Fatty Acids Covalently Bound to Erythrocyte Proteins Undergo a Differential Turnover *in Vivo*

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Recently, covalently bound fatty acids have been identified on a variety of proteins. Many of these acyl proteins are physiologically important, and the lipid modification often appears to be essential for their function. In this investigation mature erythrocytes have been used to study in detail the metabolic behavior of protein-bound fatty acids. Although deficient in protein synthesis, these cells are still able to covalently attach [1-14C]palmitic acid to proteins located at the plasma membrane and its associated cytoskeleton. Linkage analyses demonstrated that the labeled polypeptides contained ester- or thioester-bound fatty acids. The covalent binding of fatty acid was rapidly reversible. Half-lives of the protein-bound fatty acid molecules ranged from less than 30 min to more than 3 h. The results suggested an independent regulation of their lipid turnover. A number of proteins underwent dynamic fatty acid acylation, indicating that palmitylated proteins undergoing fatty acid turnover are not a rare exception.

A considerable number of proteins in eukaryotic cells are known to contain covalently bound long chain fatty acids (for reviews, see Refs. 1 and 2). Based on the fatty acid chain length as well as their linkages to the polypeptides, two classes of acyl proteins can be distinguished (3-5). Several intracellular proteins contain myristic acid which is linked via an amide bond to amino-terminal glycine residues. Myristylation is a step in the biosynthesis of proteins and occurs cotranslationally or very soon after completion of polypeptide synthesis (3-6). Accordingly, studies of p60* showed that the myristic acid once attached remains stably bound to the polypeptide (6). The second class of acyl proteins consists of proteins predominantly containing palmitic acid as well as some other usually minor fatty acids. This class is larger and more diverse, including the surface glycoproteins of several enveloped viruses (7-9), the transferrin receptor (10), the (proto)oncogene product p21* (11, 12), some HLA heavy chains (13), the HLA-associated invariant chain (14), and the membrane cytoskeletal protein ankyrin (15). In most cases palmitic acid appears to be linked through thioester bonds to internal cysteines (13, 16-19), but ester linkages may also occur (20, 21). In contrast to myristic acid modification, palmitylation is a posttranslational event. Viral membrane glycoproteins were shown to acquire palmitic acid during their biogenesis on transportation through the smooth endoplasmic reticulum/Golgi apparatus (22). Correspondingly, palmitylation of cellular polypeptides decreased considerably within 2 h after blocking protein synthesis although a small but significant incorporation of fatty acid always persisted (3, 4, 23). For the transferrin receptor, evidence suggested that it could be fatty acid-labeled many hours after its synthesis and that the fatty acid may undergo a slow turnover (24). Studies of the peripheral membrane protein ankyrin have shown that this protein incorporates fatty acid even in mature erythrocytes (15). In these cells protein synthesis has ceased long ago, and ankyrin is stably integrated into the plasma membrane-associated cytoskeleton. Therefore, its palmitylation is not related to its biogenesis. More detailed analysis indicated that the fatty acid incorporation into ankyrin does not represent a slow and continuing acylation. At least a fraction of the ankyrin-bound fatty acid turns over rapidly, indicating a possible regulatory role of this modification (25).

This study was undertaken to investigate proteins which undergo reversible palmitylation and to compare the metabolic behavior of their fatty acids. Mature erythrocytes provide an excellent system for these studies, since labeling of such proteins is not obscured by incorporation of radioactive fatty acid into newly synthesized polypeptides and the problems of using protein synthesis inhibitors are avoided. Here I show that many palmitylated polypeptides are continuously acylated and deacylated at the erythrocyte plasma membrane. The deacylation was not simply due to a chemical instability of the acyl groups, as they were quite stable after cell lysis. Kinetic analyses showed that the fatty acid turnover rates differed considerably between different polypeptides and, therefore, appear to be regulated independently. Reversible palmitylation is not exceptional among acylated proteins and may serve to modulate their functions.

EXPERIMENTAL PROCEDURES

Preparation and Labeling of Cells—Mature erythrocytes were always freshly isolated from adult rabbits. Blood was collected from the ear vein into Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.1% heparin. The cells were pelleted in a Mini-fuge (Herseaux-Christ, Osterode) for 5 min at 4000 rpm, washed four times in DMEM, and the buffy coat was removed. All manipulations were done at room temperature with the medium at 37°C. To obtain strong labeling, 80 μl of packed erythrocytes were incubated in 6 ml of DMEM supplemented with 2 mg of bovine albumin (fatty acid-free, Sigma) per ml and 1 mCi of [9,10-3H]palmitic acid (specific activity: 20 Ci/mmol).

Additional information: The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

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activity 30 Ci/mmol; E.I. DuPont de Nemours & Co., and Du Pont New England Nuclear). The organic solvent of the fatty acid was removed under vacuum and the fatty acid redisolved in the albumin solution. Cells were incubated in glass tubes on a rocker at 37°C for about 6 h. Then they were pelleted and fractionated as described below. To determine the kinetics of fatty acid incorporation 100 μl of packed cells labeled in 10 ml of DMEM containing 23 μCi of [3H]palmitic acid. Aliquots were removed after different periods of time and processed as described below. Qualitatively similar incorporation kinetics were obtained with smaller or larger amounts of [3H]palmitic acid.

Pulse-Chase Experiments—Erythrocytes (120 μl of packed cells) were labeled at 37°C in 1.2 ml of DMEM containing 720 μCi of [3H]palmitic acid and 1 mg of albumin/ml. In other experiments lower amounts of [3H]palmitic acid and albumin were used with qualitatively similar results. The use of larger amounts resulted in an increasingly inefficient chase. Cells were labeled for 10 min at 37°C and then pelleted in an Eppendorf centrifuge for chase in 24 ml of DMEM supplemented with 5 μg of albumin/ml at 37°C for 5 min. The cells were pelleted in an Eppendorf centrifuge and suspended for the chase in 24 ml of DMEM supplemented with 5 μg of albumin/ml and 5% fetal calf serum. In other experiments 100 μM unlabeled palmitic acid was added. The cells were incubated on a rocker at 37°C. Samples were taken as indicated in the figure legends.

Cell Lysis, SDS-PAGE, and Quantitation of Polypeptide-bound Label—Pelleted cells were lysed immediately in 60–100 volumes of 7.5 mM sodium phosphate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin (Boehringer Mannheim, Mannheim, FRG), 30 μg of aprotinin (Boehringer Mannheim) per ml, pH 7.5. After 2–4 min on ice, ghosts were pelleted in an Eppendorf centrifuge for 1 min. The supernatants containing most of the hemoglobin were removed, made up to 2% SDS, 100 mM Tris, pH 6.8, and 25 mM dithiothreitol, and snap-frozen in liquid nitrogen. Ghosts were dissolved in SDS-sample buffer (125 mM Tris, 1% SDS, 25 mM dithiothreitol, pH 6.8) and also frozen rapidly. After heating (95°C, 2–5 min) and centrifugation, the samples were analyzed by SDS-PAGE (10% acrylamide) (15). The gels were stained with Coomassie Brilliant Blue and destained (lane 1) contained a number of labeled polypeptides (Ref. 15 and Fig. 1, lane 5) of which the 230-kDa protein ankyrin has been identified previously (15). Treatment of the ghost polypeptides with acidic chloroform/methanol failed to remove the radioactivity, indicating a covalent attachment of the fatty acid (Fig. 1, lane 4). Most fatty acid could be removed from the polypeptides by treatment with either hydroxylamine (>70%) or methanolic KOH (>85%) (Fig. 1, lanes 2 and 3). The extent of removal with hydroxylamine varied from 71% to 140% of total label.

RESULTS

Palmitylated Proteins of Rabbit Erythrocytes—Mature rabbit erythrocytes have been shown to metabolically incorporate [3H]palmitic acid into proteins (15). Ghost fractions comprising plasma membrane and associated cytoskeleton (Fig. 1, lane 1) contained a number of labeled polypeptides (Ref. 15 and Fig. 1, lane 5) of which the 230-kDa protein ankyrin has been identified previously (15). Treatment of the ghost polypeptides with acidic chloroform/methanol failed to remove the radioactivity, indicating a covalent attachment of the fatty acid (Fig. 1, lane 4). Most fatty acid could be removed from the polypeptides by treatment with either hydroxylamine (>70%) or methanolic KOH (>85%) (Fig. 1, lanes 2 and 3). The extent of removal with hydroxylamine varied from 71% to 140% of total label.

In Vitro Incubation of Ghosts—Labeled cells (60 μl of packed cells) were washed five times with 800 μl of DMEM containing 5 μg of albumin/ml followed by two washes with 155 mM NaCl, 7.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.5. Cells were lysed, and the resulting ghosts were washed three times in 800 μl of 7.5 mM sodium phosphate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM leupeptin, 30 μg of aprotinin/ml, pH 7.5. Ghosts from about 12 μl of packed cells were suspended in 400 μl of lysis buffer supplemented with 50 μg of Triton X-100, or detergents were omitted and three times more protease inhibitor was used. The ghosts were incubated at 37°C, and samples were removed at the times indicated in Fig. 6. The samples were adjusted to 100 mM Tris, pH 6.8, 1% SDS and analyzed by SDS-PAGE.

FIG. 1. Extraction properties of fatty acid bound to erythrocyte polypeptides. Ghost fractions containing membrane and cytoskeletal proteins were prepared from erythrocytes labeled with [3H]palmitic acid. The polypeptides were separated by SDS-PAGE, stained with Coomassie Brilliant Blue and destained with a solution of hydroxylamine (lane 2), methanolic KOH (lane 3), acidic chloroform/methanol (lane 4), or remained untreated (lane 5). Fluorograms are shown (lanes 2-5). The apparent molecular weights of some polypeptides are given. A, ankyrin.
for the 82-kDa polypeptide to 98% for ankyrin (30-kDa polypeptide: 75%, 53-kDa polypeptide: 92%). While the fatty acids at most polypeptides were better cleaved with methanolic KOH (e.g. 28-kDa polypeptide: 92%, 30-kDa polypeptide: 96%), they were less well removed from the 53-kDa polypeptide and ankyrin (86% and 91%, respectively). The basis for this difference is not known. No polypeptides were lost from the gels during these treatments as demonstrated by Coommassie Brilliant Blue staining as well as by fluorography (data not shown). Under the conditions used, esterified but not amide-linked fatty acids are hydrolyzed indicating that the fatty acid forms ester or thioester linkages to the proteins.

The Kinetics of Fatty Acid Incorporation Differ between Erythrocyte Proteins—Fatty acid labeling of the erythrocyte polypeptides described above is independent of protein synthesis. It could be due to a slow and constant addition of stable acyl groups or, alternatively, a reversible fatty acid acylation. Consequently, the kinetics of incorporation during continuous labeling should either show a constantly increasing label or lead to a steady-state level of 3H-fatty acid on the polypeptides. Freshly isolated rabbit erythrocytes were labeled for various periods with [3H]palmitic acid bound to its physiological carrier albumin. Under these conditions palmitic acid rapidly distributed between albumin and the cells (Fig. 3). At different time points during the continuous labeling, aliquots were removed and the insoluble ghost fractions were analyzed by SDS-PAGE. The fluorogram (Fig. 2A) and the corresponding Coommassie Brilliant Blue-stained gel (Fig. 2B) are shown. Fig. 3 depicts the quantities of radioactivity incorporated into six typical polypeptides.

The amount of labeled fatty acid found on all acyl proteins initially increased although sometimes only after a short lag. This increase did not continue as would have been expected if the labeling represented a constant addition of stable acyl groups; instead, different maxima of protein-bound 3H-fatty acid were reached. This was not due to a limiting amount of available [3H]palmitic acid. At any time about 40% or more of the originally added palmitic acid remained in the medium (data not shown) and the amount of cell-bound free 3H-fatty acid stayed roughly constant after 3–4 h (Fig. 3). Later, during pulse labeling, the polypeptide-bound 3H-fatty acid even decreased and the values approached plateaus below the maxima. Most of this decrease cannot be explained by a decreasing acylation capacity of the cells. The 140- and 53-kDa polypeptides already exhibited dropping values when the 3H-fatty acid bound to ankyrin and to the 30-kDa polypeptide was still increasing. Likewise, if additional [3H]palmitic acid was added after a 20-h pulse labeling the amount of label on all polypeptides increased again (data not shown). That the fatty acid incorporation approached a steady-state level of labeling, therefore, is consistent with a reversible acylation.

The incorporation kinetics suggest that the specific activity of the precursor for acylation (e.g. palmityl-coenzyme A) changed during the labeling period. Due to the addition of [3H]palmitic acid it was high initially but became lower at later time points. The exogenously added [3H]palmitic acid seemingly was diluted during the incubation time by unlabeled fatty acid. This fatty acid might originate from the phospholipids if they undergo fatty acid turnover. During labeling of mature erythrocytes [3H]palmitic acid was incorporated into three major lipids comigrating by TLC analysis with phosphatidylethanolamine, phosphatidylcholine, and sphingomyelin (data not shown). In the continuous pulse labeling experiment described above (Fig. 3) phosphatidylethanolamine initially incorporated slightly less 3H-fatty acid than phosphatidylcholine/sphingomyelin. The amount incorporated reached a maximum at about 9 h. For phosphatidylcholine/sphingomyelin incorporation started to level off earlier. A steady state was reached for all phospholipids at about 11–14 h suggesting that fatty acid incorporation into phospholipids was reversible. At steady state, about 38.6% of the 3H-fatty acid was present in the cellular lipids (fatty acid 6.4%; phosphatidylethanolamine: 25.5%; phosphatidylcholine/sphingomyelin: 20.3%; others: 8.7%), and about 0.5% was protein-bound. These data show that the phospholipids incorporated the vast majority of the labeled fatty acid. A corresponding amount of unlabeled fatty acid was set free during pulse labeling and continuously lowered the specific activity of the free fatty acid pool providing an explanation for the decrease in label at the acyl proteins.

The time required to reach maximum label of the different polypeptides varied from about 4 h (140-kDa polypeptide) to about 11 h (30-kDa polypeptide) (Fig. 3). Furthermore, the
Fig. 3. Quantitation of fatty acid incorporated into polypeptides and phospholipids during continuous labeling. Typical polypeptides were selected from the experiment shown in Fig. 2. Suited fluorograms (see "Experimental Procedures") were scanned and the peak areas integrated. Values were normalized for the protein content of each lane (relative peak area). For analysis of phospholipids and free fatty acid, chloroform/methanol extracted lipids were separated on TLC plates, the radioactivity was determined directly with a thin-layer analyzer, and the phospholipid and fatty acid peaks were integrated. Phosphatidylcholine and sphingomyelin were taken together as they did not give rise to well separated peaks. PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; FA, fatty acid.

**The Fatty Acid Moieties of Erythrocyte Acyl Proteins Show Different Turnover Rates**—To directly demonstrate a turnover of the protein-bound fatty acids pulse-chase experiments were performed. A successful chase required a small precursor pool for protein acylation which could be diluted appropriately. The huge potential pool of labeled palmitic acid in the phospholipids constituted a considerable problem. Nevertheless, preliminary experiments to optimize the conditions indicated that a chase was possible for a limited time. Freshly isolated rabbit erythrocytes were pulse-labeled for 10 min with \(^{3}H\)palmitic acid bound to albumin. Compared to labeling with carrier-free palmitic acid much less free fatty acid associated with the cells under these conditions, although protein labeling was almost as efficient. The cells were then pelleted and washed seven times in medium containing 5 mg of albumin/ml to remove most of the cell-bound free fatty acid (more than 95%). Thereafter, the erythrocytes were added to carrier-free unlabeled palmitic acid about 90% of which associated with the cells during a 5-min incubation. The cells were chased in medium with albumin lacking unlabeled palmitic acid, since it was found that the labeled fatty acid set free from lipids and proteins during the chase was most efficiently removed from the cells under these conditions. This was a slight advantage during the initial time of the chase. Later no differences were found, irrespective of the addition of unlabeled palmitic acid to the chase medium. During the washing steps and the chase, aliquots were removed and the ghost fractions of the cells analyzed by SDS-PAGE (Fig. 4). The radioactivity of typical polypeptides was quantitated (Fig. 5).

After the pulse, during the time needed for washing and loading of the cells with unlabeled palmitic acid (about 60 min), the label on all polypeptides still increased although most of the fatty acid had already been removed with the first
wash. However, at the beginning of the chase a decrease was obvious, and the amount of 140-kDa polypeptide-bound label started to go down even earlier. This directly demonstrated a turnover of the fatty acid molecules on the polypeptides. The fatty acids on most of the polypeptides (28-kDa, 30-kDa, 150-kDa, and ankyrin) had biphasic turnover kinetics. The first phase was characterized by a rapid decline while the label showed a slow, linear decrease during the second phase. For the 53-kDa polypeptide a biphasic fatty acid turnover was less obvious, and for the 140-kDa polypeptide only the first phase was detectable. The decrease in $^{3}$H-fatty acid on both polypeptides also did not follow simple exponential kinetics. The rate of decrease progressively slowed down during the chase. This indicated that the chase was not ideal and became less efficient after longer periods.

This conclusion was supported by analyzing the fatty acid turnover of the phospholipids in the same experiment (Fig. 5). The turnover was biphasic, with an initially more rapid rate (apparent half-lives about 7-9 h). After about 2 h the decrease was linear and very slow. Only a small fraction of the phospholipids exhibited the more rapid turnover, while the larger fraction turned over slowly. This could reflect the existence of two populations of phospholipids. However, given the slow turnover rate during the second phase, the phospholipid-bound fatty acid would not reach an equilibrium during continuous pulse labeling already at about 14 h (Fig. 3).

Therefore, the apparent slow turnover was most likely caused by a reuse of $^{3}$H-fatty acid. The decrease during this second phase of turnover was similar for both proteins and phospholipids (Fig. 5). The loss of label was due to the export from the cells since the labeled fatty acid was found in the medium (data not shown). Therefore, the biphasic kinetics did not reflect the existence of two subpopulations of the 28-, 30-, and 150-kDa polypeptides and ankyrin but reflected an increasing and, after longer times, extensive reuse of labeled fatty acid.

Although the reuse of labeled fatty acid became dominant only after some time, the rates of deacylation decreased from the beginning of the chase. For this reason it was difficult to determine the exact half-lives of the protein-bound acyl groups, but assuming the initial decrease (up to 60 min) approximated the actual turnover an estimation of half-lives is possible: 140-kDa polypeptide, 27 min; 53-kDa polypeptide, 33 min; 28-kDa polypeptide, 42 min; 150-kDa polypeptide, 48 min; ankyrin, 72 min; 30-kDa polypeptide, 3 h 15 min. Although the values should be regarded only as an approximation they demonstrate that the turnover of the fatty acid molecules on the erythrocyte proteins analyzed is quite rapid. This is especially true compared to the stability of the polypeptides. No degradation was observed during the experiments and the proteins presumably are stable even throughout rabbit erythrocyte life (about 60 days) (30). The half-life estimations also show that fatty acids on different polypeptides exhibit different turnover rates. This conclusion is further supported by the variations in percentage of labeled fatty acid left on the polypeptides when the second phase began (Fig. 5). The portion left from maximal label must be larger for those polypeptides with a slower turnover rate. Therefore, the variations observed also indicate differences in the fatty acid turnover rates.

**Chemical Stability of Fatty Acid on Erythrocyte Acyl Proteins**—The experiments described above show that distinct proteins in mature erythrocytes are acylated with fatty acid and deacylated. The deacylation could be a physiologically induced process (enzyme-catalyzed) with a possible regulatory significance. Alternatively, the linkage of fatty acid and protein might be labile resulting in spontaneous hydrolysis. Fatty acid acylation then would serve only to repair this defect. It was, therefore, important to investigate the chemical stability of the protein-bound fatty acid. Erythrocytes were labeled with $[^{3}$H]palmitic acid and subsequently washed in medium containing albumin to remove unincorporated fatty acid. Ghosts were prepared by hypotonic lysis and incubated in vitro at 37°C in a hypotonic buffer (without salt) containing 50 mM glutathione, protease inhibitors, and 1% SDS. This buffer was used to inactivate any deacylating enzyme activities, minimize proteolysis, and provide a reducing environment as this may facilitate the release of thioester-bound fatty acids. Samples were removed after different periods of time and analyzed by SDS-PAGE and fluorography (Fig. 6), and the polypeptide-bound label was quantitated (data not shown). Over a period of 23 h no significant loss of labeled

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**Fig. 4. Metabolic behavior of polypeptide-bound fatty acid in erythrocytes.** Cells were pulse-chase-labeled with $[^{3}$H]palmitic acid and ghosts were isolated at the time indicated (pulse; p; wash; w; wash; see text). The polypeptides were separated by SDS-PAGE and visualized by fluorography, exposure time = 1500 h (A), and Coomassie Brilliant Blue staining (B). Polypeptides are designated as in Fig. 2.
fatty acid from any of the polypeptides analyzed was detectable. Similar results were obtained when SDS was substituted with Triton X-100, detergents were omitted completely, or other reducing agents were used (data not shown). However, palmitoyl-CoA, which is very reactive, was cleaved under these conditions. Although an unidentified problem preventing detection of fatty acid lability cannot be excluded completely, these results strongly suggest that the protein-bound fatty acids resist long incubation at 37°C with about 10 times the physiologic concentration of reducing agent. Most likely, deacylation of the erythrocyte acyl proteins studied here is not due to an inherent chemical instability of their fatty acids but requires catalysis.

**DISCUSSION**

In this study I have examined in some detail the metabolic behavior of fatty acids attached to acyl proteins. Fatty acid acylation was found to be rapidly reversible for quite a number of proteins in erythrocytes. While the polypeptides are stable throughout erythrocyte life, which lasts for about 60 days in rabbits (30), the fatty acid modification is a very dynamic event. A polypeptide undergoes a large number of acylation and deacylation steps during its life time. The half-lives of the fatty acid molecules as determined by pulse-chase experiments ranged from less than 30 min to more than 3 h and, therefore, clearly differed among the proteins within the same cell type. This is evident although the absolute half-life values should be regarded as approximations only. A chase with fatty acid even under optimized conditions has its technical limitations in that fatty acids are extensively reused from the huge phospholipid pool after a certain time. Eventually, a similar turnover of fatty acid on all proteins and in the phospholipids is apparent. The percentage of fatty acid label left on a polypeptide at the beginning of this phase depends on the turnover rate. These values became increasingly smaller for polypeptides with shorter fatty acid half-lives further indicating differences in the acyl group turnover. The conclusion is also supported by results from a complementary approach. During continuous pulse-labeling with fatty acid the time required to reach maximum label depends on the fatty acid turnover rate. This holds true also for the extent of decrease in label observed thereafter, which is due to the equilibration of fatty acid at proteins and in phospholipids where their turnover is much slower. The polypeptides ana-
Erythrocytes were labeled with \[^{14}C\]palmitic acid, ghosts were prepared, and dissolved in a hypotonic buffer containing thionine and 1% SDS. They were incubated in vitro at 37°C and samples were removed at the times indicated and analyzed by SDS-PAGE. A, fluorogram; B, Coomassie Brilliant Blue-stained gel. Designation of polypeptides is as in Fig. 2.

FIG. 6. In vitro decylation of erythrocyte polypeptides. Erythrocytes were labeled with \[^{14}C\]palmitic acid, ghosts were prepared, and dissolved in a hypotonic buffer containing 50 mM glutathione and 1% SDS. They were incubated in vitro at 37°C and samples were removed at the times indicated and analyzed by SDS-PAGE. A, fluorogram; B, Coomassie Brilliant Blue-stained gel. Designation of polypeptides is as in Fig. 2.

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turnover of protein-bound fatty acids