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Fatty acid acylation of membrane skeletal proteins in human erythrocytes

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Fatty acid acylation of membrane proteins was studied on human erythrocytes by measuring incorporation of [^{3}H]palmitate at different specific radioactivities. A 55 kDa polypeptide within the band 4.5 region was the main acceptor protein for acylation by fatty acids (palmitate, stearate, oleate), while other polypeptides (80, 65, 48, 30 kDa) incorporated [^{3}H]palmitate slowly, in substoichiometric amounts. Integral membrane proteins were preferentially fatty acid acylated. Skeletal membrane proteins were, however, poorly labeled. Neither purified ankyrin nor band 4.1 protein were fatty acid acylated in human erythrocytes. On the other hand, label associated with high molecular weight skeletal proteins resisted low and high ionic strength extractions, and was extracted selectively by uran along with a small subpopulation of spectrin which was also tightly associated with the membrane.

Protein acylation; Fatty acid; Membrane skeleton; Human erythrocyte; Spectrin; Ankyrin

1. INTRODUCTION

Proteins from eukaryotic cells can be modified by transfer of fatty acid groups (for review see [1]). Myristoylation by protein *N*-myristoyl-CoA-transferase is a cotranslational modification of amino-terminal glycine residues [1]. This capacity is lost during maturation of reticulocytes to erythrocytes. Mature red cells retain evidently an enzyme catalyzed acylation of proteins with long chain fatty acids, which was detected in most cells: it requires ATP and acyl-coenzyme A and involves cysteine and serine (or threonine) residues on acceptor membrane proteins [1]. Marinetti and Cattieu [2] have first reported on incorporation of different types of radiolabeled fatty acids into human erythrocyte membrane proteins. Label was associated with the spectrin/ankyrin region, band 3 protein, and mainly with a polypeptide between bands 4.2 and 5 by analysis on SDS-polyacrylamide gels. Band 3 protein and band 4.1 were also palmitoylated in rat erythrocytes [3]. More recently Staufenbiel and Lazarides reported on palmitoylation of the peripheral membrane protein ankyrin in embryonic chicken and rabbit erythrocytes [4,5]. These authors and others [6] showed evidence for ester- or thioester bonds between fatty acids and membrane proteins by demonstrating their release by hydroxylamine.

These results suggest that posttranslational fatty acid acylation occurs on integral and even some peripheral proteins. Fatty acid acylation of peripheral proteins could condition and/or regulate the association of these proteins with the plasma membrane. Thus, knowledge of the acylated proteins among the cytoskeletal proteins is required to understand membrane architecture and membrane dynamics. So far acylated polypeptides were assigned by studying incorporation of label into electrophoretically spread polypeptides of isolated membranes. In this report we studied the extent of fatty acid incorporation into membranes and its recovery in extracts and purified cytoskeletal proteins.

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2. EXPERIMENTAL PROCEDURES

2.1. *Preparation and labeling of erythrocytes*

Fresh human blood (O, Rh+) was collected in a heparin substitute, liquemin, and was provided by the Swiss Red Cross. Erythrocytes were freed from leucocytes by filtration through cellulose [7] and washed three times with PBS-glucose (10 mM phosphate, 140 mM NaCl, 5 mM KCl, 0.5 mM EDTA, 5 mM glucose, pH 7.4). Red cells were pretreated with fatty acid-free BSA (Sigma A 6003) to lower the endogenous content of free fatty acids in their membrane pools. Cells were treated twice for 15 min at room temperature with 10 vols of incubation buffer (without pyruvate and coenzyme A) containing 0.2% BSA and finally washed once with incubation buffer for fatty acid labeling: 40 mM imidazole, 90 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, 15 mM D-glucose, 0.5 mM EGTA, 30 mM sucrose, 5 mM sodium pyruvate, 5 mM Coenzyme A, 50 mg PMSF/ml and 200 U penicillin G (320 mOsm, pH 7.6). [9,10 (*n*)-^{3}H]palmitic acid (54 Ci/mmol, Amersham) was dissolved in ethanol and diluted with incubation buffer. For labeling 100 μl of BSA-treated red cells were suspended to a hematocrite of 0.1 with 200 μCi [^{3}H]palmitate/ml and incubated for up to 12 h at 37°C. Incubation was stopped by addition of ice-cold PBS-glucose. Extracellular [^{3}H]palmitate was removed by two washes of the cells with buffer containing 0.2% BSA instead of palmitate. In some experiments the following labeled fatty acids were used instead of palmitate: [^{3}H]oleic acid (5 Ci/mmol), [^{3}H]arachidonic acid (202

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Ci/mmol) and [^{14}C]stearic acid (56 mCi/mmol) obtained from Amersham. For preparative purposes 15 ml BSA-treated erythrocytes were labeled with [^{3}H]palmitate (200 μCi/ml) at a hematocrite of 0.3.

2.2 *Extraction of peripheral membrane proteins*

Erythrocyte membranes were prepared from labeled cells in hypotonic phosphate as previously described [8], including one wash of ghosts with isotonic solutions to remove glyceraldehyde-3-phosphate dehydrogenase (band 6) [9]. Low ionic strength-extractable spectrin and actin were obtained according to ref. [10], after washing membranes with extraction buffer in the cold. Ankyrin was extracted with a buffer containing 1 M KCl [9]. This extraction procedure coextracted protein band 4.1. The pellets (KCl-stripped IOV) were washed once with hemolysis buffer containing 1 mM dithiothreitol. The residual amount of spectrin retained in KCl-stripped IOV [11] was selectively extracted by 5 M urea in the presence of 5 mM NaCl, 2.5 mM phosphate (pH 7.4), and is characterized elsewhere (Mariani and Lutz, unpublished and Mariani, PhD Thesis, ETH 1989). Ankyrin and protein band 4.1 were partially purified from the high ionic strength extracts of IOV from [^{3}H]palmitate labeled cells. The two proteins were purified by ion exchange chromatography as previously described [12].

2.3. *Separation and detection of radiolabeled polypeptides*

Analytical scale: erythrocyte membranes were prepared by hemolysis of 100 μl of radiolabeled cells in Eppendorf tubes. Suspensions were centrifuged with an Eppendorf desk centrifuge for 2 min at 4°C. Membranes and extracted fractions were supplemented with 1% SDS and 5 mM *N*-ethylmaleimide prior to freezing. Samples were analyzed by SDS polyacrylamide gel electrophoresis (8% acrylamide) on a slightly modified Neville system [13], in which ankyrin migrates partially within spectrin band 2, essentially as described for the Laemmli system [14]. Samples were reduced for 30 min at 37°C and alkylated with *N*-ethylmaleimide before electrophoresis [15]. Coomassie blue stained gels were soaked with PPO (20%) in DMSO and dried gels exposed to Kodak X-100 film at −70°C [16]. Two identical gels were run in parallel, one was stained and used for fluorography, from the other gel pieces were excised for determining incorporated radioactivities. The gel pieces were treated with 0.75 ml Amersham-NCS-solubilizer/water (9:1, v/v) for 24 h at room temperature. After neutralization with acetic acid the samples were kept overnight before measuring radioactivity by liquid scintillation counting. The amount of incorporated palmitate was calculated from three different specific radioactivities of [^{3}H]palmitate in the incubation mixtures with erythrocytes and from the radioactivity incorporated into separated polypeptide bands from SDS polyacrylamide gels. The counting efficiency for [^{3}H]palmitate was 44%. The protein content in membrane preparations was determined by measuring absorbance at 280 nm in samples solubilized with 1% SDS using BSA as a reference.

3. RESULTS

3.1. *Palmitoylated polypeptides of human erythrocyte membranes*

Human erythrocytes were washed with fatty acid-free BSA to reduce the unknown pool of endogenous free fatty acids before incorporation with [^{3}H]palmitate. Erythrocytes were incubated with [^{3}H]palmitate at 3 specific activities and incorporation of label into membrane proteins was studied by SDS PAGE and fluorography (fig.1). Membranes revealed a set of at least 8–10 labeled polypeptides. A 55 kDa polypeptide within the region of band 4.5 on Coomassie blue stained gels and low molecular weight polypeptides at the tracking dye were the major substrates for palmitoylation. Other heavily labeled polypeptides migrated with apparent molecular masses of 80, 65, 48, 40 and 30 kDa. Minor labeling was found in the high molecular weight poly-

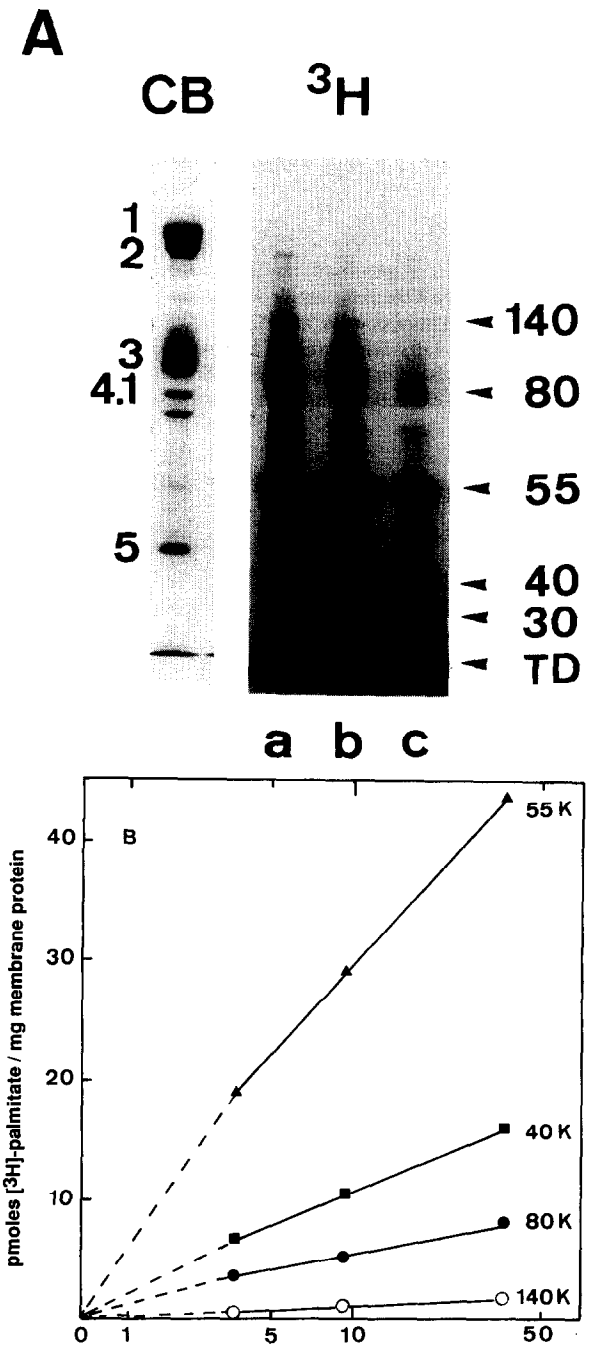

Fig.1. Labeling of membrane polypeptides after incubation of erythrocytes with [^{3}H]palmitic acid at different specific activities: a, 23.8; b, 9.5; c, 2.4 nCi/pmol palmitic acid added. (A) Labeling pattern (4 h incubation) of membrane polypeptides revealed by fluorography (^{3}H, exposed for 4 days) together with the corresponding stained gel (CB). The numbers on the right side give the apparent molecular masses of the polypeptides in kDa. The polypeptides of erythrocyte membranes were numbered 1–5 as in [9–12]. (B) Incorporation of label into several membrane polypeptides as a function of the concentration of [^{3}H]palmitate (12 h incubation).

peptides of the spectrin/ankyrin region and in a 140 kDa polypeptide.

Incorporation of [^{3}H]palmitate was proportional to the concentration of added palmitate at constant total radioactivity. The linear dose–response relationship indicates that the pool of endogenous substrates was successfully reduced by the BSA treatment (fig.1B). Enhanced palmitoylation of membrane proteins with increasing concentrations of palmitate reflects a low affinity of the involved enzyme(s) for the substrate in intact cells.

Most of the incorporated palmitate was cleaved from electrophoretically separated polypeptides when gels were treated with hydroxylamine at pH 7.0 suggesting ester or thioester linkages (not shown). Quantitation of incorporated [^{3}H]palmitate revealed even after an incubation for 12 h a small extent of modification of most polypeptides, except for the 55 kDa protein (table 1). Time courses of [^{3}H]palmitate incorporation display a rapid labeling of the 55 kDa polypeptide (fig.2) and of the proteolipids (PLP) at the tracking dye (not shown), but a retarded labeling of other analyzed polypeptides (80 and 40 kDa) (fig.2). The total amount of incorporated palmitate reached only 1.4 nmol palmitate/ml cells within 12 h at a concentration of 37 μM of added palmitate.

3.2. *Incorporation of different fatty acid substrates*

[^{14}C]Stearic acid which is commercially available at a specific activity of 1/1000 of that of [^{3}H]palmitate was chosen to test whether this saturated fatty acid showed the same specificity of labeling as palmitate. It labeled identical polypeptides. Double labeling experiments further revealed simultaneous incorporation of both fatty acids at a ratio of 3/1 for palmitate into the 55 kDa polypeptide (not shown). This indicates a non-specific use of substrates by the enzymes catalyzing membrane protein acylation.

Unsaturated fatty acids were incorporated into erythrocyte membrane proteins to some extent. A time course for [^{3}H]oleic acid incorporation into membrane polypeptides with about 1/10 of the specific radioactivity of palmitate yielded less labeling but the same subset of radiolabeled proteins. Incorporation of [^{3}H]arachidonic acid was considerably lower and was about 3% of the palmitate incorporation at comparable specific radioactivity (not shown).

Table 1

Quantitation of palmitate incorporation into human erythrocyte membrane proteins

Relative mass of polypeptide (kDa)	[^{3}H]palmitate pmol/mg membrane protein	Palmitate groups incorporated/cell
240/220	2.1	800
140	4.6	1800
80	17.4	6800
65	9.0	3500
55	84.9	33000
48	21.1	8000
40	46.1	18000
30	23.3	9000

Incorporation of palmitate into erythrocyte membrane polypeptides was determined on intact cells incubated for 12 h at 37°C with [^{3}H]palmitate. Incorporated pmol of palmitate were averaged from four determinations for each band at a specific radioactivity of 2.4 nCi/pmol at a concentration of 37 μM of added palmitate (see fig.1). The palmitate groups incorporated per cell were estimated based on the membrane protein content per erythrocyte as given in [8] (6.5×10^{-10} mg). For most polypeptides listed the number of copies per cell is not known. It is known for α-glycophorin 600000 [17], which probably corresponds to the 80 kDa polypeptide and for the Rh antigen that exists in about 60000 copies per cell [18]

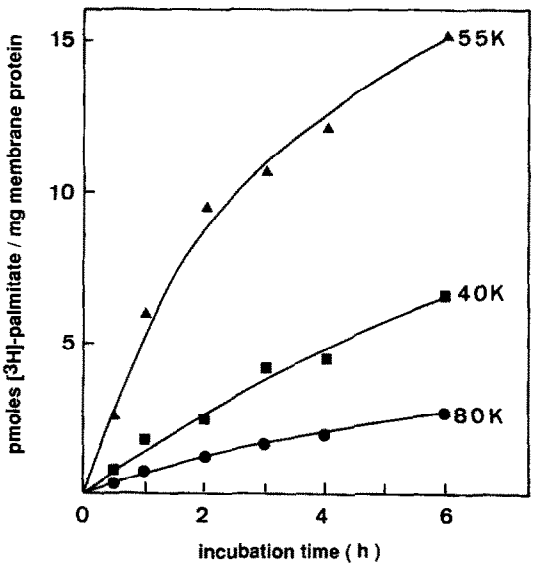

Fig.2. Time course of palmitate incorporation into the three major fatty acid acylated polypeptides of human erythrocyte membranes. Erythrocytes were incubated with 3.4 μM palmitic acid (23.8 nCi/pmol).

3.3. *Palmitate incorporation into extracted skeletal membrane proteins*

Ankyrin and band 4.1 appeared to be fatty acid acylated in membranes of chicken and rabbit erythrocytes [4,5]. Human erythrocytes incorporated very little [^{3}H]palmitate into the spectrin/ankyrin region (fig.1) and the labeled polypeptide with an apparent mass of 80 kDa did not completely coincide with band 4.1. Thus, extracts enriched in spectrin or ankyrin and band 4.1 protein were studied for their content of [^{3}H]palmitate. Low ionic strength extracted spectrin from [^{3}H]palmitoylated erythrocytes was not labeled (fig.3A,a). Some label appeared to be associated with coextracted band 4.1, while most label was localized in a 55 kDa polypeptide and at the tracking dye. Extracts obtained with 1 M KCl from IOV were enriched in ankyrin and band 4.1 (fig.3A,b). They did neither contain labeled ankyrin nor band 4.1. Instead, they revealed label in the 55 kDa

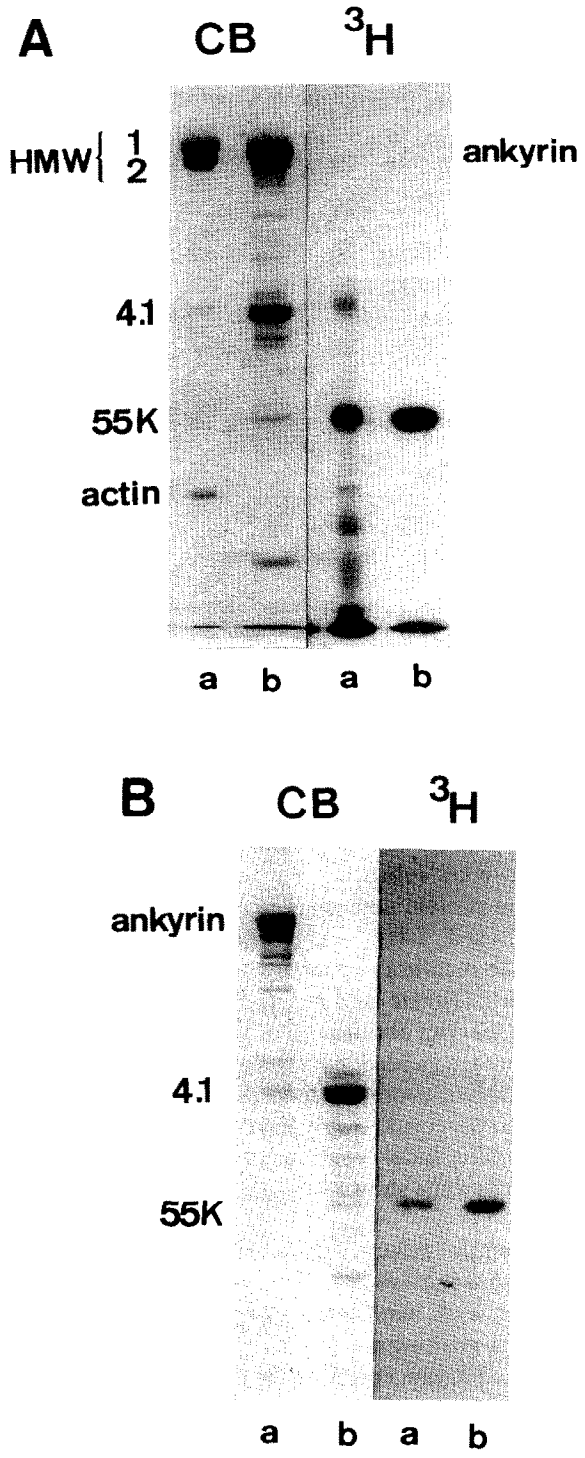

Fig.3. Low ionic strength extractable spectrin, ankyrin, and band 4.1 protein are not fatty acid acylated in human erythrocytes. Intact cells were incubated for 12 h with [^{3}H]palmitate as outlined. Membranes prepared from these cells were extracted with low ionic strength and the remaining vesicles with high ionic strength buffers. (A) Extracts were analysed on SDS PAGE (CB) and by fluorography [^{3}H]. a, low-ionic strength extract from membranes, containing mainly spectrin and actin; b, KCl-extract from IOV, containing primarily ankyrin and band 4.1. (B) Ankyrin and band 4.1 protein were purified from KCl-extracts. Fluorograms of the purified preparations did neither show label comigrating with ankyrin, nor with band 4.1. a, purified ankyrin; b, purified band 4.1 protein.

polypeptide and in low molecular weight polypeptides at the tracking dye. Separation of ankyrin from band 4.1. by ion exchange chromatography confirmed the findings. No label remained associated with either ankyrin or band 4.1 (fig.3B), and label associated with the containing 55 kDa protein was diminished.

Thus, the minor labeling of HMW proteins (fig.1) had to be confined to other proteins. Extraction of membranes by low and high ionic strength buffers yielded KCl-stripped IOV, which yet contained the label (fig.4a). Urea (5 M) extraction of KCl-stripped IOV liberated selectively a tightly membrane-bound spectrin fraction [11] and the minor label associated with HMW polypeptides (fig.4c). Most of the incorporated palmitate label associated with KCl-stripped IOV remained, however, membrane-bound after urea extraction. The urea-extract contained primarily two polypeptides reminiscent of spectrin bands 1 and 2, since they comigrated with spectrin bands 1 and 2, respectively (fig.4c). The second band carried [^{3}H]palmitate (fig.4c).

4. DISCUSSION

Incorporation of radiolabeled fatty acids into specific membrane polypeptides comprised at least 81 000 palmitate groups/cell (table 1) at high palmitic acid concentrations. The majority of proteins that were fatty acid-acylated in human erythrocytes are integral membrane proteins, since inside-out vesicles (IOV) as well as

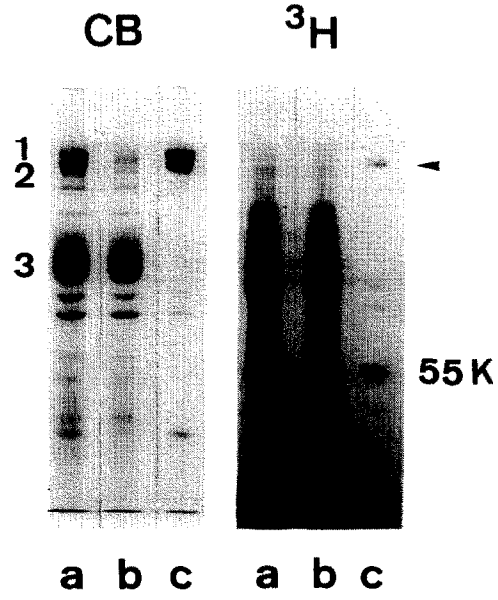

Fig.4. Extraction of ankyrin-depleted KCl-stripped IOV by urea. Vesicles and extracts were analyzed on SDS-PAGE by staining (CB) and by fluorography [^{3}H]. a, KCl-stripped IOV retained a portion of tightly membrane-associated spectrin; b, vesicles obtained after extraction with urea (5 M); c, urea extract containing predominantly polypeptides comigrating with spectrin band 1 and 2 and associated with band 2 labeled palmitate (see arrow).

KCl-stripped IOV retained the entire set of fatty acid acylated polypeptides (fig.4). The main acyl acceptor protein is a 55 kDa polypeptide in the band 4.5 region, which rapidly incorporated [^{3}H]palmitate (fig.2), [^{3}H]oleate and [^{14}C]stearate (not shown) and contained about 40% of the total amount of fatty acids attached to membrane proteins. Incorporation of a variety of substrates into membrane proteins and competition experiments suggest a nonspecific use of fatty acids in this posttranslational acylation/deacylation cycle in human red blood cells. The nature and function of the 55 kDa polypeptide and of its fatty acid modification is unknown.

Recently one of the fatty acid acylated integral proteins was identified as the Rh polypeptide in human erythrocyte membranes [6]. Its apparent molecular weight was 32 kDa, it could be identical with the 30 kDa polypeptide described here which remained bound to vesicles after extraction of peripheral membrane proteins.

No data were reported hitherto about the stoichiometry of palmitate/mol membrane protein. Based on the estimated incorporation of palmitate groups (table 1), and the total number of proteins per cell (where known) most of the labeled proteins undergo a substoichiometric protein modification by fatty acid acylation. In fact, [^{3}H]palmitate incorporation into peripheral membrane proteins of human erythrocytes was minute. The high extent of labeling within the band 4.1 region (fig.1) was not associated with the skeletal protein band 4.1. Extracted and purified band 4.1 protein contained no label (fig.3). Thus, label associated with the 80 kDa region probably resides in α-glycophorin [19], which is known to run as a dimer in the band 4.1 region [20], and migrates predominantly as a dimer, when samples were denatured at 37°C [21].

Ankyrin was clearly not labeled in human erythrocytes. Label associated with high molecular weight polypeptides was neither extracted by low nor by high salt solutions and remained bound to KCl-stripped IOV. This is in contrast to results reported for embryonic chicken and rabbit erythrocytes [4,5], where goblin [22] (chicken) and ankyrin (rabbits) were apparently fatty acid acylated in isolated membranes. The discrepancy could be due to species differences as far as it concerns chicken and human red cells. This is less likely for rabbit and human red cells which both contain ankyrin. In case of rabbit red cells Staufenbiel assigned label associated with high molecular weight proteins on fluorograms of SDS PAGE of membranes to ankyrin on the basis of analogy rather than following extraction and purification of ankyrin [5].

It was interesting that a [^{3}H]palmitate labeled polypeptide in the high molecular weight region resisted low and high ionic strength extractions. Particularly its resistance to high ionic strength extraction differentiates this component from ankyrin. Its selective solubilization by urea suggests that urea, which can perturb hydrophobic interactions [23], extracted a fatty acid acylated and thus tightly membrane associated polypeptide. Its coextraction with spectrin band 1 and 2 from KCl-stripped IOV can have several reasons: released spectrin may originate from right-side-out vesicles (ROV) and/or from a tightly membrane-associated subpopulation of spectrin [11,24]. Since low ionic strength-extracted spectrin was not fatty acid acylated at all, mere entrapment of a fraction of it in ROV and its release by urea would not explain the findings. Thus, the fatty acid label was either confined to a tightly membrane-associated subpopulation of spectrin or, less likely, of ankyrin. If the fatty acid label were confined to spectrin band 2, the 800 copies of labeled polypeptide (table 1) had to originate exclusively from that fraction of spectrin which was not extracted by low ionic strength. Since this fraction comprised at the most 8.5% [25] of the total spectrin, roughly 10% of the it would have carried the label. Conclusive evidence awaits purification and peptide analysis of the extracted protein.

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REFERENCES

[1] Towler, D.A., Gordon, J.I., Adams, S.P. and Glaser, L. (1988) Annu. Rev. Biochem. 57, 69–99.
[2] Marinetti, G.V. and Cattieu, K. (1982) Biochim. Biophys. Acta. 685, 109–116.
[3] Keenan, T.W., Heid, H.W., Stadler, J., Jarasch, E.-D. and Franke, W.W. (1982) Eur. J. Cell Biol. 26, 270–276.
[4] Staufenbiel, M. and Lazarides, E. (1986) Proc. Natl. Acad. Sci. USA 83, 318–322.
[5] Staufenbiel, M. (1988) J. Biol. Chem. 263, 13615–13622.
[6] DeVetten, M.P. and Agre, P. (1988) J. Biol. Chem. 263, 18193–18196.
[7] Beutler, E., West, C. and Blume, K.-G. (1976) J. Lab. Clin. Med. 88, 328–333.
[8] Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1963) Arch. Biochem. Biophys. 100, 119–130.
[9] Tyler, J.M., Hargreaves, W.R. and Branton, D. (1979) Proc. Natl. Acad. Sci. USA 76, 5192–5196.
[10] Bennett, V. and Branton, D. (1977) J. Biol. Chem. 252, 2753–2763.
[11] Lutz, H.U., Maretzki, D. and Mariani, M. In: Cytoskeletal and extracellular proteins. Springer Series in Biophysics. Vol. 3, (Aebi, U. and Engel, J., eds) Springer, Berlin, 1989, p. 328–336.
[12] Bennett, V. and Stenbuck, P.J. (1980) J. Biol. Chem. 255, 2540–2548.
[13] Neville, D.M., jr. (1971) J. Biol. Chem. 246, 6328–6334.
[14] Thompson, S., Rennie, C.M. and Maddy, A.H. (1980) Biochim. Biophys. Acta 600, 756–768.
[15] Fasler, S., Skvaril, F. and Lutz, H.U. (1988) Anal. Biochem. 174, 593–600.
[16] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[17] Merry, A.H., Hodson, C., Thomson, E., Mallinson, G. and Anstee, D.J. (1986) Biochem. J. 233, 93–98.
[18] Agre, P., Saboori, A.M., Asimos, A. and Smith, B.L. (1987) J. Biol. Chem. 262, 17497–17503.

[19] Dolci, E.D. and Palade, G.E. (1985) J. Biol. Chem. 260, 10728–10735.
[20] Furthmayr, H. (1978) J. Supramol. Struct. 9, 79–95.
[21] Lutz, H.U., von Däniken, A., Semenza, G. and Bächi, T. (1979) Biochim. Biophys. Acta 552, 262–280.
[22] Moon, R.T. and Lazarides, E. (1984) J. Cell. Biol. 98, 1899–1904.
[23] Kamoun, P.P. (1988) Trends Biol. Sci. 13, 424–425.
[24] Lutz, H.U., Stringaro-Wipf, G. and Maretzki, D. (1986) J. Cardiovasc. Pharmacol. 8 (Suppl.8), 76–79.
[25] Lux, S.E., John, K.M. and Ukena, T.E. (1978) J. Clin. Invest. 61, 815–827.