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

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This article has been withdrawn by the authors following concerns raised about data integrity in supplemental Figs. S1 and S2 prepared by one of the authors. Although these results are only of supportive nature and do not affect the main results and conclusions presented in the published paper, which were supported additionally by later findings in several experimental systems and published in other papers from 2013 to 2017. Nevertheless, we believe that the responsible course of action is to withdraw the article in the interests of maintaining the publication standards of the journal. We apologize for any inconvenience this may have caused.
Role of (Palmitoylated) MPP1 in Membrane Lateral Organization

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-BrP10 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 (L0) 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 and lipids, which leads to the fusion of small rafts to the formation of larger lipid-based protein assemblies that now could be considered rafts (resting).

We have identified two patients with hereditary hemolytic anemia wherein the MPP1 was absent in RBC. This was caused due to deletion of DHHC17 protein, which we identified to be isoform present in normal RBC. Since 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 might be crucial for lateral membrane organization or is directly involved in regulation of domain formation.

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 worsened 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 3.56×1012/μl, 3.47×1012/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 6.75×109/liter, 5.29×109/liter; PLT 226×109/μl, 269×109/μl; RET 84.1%, 299.4×109/liter, 72.7%, 252.3×109/liter; blood smear: RBC, anisocytosis, azurophilic stipplings, stomatocytes, and WBC, neutrophils 40 and 40%; eosinophils 2 and 4%; monocytes 8 and 7%; lymphocytes 30 and 48%; osmotic resistance of erythrocytes: 0.33 and 0.40–0.30%. BUN, creatinine, uric acid, sodium, AST and ALT activities were normal. Total protein 5.9 g/liter and 7.9 g/liter; iron, total iron 169 μmol/liter and 163 μmol/liter; transferrin saturation 51.3 and 31.3%; soluble transferrin receptor, ferritin 75.1 and 23.7 μg/liter. Na+/K+ Measurements—Peripheral blood from a healthy donor and from two patients with HA was collected 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 resealed 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 x g, 4 °C). For isolation of Na2CO3-treated DRM (32) RBC membrane, 80% sucrose contained 0.3 mM Na2CO3. 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.

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10 The abbreviations used are: 2-BrP, 2-bromopalmitate; AchE, acetylcholine esterase (GPI-anchored); DHHC, aspartate-histidine-histidine-cysteine domain; di-4, di-4-ANEPPDHQ (order-sensing dye); DRM, detergent-resistant membrane; FLIM, fluorescence lifetime imaging microscopy; HA, hemolytic anemia; L0, liquid ordered phase; MAGUK, membrane-associated guanylyl kinase; MBD, methyl-β-cyclodextrin; PAT, palmitoyl acyltransferase; BisTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol.
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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 luminal and coumaric acid (both from Sigma) as substrates and exposed on a UVP 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 phosphor screen on which was scanned in a Typhoon 8600 scanner.

2-BrP Treatment and Labeling of Palmitoylated Proteins—Radioactive labeling of palmitoylated protein-[^14C]palmitic acid (Hartmann Analytic) was performed as described (13). For inhibition of palmitoylation, RBC were 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. The obtained reticulocyte fraction was washed several times in a solution of 0.13 M NaCl, 5 mM KCl, and 7.4 mM Na-HEPES pH 7.4; RBC was isolated using the RNase Mini kit (Qiagen) according to the manufacturer’s instructions with the exception that the erythrocyte lysis step was omitted. After precipitation with TCA, the sequence of the primers was used to identify the erythrocyte isoform of STOM (H9262) and/or GYPC (glycophorin C), and isoforms were included in the supplement.

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 in a solution of 0.13 M NaCl, 5 mM KCl, and 7.4 mM Na-HEPES pH 7.4; RBC was isolated using the RNase Mini kit (Qiagen) according to the manufacturer’s instructions with the exception that the erythrocyte lysis step was omitted. After precipitation with TCA, the sequence of the primers was used to identify the erythrocyte isoform of STOM (H9262) and/or GYPC (glycophorin C), and isoforms were included in the supplement.

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 dideoxy termination method of Sanger (41) by the DNA Sequencing and Oligonucleotide Synthesis Service of the Institute of Biochemistry and Biophysics, Warsaw, Poland.

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
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A complete description of the patients is given under “Experimental Procedures.” The parents of the affected male individual were asymptomatic. As the patients showed the anemia that did not fit the pattern of inheritance of the studied family, where only male individuals were affected, the possibility of mutation in the MPP1 gene seemed very likely, as this gene locus is in the X chromosome, which would fit the pattern of inheritance of the studied family, where only male individuals were found to be affected. To search for mutations, fragments coding for MPP1 were amplified from cDNA obtained from the reticulocyte mRNA of the patients and sequenced (for primer sequences see 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 database (code H11022) the RBC major palmitoylated protein, MPP1, was present in similar levels in the patients as in the control sample. As shown in the Western blot (WB) (lower) present in the reticulocytes of healthy subjects and patients. For comparison the reverse transcriptase-PCR products for HBB (high abundance; bottom) and peroxisome proliferator-activated receptor α (PPARA) (low abundance; middle) genes are shown (for primer sequences see Tables S1 and 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 gene in erythrocyte precursors. The presence of a DHHC17-CAR product was detected in the erythrocyte precursors (Fig. 1C). In contrast, other CARs were not detected in lymphoid or myeloid cells, although several other DHHC isoforms were expressed in lymphoid or myeloid tissues (supplemental Fig. S2B). Reticulocyte cDNA quality was assessed by three different means. (i) Expression of the HBB gene seemed very likely, as this gene locus is in the X chromosome, which would fit the pattern of inheritance of the studied family, where only male individuals were found to be affected. To search for mutations, fragments coding for MPP1 were amplified from cDNA obtained from the reticulocyte mRNA of the patients and sequenced (for primer sequences see supplemental Table S2). Only a silent mutation (ACG > ACT; T835T) was identified in these patients (see supplemental Table S6). No mutations were found in the nucleotide sequences coding for the cytoplasmic domain of glycoporphin C (GPC1) or in the coding sequences of genes coding for protein 4.1 (EPB4.1R). Moreover, sequencing the stomatin 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 S1). 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 gene in erythrocyte precursors. The presence of a DHHC17-CAR product was detected in the erythrocyte precursors (Fig. 1C). In contrast, other CARs were not detected in lymphoid or myeloid tissues, although several other DHHC isoforms were expressed in lymphoid or myeloid tissues (supplemental Fig. S2B). Reticulocyte cDNA quality was assessed by three different means. (i) Expression of the HBB gene seemed very likely, as this gene locus is in the X chromosome, which would fit the pattern of inheritance of the studied family, where only male individuals were found to be affected. To search for mutations, fragments coding for MPP1 were amplified from cDNA obtained from the reticulocyte mRNA of the patients and sequenced (for primer sequences see supplemental Table S2). Only a silent mutation (ACG > ACT; T835T) was identified in these patients (see supplemental Table S6). No mutations were found in the nucleotide sequences coding for the cytoplasmic domain of glycoporphin C (GPC1) or in the coding sequences of genes coding for protein 4.1 (EPB4.1R). Moreover, sequencing the stomatin transcript, which was at the normal level, did not reveal mutations or polymorphisms (for primer sequences see supplemental Table S3).

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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 Pt-61 and Pt-62, Western blot analysis using anti-DHHC17 antibodies showed 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 the 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.

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
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Support (supplemental Fig. S4B). Moreover, the inhibition of palmitoylation by 2-BrP treatment blocked adducin (a protein of ~90 kDa) phosphorylation in response to the Gαs-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 μM 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 with 2-BrP treatment induced a considerable decrease in the protein and cholesterol content (Fig. 3A) 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. The net protein content of 2-BrP-treated cells was more than 6-fold lower than in control cells.

Protein and cholesterol content as well as AchE activity were recovered in fractions 7–10 (Fig. 3A). Increased protein and cholesterol content in control and DRM isolation. Thus, formation/stabilization 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 quantitatively. 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 (~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.

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 (~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...
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 patterns of some DHHC enzymes is tissue-dependent/specific with one isofom present in any particular cell/tissue due to overlapping activities (2, 4). Thus it is not surprising that the disease state is limited only to RBC as the presence of other DHHC enzymes palmitoylation is important for protein targeting and is suggested to play a role in the sorting 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 (supplemental Fig. S6C) antibodies indicated that the DHHC17 siRNA-transfected reticulocyte DRM low-density fraction contained much less MPP1 than the same fraction obtained from reticulocytes transfected with control RNA of a scrambled sequence (the total protein content was equal in both samples). By analogy with RBC DRM, we may conclude...
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

11 A. Łach, J. Podkalicka, M. Majkowski, K. Augoff, J. Korzeniewski, and A. F. Sikorski, manuscript in preparation.
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 ressealed 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 MβCD (58). Moreover, in human erythroleukemia cells upon inhibition of palmitoylation and in which the expression of p55/MPP1 was silenced.11 It should be noted that the fluorescence lifetime image corresponds to dye residing in subresolution domains in the membrane. Such domain is in the 10–20 nm range below the resolution limit of the microscope (60). 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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