Biochimica et Biophysica Acta 1828 (2013) 924–931

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Freely turning over palmitate in erythrocyte membrane proteins is not responsible for the anchoring of lipid rafts to the spectrin skeleton: A study with bio-orthogonal chemical probes

Annarita Ciana [a,1], Cesare Achilli [a,1], Rami N. Hannoush [b], Angela Risso [c], Cesare Balduini [a], Giampaolo Minetti [a,*]

[a] University of Pavia, Department of Biology and Biotechnology "Lazzaro Spallanzani", Laboratories of Biochemistry, via Bassi 21, 27100 Pavia, Italy
[b] Genentech, Department of Early Discovery Biochemistry, 1 DNA Way, MS 27, South San Francisco, CA 94080, USA
[c] University of Udine, Department of Agricultural and Environmental Sciences, via delle Scienze 91, 33100 Udine, Italy

ARTICLE INFO

Article history:
Received 4 June 2012
Received in revised form 31 October 2012
Accepted 23 November 2012
Available online 3 December 2012

Keywords:
Fatty acylation
Detergent-resistant membrane
2-bromopalmitate
Cerulenin
Click chemistry
Omega-alkynyl

ABSTRACT

Erythrocyte lipid rafts are anchored to the underlying spectrin membrane skeleton [A. Ciana, C. Achilli, C. Balduini, G. Minetti, On the association of lipid rafts to the spectrin skeleton in human erythrocytes, Biochim. Biophys. Acta 1808 (2011) 183–190]. The nature of this linkage and the molecules involved are poorly understood. The interaction is sensitive to the increase in pH and ionic strength induced by carbonate. Given the role of palmitoylation in modulating the partitioning of certain proteins between various sub-cellular compartments and the plasma membrane, we asked whether palmitoylation of p55, a peripheral protein located at the junctional complex between spectrin-actin-protein 4.1 that anchors the membrane skeleton to the lipid bilayer via the transmembrane protein glycophorin C, could contribute to the anchoring of lipid rafts to the membrane skeleton. We adopted a new, non-radioactive method for studying protein palmitoylation, based on bio-orthogonal chemical analogues of fatty acids, containing an omega-alkynyl group, to metabolically label cell proteins, which are then revealed by a "click chemistry" reaction of the alkynyl moiety with an azide-containing reporter tag. We show that the membrane localization and palmitoylation levels of p55 did not change after carbonate treatment. 2-bromopalmitate and cerulenin, two known palmitoylation inhibitors, completely inhibited p55 palmitoylation, and protein palmitoyl thioesterase-1 (PPT1) reduced it, without affecting the association between lipid rafts and membrane-skeleton, indicating, on the one hand, that p55 palmitoylation is enzymatic, and, on the other, that it is not involved in the modulation of the linkage of lipid rafts to the membrane-skeleton.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Lipid rafts are membrane nano-micro-domains enriched in sphingolipids and cholesterol in liquid ordered state, and selected classes of proteins, such as GPI-anchored, signal transducing and other acylated proteins [1]. The biochemical characterization of lipid rafts has historically been based on their isolation as Detergent-Resistant-Membranes (DRMs), which are portions of the membrane insoluble in non-ionic detergents, such as Triton X-100 (TX100), at low temperatures [2].

We have shown previously that, differently from what observed in other cell types, erythrocyte DRMs, with the properties of canonical lipid rafts, where lipids are in liquid-ordered state and specific marker proteins are enriched [3], are tightly anchored to the spectrin membrane skeleton. The incubation with the detergent alone is not sufficient to allow the isolation of DRMs as a band of low density in sucrose gradients, unless a simultaneous increase in pH and ionic strength is induced, for instance by using a carbonate salt [4].

The molecules involved in the linkage between erythrocyte DRMs and the membrane skeleton are not known. The connection appears not to involve the two main vertical linkages, mediated by ankyrin and protein 4.1, which anchor the spectrin skeleton to the integral membrane proteins band 3 and glycophorin C (GPC), respectively. These proteins are in fact excluded from the raft compartment [5]. The requirement for carbonate in the protocol for their isolation would indicate that lipid rafts are anchored by an electrostatic mechanism. This may involve the lipid raft marker protein stomatin (alone or together with stomatin-like-protein-2) [6,7], which has been found in relatively large amounts in erythrocyte lipid rafts [8], or a direct binding of membrane phospholipids to spectrin [9,10].

It is also conceivable that palmitoylated proteins in the membrane, such as p55 (also known as membrane palmitoylated protein

Abbreviations: Alk-C16, ω-alkynyl palmitic acid; 2BP, 2-bromopalmitic acid; DFP, di-isopropyl fluoro phosphate; DRM, detergent-resistant membranes; GPC, glycophorin C; HRP, horseradish peroxidase; PPT1, protein palmitoyl thioesterase 1; TX100, Triton X-100

* Corresponding author at: University of Pavia, Department of Biology and Biotechnology "Lazzaro Spallanzani", Laboratories of Biochimica, via Bassi, 21, 27100 Pavia, Italy. Tel.: +39 0382 987891; fax: +39 0382 987240.
E-mail address: minetti@unipv.it (G. Minetti).

[1] These two authors contributed equally to the work.

0005-2736/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbamem.2012.11.029

1, MPP1) [11], spectrin [12], ankyrin [13], protein 4.2 [14], and stomatin itself [15], could be responsible for the anchoring of lipid rafts. If this were the case, the mechanism of action of carbonate in releasing the lipid rafts from the membrane skeleton could be related, rather than to the perturbation of electrostatic interactions, to the hydrolysis, in the alkaline environment generated by carbonate itself, of the relatively weak thioester bond that links palmitic acid to the protein.

There is increasing evidence for a role of protein lipidation in the process of protein sorting [16] and in modulating the partitioning of proteins into the sphingolipid rich domains of the membrane [17]. In particular, protein S-palmitoylation has been shown to be a means of reversibly regulating the targeting of membrane proteins to lipid rafts [18]. The study of protein palmitoylation has been generally affected by the limitations in the assay methods. Original evidence of S-palmitoylation of erythrocyte proteins, in particular of p55, was obtained after metabolic labeling of erythrocytes with tritium-labeled palmitate, protein separation by electrophoresis, and days-long autoradiography. Recent developments have made available novel approaches for the study of protein fatty-acylation [19–24]. One of these is based on chemical analogues of fatty acids containing an alkynyl group in the omega position of the hydrocarbon chain, which have been demonstrated to be bio-orthogonal, i.e. to behave as the unmodified, natural compound, in being metabolically incorporated by the cells into the target proteins, and to undergo a chemical reaction without cross reactivity with biochemical processes. Following the incorporation of the analogue, this is made to react with an azide-containing reporter tag that could be then visualized via fluorescence or chemiluminescence approaches.

We have explored the palmitoylation turnover in erythrocyte membrane proteins by this new method. The localization and palmitoylation level of p55 have been thus investigated in the presence and in the absence of carbonate, and after incubation with inhibitors of protein palmitoylation, in search for a possible role of p55 palmitoylation in the anchoring of lipid rafts to the membrane skeleton.

2. Materials and methods

2.1. Materials

ω-alkynyl palmitic acid (Alk-C16) and biotin-azide were synthesized as described earlier [19] and supplied by Genentech, South San Francisco, California, USA; Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) (code C4706), Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl]amine (TBTA) (code 678937), 2-Bromopalmitic acid (code 21604), cerulenin (code C2389), di-isopropylfluorophosphate (DFP), Na-pyruvate (code P8574), Coenzyme A (code C3019), penicillin G and horseradish peroxidase (HRP)-conjugated avidin (code A7419) were from Sigma-Aldrich. Goat polyclonal (N-19) anti-human p55 (code sc-13490), mouse monoclonal (BRIC10) anti-human GPC (code sc-59183) and HRP-conjugated mouse anti-goat IgG (code sc-2354) were from Santa Cruz Biotechnology. Mouse polyclonal anti-human protein 4.1 (code H00002035-A01) was from Abnova. HRP-conjugated goat anti-mouse IgG (code 170-6516), PVDF membranes 0.2 μm pore diameter (code 162-0177), and protein molecular weight standards (Precision Plus Protein™ Standards All Blue, code 161-0373; Prestained SDS-PAGE Standards Low Range, code 161-0305; SDS-PAGE Standards Broad Range, code 161-0317), were from Bio-Rad Laboratories.

2.2. Blood processing

Blood was freshly collected from healthy human donors in ACD-B (Vacuette®, Greiner bio-one) or in 0.1 vol of 3.8% (w/v) sodium citrate as anticoagulants. Erythrocytes were purified from leukocytes and platelets by filtration on a mixture made of equal parts, by weight, of α-cellulose and microcrystalline cellulose equilibrated with PBS (5 mM Na-phosphate, 154.5 mM NaCl, 4.5 mM KCl, pH 7.4) [25]. Filtered cells were washed three times with PBS before use.

2.3. Metabolic labeling of cellular proteins with Alk-C16

Purified erythrocytes were incubated at 10% hematocrit at 37 °C for 16–18 h under gentle end-over-end agitation, with 20–25 μM Alk-C16 (stock solution 50 mM in DMSO), or 0.05% (v/v) DMSO, in the following buffer: 40 mM imidazole, 90 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 15 mM glucose, 30 mM sucrose, 5 mM Na-pyruvate, 5 μM Coenzyme A, 200 U/ml penicillin G, pH 7.6, 300–320 mosmol/kg H_2O [26]. After incubation, erythrocytes were washed once in cold PBS, treated with 3.5 mM (final concentration) DFP in PBS for 10 min, washed with PBS containing 0.2% BSA to remove unbound Alk-C16 and finally with PBS. Cells were next lysed with hypotonic buffer (5 mM Na-phosphate, pH 8) according to Dodge [27], but without EDTA, in order to avoid chelation of copper ions during the subsequent cycloaddition reaction (see below), and washed until white ghost membranes were obtained. The volume of the ghost suspension was adjusted to that of the original sample of packed erythrocytes, resulting in a protein concentration of approximately 5 mg/ml in the ghost suspension.

2.4. Alkyne-azide cycloaddition reaction (click chemistry)

Freshly prepared ghosts were first diluted with one vol of hypotonic buffer, then to 21 μl of this diluted sample the following reagents were added, from concentrate stock solutions, and in the exact order given, for the Cu(I)-catalysed (3 + 2) Huisgen cycloaddition reaction or click reaction (final concentrations): SDS (0.5%), biotin-azide (0.1 mM), TCEP (1 mM) (to generate Cu^+ in situ from the reduction of Cu^{2+}), TBTA (0.2 mM) (to complex Cu^+ and allow the regio-selective cycloaddition), $CuSO_4$ (1 mM) [19]. The final volume was 25 μl, containing approximately 50 μg of membrane proteins. Higher sample concentration (4–5 μg proteins/μl) resulted in a weaker signal after Western blotting and chemiluminescence detection (see below). After 1 h at room temperature the samples were diluted with one vol of PBS, mixed with 0.5 vol of reducing SDS-PAGE sample buffer [50 mM Tris–HCl pH 6.8, 5% (w/v) SDS, 5 mM EDTA, 200 mM DTT, 35% (w/v) sucrose, 0.01% bromophenol blue], heated at 60 °C for 15 min and loaded (6–20 μg proteins/lane) on 5–15% (w/v) linear polyacrylamide gradient SDS-PAGE gels [28] for electrophoresis and Western blotting (see below).

2.5. Preparation of DRMs and processing of sucrose gradient fractions

DRMs were extracted from whole erythrocytes as previously described [5]. Briefly, filtered, DFP-treated erythrocytes (1.25×10^9) were diluted in TX100-containing HKM buffer (5 mM HEPES, 150 mM KCl, 4.5 mM NaCl, 2 mM $MgCl_2$, pH 7.4), so that the final volume was 0.625 ml and the final detergent concentration 1% (w/v). Samples were incubated on ice for 30 min, mixed with an equal vol of 80% (w/v) sucrose in HKM or in 0.3 M K_2CO_3, transferred to ultracentrifuge tubes (5 ml, Ultra-clear™, code N. 344057, Beckman Coulter), layered with 2.5 ml of 30% sucrose and 1.25 ml of 5% sucrose solutions in HKM, and spun at $225{,}000 \times g_{max}$ at 4 °C in a bench-top ultracentrifuge (Optima-Max, equipped with a swinging-arm MLS50 rotor, Beckman Coulter). After ultracentrifugation, DRMs floating at the interface 5–30% sucrose solutions were collected in 1 ml fraction, while the 40% sucrose, Hb-rich bottom of the gradient was split and collected in four 0.34 ml fractions. Proteins in the fractions were analysed by SDS-PAGE in 10% (w/v) isocratic or 5–15% (w/v) linear gradient polyacrylamide gels. The DRM fraction was mixed with 0.5 vol of reducing SDS-PAGE sample buffer, while the bottom fractions were dissolved in 9 vol of dilute SDS-PAGE sample buffer [1 vol of reducing SDS-PAGE sample buffer plus 1.7 vol of 5% (w/v) SDS in water],

heated at 60 °C for 15 min and subjected to Western blotting and chemiluminescence detection. Alternatively, samples were processed as follows for SDS-PAGE and Coomassie staining: bottom fractions were heated in 3 vol of dilute SDS-PAGE sample buffer and proteins separated by SDS-PAGE. Gels were stained with 0.2% (w/v) Coomassie blue R250 in 20% (w/v) trichloroacetic acid (TCA) and destained with 7.5% (v/v) acetic acid.

For detecting the palmitoylation of proteins contained in the bottom fraction of the gradient, white ghost membranes, not whole erythrocytes, had to be subjected to TX100 treatment, because of Hb interference with the click chemistry. Ghosts were purified from Alk-C16-labeled erythrocytes (1.25×10^9) and incubated in final 1% TX100 in PKM (5 mM Na-phosphate, 150 mM KCl, 4.5 mM NaCl, 2 mM $MgCl_2$, pH 7.4) at 4 °C for 30 min. Samples were mixed with an equal vol of 80% sucrose in 0.2 M K_2CO_3 and ultracentrifuged in sucrose density gradients in PKM for 1 h as described above. The bottom 40% sucrose step of the gradient was subjected to the click reaction after dilution 1:10 in 5 mM Na-phosphate and subsequent concentration to one tenth of the original volume in a Centricon centrifugal filter device, cut-off 3000 Da (Millipore). This dialysis step was required to remove sucrose, carbonate, and K^+ (which induces the precipitation of the SDS used in the click reaction).

2.6. Incubation of erythrocytes with inhibitors of protein palmitoylation: 2-bromopalmitic acid (2BP) and cerulenin

Erythrocytes were incubated at 10% hematocrit at 37 °C for 4 h with or without 50 μM 2BP or 200 and 400 μM cerulenin, added from stock solutions of, respectively, 100 mM and 90 mM concentration in DMSO. After incubation with 2BP, cells were washed three times in the incubation buffer without 2BP, before adding Alk-C16 and metabolic labeling as described above. After incubation with cerulenin, cells were either washed free of the inhibitor or not, before adding Alk-C16 and metabolic labeling as described above. Ghost membranes were purified from the labeled erythrocytes and subjected to the click chemistry reaction as described above.

2.7. Incubation of ghost membranes with palmitoyl-protein thioesterase-1 (PPT1)

Recombinant PPT1 was a kind gift from Prof. Sandra L. Hofmann, University of Texas Southwestern Medical Center, Dallas, Texas, USA [29]. Ghost membranes purified from Alk-C16-labeled erythrocytes, pre-diluted with one vol of hypotonic buffer, were incubated at 37 °C for 1 h in the presence of 0.05 μg/μl PPT1 at pH 8, and then subjected to the click chemistry reaction and Western blotting detection as described above.

2.8. Western blotting and chemiluminescence detection

Following SDS-PAGE, proteins were electro-transferred to PVDF membranes [30]. For detection of biotin-labeled proteins, PVDF membranes were incubated overnight at 4 °C in blocking buffer [20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween-20, 5% (w/v) skimmed milk], then washed three times (5 min each) with PBS containing 0.1% Tween-20, incubated with horseradish-peroxidase-conjugated avidin 1:10,000 for 1 h at 25 °C and developed using chemiluminescence detection reagent Amersham™ ECL plus Western blotting detection system (GE Healthcare).

The same membranes, after at least 1 h at 25 °C incubation with 0.02% NaN_3 in washing buffer [50 mM Tris-HCl pH 7.5, 0.2 M NaCl, 1% (w/v) polyethylene glycol 20000, 1% (w/v) BSA, 0.5% (v/v) Tween-20], were re-probed for p55 detection. For detection of proteins with specific antibodies, PVDF membranes were dipped for 1 h at 25 °C in blocking buffer, then overnight at 4 °C in the primary antibody (goat anti-p55 diluted 1:200, mouse anti-GPC diluted 1:4000 and mouse anti-protein 4.1 diluted 1:2000) in washing buffer. Secondary antibodies were diluted in washing buffer (mouse anti-goat 1:4000, goat anti-mouse 1:10,000 and goat anti-mouse 1:4000, respectively) and added for 1 h at 25 °C after PVDF membranes were properly washed in the same solution. Finally, proteins were revealed with the Amersham detection reagent.

For palmitoylation experiments, that palmitate was actually bound to proteins via a thioester bond was assessed by evaluating the sensitivity of the acylation to hydroxylamine, a reagent which specifically removes fatty acid incorporated into proteins via thioester but not amide linkage. After SDS-PAGE and Western blotting, PVDF membranes were soaked for 72 h at room temperature in 5% (w/v) hydroxylamine (NH_2OH) in PBS containing 0.1% Tween-20, and then subjected to chemiluminescence detection.

3. Results

3.1. Development of the Alk-C16 labeling protocol in human erythrocytes

First, it was necessary to verify that the ω-alkynyl analogue of palmitic acid was bio-orthogonally incorporated into acyl-modified proteins also in the erythrocyte model. Our initial hope was that the cycloaddition reaction required for the linking of the ω-alkynyl moiety to the detection probe (biotin-azide in our case) could be conducted on a whole cell lysate. However, it became immediately clear that this could not be done, as the presence of even trace amounts of Hb strongly inhibited the reaction. Therefore, following metabolic labeling by incubation at 10% hematocrit with 20 μM Alk-C16 for 16 h at 37 °C [12], erythrocytes were processed for preparation of ghost membranes by hypotonic hemolysis. Only highly purified ghost membrane preparation, i.e. ghost membranes that were carefully washed free of Hb (Fig. 1A, “white gh.”), could be successfully subjected to the cycloaddition reaction, whereas “pink ghost” membranes showed no signal (Fig. 1A, “pink gh.”). With this procedure an intense band of approximately 55 kDa was visible, together with other, less intense bands, in the samples incubated with Alk-C16 (Fig. 1A, top panel, “Alk-C16 white gh.”). Apart from several other minor bands, whose identity deserves further analysis, at least the 55 kDa palmitoylation signal was sensitive to treatment with hydroxylamine, indicative of the analogue being metabolically incorporated via a thioester bond into this protein (Fig. 1B, top panel). Re-probing of the two membranes with anti-p55 confirmed that the most intensely palmitoylated band was indeed p55 (Fig. 1A, bottom panel), and that the hydroxylamine treatment is really effective in only cleaving the thioester bond without affecting the amount of p55 on the blotting membrane (Fig. 1B, bottom panel). Moreover, the identity of p55 with the 55 kDa palmitoylated band was confirmed by immunoprecipitation of p55 with the sc-13490 antibody (not shown).

As the conventional protocols for ghost preparation contemplate the use of EDTA, which is a strong inhibitor of the cycloaddition reaction because it chelates the required copper ions, special care had to be taken to avoid EDTA in all the buffers (not shown). Although the original protocol contemplated the use of a non-ionic detergent (0.9% TX100) in the sample-processing buffer prior to the cycloaddition reaction, we observed that the inclusion of 0.5% SDS together with the non-ionic detergent strongly improved the signal (not shown). As a further improvement to the protocol, it was observed that an additional one-fold dilution of the “white” ghost membranes to be subjected to the cycloaddition reaction significantly improved the subsequent signal response (Fig. 1C and D, compare “undil.” *vs.* “dil.” ghosts), probably because of additional dilution of traces of contaminating Hb. As a result of the above described optimization procedures, the sensitivity of the method was greatly increased: less than 20 μg total ghost proteins were loaded in each lane.

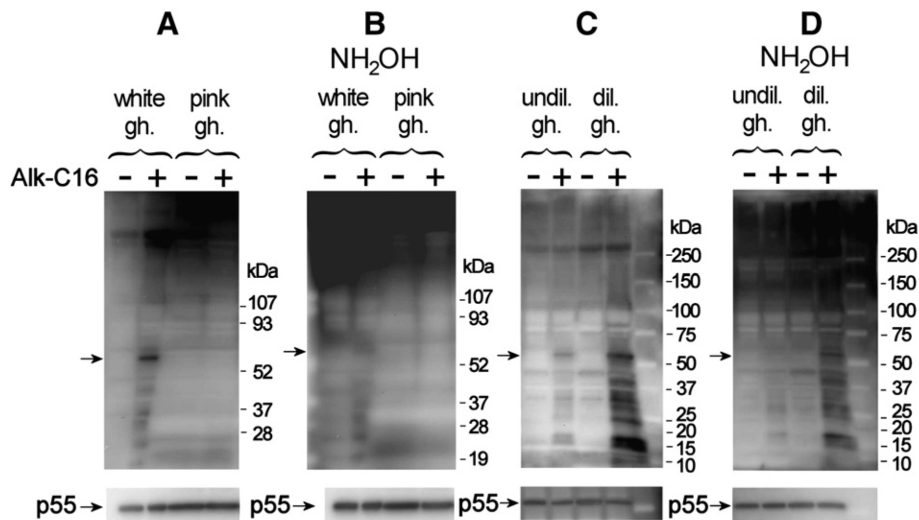

Fig. 1. Detection of erythrocyte membrane palmitoylated proteins by Alk-C16 metabolic labeling and click chemistry. Erythrocytes were metabolically labeled as described in Materials and methods. (A, B) Two parallel PVDF membranes from gels that were loaded with ghosts prepared from labeled cells by hypotonic hemolysis and repeated washings in hypotonic buffer until membranes turned white ("white gh."); "pink gh." refers to partially washed ghosts, not completely free of Hb. Alk-C16 labeled membrane proteins were revealed by click chemistry reaction of the alkynyl moiety with biotin-azide, followed by SDS-PAGE and Western blotting. (C, D) Two parallel PVDF membranes from gels loaded with white ghosts (prepared from labeled cells) were subjected to the click chemistry reaction without ("undil. gh.", protein concentration 4–5 μg/ml) or with pre-dilution 1:1 in buffer ("dil. gh.", protein concentration 2 μg/ml), then to SDS-PAGE and Western blotting. *Top panels*: chemiluminescence detection with HRP-conjugated avidin without (A, C) or with (B, D) pre-incubation of the blotting membranes in hydroxylamine. Hydroxylamine-treated membranes were purposely overexposed to better show the differential in the hydroxylamine-sensitive signals. Arrows indicate palmitoylated protein p55. *Bottom panels*: Immunodetection with anti-p55 antibody of the membranes shown in the top panels.

3.2. Palmitoylated p55 remains localized with the membrane-skeleton after DRM extraction

Protein p55 is believed to be localized at the junctional complex of the membrane skeleton, where it associates on the one side with the spectrin skeleton, via protein 4.1, and on the other with the integral membrane protein GPC. However, little information exists on the partition of p55 among the various fractions resulting from detergent treatment of erythrocytes. We have recently shown that the lipid raft fraction obtained under optimized conditions from whole erythrocytes by TX100 + carbonate treatment contains only trace amounts of p55 [5]. In order to explore the possible role of p55 palmitoylation in the linkage of lipid rafts to the membrane skeleton, we have analysed the localization of p55 relative to spectrin and GPC, before and after detaching lipid rafts from the membrane skeleton by carbonate.

Purified, DFP-treated erythrocytes were incubated with 1% TX100 at 4 °C, mixed with 80% sucrose in buffer or in carbonate, and subjected to ultracentrifugation for 16 h in sucrose density gradient (Fig. 2A). After treatment with TX100 alone, the linkage between lipid rafts and the membrane skeleton is preserved [5] (Fig. 2A, tube 1, fraction "a"). Protein p55 segregates together with GPC, protein 4.1 and spectrin in the high-density DRM material of the sucrose gradient (Fig. 2A, bottom panels). Following addition of carbonate, the lipid rafts detach from the membrane skeleton and float at the 5–30% sucrose interface in the gradient (Fig. 2A, tube 2, arrow), whereas p55 remains completely localized with GPC, protein 4.1 and spectrin in the dense fractions of the gradient (Fig. 2A, bottom panels).

We then asked whether the release of lipid rafts from the membrane skeleton under the action of carbonate was related to depalmitoylation of p55. For this experiment, erythrocytes were first metabolically labeled with Alk-C16. White ghosts were then prepared from these cells and subjected to TX100 treatment with the addition of carbonate and ultracentrifugation in sucrose gradient. The p55-containing bottom of the tube was collected and subjected to the "click chemistry" reaction as described in Materials and methods. As shown in Fig. 2B, the p55 palmitoylation signal in the bottom of the gradient (Fig. 2B, lane "bottom") is quantitatively similar to that detected in the corresponding control ghost sample (Fig. 2B, lane "Alk-C16 +"). This indicates that the p55 protein, which remains associated with the membrane skeleton, and which amounts to the full complement of p55 molecules of the cell, is also still fully palmitoylated even after carbonate treatment. Therefore, carbonate, which is known to affect the linkage between DRMs and the spectrin skeleton, does not so by cleaving the thioester bond between p55 and its palmitic moiety.

3.3. p55 palmitoylation is inhibited by 2-bromopalmitate and cerulenin

To further investigate the role of p55 palmitoylation in anchoring the lipid raft to the membrane skeleton, we adopted a different strategy, consisting in inhibiting protein palmitoylation. To this purpose, we tested two known pharmacological inhibitors of protein palmitoylation: 2-bromopalmitate (2BP), a non metabolizable palmitate analogue, and cerulenin, a natural anti-fungal antibiotic.

Cells were incubated with the inhibitors for 4 h at 37 °C prior to the metabolic labeling with Alk-C16. p55 palmitoylation was completely prevented by 50 μM 2BP (Fig. 3A), or 200 μM cerulenin (Fig. 3B). 2BP was irreversibly incorporated into the cell compartment, and the inhibitory effect persisted even when cells were washed before metabolic labeling with Alk-C16. On the contrary, cerulenin had to be left in the incubation medium, because washing of the cells before metabolic labeling resulted in the loss of the inhibitory effect on palmitoylation (Fig. 3C).

That Alk-C16 was indeed linked via a thioester bond was shown by the selective removal of the label following incubation of the purified ghost membranes (derived from erythrocytes metabolically labeled with Alk-C16), with recombinant PPT1, a lysosomal enzyme characterized by insensitivity to serine-modifying reagents like DFP [29], followed by the "click chemistry" reaction. As shown in Fig. 3D, this resulted in a significant decrease of p55 palmitoylation.

3.4. The p55 lipid moiety is not involved in anchoring lipid rafts to the membrane skeleton

We further demonstrated that p55 palmitoylation is not responsible for the interaction between lipid rafts and the membrane skeleton by using 2BP. Erythrocytes were subjected to metabolic labeling with

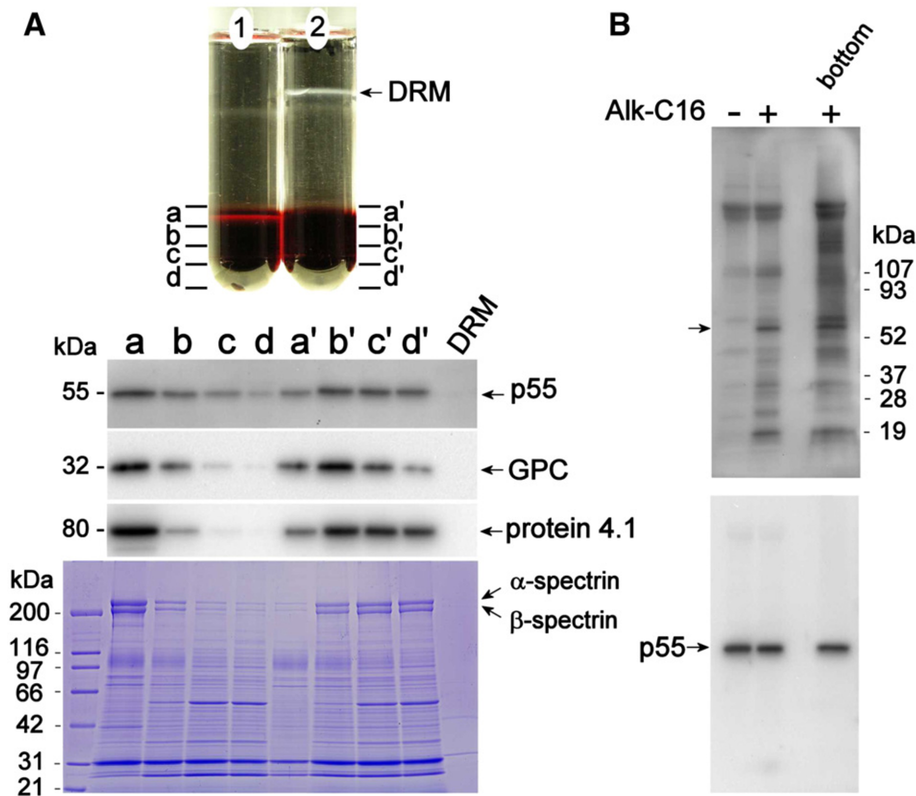

Fig. 2. Localization and palmitoylation state of p55 after isolation of lipid rafts. (A) Erythrocytes were treated with 1% Triton X-100 at 4 °C for 30 min, then mixed with 80% sucrose in HKM buffer (tube 1) or in 0.3 M K_2CO_3 (tube 2) and ultracentrifuged for 16 h in sucrose gradients. DRMs were recovered only in the presence of carbonate as a low density band floating at the interface 5–30% sucrose (arrow). The 40% sucrose bottoms of the gradients were collected in four fractions (a–d from tube 1; a'–d' from tube 2) from the top and protein content in each fraction, and in the DRM fraction ("DRM"), were analysed by Western blotting (p55, GPC, protein 4.1) and Coomassie blue staining. (B) p55 palmitoylation after carbonate treatment was assayed in the 40% sucrose fraction of a separate gradient, in which Alk-C16-labeled ghosts were loaded. *Top panel*: Palmitoylated proteins in ghost samples purified from Alk-C16-unlabeled or Alk-C16-labeled erythrocytes, and in the gradient bottom fraction ("bottom"). The arrow indicates palmitoylated p55. *Bottom panel*: Immunodetection of p55 in the membrane shown in the top panel.

Alk-C16 after preincubation with 2BP. Whole cells were subsequently lysed in 1% TX100 at 4 °C and subjected to the flotation experiment in sucrose gradient without addition of carbonate. The effectiveness of 2BP in inhibiting palmitoylation was checked by parallel analysis of a sample of purified white ghosts prepared from the treated cells. The metabolic labeling with Alk-C16 itself does not interfere with the recovery of DRMs as a band of low density material, as verified by control samples treated with carbonate (Fig. 4, compare tubes 2 and 4). However, no low density DRM material was observed after ultracentrifugation in the sucrose gradient of 2BP-treated cells in

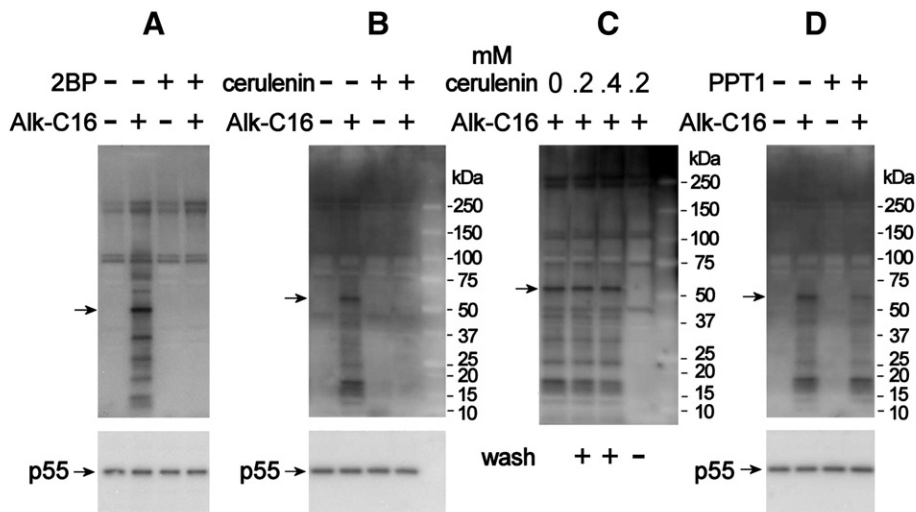

Fig. 3. Effect of protein palmitoylation inhibitors 2BP and cerulenin, and of PPT1 enzyme, on p55 palmitoylation. *Top panels*: (A) Erythrocytes were incubated at 37 °C for 4 h with 50 μM 2BP, washed, labeled with Alk-C16, and palmitoylated proteins detected by click reaction and Western blotting. (B) Erythrocytes were incubated at 37 °C for 4 h with 200 μM cerulenin, then subjected to Alk-C16 labeling in the presence of the same concentration of cerulenin, followed by detection of palmitoylated proteins. (C) Washing the cells to remove the unbound cerulenin before metabolic labeling, resulted in the loss of the inhibitory effect, even at higher (400 μM) inhibitor concentration. (D) PPT1 effect on p55 palmitoylation was assayed on Alk-C16 labeled ghosts incubated with 0.05 μg/ml PPT1 at 37 °C for 1 h. *Bottom panels*: the amount of p55 in the same samples shown in panels A, B, D was revealed by re-probing the same blotting membranes with anti p55 antibody.

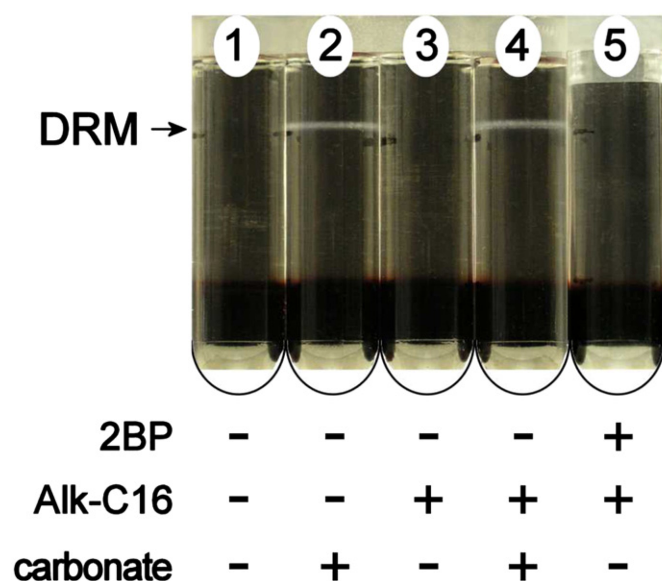

Fig. 4. Effect of the inhibition of p55 palmitoylation on the association between lipid rafts and the membrane skeleton. Control and Alk-C16-labeled erythrocytes were treated with 1% TX100 at 4 °C for 30 min and ultracentrifuged in sucrose gradients in the absence or in the presence of carbonate, as indicated. Pre-treatment of cells with 2BP does not induce detachment of DRMs from the membrane skeleton (tube 5).

the absence of carbonate (Fig. 4, tube 5), again excluding an involvement of p55 palmitoylation in modulating the linkage between lipid rafts and the membrane skeleton.

4. Discussion

The study of protein lipidation has been classically hampered by technical difficulties connected mostly with the requirement for radio-labeled compounds and time-consuming detection protocols. Recent technical developments have made accessible a flourishing number of new molecular tools and novel chemistries for studying this type of post-translational modification of proteins [19–24]. Among the new techniques, the one based on ω-alkynyl analogues of long-chain fatty acids for studying protein fatty acylation looks promising, especially for investigating reversible cellular processes such as protein S-palmitoylation. The bio-orthogonal ω-alkynyl analogue of palmitic acid is readily incorporated into proteins undergoing active palmitate turnover.

Two fundamentally different methods exist for studying protein S-palmitoylation: one is based on the metabolic utilization of a suitable palmitic acid probe, whether a radioactively-labeled [31] or bio-orthogonal analogue [19], followed by separation and visualization of the labeled protein. The second, exemplified by the so called acyl-biotin-exchange (ABE) method [22,32], consists in a series of passages through which the naturally-occurring palmitic acid residue bound to a given protein via a thioester bond is replaced by a chemical probe that binds to the cysteine's thiol group originally linked to the palmitic acid. While the first method is only useable for proteins that undergo active palmitate turnover in vivo, the second method can also detect stably palmitoylated proteins, although its implementation may be more difficult, due to the several critical steps required for processing the samples [32].

Be as it may, the new techniques may re-open old fields of study that were abandoned because of the intrinsic limitations of the experimental methodology. For instance, erythrocytes were among the first cell types where protein palmitoylation was studied [13,33–35]. It became immediately clear that this cell type, which does not perform protein synthesis, still retains the ability of reversibly palmitoylating a number of proteins, in a way that is apparently regulated physiologically, because different proteins display a different rate of palmitate incorporation and different rates of depalmitoylation, suggesting an independent regulation of the turnover [36]. Ankyrin was the first protein to be shown to undergo reversible palmitoylation in avian and rabbit erythrocytes [13,37] but, since human erythrocyte ankyrin does not appear to be palmitoylated, those results were criticized [26]. Other palmitoylated proteins identified to a reasonable degree of certainty in the human erythrocytes are the Rh polypeptide [35], a subpopulation of spectrin [12], the anion exchanger protein (AE1) band 3 [38,39], although band 3 does not undergo palmitate turnover to any appreciable extent, suggesting that it may be stably palmitoylated, and no biological role for this modification has been demonstrated [40,41], stomatin [15,42], protein 4.2 [14], and p55. Before its identification and characterization [11], p55 was long known to be the protein with the highest palmitate incorporation on metabolic labeling [13,26,33,35], and at a certain time it was proposed that this protein band coincided with the glucose transporter [43], which was subsequently proved wrong [44]. Although the exact disposition and function of p55 in the erythrocyte membrane are still not known [45,46], subsequent characterization of this protein suggested a role for bridging the membrane skeleton to the bilayer by association with GPC [47], in a ternary complex involving protein 4.1, p55 and GPC. p55 would bind, with its PDZ domain, to the cytoplasmic tail of GPC, and, with a central domain of 39 residues, to a region of the 30 kDa domain in protein 4.1 which also contains a binding site for GPC [48,49]. In a recent article, one possible site for palmitoylation of p55 has been mapped at Cys 242 [22]. Interestingly, only one palmitoylation site could be identified in this protein, which was sometimes defined, in the older literature, as “multiply” or “heavily” palmitoylated [11,46,50]. Either the latter were arbitrary definitions, originating from the relatively faster palmitate turnover that this protein undergoes in erythrocytes, or there exist in fact more than one palmitoylated cysteines in erythrocyte p55, owing to the presence in this cell type of different palmitoylating enzyme(s) with respect to the one(s) expressed in the cell type used for the proteome-scale analysis of S-acylated proteins (see below) [22]. It is interesting to note that, if confirmed, the palmitoylated Cys in p55 lies in the middle of the 39-residue domain that binds protein 4.1, suggesting a possible role for palmitoylation in modulating this linkage and thus one of the vertical interactions of the spectrin skeleton with the lipid bilayer.

The enzymatic machinery which maintains the palmitate turnover on a selected subset of membrane proteins is not characterized in erythrocytes. After the discovery in yeast of enzymes that catalyse palmitoylation reactions (PATs, protein acyl transferases), the long standing issue whether palmitoylation is a spontaneous [51] or enzyme-mediated reaction appears to be solved in favour of the latter hypothesis [52]. This research has culminated in the discovery of approximately 23 genes in the human genome that encode S-palmitoyltransferase proteins, belonging to the so called DHHC family (from the consensus sequence Asp-His-His-Cys that the members of this group have in common) [52–54]. Yet, apparently no DHHC protein is expressed in human erythrocytes, based on a survey of the most recent and accurate proteome analysis of this cell type [55–60]. This is despite a report was published about the purification from human erythrocytes of a protein-palmitoyl acyltransferase, whose relation to the now characterized DHHC family remains to be examined [61]. On the other hand, it is still possible that other palmitoyl transferases exist, that do not belong to the DHHC family [52].

Only recently, results have been published that claim that DHHC 17 is the isoform of PAT expressed in erythrocytes [62]. This finding awaits future confirmation. In fact, there is serious concern that those data may be affected by a major flaw, consisting in the contamination by granulocytes of the erythrocyte preparations used by the Authors. Published results of our recent work on erythrocyte DRMs have unquestionably demonstrated that the isolation of lipid rafts as a low-density

fraction in sucrose gradients is only possible with the addition of carbonate to the detergent-containing medium [5]. The lipid raft fraction isolated by the Authors was dislodged from the anchored membrane skeleton thanks to the action of proteases released from contaminating neutrophils. This contamination could also have affected the identification of DHHC 17 as the isoform expressed in erythrocytes. The evidence presented in our work, and in that of Lach and collaborators, on the effects of 2BP, together with our additional demonstration that another inhibitor, cerulenin [63], is also effective in inhibiting MPP1 palmitoylation, would support the enzymatic nature for the mechanism of protein palmitoylation in the human erythrocyte. 2BP has been shown to act as a strong, irreversible inhibitor of DHHCs in vitro [64]. This could explain its higher effectiveness, with respect to cerulenin, in blocking incorporation of Alk-C16 in erythrocytes. As only one enantiomer of 2BP was shown to be effective in inhibiting acyl-CoA synthetase [65] (which was also found expressed in erythrocytes [66]), and probably other fatty-acid utilizing enzymes, and because we used a commercial racemic version of 2BP, the concentration of the effective inhibitor in our experiments was 25 μM. The mechanism of inhibitory action of cerulenin is not understood. Evidence for a covalent modification, by cerulenin, of critical thiols in either the protein acceptor substrates or in the palmitoyl acyl transferase has been reported [63]. However, this proposed mechanism of action is difficult to reconcile with the results presented here, where cerulenin had to be present all along the incubation with Alk-C16 to inhibit its incorporation into proteins. It cannot be ruled out, on the other hand, that chemical instability of cerulenin under the experimental conditions described, was the cause of its loss of effectiveness upon washing. Given the ease with which palmitoylation of erythrocyte proteins can be studied using the click chemistry method, the erythrocyte model could be successfully adopted for screening new inhibitors of palmitoyl-transferase enzymes, and to elucidate their mechanism of action.

We do not know whether there are additional residues of palmitate bound to p55 in a stable manner that could be responsible for performing the anchoring function to lipid rafts. The answer to this question, which can be posed also for other potentially palmitoylated proteins, would entail changing the experimental approach from that based on palmitate turnover to one which directly assays the bound fatty acid, for example after pulling down the proteins of interest with suitable procedures, followed by proteomic/MS analysis.

In summary, we have presented here for the first time the experimental evidence for the feasibility of studying protein palmitoylation in the erythrocyte cell model with the new click chemistry techniques employing bio-orthogonal analogues of fatty acids. The method was implemented to address a specific issue (the role of p55 palmitoylation for anchoring the lipid rafts to the membrane skeleton), but it is open to further investigation on the still unresolved role of protein palmitoylation in erythrocytes. Work is in progress in our laboratory to study p55 and other palmitoylated proteins in erythrocytes of different age. Erythrocytes contain all the machinery necessary for acylating and deacylating selected protein species in the membrane. In fact, not only p55, but also a number of other important integral and peripheral proteins are reversibly palmitoylated. It is unlikely that this is simply the vestige of a machinery that operated in the maturation of erythroid precursors, but the discovery of a possible functional role in the mature erythrocyte awaits future investigations.

Acknowledgements

This work was supported by the “Ministero dell'Università e della Ricerca”, Italy, with PRIN2008 funds to G.M. and by the University of Pavia with FAR funds to G.M. We wish to thank Prof. Sandra L. Hofmann for kindly providing a sample of recombinant PPT1.

References

[1] D.A. Brown, E. London, Structure of detergent-resistant membrane domains: does phase separation occur in biological membranes? Biochem. Biophys. Res. Commun. 240 (1997) 1–7.
[2] D.A. Brown, J.K. Rose, Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface, Cell 68 (1992) 533–544.
[3] C.C. Domingues, A. Ciana, A. Buttafava, B.R. Casadei, C. Balduini, E. de Paula, G. Minetti, Effect of cholesterol depletion and temperature on the isolation of detergent-resistant membranes from human erythrocytes, J. Membr. Biol. 234 (2010) 195–205.
[4] A. Ciana, C. Balduini, G. Minetti, Detergent-resistant membranes in human erythrocytes and their connection to the membrane-skeleton, J. Biosci. 30 (2005) 317–328.
[5] A. Ciana, C. Achilli, C. Balduini, G. Minetti, On the association of lipid rafts to the spectrin skeleton in human erythrocytes, Biochim. Biophys. Acta 1808 (2011) 183–190.
[6] Y. Wang, J.S. Morrow, Identification and characterization of human SLP-2, a novel homologue of stomatin (band 7.2b) present in erythrocytes and other tissues, J. Biol. Chem. 275 (2000) 8062–8071.
[7] L. Lapatsina, J. Brand, K. Poole, O. Daumke, G.R. Lewin, Stomatin-domain proteins, Eur. J. Cell Biol. 91 (2012) 240–245.
[8] L. Snyers, E. Umlauf, R. Prohaska, Oligomeric nature of the integral membrane protein stomatin, J. Biol. Chem. 273 (1998) 17221–17226.
[9] A.F. Sikorski, K. Białkowska, Interaction of spectrins with membrane intrinsic domain, Cell. Mol. Biol. Lett. 1 (1996) 97–104.
[10] X. An, X. Guo, H. Sum, J. Morrow, W. Gratzer, N. Mohandas, Phosphatidylserine binding sites in erythroid spectrin: location and implications for membrane stability, Biochemistry 43 (2004) 310–315.
[11] P. Ruff, D.W. Speicher, A. Husain-Chishti, Molecular identification of a major palmitoylated erythrocyte membrane protein containing the src homology 3 motif, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 6595–6599.
[12] M. Mariani, D. Maretzki, H.U. Lutz, A tightly membrane-associated subpopulation of spectrin is 3H-palmitoylated, J. Biol. Chem. 268 (1993) 12996–13001.
[13] M. Staufenbiel, E. Lazarides, Ankyrin is fatty acid acylated in erythrocytes, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 318–322.
[14] A.K. Das, R. Bhattacharya, M. Kundu, P. Chakrabarti, J. Basu, Human erythrocyte membrane protein 4.2 is palmitoylated, Eur. J. Biochem. 224 (1994) 575–580.
[15] L. Snyers, E. Umlauf, R. Prohaska, Cysteine 29 is the major palmitoylation site on stomatin, FEBS Lett. 449 (1999) 101–104.
[16] J. Greaves, L.H. Chamberlain, Palmitoylation-dependent protein sorting, J. Cell Biol. 176 (2007) 249–254.
[17] I. Levental, D. Lingwood, M. Grzybek, U. Coskun, K. Simons, Palmitoylation regulates raft affinity for the majority of integral raft proteins, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 22050–22054.
[18] I. Levental, M. Grzybek, K. Simons, Greasing their way: lipid modifications determine protein association with membrane rafts, Biochemistry 49 (2010) 6305–6316.
[19] R.N. Hannoush, N. Arenas-Ramirez, Imaging the lipidome: omega-alkynyl fatty acids for detection and cellular visualization of lipid-modified proteins, ACS Chem. Biol. 4 (2009) 581–587.
[20] R.N. Hannoush, J. Sun, The chemical toolbox for monitoring protein fatty acylation and prenylation, Nat. Chem. Biol. 6 (2010) 498–506.
[21] M.C. Yap, M.A. Kostiuk, D.D. Martin, M.A. Perinpanayagam, P.G. Hak, A. Siddam, J.R. Majjigapu, G. Rajaiah, B.O. Keller, J.A. Prescher, P. Wu, C.R. Bertozzi, J.R. Falck, L.G. Berthiaume, Rapid and selective detection of fatty acylated proteins using omega-alkynyl-fatty acids and click chemistry, J. Lipid Res. 51 (2010) 1566–1580.
[22] W. Yang, D. Di Vizio, M. Kirchner, H. Steen, M.R. Freeman, Proteome scale characterization of human S-acylated proteins in lipid raft-enriched and non-raft membranes, Mol. Cell. Proteomics 9 (2010) 54–70.
[23] H.C. Hang, M.E. Linder, Exploring protein lipidation with chemical biology, Chem. Rev. 111 (2011) 6341–6358.
[24] R.N. Hannoush, Profiling cellular myristoylation and palmitoylation using ω-alkynyl fatty acids, Methods Mol. Biol. 800 (2012) 85–94.
[25] E. Beutler, C. West, K.G. Blume, The removal of leukocytes and platelets from whole blood, J. Lab. Clin. Med. 88 (1976) 328–333.
[26] D. Maretzki, M. Mariani, H.U. Lutz, Fatty acid acylation of membrane skeletal proteins in human erythrocytes, FEBS Lett. 259 (1990) 305–310.
[27] J.T. Dodge, C. Mitchell, D.J. Hanahan, The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes, Arch. Biochem. Biophys. 100 (1963) 119–130.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature (London) 227 (1970) 680–685.
[29] J.Y. Lu, J. Hu, S.L. Hofmann, Human recombinant palmitoyl-protein thioesterase-1 (PPT1) for preclinical evaluation of enzyme replacement therapy for infantile neuronal ceroid lipofuscinosis, Mol. Genet. Metab. 99 (2010) 374–378.
[30] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. U. S. A. 76 (1979) 4350–4354.
[31] M.F. Schmidt, Fatty acylation of proteins, Biochim. Biophys. Acta 988 (1989) 411-246.
[32] R.C. Drisdel, W.N. Green, Labeling and quantifying sites of protein palmitoylation, Biotechniques 36 (2004) 276–285.
[33] G.V. Marinetti, K. Cattieu, Tightly (covalently) bound fatty acids in cell membrane proteins, Biochim. Biophys. Acta 685 (1982) 109–116.

[34] B.M. Sefton, J.E. Buss, The covalent modification of eukaryotic proteins with lipid, J. Cell Biol. 104 (1987) 1449–1453.
[35] M.P. de Vetten, P. Agre, The Rh polypeptide is a major fatty acid-acylated erythrocyte membrane protein, J. Biol. Chem. 263 (1988) 18193–18196.
[36] M. Staufenbiel, Fatty acids covalently bound to erythrocyte proteins undergo a differential turnover in vivo, J. Biol. Chem. 263 (1988) 13615–13622.
[37] M. Staufenbiel, Ankyrin-bound fatty acid turns over rapidly at the erythrocyte plasma membrane, Mol. Cell. Biol. 7 (1987) 2981–2984.
[38] E. Zdebska, J. Antoniewicz, J. Koscielak, Characterization and quantitation of fatty acids covalently bound to erythrocyte membrane proteins: anion transporter contains 1 mol of fatty acid thiol ester, Arch. Biochem. Biophys. 273 (1989) 223–229.
[39] K. Okubo, N. Hamasaki, K. Hara, M. Kageura, Palmitoylation of cysteine 69 from the COOH-terminal of band 3 protein in the human erythrocyte membrane, J. Biol. Chem. 266 (1991) 16420–16424.
[40] D. Kang, D. Karbach, H. Passow, Anion transport function of mouse erythroid band 3 protein (AE1) does not require acylation of cysteine residue 861, Biochim. Biophys. Acta 1194 (1994) 341–344.
[41] J.C. Cheung, R.A. Reithmeier, Palmitoylation is not required for trafficking of human anion exchanger 1 to the cell surface, Biochem. J. 378 (2004) 1015–1021.
[42] D. Wang, W.C. Mentzer, T. Cameron, R.M. Johnson, Purification of band 7.2b, a 31-kDa integral phosphoprotein absent in hereditary stomatocytosis, J. Biol. Chem. 266 (1991) 17826–17831.
[43] J.M. May, Thiol-fatty acylation of the glucose transport protein of human erythrocytes, FEBS Lett. 274 (1990) 119–121.
[44] A.K. Das, M. Kundu, P. Chakrabarti, J. Basu, Fatty acylation of a 55 kDa membrane protein of human erythrocytes, Biochim. Biophys. Acta 1108 (1992) 128–132.
[45] N. Mohandas, P. Gascard, L. Peters, J. Barker, What do mouse gene knockouts tell us about the structure and function of the red cell membrane? Baillieres Clin. Haematol. 12 (1999) 605–620.
[46] A.H. Chishti, Function of p55 and its nonerythroid homologues, Curr. Opin. Hematol. 5 (1998) 116–121.
[47] N. Alloisio, N. Dalla Venezia, A. Rana, K. Andrabi, P. Texier, F. Gilsanz, J.P. Cartron, J. Delaunay, A.H. Chishti, Evidence that red blood cell protein p55 may participate in the skeleton-membrane linkage that involves protein 4.1 and glycophorin C, Blood 82 (1993) 1323–1327.
[48] S.M. Marfatia, R.A. Lue, D. Branton, A.H. Chishti, In vitro binding studies suggest a membrane-associated complex between erythroid p55, protein 4.1, and glycophorin C, J. Biol. Chem. 269 (1994) 8631–8634.
[49] W. Nunomura, Y. Takakuwa, M. Parra, J. Conboy, N. Mohandas, Regulation of protein 4.1R, p55, and glycophorin C ternary complex in human erythrocyte membrane, J. Biol. Chem. 275 (2000) 24540–24546.
[50] S.M. Marfatia, J.H. Morais-Cabral, A.C. Kim, O. Byron, A.H. Chishti, The PDZ domain of human erythrocyte p55 mediates its binding to the cytoplasmic carboxyl terminus of glycophorin C. Analysis of the binding interface by in vitro mutagenesis, J. Biol. Chem. 272 (1997) 24191–24197.
[51] L.E. Dietrich, C. Ungermann, On the mechanism of protein palmitoylation, EMBO Rep. 5 (2004) 1053–1057.
[52] R. Tsutsumi, Y. Fukata, M. Fukata, Discovery of protein-palmitoylating enzymes, Pflugers Arch. 456 (2008) 1199–1206.
[53] C. Salaun, J. Greaves, L.H. Chamberlain, The intracellular dynamic of protein palmitoylation, J. Cell Biol. 191 (2010) 1229–1238.
[54] J. Korycka, A. Lach, E. Heger, D.M. Boguslawska, M. Wolny, M. Toporkiewicz, K. Augoff, J. Korzeniewski, A.F. Sikorski, Human DHHC proteins: a spotlight on the hidden player of palmitoylation, Eur. J. Cell Biol. 91 (2012) 107–117.
[55] E.M. Pasini, M. Kirkegaard, P. Mortensen, H.U. Lutz, A.W. Thomas, M. Mann, In-depth analysis of the membrane and cytosolic proteome of red blood cells, Blood 108 (2006) 791–801.
[56] S.R. Goodman, A. Kurdia, L. Ammann, D. Kakhniashvili, O. Daescu, The human red blood cell proteome and interactome, Exp. Biol. Med. (Maywood) 232 (2007) 1391–1408.
[57] F. Roux-Dalvai, A. Gonzalez de Peredo, C. Simó, L. Guerrier, D. Bouyssié, A. Zanella, A. Citterio, O. Burlet-Schiltz, E. Boschetti, P.G. Righetti, B. Monsarrat, Extensive analysis of the cytoplasmic proteome of human erythrocytes using the peptide ligand library technology and advanced mass spectrometry, Mol. Cell. Proteomics 7 (2008) 2254–2269.
[58] A. De Palma, A. Roveri, M. Zaccarin, L. Benazzi, S. Daminelli, G. Pantano, M. Buttarello, F. Ursini, M. Gion, P.L. Mauri, Extraction methods of red blood cell membrane proteins for Multidimensional Protein Identification Technology (MudPIT) analysis, J. Chromatogr. A 1217 (2010) 5328–5336.
[59] E.M. Pasini, H.U. Lutz, M. Mann, A.W. Thomas, Red blood cell (RBC) membrane proteomics—Part I: proteomics and RBC physiology, J. Proteomics 73 (2010) 403–420.
[60] E.M. Pasini, H.U. Lutz, M. Mann, A.W. Thomas, Red blood cell (RBC) membrane proteomics—Part II: comparative proteomics and RBC patho-physiology, J. Proteomics 73 (2010) 421–435.
[61] A.K. Das, B. Dasgupta, R. Bhattacharya, J. Basu, Purification and biochemical characterization of a protein-palmitoyl acyltransferase from human erythrocytes, J. Biol. Chem. 272 (1997) 11021–11025.
[62] A. Lach, M. Grzybek, E. Heger, J. Korycka, M. Wolny, J. Kubiak, A. Kolondra, D.M. Boguslawska, K. Augoff, M. Majkowski, J. Podkalicka, J. Kaczor, A. Stefanko, K. Kuliczkowski, A.F. Sikorski, Palmitoylation of MPP1 (membrane-palmitoylated protein 1)/p55 is crucial for lateral membrane organization in erythroid cells, J. Biol. Chem. 287 (2012) 18974–18984.
[63] M.D. Resh, Use of analogs and inhibitors to study the functional significance of protein palmitoylation, Methods 40 (2006) 191–197.
[64] B.C. Jennings, M.J. Nadolski, Y. Ling, M.B. Baker, M.L. Harrison, R.J. Deschenes, M.E. Linder, 2-Bromopalmitate and 2-(2-hydroxy-5-nitro-benzylidene)-benzo[b] thiophen-3-one inhibit DHHC-mediated palmitoylation in vitro, J. Lipid Res. 50 (2009) 233–242.
[65] N.D. Oakes, A. Kjellstedt, G.B. Forsberg, T. Clementz, G. Camejo, S.M. Furler, E.W. Kraegen, M. Olwegård-Halvarsson, A.B. Jenkins, B. Ljung, Development and initial evaluation of a novel method for assessing tissue-specific plasma free fatty acid utilization in vivo using (R)-2-bromopalmitate tracer, J. Lipid Res. 40 (1999) 1155–1169.
[66] B.C. Davidson, R.C. Cantrill, Erythrocyte membrane acyl:CoA synthetase activity, FEBS Lett. 193 (1985) 69–74.