Palmitoylation of MPP1 (Membrane-palmitoylated Protein 1)/p55 Is Crucial for Lateral Membrane Organization in Erythroid Cells

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Background: Lateral membrane organization is important in many essential cellular functions.

Results: Lack of palmitoylation of normal or unknown anemia erythrocyte membrane proteins, specifically MPP1, leads to changes in lateral membrane organization.

Conclusion: Palmitoylation of MPP1 is crucial for membrane organization and is linked to the pathogenesis of hemolytic anemia.

Significance: This might be the first mechanism of biological control of membrane lateral organization.

S-Acetylation of proteins is a ubiquitous post-translational modification and a common signal for membrane association. The major palmitoylated protein in erythrocytes is MPP1, a member of the MAGUK family and an important component of the ternary complex that attaches the spectrin-based skeleton to the plasma membrane. Here we show that DHHC17 is the only acyltransferase present in red blood cells (RBC). Moreover, we give evidence that protein palmitoylation is essential for membrane organization and is crucial for proper RBC morphology, and that the effect is specific for MPP1. Our observations are based on the clinical cases of two related patients whose RBC had no palmitoylation activity, caused by a lack of DHHC17 in the membrane, which resulted in a strong decrease of the amount of detergent-resistant membrane (DRM) material. We confirmed that this loss of detergent-resistant membrane was due to the lack of palmitoylation by treatment of healthy RBC with 2-bromopalmitic acid (2-BrP, common palmitoylation inhibitor). Concomitantly, fluorescence lifetime imaging microscopy (FLIM) analyses of an order-sensing dye revealed a reduction of membrane order after chemical inhibition of palmitoylation in erythrocytes. These data point to a pathophysiological relationship between the loss of MPP1-directed palmitoylation activity and perturbed lateral membrane organization.

Protein palmitoylation is a reversible, dynamic post-translational modification that occurs mostly as a thioester linkage of palmitate to cysteines (S-palmitoylation) and acts as a switch, regulating protein function and/or subcellular distribution (1–3). The transfer of palmitate to cysteine residues is performed by S-acyltransferases, which belong to the DHHC family of proteins (DHHC 1–9 and 11–24) (2, 4). Dysregulated palmitoylation may lead to neurological disorders, cancer, and inhibition of normal platelet accumulation into thrombi (5–7). However, to date there have been no reports that RBC diseases are linked to protein hypopalmitoylation. The major target of palmitoylation in RBC membrane is MPP1 (p55), a MAGUK family member, which is an important protein of the membrane skeleton ternary complex (8, 9). In this role, the PDZ domain of MPP1 interacts with the cytoplasmic domain of glycophorin C (9), whereas the central region (D5-domain) is responsible for the interaction with protein 4.1R (10). The functional purpose of MPP1 palmitoylation is currently unclear. Moreover, no PAT enzyme has yet been identified in this system (11, 12), although palmitoyltransferase activity has been shown (13).
Because palmitoylation has been proposed to enhance the affinity of proteins for raft domains (14), much attention has been paid to its possible role in mechanism(s) that regulate protein associations with rafts. Evidence has shown palmitoylation-dependent raft associations of soluble and transmembrane proteins including H-Ras, Src family kinases Fyn and Lyn, LAT, CD4, and CD8 (14–24). Although studies on inhibition of protein palmitoylation by either site-directed mutagenesis or use of pharmacological inhibitors such as 2-BrBu demonstrated that appropriate targeting of protein to membrane rafts via palmitoylation is crucial for function (16, 23, 25), there are no published data indicating that palmitoylation of proteins might be crucial for lateral membrane organization or is directly involved in regulation of domain formation.

Membrane rafts are currently defined as dynamic, sterol-sphingolipid-enriched, ordered nanoscale assemblies of proteins and lipids, with a lipid structure that is equivalent to the liquid ordered (L_{o}) phase (26, 27) of model membranes (for review, see Refs. 28–30). Although it has been well documented that the functions of membrane rafts are important in many different cellular processes, the molecular mechanisms by which cells regulate raft formation remain unclear. Hancock (28) proposed that small (<10 nm), short lived (<0.1 ms), laterally mobile liquid ordered nanoclusters form spontaneously in the plasma membrane as occurred in the model systems. These unstable “precursor” rafts can, however, be captured and stabilized by lipid-anchored or transmembrane proteins or lipid domains, which leads to the fusion of small domains and the formation of larger lipid-based protein assemblies, which now could be considered rafts (resting state rafts).

We have identified two patients with an unusual form of hemolytic anemia wherein no PAT activity is detectable in their RBC. This was caused due to an absence of membrane DHHC17 protein, which we identified as the only DHHC isoform present in normal RBC. Surprisingly, lack of protein palmitoylation leads to marked changes in lateral membrane organization revealed by a decrease in detergent-resistant membrane fraction and a decrease in membrane order. Taken together, our data suggest that absence of palmitoylated MPP1 results in a loss of ordered raft domains. To the best of our knowledge, this is the first case of “raftopathy,” and the presented data may help to understand the pathophysiological consequences of perturbed membrane raft organization.

**EXPERIMENTAL PROCEDURES**

**Clinical Case of Two Brothers with Hemolytic Anemia**—Two brothers presenting with hemolytic anemia were seen in an outpatient clinic of the Hematology Department of the Medical University of Wroclaw. The older (Pt-61, 34 years old) was hospitalized as a child at the age of 6 in the city in which they lived. Congenital spherocytosis was diagnosed and a splenectomy was proposed, but the patient’s parents refused permission. The second brother (Pt-62, 29 years old) reported that his hemolytic anemia was diagnosed when he was 4 years old. Four years later, he was hospitalized, but no conclusive diagnosis was made. Physically, yellow skin and a high hard palate were the only abnormalities. Both brothers’ heart rate and RR were normal. Currently, they are monitored at an outpatient clinic and are also treated sporadically with folic acid and iron. According to the patients, the symptoms of the anemia worsen during periods of infection and fatigue. Laboratory (data for Pt-61 and Pt-62, respectively) results were: CBC, Hb 12.8 g/dl, 11.1 g/dl; Hct 36.9%, Hct 35.0%; RBC 3.56 × 10^{12}/μl, 3.47 × 10^{12}/μl; MCV 103.7 fl, 100.9 fl; MCH 36.0 pg, 32.0 pg; MCHC 34.7 g/cl, RDW-SD 59.8 fl, 62.4 fl; RDW-CV 16.1%, 17.6%; WBC 6.75 × 10^{9}/liter, 5.29 × 10^{9}/liter; PLT 226 × 10^{12}/μl, 269 × 10^{12}/μl; RET 84.1%, 299.4 × 10^{3}/liter, 72.7%, 252.3 × 10^{3}/liter; blood smear: RBC, anisocytosis, aurophilic stipplings, stomatocytes, and WBC, neutrophils 60 and 40%; eosinophils 2 and 4%; monocytes 8 and 7%; lymphocytes 30 and 48%; osmotic resistance of erythrocytes: 0.43–0.33 and 0.40–0.30%. BUN, creatinine, uric acid, potassium, and sodium, AST and ALT activities were normal. Bilirubin was: total 3.58 and 8.22 mg/dl; direct 0.37 and 0.32 mg/dl; indirect 3.21 and 7.90 mg/dl; iron, total 27.2 and 16.3 μmol/liter; TIBC, 53.0 and 16.3 μmol/liter; UIBC, 25.8 and 35.8 μmol/liter; saturation 51.3 and 31.3%; soluble TFR, 4.14 and 5.30 μg/liter; and ferritin, 75.1 and 23.7 μg/liter.

Isolation of RBC and Na^{+}/K^{+} Measurements—Peripheral blood from a healthy donor and from two patients with HA was preserved from clotting with EDTA and erythrocyte ghosts were obtained as described previously (31). Na^{+}/K^{+} measurements were carried out using Electrolyte Analyzer 9180 (Roche Applied Science).

**Isolation of Detergent-resistant Membrane (DRM)—** DRMs from RBC were isolated after obtaining resuspended RBC ghosts (150 μl) as described (Ref. 31 and references therein) by treatment with an equal volume of ice-cold DRM-isolation buffer (10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 100 μM PMSF, protease inhibitor mixture, Sigma), incubated on ice for 20 min, and vortexed occasionally. The samples were then mixed with an equal volume of 80% sucrose 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 × g, 4 °C). For isolation of Na_{2}CO_{3}-treated DRM (32) RBC membrane, 80% sucrose contained 0.3 μl Na_{2}CO_{3}. For isolation of DRM after latrunculin A treatment (33, 34), RBC were incubated with 1 μM latrunculin A (Sigma) in PBS/glucose buffer at 37 °C for 1 h. After incubation cells were washed and DRMs were obtained from RBC ghosts as described above.

**Biochemical Analysis of DRM Fraction—** After DRM isolation, 10 fractions (420 μl) were collected from the top of the gradient. In each fraction, protein (BCA, Sigma), cholesterol (AmplexRed, Invitrogen), and AChE activity (AmplexRed, Invitrogen) were analyzed as described by the manufacturers’ instructions. Measurements were performed using a Carry 1E UV-visible spectrophotometer and a Cary Eclipse Spectrofluorimeter.
Role of (Palmitoylated) MPP1 in Membrane Lateral Organization

Protein Electrophoresis, Immunodetection, and Detection of Radiolabeled Polypeptides—Proteins were electrophoresed by SDS-PAGE (10% gel) (35) or NuPAGE BisTris 4–12% (Invitrogen), stained in 0.01% Coomassie Blue in 5% acetic acid, 10% ethanol, or transferred onto a nitrocellulose membrane (PROTRAN®, Schleicher & Schuell Bioscience) in transfer buffer (192 mM glycine, 0.01% SDS, 20% methanol, pH 9.2) (36). The proteins from sucrose gradient fractionation in each collected fraction prior to electrophoresis were precipitated with 10% TCA. TCA precipitates were electrophoresed as above.

Primary antibodies used for protein detection were as follows: monoclonal mouse anti-MPP1, polyclonal rabbit anti-flotillin 1, and polyclonal goat anti-flotillin 2 polyclonal rabbit anti-DHHC17 were from Abcam (1 μg/ml); polyclonal goat anti-stomatin and goat anti-actin were from Santa Cruz (0.2 μg/ml); anti-spectrin and anti-ankyrin were obtained in our laboratory. Secondary antibodies (anti-goat, anti-rabbit, or anti-mouse; 0.04 μg/ml) were conjugated with horseradish peroxidase (Jackson ImmunoResearch). Reactions were developed using ECL procedures using luminol and coumaric acid (both from Sigma) as substrates and exposed on a UV-S Multispectral Imaging System or on x-ray film (Amersham Biosciences Hyperfilm, GE Healthcare Life Sciences). The detection of radiolabeled peptides was done by exposure of the nitrocellulose or dried gels to a GE Healthcare storage phosphorscreen, which was scanned in a Typhoon 8600 scanner.

2-BrP Treatment and Labeling of Palmitoylated Proteins—Radioactive labeling of palmitoylated proteins in RBC with [14C]palmitic acid (Hartmann Analytic) was performed as described (13). For inhibition of palmitoylation, RBC were pre-treated with fatty acid-free BSA (Roth) and incubated with 1–100 μM 2-BrP and/or [14C]palmitic acid (depending on the experiment) in PBS containing glucose for 20 h at 37 °C.

RBC Stimulation via Adenosine Receptor—Stimulation of control and 2-BrP-treated cells using 50-N-ethylcarboxamidoadenosine (Sigma) was performed as described by Kamata et al. (37).

Morphology of Resealed Ghosts—To evaluate the effect of inhibition of palmitoylation on the ability to retain the correct shape, resealed RBC ghosts were stained with DiD (Molecular Probes) using the previously described protocol (31). The images were acquired on an Olympus Fluoview 500 confocal or Zeiss LSM 510 scanning microscope. A PLAPO × 60 oil immersion objective was used.

Resealing of RBC Ghosts in Presence of Antibodies—For resealing of the RBC ghosts in the presence of antibodies, resealing buffer containing anti-MPP1 or anti-stomatin antibodies at a final concentration of 8 μg/ml were used. For microscopic observation, resealed ghosts were resuspended in resealing buffer containing DiD (Molecular Probes), and incubated (30 min, 37 °C) (31). Ghosts were then washed, mixed with low melting agarose solution, placed on glass slides, and covered with coverslips. Images were acquired on a confocal scanning microscope (see above).

Fluorescence Lifetime Measurement by Time-correlated Single Photon Counting (TCSPC) FLIM—RBC were labeled with 7.5 μM di-4-ANEPPDHQ (di-4) (Invitrogen) according to the protocol published by others (38, 39). Fluorescence lifetime imaging microscopy (FLIM) was performed at 23 °C with an LSM 510 microscope (Carl Zeiss) equipped with a FLIM dedicated system (PicoQuant). The probe was excited with a 470-nm pulsed laser diode (40 MHz) 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 104–105 photons to avoid the pile-up effect. Acquisition time was of the order of 200 s to achieve at least 103 photons per pixel. Each pixel in the image was pseudo-colored according to the average fluorescent lifetime. Improvement of the fluorescent lifetime estimation was achieved by 2 × 2 pixel binning.

Purification of Reticulocytes, RNA, and cDNA—The reticulocyte fraction was obtained from fresh blood of three healthy volunteers by centrifugation through the FicollPack (GE Healthcare) (1.077 g/cm3) at 400 × g for 35 min at 20 °C in a swinging-bucket rotor (without brake) to separate white blood cells. Next, platelets were separated by centrifugation at 200 × g for 15 min at 20 °C. The obtained reticulocyte fraction was washed several times with a solution of 0.13 M NaCl, 5 mM KCl, and 7.4 mM MgCl2, (mRNA was isolated using the RNEasy Mini kit (Qiagen) according to the manufacturer’s instructions with the exception that the erythrocyte lysis step was omitted. Synthesis of the cDNA was carried out by using a DyNaMo cDNA Synthesis Kit (Finnzymes). The sequence of the primers used in amplifying the major erythrocyte isoform of STOM (stomatin), EPB4.1R (protein 4.1R), GYPC (glycophorin C), MPP1, and all ZDHHC isoforms are included in the supplemental Tables S2–S4.

Identification of DHHC Isoform—“Control” cDNA was prepared from the reticulocyte fraction obtained from fresh blood of three healthy volunteers, as described above. A series of PCRs using primers specific for individual DHHC isoforms was carried out. Sequences of primers are included in supplemental Table S4.

Purification of Genomic DNA for Sequencing ZDHHC17 Gene—Genomic DNA for the sequencing of ZDHHC17 was isolated from nucleated blood cells using Blood Mini® (A&A Biotechnology). All the coding for exons and exon-intron boundaries of the ZDHHC17 gene (supplemental Table S5) were PCR amplified, using the primers listed in supplemental Table S5, and sequenced on both strands using the dyeode termination method of Sanger (41) by the DNA Sequencing and Oligonucleotide Synthesis Service of the Institute of Biochemistry and Biophysics, Warsaw, Poland.

Lipid Extraction and TLC—Lipids were extracted with chloroform/methanol (10:1 followed by 2:1). As the running phase for the TLC, the chloroform/methanol/NH4OH (9/7/2) solution was used. The TLC was run on glass plates covered with silica. Cholesterol content was measured using the AmplexRed (Invitrogen) kit and phospholipids were measured using a phosphate assay.

RESULTS

Unknown Hemolytic Anemia Is Caused by Loss of Protein Palmitoylation Activity—During our studies on hereditary spherocytosis, we identified a family in which 2 members (brothers Pt-61 and Pt-62) displayed symptoms of hemolytic
anemia that did not fit the characteristics of the known disease. A complete description of the patients is given under “Experimental Procedures.” The parents of the brothers and their sister (who was included in this study, Pt-63) and the family on both sides of parents were asymptomatic. As the patients showed the presence of stomatocytes in their blood smears, we checked the sto¬matin transcript, which was at the normal level, did not reveal mutations or polymorphisms (for primer sequences see supplemental Table S3).

Loss of Palmitoylation Is Attributable to Absence of DHHC17 Protein in RBC Membrane—Proteomic analyses (11, 12) have failed to identify the gene product responsible for the palmitoylation reaction in RBCs, although this activity has been shown to be present (13). Therefore, a systematic search for ZDHHC gene transcripts in mRNA isolated from reticulocytes was performed. In the reticulocyte cDNA, a series of PCRs using primers specific for individual ZDHHC gene transcripts was carried out (see “Experimental Procedures” and supplemental Table S4). Only the reaction based on ZDHHC17 primers gave a visible PCR product (see Fig. 1B and supplemental Fig. S2). The sequence of the PCR product was verified by DNA sequence analysis. PCR products corresponding to the other 22 isoforms’ signals were not detected, indicating that the transcripts were not present at a level above the detection threshold. Although the threshold is difficult to define, we were able to easily detect a PCR product corresponding to the “low abundance” transcript of peroxisome proliferator-activated receptor α (PPARA) (Fig. 1B and supplemental Fig. S1). Based on this observation we have confidence that the ZDHHC17 gene is the major PAT expressed in erythrocyte precursors. The presence of a ZDHHC17 gene product was also detected in the erythrocyte membrane by immunoblotting (Fig. 1C). In contrast, ZDHHC17 transcripts were not detected in lymphoid or myeloid cell lines, although several other DHHC isoforms were detected (supplemental Fig. S2B). Reticulocyte cDNA quality was checked by three different means. (i) Expression of the HBB gene (β-globin) (Fig. 1B). As can be seen, expression of the HBB gene is visible in all samples of cDNA. (ii) Testing the possible presence of impurities derived from contaminating platelets or white blood cell primers (supplemental Table S1) specific for integrin β3 (ITGB3) and CD42c (GP1BB) was used to detect platelet contamination; and primers specific for CD11a (ITGAL) and CD45 (PTRPC) were used to detect leukocyte contamination. As a loading control, β-actin (ACTB) primers were used. The results showed no presence of integrin β3, CD42c, CD11a, or CD45 products (supplemental Fig. S1). (iii) The above mentioned testing for the presence of a low abundance transcript, PPARA (reticulocyte cDNA library, NCBI, Library 11923). The ZDHHC17 gene transcript was also present in the reticulocytes of the patients (Fig. 1B). To search for possible mutations in the ZDHHC17 gene, we performed a series of PCRs on the genomic DNA from the patients using primers for individual exons and the promoter sequence (supplemental Table S5). Among several polymorphisms in the exons and introns, the most interesting change was a heterozygous exon 12 polymorphism (codon 383 AAC → AGC), which results in N383S substitution (supplemental Fig. S3 and Table S6), a polymorphism already present in the SNP data base (code rs33996476). In the studied family, this substitution was detected in the affected brothers but not in their healthy sister (Pt-63) or in three unrelated control individuals. As the polymorphism is heterozygous, it is unlikely that this is a back¬ground mutation of the observed effect.
Despite the presence of the message in reticulocyte mRNA from Pt-61 and Pt-62, Western blot analysis of RBC membranes using anti-DHHC17 antibodies demonstrated an absence of this gene product in the membrane of mature RBC (Fig. 1C, left), which might explain the lack of PAT activity in these cells. One should note that this enzyme is present in rather a low copy number as it can be detected by the used antibodies only when the gels are “overloaded” (Fig. 1C, right).

Taken together, these observations clearly explain the absence of protein palmitoylation activity in the RBCs of the patients and suggest that ZDHHC17 is the only ZDHHC gene transcribed in normal human erythroid cells.

**Loss of Palmitoylation Is Linked to Marked Decrease in DRM Fraction of RBC Membrane**—As protein palmitoylation is suggested to be one of the signals that target proteins to membrane rafts, we decided to isolate the DRM fraction from the RBC from the patients. To our surprise, there were essentially no DRMs in the gradient position where they are found in analyses of normal membranes (5/30% sucrose interface; fractions 2–4). The control DRM fraction was, as expected, enriched in cholesterol and AchE, whereas only a residual amount (4–6 fold smaller) of these DRM components was found in this fraction derived from the RBC membranes from the patients (Fig. 2A).

Western blot analysis using anti-stomatin antibodies (a commonly used DRM marker, Fig. 2B) showed that in the control sample stomatin was present in the low-density fractions (2–4), whereas in both patients the pattern was different, with the bulk of stomatin in the bottom of the tubes (high density fractions). Similar results were obtained for MPP1. Its association with DRM (2–4 fractions) was observed only in the control sample, whereas in both patients MPP1 was located mainly at the bottom of the gradient (Fig. 2B). As MPP1 is the major target of palmitoylation in the RBC, it appears that the unpalmitoylated form of this protein remained attached to the membrane skeleton. Moreover, the changes in membrane solubility in cold 1% Triton X-100 solution was not an effect of differences in lipid composition as both the TLC and chemical analysis of lipids in lipid extracts from RBC ghosts showed no significant variations among the major phospholipid classes between controls and patients (Fig. 2C). Also the cholesterol phospholipid ratio was unchanged for control and patient RBC membranes.

We observed a correlation between the lack of protein palmitoylation activity and the drastic reduction in DRM fraction. To test the possible causal relationship between these observations, the effect of palmitoylation inhibition on RBC membrane lateral organization was analyzed. SDS-PAGE followed by autoradiography of the membrane proteins from RBC incubated with [¹⁴C]palmitate indicated that the presence of 100 µM 2-BrP completely suppressed protein palmitoylation (supplemental Fig. S4A). Treatment with 2-BrP, a commonly used palmitoylation inhibitor, resulted in morphological changes of the resealed RBC ghosts from the characteristic biconcave shape to a more spherical morphology, consistent with delamination of the membrane bilayer from its membrane skeletal...
support (supplemental Fig. S4B). Moreover, the inhibition of palmitoylation by 2-BrP treatment blocked adducin (a protein of $\sim 90$ kDa) phosphorylation in response to the $G_{\alpha_5}$-mediated stimulation of PKA (supplemental Fig. S4D), which is known to be a palmitoylation and raft-dependent signaling pathway (37).

Membranes obtained from RBC incubated with 100 $\mu$M 2-BrP for 20 h were extracted with cold Triton X-100-containing buffer, and DRMs were isolated by sucrose gradient centrifugation. As shown in Fig. 3A, inhibition of palmitoylation produced a considerable decrease in the amount of DRM (fractions 2–4), which was estimated quantitatively from the protein and cholesterol content and AchE activity. The net protein content was more than 6-fold lower in 2-BrP-treated cells. Similar decreases were observed for cholesterol content and AchE activity (Fig. 3A). Increased protein and cholesterol content as well as AchE activity were recovered in fractions 7–10 (Fig. 3A). Proper DRM localization in the density gradient was observed when the experiments were carried out in the presence of 100 $\mu$M 2-BrP. After ultracentrifugation, 10 fractions from the top of the gradient were collected and in each fraction the protein and cholesterol contents and AchE activity were measured ($n = 5$). 2-BrP treatment changes the distribution profile of MPP1 and stomatin during DRM isolation as revealed by Western blot.

![FIGURE 3. Inhibition of palmitoylation with 2-bromopalmitate affects the DRM profile of normal erythrocytes. A, characterization of fractions collected after DRM isolation (gray box on the graphs) from RBC incubated with 100 $\mu$M 2-BrP. After ultracentrifugation, 10 fractions from the top of the gradient were collected and in each fraction the protein and cholesterol contents and AchE activity were measured ($n = 5$). B, 2-BrP treatment changes the distribution profile of MPP1 and stomatin during DRM isolation as revealed by Western blot.](http://www.jbc.org/)

As shown in Fig. 4A, DRM from control RBC membranes contains actin and spectrin in addition to MPP1 and raft marker proteins. The DRM fraction from RBC in the presence of 0.3 M Na$_2$CO$_3$ did not contain actin (Fig. 4B) but it did contain MPP1, raft marker proteins, and small amounts of spectrin. A similar result (Fig. 4C) was obtained when RBCs were treated with 1 $\mu$M latrunculin A prior to ghost and DRM isolation. Thus, formation of DRM appears to be independent of the presence of membrane skeleton and polymerized actin. It should be stressed that MPP1 remains associated with the DRM fraction together with raft marker proteins upon extraction with alkaline solution or actin depolymerization, which indicates rather strong, nonionic interactions with the membrane/membrane rafts of this otherwise peripheral protein.

**Presence of DRM Is Linked to Presence of (Palmitoylated) MPP1**—To evaluate the direct connection between normal DRM content and MPP1, we tested whether blocking of endogenous MPP1 by antibodies would specifically affect DRM quantity. Ghosts resealed with anti-MPP1 antibodies exhibited noticeable membrane deformations, presumably resulting from steric blockage of endogenous MPP1 by the antibodies, whereas the control and ghosts resealed with anti-stomatin antibodies exhibited a characteristic biconcave shape (Fig. 5A). Moreover, we observed a decrease in the amount of protein and lipid retrieved in low-density fractions when DRMs were isolated from ghosts resealed with anti-MPP1 antibodies. The amount of protein that was recovered in fractions 2–4 was reduced almost 2-fold, and the amount of cholesterol was reduced 4-fold. Only a slight decrease ($\sim 10\%$) could be observed when anti-stomatin antibodies were present during resealing (Fig. 5B), indicating that cross-linking of raft proteins was not responsible for the decrease in the DRM fraction.

**Order Imaging of RBC Membranes Using di-4 Probe**—Our data on isolation of DRM suggested that protein palmitoylation may significantly affect membrane order. The fluorescent probe di-4 exhibits a lifetime shift between liquid disordered and liquid ordered phases and has recently been used to study the distribution in membrane order in living cells (38, 39). We
Role of (Palmitoylated) MPP1 in Membrane Lateral Organization

used FLIM to investigate the changes in RBC membrane order following treatment with 2-BrP. As can be seen in Fig. 6, the probe lifetime was reduced on average by 0.3 ns in membranes of cells treated with 2-BrP compared with control, untreated RBC. Dimethyl sulfoxide or palmitate did not affect the lifetime values of the probe (see supplemental Fig. S5). For comparison, removal of cholesterol from the membranes by incubation with 10 mM MβCD induced a fluorescence lifetime reduction by 1–1.2 ns (Fig. 6). Thus, these observations demonstrate that inhibition of protein (specifically MPP1) palmitoylation not only induced a substantial decrease in the amount of DRM fraction, but also altered the physicochemical state of the membrane bilayer which, although much smaller, occurs in the same direction as that resulting from the reduction in cholesterol content (Fig. 6, B and C).

DISCUSSION

Although there are quite reasonable literature data concerning the connection of rafts/raft platforms to pathology (47–51), the evidence of this linkage is rather indirect. Here we present, for the first time, data providing evidence that principles of membrane lateral organization are directly linked to a disease state.

Our first experiments characterizing RBC membranes of HA patients showed changes in membrane lateral organization (changed DRM profile, Fig. 2), which was connected with the lack of S-acylation activity in their RBC membranes (Fig. 1A). The lack of DHHC17 (which, as shown here, is the only ZDHHC gene product found in erythrocytes) in patient RBC membranes was the reason for the lack of PAT activity in the RBCs of the patients (Fig. 1). As the ZDHHC17 message was easily detected in reticulocytes from the patients (Fig. 1B), we hypothesize that the molecular basis for the anemia involves regulation of translation, sorting, or integrating the protein into the membrane during biosynthesis. It should be noted that the expression pattern of various DHHC enzymes is tissue-dependent/specific, with more than one isoform present in any particular cell with possible overlapping activities (2, 4). Thus it is not surprising that the disease state is limited only to RBC as the absence of PAT activity of DHHC17 is probably compensated in other cells and tissues by the presence of other DHHC enzymes.

It is known that protein palmitoylation is important for proper membrane targeting and is suggested to play a role in raft localization of several proteins (20, 52–54). The main target of palmitoylation among RBC membrane proteins is MPP1. This protein was not palmitoylated and was absent from the DRM fraction (Figs. 1 and 2B), whereas its unpalmitoylated form was detected to the same level as in the control RBC, indicating an important role of MPP1 palmitoylation in determination of the membrane properties of the RBC. Moreover, we note that the general lipid composition of the patient RBC membranes was comparable with normal: cholesterol and major lipid classes composition remained unchanged (Fig. 2, C and D). Therefore our data showing a specific decrease in the amount of DRM observed upon the absence of protein palmitoylation suggests that, in addition to raft recruitment, this modification is indispensable for lateral membrane organization. This notion could also be supported by observations of the effect of DHHC17-directed siRNA on the reticulocyte lateral membrane organization (supplemental Fig. S6). Namely, knowing that PAT activity is important for RBC membrane function, we assumed that reticulocytes would still synthesize the DHHC17 protein, so DHHC17-directed siRNA treatment was done on reticulocytes isolated from umbilical cord blood (40) (for experimental details, see supplemental Fig. S6 legend). Western blot analysis of the sucrose density gradient fractions using anti-MPP1, flotillin 1, flotillin 2, stomatin, actin, and spectrin antibodies.

FIGURE 4. Presence of assembled spectrin-actin membrane skeleton does not affect the presence of raft marker proteins and MPP1 in DRM fraction. Western blot analysis of sucrose gradient fractions (DRM isolation). A, fractionation of DRM from control ghosts. B, fractionation of DRM obtained in the presence of 0.3 M Na2CO3. C, fractionation of DRM obtained from latrunculin A-treated RBC (for details see “Experimental Procedures”). Western blot analysis was performed using anti-MPP1, flotillin 1, flotillin 2, stomatin, actin, and spectrin antibodies.

FIGURE 4. Presence of assembled spectrin-actin membrane skeleton does not affect the presence of raft marker proteins and MPP1 in DRM fraction. Western blot analysis of sucrose gradient fractions (DRM isolation). A, fractionation of DRM from control ghosts. B, fractionation of DRM obtained in the presence of 0.3 M Na2CO3. C, fractionation of DRM obtained from latrunculin A-treated RBC (for details see “Experimental Procedures”). Western blot analysis was performed using anti-MPP1, flotillin 1, flotillin 2, stomatin, actin, and spectrin antibodies.
that the decreased MPP1 content in the low-density fraction may correspond to lower DRM content. Moreover, FLIM analysis performed using the di-4 probe (supplemental Fig. S6, D–F) may indicate an increase in the lower order fraction in the membrane of DHHC17 siRNA-transfected reticulocytes, as was shown by a significant reduction of the average lifetime (3.28 ns) compared with the control (scrambled RNA sequence)-treated reticulocytes (3.42 ns). Overall, these data may support our observation that a lack or marked decrease of the DHHC17 protein might lead to changes in membrane lateral organization.

We speculate that global inhibition of palmitoylation, as presented here for the RBC, may lead to dramatic changes in cholesterol binding by raft-organizing PHB domain-containing proteins such as stomatin and flotillins, as has been observed for the PHB protein podocin (55). The mutual interactions between MPP1, flotillins, and cholesterol confirm that these components show the potential for such function.11

Similar observations of a loss of DRM material from RBC membranes, for example, following MβCD (56) or lidocaine (57) treatment, have been previously published. RBC treated with these compounds were also highly resistant to infection by Plasmodium, as were elliptocytic RBCs lacking MPP1 (58). As no molecular mechanism of this resistance has yet been provided, and given that most of the changes observed here upon inhibition of MPP1 palmitoylation resembled changes induced by other means of perturbing lateral membrane organization, it

FIGURE 5. Resealing of the RBC ghosts in the presence of anti-MPP1 IgG leads to a decrease in the DRM fraction, whereas resealing in the presence of anti-stomatin IgG induces only minor change in DRM fraction. A, ghosts resealed with anti-MPP1 antibodies (right) were deformed and showed membrane blebbing compared with controls (middle and left), details as described in the legend to Fig. 3. B and C show the mean ± S.D.; n = 3.

FIGURE 6. FLIM of RBC and using di-4 probe. A, the FLIM images, and B, fluorescence lifetime histograms for di-4 probe of control, 2-BrP, or MβCD-treated RBC. C, quantitative analysis shows significant reduction of average lifetime values for both 2-BrP (n = 20) and MβCD (n = 12)-treated RBC (p < 0.0001). Statistical analysis was performed using unpaired t test. All scale bars represent 10 μm.

Role of (Palmitoylated) MPP1 in Membrane Lateral Organization

would be of interest to determine whether inhibition of palmitoylation induces resistance to Plasmodium.

Our experiments with binding of MPP1 in RBC ghosts with specific antibodies also resulted in a decreased amount of DRM when compared with untreated ghosts or ghosts resealed in the presence of anti-stomatin antibodies. Moreover, our data on human erythroleukemia cells (erythroid-precursor cell line) with MPP1 expression silenced with lentiviral-derived vector bearing siRNA sequences also indicated a marked decrease in DRM fraction,11 confirming the crucial role of (palmitoylated) MPP1 in lateral membrane organization.

It should be emphasized that although DRMs cannot be directly viewed as membrane rafts and that data obtained via this method should be treated with caution, it remains a useful tool and provides at least some insight into possible raft organization (59). Moreover, the significance of protein palmitoylation in lateral membrane organization was confirmed using a more advanced method, which was FLIM combined with a novel Laurdan family probe, di-4. Results obtained using this method allowed us to detect changes in lateral membrane ordering and revealed a decrease in fluorescence lifetime upon 2-BrP treatment compared with controls (Fig. 6 and supplemental Fig. S5). As was previously shown by others (38, 39), lower lifetime values of di-4 were accompanied by increasing membrane disorder. The direction of changes, i.e. increase in membrane disorder, although quantitatively much smaller, is similar following treatment of cells with MJβCD (Fig. 6) and, moreover, in human erythrocytes cells upon inhibition of palmitoylation and in which the expression of MPP1 was silenced.11 It should be noted that each pixel in a collected image corresponds to dye residing in ordered and disordered subresolution domains in the membrane (the diameter of the domain is in the 10–20 nm range, which is much smaller than the resolution limit of the microscope). Therefore in natural membranes, due to the large diversity of their composition, fluidity values never reach the extremes observed in artificial systems (60).

Dependence of resting raft/DRM formation/stabilization on the cortical actin/membrane skeleton is a topic that is interesting from two points of view: whether, as mentioned above (see “Results”), membrane skeleton is implied in the lateral membrane organization; and whether the (palmitoylated) MPP1 pool (DRM) is still engaged in the interactions with membrane skeleton complexes. The results presented here (Fig. 4) may suggest that DRM formation/stabilization at least in the erythrocyte model seems to be independent of the presence of the assembled membrane skeleton and that the MPP1 pool, which is present in the DRM fraction is not associated with the assembled spectrin-actin membrane skeleton. The lack of relationship of the DRM-membrane skeleton may in essence be in accordance with the proposal of the “hierarchical mesoscale domain organization of the plasma membrane” by Kusumi et al. (61).

CONCLUSIONS

Dynamic protein palmitoylation was previously shown to regulate cellular localization or membrane partitioning of certain proteins. Here we show that this process additionally affects membrane order, as revealed by differences in DRM profiles or changes of lifetime values of the di-4 probe.

In conclusion, MPP1 palmitoylation appears to be a crucial event involved in raft formation, which might be directly linked to RBC pathology. To the best of our knowledge, the described anemia is the first case of raftopathy that together with the data derived from the model system may help to understand the biological mechanism of membrane lateral domain organization. It also points to the importance of membrane lateral domain organization in whole organism biology.

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Role of (Palmitoylated) MPP1 in Membrane Lateral Organization

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Role of (Palmitoylated) MPP1 in Membrane Lateral Organization


Palmitoylation of MPP1 (Membrane-palmitoylated Protein 1)/p55 Is Crucial for Lateral Membrane Organization in Erythroid Cells

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