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Fatty acylation of a 55 kDa membrane protein of human erythrocytes

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The major palmitoylated human erythrocyte membrane protein has an M_r of 55000. It is distinct from the glucose transporter and is not derived from band 3 or ankyrin. It resists salt extraction suggesting a high affinity for the membrane. Pulse chase experiments demonstrate that palmitoylation is a dynamic process, and it may therefore have regulatory significance in membrane protein–protein or protein–lipid interaction. Slower dynamics of palmitoylation in erythrocytes from patients suffering from chronic myelogenous leukemia, which are less stable than normal erythrocytes, strengthen this view.

Introduction

The fatty acylation of proteins, usually through the attachment of myristic acid in amide linkage or palmitic acid in ester or thioester linkage, has received much attention in recent years [1,2]. Post-translational modification of proteins by [^{3}H]palmitate has been studied in mature avian [3], rabbit [4] and human [5] erythrocytes. Whereas the cytoskeletal protein ankyrin has been proposed to be palmitoylated in avian and rabbit erythrocytes; in human erythrocytes, the functional identity of the major palmitoylated protein with M_r 55000, is still not known. Although it has been suggested to be the membrane-spanning glucose transporter [5], our studies point to the contrary. Moreover, the dynamic nature of the acylation observed in the present study, suggests that this kind of modification may serve to modulate the functions of the acylated protein; and the altered fatty acylation kinetics of this protein in erythrocytes from patients suffering from chronic myelogenous leukemia, is reported here.

Materials and Methods

Materials. Bovine serum albumin was purchased from Sigma (Cat. No. A 7030) and was essentially fatty acid free; octyl glucoside, DTT, aprotinin, leupeptin, PMSF, fetal calf serum and DMEM were also purchased from Sigma. [9,10(n)-^{3}H]palmitic acid was purchased from Du Pont/NEN, USA. All other reagents were of analytical grade.

Preparation and labeling of cells. Blood was collected from normal, healthy human volunteers in Dulbecco's modified essential medium (DMEM). Cells were collected by centrifugation and washed with DMEM to remove the buffy coat. Packed erythrocytes (60 μl) were incubated at 37°C with DMEM (4 ml) containing 10 μCi [^{3}H]palmitic acid and 80.4 μg bovine serum albumin (BSA) (fatty acid free). Aliquots were removed at different time intervals, as indicated in the figure legends, washed five times with DMEM containing 5 mg/ml BSA, twice with 155 mM NaCl, 7.5 mM sodium phosphate, 0.1 mM Na_2EDTA (pH 7.5) and lysed with lysis buffer (7.5 mM sodium phosphate, pH 7.5) containing 1 mM Na_2EDTA, 30 μg/ml aprotinin, 1 mM PMSF, 0.1 mM leupeptin. Ghosts were precipitated by centrifugation at 15000 rpm (Sorvall SS-34) for 10 min followed by two washes in lysis buffer, and run on SDS-gels. Gels were treated with chloroform/methanol (2:1, v/v) containing 10 μM HCl for 6 h, to remove palmitic acid that was not protein-bound; immersed in water for one hour and then prepared for fluorography as described by Bonner and Laskey [6] and developed after 96 h.

Preparation of cytoskeletal protein extracts and membrane vesicles. This was done essentially as described by Bennett [7]. Briefly, ghosts were washed once with spectrin extraction buffer (0.2 mM sodium EDTA, pH

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Abbreviations: DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco's modified Eagle's medium; CML, chronic myelogenous leukemia.

7.5) and incubated for 30 min at 37°C in 10 volumes of the extraction buffer. The supernatant obtained after centrifugation contains primarily spectrin and will be referred to as the low ionic strength extract. Spectrin-depleted vesicles were further extracted with KI extraction buffer (1 M KI, 7.5 mM sodium phosphate, 1 mM sodium EDTA, 1 mM DTT (pH 7.5)) for 30 min at 37°C. The resulting supernatant containing ankyrin, bands 4.1, 4.2 and 6, will be referred to as the KI extract, and the vesicles as KI-extracted vesicles.

Labeling of erythrocytes and purification of the glucose transporter. Erythrocytes (15 ml) were labeled as described above, lysed, and the glucose transporter was purified as described by Baldwin et al. [8]. Ghosts were stripped of peripheral proteins by treatment with 15.4 mM NaOH, 0.1 mM DTT, 2 mM Na_2EDTA for 10 min on ice. After centrifugation at $48000 \times g$ for 15 min, the extract was stored. This will be termed the peripheral protein extract. The pellet was suspended in 50 mM Tris-HCl (pH 6.8), centrifuged, the pellet resuspended in the same buffer at 4 mg/ml and stored at −70°C. Subsequently, octyl glucoside was added to the vesicles to give a final concentration of 46 mM in 50 mM Tris-HCl, 2 mM DTT (pH 7.4). After shaking on ice for 20 min, and centrifugation at $13000 \times g$ for 60 min, the supernatant was brought to 25 mM in NaCl, and loaded on a DE-52 column equilibrated in 50 mM Tris-HCl, 25 mM NaCl, 34 mM octyl glucoside, 2 mM DTT (pH 7.4). The flow through was dialyzed for 48 h against 50 mM Tris-HCl, 100 mM NaCl, 1 mM Na_2EDTA (pH 7.4) with changes every 12 h. This preparation of the glucose transporter was stored at −70°C.

Raising of antibodies against ankyrin and band 3. Ankyrin and band 3 were purified from human erythrocytes as described by Bennett [9] and Lodish and Braell [10], respectively. Antibodies to the purified proteins were raised in adult male rabbits.

Collection of blood and labeling of erythrocytes from patients suffering from chronic myelogenous leukemia (CML). Blood was collected from CML patients from the NRS Medical College and Hospital, Calcutta. Erythrocytes were separated from whole blood on Ficoll, and labeled as described above. Complete separation of erythrocytes from whole blood in CML patients necessitated the use of Ficoll due to the high white blood cell count. CML patients studied had not received therapy.

Pulse chase experiments. Erythrocytes (120 µl) were labeled at 37°C with 480 µCi [^{3}H]palmitic acid in 1.2 ml DMEM containing 1 mg BSA, for 10 min. Cells were pelleted and washed seven times with DMEM containing 5 mg/ml BSA. Thereafter, cells were resuspended in 1.2 ml DMEM, added to 250 nmol solid palmitic acid and incubated for 5 min at 37°C. Cells were then pelleted and suspended for the final chase in 24 ml of DMEM supplemented with 1 mg/ml of BSA and 5% fetal calf serum. Cells were incubated at 37°C and 1.5 ml aliquots were removed at time intervals indicated in the figure legends. Ghosts were prepared as described previously, run on SDS gels and subjected to fluorography.

Results and Discussion

Fatty acylation of human erythrocyte membrane proteins

Labeling of human erythrocyte membrane protein with [^{3}H]palmitic acid revealed that the major palmitoylated protein was one of M_r 55000. The present study focuses on this 55 kDa protein.

When cells were labeled with [^{3}H]palmitic acid followed by low ionic strength extraction and extraction with 1 M KI, most of the label associated with the 55 kDa protein remained in the KI-extracted vesicles, and some label was found in the KI extract (Fig. 1). May [5] has suggested that the 55 kDa protein may be the glucose transporter, since the labeled protein was crossreactive with monoclonal antibodies against epitopes of the glucose transporter. However, in our experiments, purification of the glucose transporter showed little or no label associated with this protein. On the other hand, label was found primarily in the peripheral protein extract obtained during the initial steps of glucose transporter purification by extraction with NaOH (Fig. 2). While this work was in progress, a recent paper by Ruff et al. [11] has reported similar findings. A hydropathy plot of the deduced amino acid sequence of the cloned p55 protein [11] has shown a predominance of β sheets with little α-helical content, consistent with the properties of a peripheral mem-

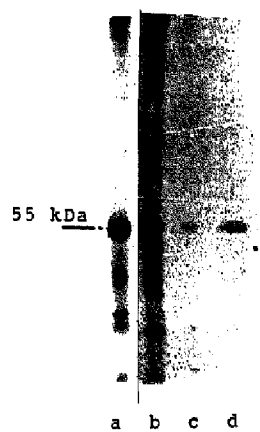

Fig. 1. Erythrocytes were labeled with [^{3}H]palmitic acid as described in Materials and Methods. Lanes a, b, c and d represent intact erythrocyte ghosts, KI-extracted vesicles, low ionic strength extract and KI extract, respectively.

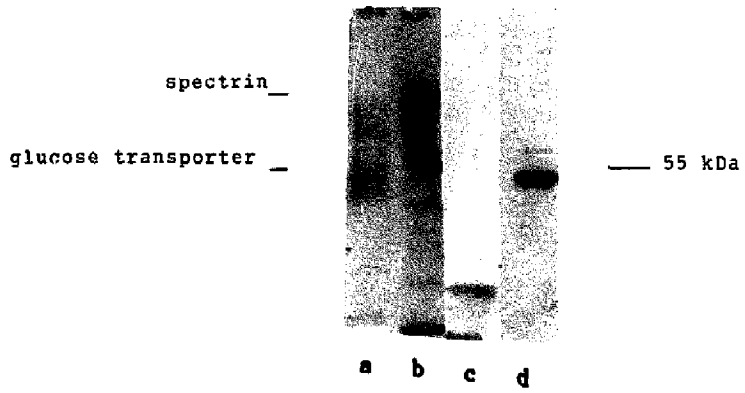

Fig. 2. Extraction of the 55 kDa palmitoylated protein. Lanes a, b represent Coomassie blue-stained gels of the purified glucose transporter and the peripheral extract prepared by treatment with NaOH, respectively. Lanes c and d represent the corresponding fluorograms.

brane protein. The tight association of the 55 kDa protein with the membrane is therefore, probably strengthened by palmitoylation.

Cross-reactivity of the palmitoylated 55 kDa protein with anti-band 3 and anti-ankyrin antibodies

To rule out possibilities that the 55 kDa palmitoylated protein may be derived from band 3, which has been reported to contain covalently bound fatty acid [12]; or ankyrin, which has been reported to be palmitoylated in avian erythrocytes [3], antibodies were raised against purified ankyrin and band 3. These antibodies were checked for specificity in immunoblots using erythrocyte ghosts (Fig. 3). The antibodies against ankyrin and band 3 were found to be specific for ankyrin and band 3 respectively, in immunoblots. When immunoblots were performed with the extract obtained after NaOH treatment, described before, which contained the 55 kDa palmitoylated protein, no bands were visible in the 55 kDa region in immunoblots using the anti-ankyrin and anti-band 3 antibodies. The possibility of the 55 kDa palmitoylated protein being derived from ankyrin or band 3, was therefore ruled out.

Dynamics of palmitoylation of the 55 kDa protein in normal and CML erythrocytes.

Chronic myelogenous leukemia is a hematologic malignancy characterized by excessive growth of myeloid cells and their progenitors [13]. Over the last few years, our laboratory has been interested in studying the causes underlying decreased erythrocyte membrane stability in CML erythrocytes accounting for premature removal of these erythrocytes from the circulation [14–16]. CML erythrocytes are thermally less sensitive than normal erythrocytes [14]. In the present study, it was of interest to note that the time course of fatty acylation

Fig. 3. Specificity of polyclonal antibodies raised against ankyrin and band 3. Lane b, Coomassie blue-stained gel of erythrocyte ghost; lane a, immunoblot of erythrocyte ghost using anti-ankyrin antibodies; lane c, immunoblot of erythrocyte ghost using anti-band 3 antibodies.

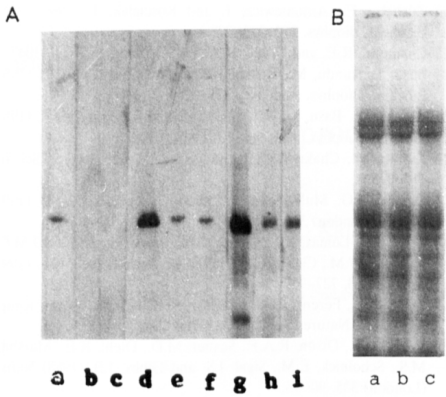

Fig. 4. (A) Labeling of normal and CML erythrocytes with [^{3}H]palmitate. Cells were labeled with [^{3}H]palmitate and 0.5-ml aliquots were removed at 30 min (lanes a–c), 1 h (lanes d–f) and 2 h (lanes g–i), ghosts prepared and analyzed by fluorography. Data obtained for two CML patients (lanes b, e, h and lanes c, f, i) and one normal volunteer (lanes a, d, g) are shown). (B) Coomassie blue-stained gels of normal and CML erythrocyte ghosts. (lane a: normal; lanes b and c: CML). Equal amounts of proteins were loaded on all gels.

of CML erythrocyte membrane 55 kDa protein was significantly different from the normal 55 kDa protein. In preliminary studies it was found that over a period of 2 h the incorporation of [^{3}H]palmitic acid into CML erythrocyte 55 kDa protein was slower than in the case of the normal protein (Fig. 4). The Coomassie blue-stained gels did not reveal any differences in band pattern between normal and CML erythrocytes during this time. To directly demonstrate the turnover characteristics of the protein-bound fatty acid, pulse-chase experiments were performed. As in the case of ankyrin in rabbit erythrocytes [4], a gradual decrease of label associated with the 55 kDa protein was observed over a period of time (Fig. 5). Since Coomassie blue-stained gels showed an identical pattern during this period of time (data not shown), the decrease in protein-bound

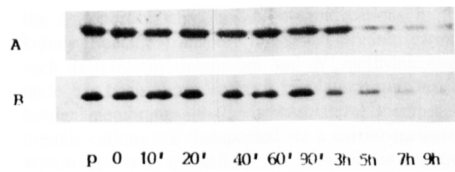

Fig. 5. Pulse chase of [^{3}H]palmitate-labeled 55 kDa protein. Cells were pulse-labeled with [^{3}H]palmitic acid and chased with nonlabeled palmitic acid as described in Materials and Methods. Ghosts were isolated at times indicated (p, pulse) and analyzed by SDS-PAGE and fluorography (exposure 630 h). (A) CML, (B) normal. Coomassie blue-stained gels showed equal intensities of erythrocyte membrane proteins at all the time periods indicated.

fatty acid could not be attributed to degradation of proteins. This obviously suggests that fatty acylation of the 55 kDa protein is accompanied by a deacylation process. It was of interest to observe that the decrease in label of the 55 kDa protein occurred more slowly in CML erythrocytes (Fig. 5A) compared to normal erythrocytes (Fig. 5B). From densitometric scanning of the fluorograms, it was observed that the label associated with the 55 kDa peptide decreased by 60% in 3 h and by 88% in 5 h in normal erythrocytes, whereas for CML erythrocytes the decrease was by 6% in 3 h and by 68% in 5 h. These observations were consistently observed for four normal volunteers and five CML patients. The turnover of the 55 kDa-associated fatty acid was, therefore, slower in CML compared to normal erythrocytes. This is the first report of the slower dynamics of palmitoylation of an erythrocyte membrane protein in pathological red blood cells.

Conclusion

The major palmitoylated protein of the human erythrocyte is a 55 kDa peptide which is a peripheral protein with a tendancy to remain tightly associated with the membrane. Using polyclonal antibodies, at appears that the 55 kDa peptide is not derived from ankyrin or band 3. It has also been conclusively proved that the 55 kDa protein is distinct from the glucose transporter which is a transmembrane protein. It resists extraction with high or low salt concentration, but is extracted with NaOH, a fact which has been corroborated in parallel studies by Ruff et al. [11]. The present observation of the slower turnover of the 55 kDa-associated fatty acid in CML erythrocytes compared to normal erythrocytes, suggests possible implications of changes in fatty acylation kinetics of membrane proteins in relation to altered membrane stability and functions in pathology; since the acylation-deacylation process is likely to be of regulatory significance. This is strengthened by the fact that the predicted amino acid sequence of the 55 kDa peptide reveals the presence of the *src* homology 3 (SH-3) motif [11] found in the non-catalytic domains of oncogene-encoded tyrosine kinases. the SH-3 motif has been found in several proteins likely to play important roles in signal transduction [17–20]. The results obtained in the present study, warrant further investigation into the nature of the ligands interacting with the 55 kDa protein, the role of fatty acylation in these interactions, and their significance in relation to erythrocyte membrane stability and functions.

Acknowledgements

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