylamide gel electrophoresis and Coomassie Blue staining (see Supplementary Fig. 2) and used for in vitro binding experiments.

2.6. Pull-down assays

For indirect pull-down, MPP1-His was immobilized on beads (Pierce™ Pull-Down PolyHis Protein:Protein Interaction Kit, Thermo Fisher Scientific) and incubated overnight at 4 °C with RBC membrane lysates or DRM fraction, according to the manufacturer’s protocol. Bound proteins were eluted in 5 × SDS sample buffer as was mentioned above and analyzed by Western blot. As a negative controls beads without immobilized MPP1-His were incubated with RBC membrane lysates or DRM fraction respectively. For direct pull-down assay, fotorillin-1-His/fotorillin-2-His were immobilized on Talon beads and incubated overnight with recombinant untagged MPP1 at 4 °C. Bound proteins were eluted as described above and analyzed via Silver staining and Western blot. For all experiments 25 μg of each protein was used. To verify non-specific binding of MPP1 to the resin, Talon beads were incubated with untagged MPP1 only and used as a negative control.

2.7. Overlay assay

Proteins from DRM fraction separated in SDS-PAGE were transferred onto nitrocellulose membrane, and each lane was cut out, incubated overnight either with purified MPP1-His (25 μg) in TBS-T buffer and probed with anti-His antibodies and subjected to ECL detection. Endogenous DRM marker proteins (fotorillin 1, fotorillin 2, stomatin) or MPP1 were detected in DRM fraction by using specific antibodies.

2.8. Dot-blot binding assay

Recombinant fotorillin-1-His/fotorillin-2-His were dotted separately or in a (1:1) mixture onto a nitrocellulose membrane, blocked and incubated overnight with untagged-MPP1 (100 nM) recombinant protein in TBS-T buffer at 4 °C. Detection of bound MPP1 was performed by using anti-MPP1 antibodies followed by chemiluminescence analysis performed on a UVP Imaging System.

2.9. Proximity ligation assay

RBC from freshly drawn blood were fixed and permobilized with Triton X-100 as described by others [26], spun on poly-L-lysine coated cover-slips, then blocked with Blocking solution (Sigma) for 30 min at RT and subsequently probed with the two primary antibodies: anti-MPP1/fotorillin 1 or anti-MPP1/fotorillin 2, respectively, at 4 °C overnight in a humid chamber. After incubation cover-slips were washed and processed with the Duolink-PLA proximity ligation assay (Sigma) according to the manufacturer’s protocol. As a positive control, fixed RBC were incubated with two primary antibodies: mouse anti-MPP1 and rabbit anti-protein 4.1, whereas negative controls were performed by omitting primary antibodies using fixed RBC treated with PLA probes only, according to the manufacturer’s protocol. Imaging was performed with a LSM 510 META confocal microscope (Carl Zeiss, GmbH Germany) using a PLAN-APochromat 63x/1.4 OIL DICM27 objective. All images were contrast-enhanced using ZEN 2009 Light Edition software.

2.10. Resealing of RBC ghosts in the presence of antibodies and FLIM analysis

Red blood cells (20 μl per each sample) were washed three times with 10 mM Tris buffer (pH 7.4) containing 120 mM KCl. Intact cells were lysed (1:10) in ice-cold lysis buffer containing MgATP (5 mM Tris, 5 mM KCl, 1 mM MgCl2, 0.6 mM MgATP, pH 7.4) and centrifuged twice (15,000 g, 15 min, 4 °C) until the ghosts were pale pink. For resealing of the RBC ghosts in the presence of antibodies, ghosts were resuspended in rescaling buffer (5 mM Tris, 150 mM KCl, 1.6 mM MgCl2, 1 mM DTT, 0.6 mM MgATP, pH 7.4) only (control), or the same buffer containing 1 μg of nonimmune rabbit IgG or anti fotorillin 1/fotorillin 2 (0.5 μg of each) antibody, incubated for 10 min on ice, and then incubated with gentle rotation (100 RPM) for 1 h at 37 °C.

FLIM was used to measure fluorescence-lifetime using the membrane-order sensitive probe, di-4 (Life Technologies). RBC ghosts (control, nonimmune IgG or anti fotorillin 1/fotorillin 2 treated) were stained with 0.5 μM di-4 in rescaling buffer for 15 min at RT and transferred onto poly-L-lysine coated cover-slips and left for 20 min at RT. FLIM measurements were performed at 23 °C using an LSM 510 META microscope (Carl Zeiss, GmbH, Germany) upgraded with FLIM and FCS capabilities with a dedicated kit (PicoQuant, GmbH, Germany). The probe was excited with a 470 nm pulsed-laser diode with a 40 MHz repetition rate and observed with a 40 × C-Apochromat water immersion objective (NA 1.2) and fluorescence was collected through a 500 nm long wave pass filter. Laser power was adjusted to give an average photon rate of 105–106 photons, in order to avoid the pile-up effect. Acquisition time depended on the brightness of the sample and was in the range of 200 s, in order to achieve a sufficient number of photons per pixel (at least 105). Each pixel in the image was pseudo-colored according to the average fluorescent-lifetime. The size of the images was 256 × 256 pixels. Improvement of the fluorescence-lifetime estimation was achieved by 2 × 2 pixel binning. Statistical analysis was performed using one-way ANOVA with post hoc Tukey test applying GraphPad PRISM® 6 Software.

3. Results

3.1. Identification of novel binding partners for MPP1 in RBC membranes

First, we identified proteins co-precipitating with endogenous MPP1 from whole native human RBC membranes. Our first approach was immunoprecipitation by using anti-MPP1 antibodies. Among several well-known skeletal-proteins interacting with MPP1 (Fig. 1A–B), we have identified raft-associated proteins i.e. fotorillins and stomatin in co-precipitated complexes. Notably, the specificity of binding between MPP1 and raft-marker proteins was confirmed when anti-fotorillin 2 antibodies were used as a bait in a co-immunoprecipitation assay (Fig. 1D–E). Since SPFH members were found to serve as docking-sites for cytoskeleton components [27] membrane-skeleton depleted RBC membranes were used in an immunoprecipitation assay to verify whether their presence is due to spectrin-actin-anchorage (protein 4.1-dependent). Fig. 1C, F clearly demonstrates that fotorillins/stomatin-MPP1 association is membrane-skeleton independent, as their presence was preserved in the co-precipitates from the skeleton-depleted RBC membranes.

As both fotorillins and stomatin define a subpopulation of cholesterol-enriched microdomains (rafts) and DRM are considered to be related to these microdomains [28], in the next step, co-precipitation of MPP1 from DRM was performed. As indicated in Fig. 2A both fotorillins and stomatin were involved in binding to MPP1 in DRM whereas protein 4.1 was not bound, but present in the unbound fractions. This further supports the existence of a novel, yet unreported, interaction between MPP1 and raft-marker proteins in RBC membrane and implies also involvement of MPP1 in functional domains of RBC membrane.

Another approach to follow MPP1 interactions was in situ cross-linking analysis of MPP1-complexes in the DRM by using the cleavable bi-functional reagent, DTBP (spacer arm 11.9 A), combined with 2D electrophoresis. First, proteins from density gradient centrifugation fractions of detergent-treated membranes from cross-linker treated RBC were separated in non-reducing conditions followed by Western blot.
analysis. These experiments revealed that raft-associated proteins and MPP1 cluster in DRM (lane no 2) into high molecular-weight complexes in the range of 130–250 kDa (Fig. 2B). MPP1, flotillin 1 and stomatin monomers could be released from the clusters by 2D electrophoresis and were found to overlap as immune-positive bands in the region corresponding to 100–130 kDa (Fig. 2C). Importantly, these proteins were detected also when immunoprecipitation of MPP1 from cross-linked DRM fraction was performed (Supplemental Fig. 1B). It should be also emphasized, that the presence of MPP1 complexes observed with a higher abundance in non-DRM fraction (Fig. 2B) is not surprising, as this protein as a peripheral protein, is mainly localized in the bottom fractions in sucrose gradient together with soluble 4.1-based or spectrin-based skeletal proteins (see also ref.[21]). Therefore different MPP1-skeletal protein complexes are probably formed in non-DRM fraction independently from raft-associated proteins.

In addition, the interaction between MPP1 and raft proteins was confirmed in an in vitro indirect pull-down experiment, where recombinant His-tagged-MPP1 bound all three endogenous raft-marker proteins, i.e. flotillin 1 and 2 as well as stomatin, from native RBC membranes and DRM (Fig. 3A).

In conclusion, the above data indicate that MPP1 specifically associates with raft-marker proteins in RBC membrane, thus implying the significant potency of these associations in lateral membrane organization.

### 3.2. MPP1 binds directly to flotillin 1 and flotillin 2 in the RBC membrane and in vitro

In the search for the most preferential partner for MPP1 among the above mentioned raft-proteins, a protein-overlay assay between endogenous DRM proteins and His-tagged-MPP1 was conducted. Proteins from the DRM were therefore separated, transferred onto nitrocellulose and incubated with recombinant MPP1-His. Positive immunoreaction for MPP1 was found within the region between 48 and 50 kDa of the blot (Fig. 3B, lane 1), suggesting thereby that endogenous flotillins (lane 2 and 3), but not stomatin (lane 3), are potential MPP1 partners in RBC functional membrane domains. Thus, based on the overlay assay results and dot-blot binding analysis between MPP1 and stomatin (Supplemental Fig. 3), the presence of stomatin in MPP1-precipitates is probably due to their association with flotillins, similarly to what was shown recently by others [29].

To further investigate the individual interactions, we used direct pull-down experiments with purified recombinant His-tagged flotillins and untagged MPP1. Interestingly, our results demonstrated that MPP1
was capable of binding to both, flotillin 1 and flotillin 2 in vitro (Fig. 4A) whereas no detectible MPP1-binding to the resin was observed with the MPP1 protein alone. Indeed, these findings were confirmed by in situ proximity ligation assay, where fixed RBC were subjected to Duolink PLA using rabbit anti-flotillin 1/flotillin 2 and mouse anti-MPP1 antibodies, and endogenous close-proximity complexes between these proteins were detected as red dots by fluorescent microscopy (Fig. 4B) as compared to the controls. Taken together, these experiments document the existence of a novel MPP1-flotillins complex not only in vitro conditions, but also in the intact RBC membrane.

To confirm MPP1-flotillins interactions in the next step, an in vitro dot-blot binding assay was carried out using recombinant proteins. Increasing concentration of His-tagged-flotillin 1, His-tagged-flotillin 2 or flotillin 1/flotillin 2 (1:1) mixture were immobilized onto nitrocellulose membranes, followed by incubation with untagged MPP1. The binding of MPP1 was then monitored by using anti-MPP1 antibodies. The result obtained from the dot-blot analysis indicated that MPP1 seems to bind flotillin 2 better than to flotillin 1 (Fig. 4C), indicating therefore that the interaction of MPP1 to flotillin 2 could be stronger than that of flotillin 1. Markedly, when a flotillin 1/flotillin 2 1:1 mixture was used, and assuming that a heterodimeric complex was formed, a further increase in signal intensity was not observed, implying that flotillin 2 might be the major binding-partner responsible for the formation of the complex.

3.3. Disruption of endogenous MPP1-flotillins interactions modulate RBC membrane order

Identification of flotillins as novel binding partners of MPP1 in RBC membranes raises an important question about the physiological significance of such interactions. As both MPP1 and flotillins were shown to be crucial components of RBC functional domains, to answer the
Fig. 3. Recombinant MPP1 binds endogenous raft-marker proteins in vitro.
(A) Western blot of indirect pull-down assays was used to monitor the interactions between recombinant MPP1 and raft-marker proteins in vitro in whole RBC membrane lysates (left) and DRM fraction (right). As a negative control, lysates were incubated with the resin only. (B) Overlay assay experiment between recombinant MPP1-His and endogenous proteins from DRM fraction. Proteins from DRM fraction number 2 were resolved on SDS-PAGE, transferred to nitrocellulose and then incubated with recombinant MPP1-His (line 1) or antibodies against flotillin 1 (FL-1), flotillin 2 (FL-2), stomatin (stom) and MPP1, respectively. Anti His-tag antibodies were used for detection of bound MPP1 to endogenous DRM proteins.

Fig. 4. MPP1 interacts directly with flotillin 1 and flotillin 2.
(A) Silver staining and Western blot analysis of direct interactions between recombinant MPP1/FL-1-His or MPP1/FL-2-His revealed by a direct pull-down assay. Flotillin-1-His/flotillin-2-His were immobilized on Talon beads, incubated overnight with recombinant untagged MPP1 at 4 °C and bound proteins were eluted. For all experiments 25 μg of each protein was used. Untagged recombinant MPP1 incubated with resin was used as a negative control. (B) Visualization of direct close-proximity interactions between MPP1 and flotillins in native RBC membranes by the PLA assay. RBC were fixed and incubated with mouse anti-MPP1 and rabbit anti-flotillin 1 or anti-flotillin 2 antibodies, respectively, then incubated with PLA probes, followed by ligation and amplification cycles. Endogenous close-proximity complexes between these proteins were then detected as red dots. Rabbit 4.1R antibody was used as a positive binding partner of MPP1 in RBC. As a negative controls, fixed RBC were stained with one primary antibody (anti-MPP1) and PLA probes or with PLA probes only. Scale bar, 5 μm. (C) Dot-blot binding assay between MPP1 and flotillins. Increasing concentration of recombinant His-tagged flotillin 1 and flotillin 2, respectively, or flotillin 1-His/flotillin 2-His complex were immobilized onto nitrocellulose membranes, followed by incubation with untagged MPP1 (100 nM). The binding of MPP1 was monitored by using mouse anti-MPP1 antibodies.
In the afore-mentioned point, FLIM analysis was used to detect changes in RBC membrane-order after blocking of endogenous MPP1-binding sites with specific anti-flotillins antibodies. Briefly, RBC ghosts were resealed with an anti-flotillin 1/flotillin 2 antibody mixture (1:1) and changes in the fluorescence-lifetime values of the polarity-sensitive di-4 dye were subsequently monitored. The probe exhibits a lifetime-shift between liquid disordered (Ld) and liquid ordered (Lo) phases and, recently, has been used to detect the relative levels of membrane order in living cells [30]. As shown in Fig. 5A FLIM analysis revealed significant changes in membrane fluidity observed as a decrease in the lifetime value of the di-4 probe in RBC ghosts resealed with anti-flotillins antibodies (~3.36 ns; n = 30) compared to the controls (~3.6 ns) (Fig. 5B). No particular differences in the lifetime values were noticed between IgG-resealed (~3.62 ns; n = 30) or untreated RBC ghosts (~3.61 ns; n = 30). Notably, the characteristic shift into the lower lifetime values (~0.3 ns) observed here is in line with our previous studies performed on native RBC membranes [22] and HEL cell membranes [23], providing therefore strong evidence that loss of the endogenous MPP1-flotillins interactions has direct consequences for the physiochemical properties of RBC ghost membranes, pointing to its biological importance.

4. Discussion

Membrane rafts organize the local membrane environment allowing the spatio-temporal regulation of numerous cellular processes. However, understanding how functional domains are formed in native membranes is challenging. Importantly, we have shown previously that one of the MAGUK protein family members, MPP1, acts as a crucial tuning molecule in stabilizing local, lateral heterogeneity in erythrocytes and erythroid cells, emphasizing an entirely new direction in the current perception of the function of this protein. This picture emerged from different experimental approaches, and also based on studies of the pathological red cells of a patient with hemolytic anemia, wherein formation of MPP1-dependent domains was shown to have also functional significance in both establishing and maintaining proper RBC membrane functions as well as in modulating insulin/c-kit receptor signaling pathways and changing plasma membrane fluidity or the phase-separation behavior of GPMVs prepared from MPP1-knockdown HEL cells [22-24]. Altogether our observations point to the novel concept of engagement of MPP1, or possibly other MAGUK proteins in various cells, in the association of pre-existing lipid-protein nano-assemblies. However, the open question remains that is how a single lipid-anchored scaffold protein, MPP1, could trigger domain formation in the plasma membrane? Our previously published hypothesis assumed that palmitoylation of MPP1 is crucial for its direct binding to the plasma membrane and, subsequently, for the assembly of functional pre-existing protein-lipid nano-assemblies into resting state rafts to initiate signaling cascades. As palmitoylation has been shown to be engaged in the regulation of membrane-protein clustering and its oligomerization, we therefore postulated that MPP1 plays a role in raft-protein(s) oligomerization in erythroid cells [31-33].

Detailed exploration of MPP1 function in organizing functional domains, described here, led us to the identification of flotillin 1 and flotillin 2 as novel and direct MPP1-binding partners in the RBC membrane. Both endogenous and recombinant MPP1 were found to form complexes with flotillins in native RBC membranes. Importantly, these interactions were shown to be formed independently of the well-known protein 4.1-based complexes [18,21], providing therefore evidence for the existence of a direct linkage between MPP1 and raft-marker proteins in RBC membrane. The use of the proximity ligation assay enabled us to show the close proximity associations between MPP1 and flotillins at single-molecule resolution [33] in RBC membranes and, most significantly, cross-linking experiments revealed that these proteins form high molecular-weight complexes as they were captured at distance smaller than 1.2 nm in RBC membrane. This, in turn, indicates that MPP1-flotillins complexes might be responsible for organization of assemblies corresponding to resting state rafts (~20 nm diameter) observed in the native membranes [2,34,35]. Data obtained from our dot-blot binding assay pointed to a better binding of MPP1 to flotillin 2 than to flotillin 1. Inside the cell, flotillins 1 and 2 are known to form 1:1 hetero-complexes [8] but, notably, once a flotillin 1/flotillin 2 complex was formed, an increase in MPP1 binding was not observed, strengthening the argument that flotillin 2 is possibly the major protein partner responsible for the formation of the MPP1-flotillins complex. Nevertheless, it should be noted, that the dot-blot assay highlights only the approximate estimate of MPP1-flotillins interaction, and stronger conclusions could be drawn if more accurate kinetics analysis methods were applied in order to fully understand its nature.

Finally, the question concerning the physiological meaning of the presence of MPP1-flotillins complexes in RBC membrane was addressed to fully understand their mutual role in lateral membrane organization. To cope with this particular problem, we decided to use FLIM analysis to monitor changes in membrane fluidity after disrupting endogenous MPP1-flotillins interactions with specific antibodies, acting as competitors of the interactions. Strikingly, we noticed that impairment of the internal MPP1-flotillins interactions triggers the loss of membrane-order properties, observable as a characteristic shift in the lifetime value of the membrane-embedded polarity-sensitive dye, by around 0.3 ns. Such a shift represents a noticeable change in lipid-packing and, more importantly, is in agreement with our previous data performed on the MPP1 knockdown HEL cell line [23,24], and native RBC studies in which palmitoylation of MPP1 was blocked [22]. Indeed, when antibodies against MPP1 were used in our other experiments, a considerable decrease in the isolated DRM fraction was observed. Together, these data point towards an essential engagement of MPP1-flotillins interactions in the control of the physicochemical properties of the RBC membrane. As changes in the lateral lipid packing are believed to initiate coalescence and conformational changes of proteins and, therefore, compartmentalization of membrane constituents [1], direct MPP1-flotillins interaction appears to play a pivotal role in controlling and stabilizing lateral-membrane heterogeneity, reflecting functional domain formation in RBC membrane.

Thus, the identification of flotillins as direct partners of MPP1 seems to point to the missing link for the regulation of resting state raft formation in RBC membranes. The capacity to oligomerize is one of the essential features of flotillins that allows them to act as molecular
A good example is the polarization of various immune cells, such as T-cells and neutrophils, where the spatial segregation of proteins and lipids to distinct regions of the cells is crucial for the function of the cells and, interestingly, both flotillins and MAGUKs were shown to be actively involved in this process. The studies of Ludwig et al. performed on flotillin-1-knockout mice demonstrated that flotillin microdomains are important for neutrophil recruitment to chemotactants in vivo [36], while Quinn et al. have shown that MPP1 is directly engaged in the regulation of neutrophil polarity and chemotactic stimulation in a resting state of the Rho-GTPases, Rho, Rac and cdc42, and also affected MAP-kinase and Ras activation [39,40], while in adipocytes glucose transporter 4 (GLUT4) translocation [41].

Importantly, data from other groups show that MAGUKs and flotillins are involved in similar cellular processes, suggesting that the proposed mechanism of interaction could exist in cells other than RBCs. A good example is the polarization of various immune cells, such as T-cells and neutrophils, where the spatial segregation of proteins and lipids to distinct regions of the cells is crucial for the function of the cells and, interestingly, both flotillins and MAGUKs were shown to be actively involved in this process. The studies of Ludwig et al. performed on flotillin-1-knockout mice demonstrated that flotillin microdomains are important for neutrophil recruitment to chemotactants in vivo [36], while Quinn et al. have shown that MPP1 is directly engaged in the regulation of neutrophil polarity and chemotactic stimulation in a Mmp1 knockout (pp5−/−) mouse [37]. Moreover, in T-cells, flotillins are closely associated with the Src-family kinases, lck and fyn, and the adaptor protein LAT, and were shown to provide pre-existing priming platforms upon which multi-protein components of the TCR (T-cell receptor) signaling complexes assemble to coordinate cytoskeletal remodelling upon T-cell activation [38]. Our latest study performed on an Mpp1-knockdown HEL cell-line, demonstrated that a reduced level of MPP1 affects the insulin signaling pathway and the inhibition of signal-transduction occurs at the level of small GTPase, H-Ras (Podkalicka et al., submitted).

Significantly, the recent study of Koh et al. identified flotillitin-1 as an important regulator of H-Ras activation and breast cancer cell invasion. Flotillitin-1-knockdown was shown to be accompanied with inhibition of epidermal growth factor-induced activation of H-Ras and suppression of tumor progression [12]. Furthermore, other study has shown that down-regulation of flotillitin 2 in hippocampal neurons and N2a neuroblastoma cells caused a disruption of the normally balanced activation state of the Rho-GTPases, Rho, Rac and cdc42, and also affected MAP-kinase and Ras activation [39,40], while in adipocytes flotillins were implicated in insulin signaling pathway through active regulation of glucose transporter 4 (GLUT4) translocation [41].

Most importantly, our hypothesis might explain the latest observations of Tulodziecka et al. showing that palmitoylation of PSD-95, the most abundant MAGUK protein in neurons, facilitates the formation of postsynaptic membrane microdomains, critical for postsynaptic plasma membrane organization and synapse plasticity [42]. The authors hypothesized that, palmitoylated PSD-95 anchors a stable raft-like membrane domain via its posttranslational palmitoylation, playing therefore a key role in raft-domain nucleation, necessary for glutamate receptor clustering. As flotillins were originally identified as crucial proteins for axon regeneration and growth [43,44] and their contribution to postsynaptic density/spines in conjunction with PSD-95 has recently been published in hippocampal neurons by Bodrikov et al. [45], we postulate, that the process of postsynaptic lateral organization is based on the same pattern as those observed in RBC, where mutual MAGUK-flotillins interactions trigger the local lateral membrane heterogeneity.

On the other hand many studies have provided conclusive evidence for the strong association of flotillins with GPI-anchored proteins. In Jurkat T cells, close associations of flotillins with GPI-anchored proteins such as PrP and Thy-1 have been shown to participate in signaling leading to the recruitment of TCR components [11,46–48] whereas in neurons, flotillins-GPI-anchored proteins interactions regulate the targeted delivery of cargo proteins to specific sites of the plasma membrane by triggering the activation of src tyrosine and MAP kinases, as well as small GTP hydrolizing enzymes, controlling therefore, extension and axon growth [49]. Detailed mechanism of how such interactions might be formed is missing, however, as flotillins contain putative cholesterol recognition amino acid consensus (CRAC) motifs [50] and hydrophobic stretches [51], it is conceivable that direct interactions of flotillins with cholesterol or/and other inner leaflet lipids might mediate their associations with GPI-anchored proteins.

In conclusion, our data support hypothesis that MPP1/MAGUK proteins have the capacity to act as molecular switches for functional domains formation by interacting with flotillins. In this context, we propose a novel mechanism of lateral-membrane heterogeneity regulation, where MPP1/MAGUKs act as critical regulatory and structural partners of raft-associated proteins. This, in turn, strongly supports the idea that endogenous factors, when binding to naturally existing raft-precursors (nano-assemblies), have the ability to induce and stabilize the lateral heterogeneities in membrane organization, providing a new insight into understanding how the inner-membrane leaftlet participates in the formation of functional domains in native membranes.

Author information

A.B. and A.F.S. designed the research, analyzed the data, and wrote the paper. A.B., K.A., S.T., W.G-N. devised and performed the
experiments, analyzed the data; J.P and A.Cz. contributed to design of experiments and to the manuscript.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

Transparency Document

The http://dx.doi.org/10.1016/j.bbamem.2017.08.021 associated with this article can be found in the online version.

Acknowledgments

The work was supported by a post-doctoral research grant (DEC-2014/12/S/NZ1/00604). Publication costs were supported by Wroclaw Center of Biotechnology, program The Leading National Research Center (KNOW) for years 2014–2018.

Appendix A. Supplementary material

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2017.08.021.

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[28] J.P and A.Cz. contributed to design of experiments and to the manuscript. A.Cz. was a recipient of the Ministry of Science and Higher Education of the Republic of Poland, the promotion of quality subsidy and grants from Biotechnology Faculty 1251/M/MB/15 and 0420/1508/16. S.T. was supported by the National Science Centre, Poland via a post-doctoral research grant (DEC-2014/12/S/NZ1/00604).
A. Biernatowska et al. BBA - Biomembranes 1859 (2017) 2203–2212


