Covalent Modification of Platelet Proteins by Palmitate

By Laszlo Muszbek and Michael Laposata

Covalent attachment of fatty acid to proteins plays an important role in the interaction of proteins with hydrophobic membrane structures. In platelets, the structure of many membrane glycoproteins (GPs) has been examined in detail, but the question of fatty acid acylation of platelet proteins has not been addressed. In this study, we wished to determine (a) whether platelet proteins could be fatty acid acylated; and, if so, (b) whether these modified proteins were present in isolated platelet membranes and cytoskeletal fractions; and (c) if the pattern of fatty acid acylated proteins changed on stimulation of the platelets with the agonist thrombin. We observed that in platelets allowed to incorporate $^3$H-palmitate, a small percentage (1.37%) of radioactivity incorporated into the cells became covalently bound to protein. Selective cleavage of thioester, thioester plus O-ester, and amide-linked $^3$H-fatty acids from proteins, and their subsequent analysis by high-performance liquid chromatography (HPLC) indicated that the greatest part of $^3$H-fatty acid covalently bound to protein was thioester-linked $^3$H-palmitate. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography, at least ten major radiolabeled proteins were detected. Activation of platelets by thrombin greatly increased the quantity of $^3$H-palmitoylated proteins associated with the cytoskeleton. Nearly all radiolabeled proteins were recovered in the membrane fraction, indicating that these proteins are either integral or peripheral membrane proteins or proteins tightly associated to membrane constituents. Components of the GP IIb-IIIa complex were not palmitoylated. Thus, platelet proteins are significantly modified posttranslationally by $^3$H-palmitate, and incorporation of palmitoylated proteins into the cytoskeleton is a prominent component of the platelet response to thrombin stimulation.

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ACYLATION of proteins by fatty acid is a modification which plays an important role in the interaction of proteins with hydrophobic membrane domains.1-3 Point mutations which prevent fatty acid acylation of p60$^c$, Pr65$^c$, and p21$^c$ render these proteins unable to bind to cell membranes.4,5 Three predominant types of covalent linkages exist between fatty acids and proteins: (a) fatty acid linked to a cysteine residue through a thioester bond, (b) fatty acid amide-linked to the N-terminal glycine residue, and (c) a phosphatidylglycerol moiety attached through an intervening glycan structure to the C-terminal amino acid of a protein. The last two types of fatty acid modification of proteins relate to de novo protein synthesis. Acylation of proteins through an amide linkage occurs cotranslationally, and the fatty acid moiety does not turn over independent of the protein.6,7 The addition of glycosylphosphatidylinositol also occurs very early after synthesis of the protein.8,9 In contrast, fatty acid acylation of proteins through thioester linkages occurs posttranslationally, and the fatty acid moiety attached to protein apparently turns over independent of the protein.10-12 Most fatty acid-acylated proteins are membrane components, although some of them, predominantly those bound in amide linkages, are cytoplasmic with only temporary (if any) interaction with the membrane.13,14 Certain cytoskeletal proteins apparently involved in membrane-to-cytoskeleton association (amphiprotic proteins) are fatty acid acylated through thioester or amide linkages.15,20 Platelet membrane GPs are essential for normal hemostatic function of these cells.26,27 Although many studies have extensively addressed the structure of various platelet membrane proteins, fatty acid acylation of platelet proteins has not been investigated. Platelets, because they are anucleate cells with minimal protein synthesis, provide an excellent opportunity for specific investigation of the late posttranslational type of fatty acid modification (i.e., the covalent attachment of fatty acids to the thiol group of peptide bond cysteine residues), without introducing the potentially confounding variable of protein synthesis inhibitors. In addition, platelets are highly dynamic cells, and changes in the fatty acid acylation of proteins during cell activation are readily explored and related to specific phases of the activation process.

In this study, we incubated platelets with $^3$H-palmitate to permit covalent attachment of the radiolabeled fatty acid to proteins. We observed a small but reproducible percentage of the incorporated radiolabel covalently attached to proteins, nearly all of which was identified as thioester-linked $^3$H-palmitate. By SDS-PAGE, multiple radiolabeled proteins were detected by fluorography, and all but one were recovered in the membrane fraction. The membrane GPs IIb and IIIa were not fatty acid acylated. In whole platelets during thrombin-induced activation, an increased amount of fatty acid acylated protein was detected in the Triton-insoluble cytoskeletal fraction. Thus, posttranslational fatty acid acylation of membrane proteins, some of which are incorporated into the cytoskeleton during platelet activation, is an active process in human platelets.

MATERIALS AND METHODS

Materials. [9,10 $^3$H]-Palmitic acid (60 Ci/mmol) was purchased from DuPont-New England Nuclear (Boston). Unlabeled fatty acids (myristate, palmitate, and stearate) and fatty acid

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Blood, Vol 74, No 4 (September), 1989: pp 1339-1347

1339
methyl esters (methyl myristate, methyl palmitate, and methyl stearate) were products of Nu-Check Prep (Elysian, MN). Protein A-Sepharose CL 4B, apprse (grade VII), luciferin-luciferase reagent, human thrombin (3,000 NIH U/mg), molecular weight (mol wt) marker proteins, leupeptin, N-CBZ-glutamyl-tyroine, and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma Chemicals (St. Louis). Sepharose CL-2B was purchased from Pharmacia (Uppsala, Sweden), and protaglandin E1 (PGE1) was purchased from Advanced Magnetics (Cambridge, MA). 4C-Methylated mol wt markers and autoradiographic image enhancer (Amplify) were from Amersham (Arlington Heights, IL). High-performance liquid chromatography (HPLC) grade solvents were used in delipidation of proteins, for alkaline and acid methanolysis, and in extraction of fatty acids. Methanolic HCl (3 mol/L) was purchased from supelco (Bellefonte, PA). Scintillation solution (Ecoscint) was the product of National Diagnostics (Manville, NJ). Mouse monoclonal antibody (MoAb) A5D8 (IgG2a type) directed against the platelet membrane GPIIb/GPIIIa complex (fibronogen receptor) was a gift from Dr. Joel S. Bennett, and its characterization was reported previously.36

Preparation and activation of the 3H-palmitate-labeled platelet suspension. Human blood was anticoagulated with a one sixth volume acid-citrate-dextrose (ACD) and supplemented with 0.18 μmol/L PGE1. Platelet-rich plasma (PRP) was obtained by centrifugation (120 g, 15 minutes, 37°C). Platelets were separated from plasma components and, after radiolabeling, from unincorporated 3H-palmitate by gel filtration or by a washing procedure. Platelets were gel filtered by a modification of the method described by Livne et al.37 PRP further enriched in platelets by centrifugation (2,300 g, 15 minutes, 37°C) was passed through a Sepharose CL-2B column equilibrated with a modified Tyrode's solution containing 15 mmol/L HEPES, 0.3 μmol/L PGE1, 1 U/mL apyrase, and no added Ca2+ (pH 7.4). 3H-Palmitate was dried completely under a stream of nitrogen and solubilized by vortexing for 60 seconds in the modified Tyrode's solution containing 36 mg/mL fatty acid-free BSA. To the platelet suspension, one ninth volume solubilized 3H-palmitate was added (300 μCi/mL final concentration) and the mixture was incubated at 37°C for 45 minutes. Unincorporated radiolabeled fatty acid was removed by a second gel filtration step in which PGE1 was omitted from the elution buffer. Washed platelets were prepared by a modification of the method described by Zucker and Masiello.25 Platelets were isolated from 56 mL ACD blood, and the entire procedure, including centrifugations, was performed at 37°C. Platelets pelleted from PRP at 1,300 g (15 minutes) were resuspended in 10 mL solution A (140 mmol/L NaCl, 2.5 mmol/L KCl, 0.1 mmol/L MgCl2, 10 mmol/L NaHCO3, 0.5 mmol/L Na2HPO4, 1 g/L glucose, 10 mmol/L HEPES, pH 7.4) containing 3.6 mg/mL fatty acid-free BSA, 1 U/mL apyrase, 0.3 μmol/L PGE1, and 300 μCi/mL 3H-palmitate. 3H-Palmitate was dried completely under nitrogen and then solubilized by vortexing for 60 seconds in the above buffer. After incubation for 45 minutes, total radioactivity and platelet counts were determined, and the platelets were then pelleted by centrifugation (1,100 g, 15 minutes) and resuspended in the same volume of solution A containing 1 U/mL apyrase. After an additional 30-minute incubation, the platelet count and incorporated radioactivity were again determined, the platelet count was adjusted to 300,000/μL, and the Ca2+ concentration of the platelet suspension was brought to 1 mmol/L. The uptake of 3H-palmitate by platelets was consistent (13.0% ± 0.5% of added radioactivity, n = 10) and there was no significant difference between the two methods of handling platelets (gel filtration + centrifugation). Radioabeled platelets prepared by either method responded effectively to 0.2 U/mL thrombin when tested for aggregation and ATP release in a lumiaagrégometer (Chrono-log, Havertown, PA), although aggregation of gel-filtered platelets was somewhat diminished as compared with washed platelet suspensions. Because the results of experiments assessing linkage of radiolabeled fatty acid to protein were essentially the same with both types of platelet preparations, only the data obtained with washed radiolabeled platelets were included in this article.

The washed and radiolabeled platelets were either precipitated with 3.5 vol cold acetone (resting platelets) or first aggregated with 0.2 U/mL thrombin for 0.5, 1.5, and five minutes (constant stirring at 1,000 rpm, 37°C) and then treated with the same volume of cold acetone. Acetone-treated resting or activated platelets were kept on ice for 30 minutes, at which time the precipitate was collected by centrifugation (4,500 g, ten minutes, 0°C). The pellet was either washed with another volume of cold acetone, dried, and dissolved in SDS-PAGE sample buffer (one fifth of the volume of the final platelet suspension) or subjected to thorough delipidation (discussed below). In certain experiments, resting radiolabeled platelets without acetone treatment were also pelleted and dissolved directly in sample buffer. Acetone treatment did not remove any radiolabeled platelet protein, since with or without acetone there was no difference in the protein or fluorographic pattern.

Preparation of the cytoskeletal fraction from resting and thrombin-activated radiolabeled platelets. To a radiolabeled platelet suspension activated with 0.2 U/mL thrombin for 0 (resting platelets), 0.5, 1.5, or five minutes, an equal volume of ice-cold lysis buffer (145 mmol/L NaCl, 0.1 mmol/L MgCl2, 15 mmol/L HEPES, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 10 mmol/L EGTA, 800 μg/mL leupeptin, 20 g/L Triton X-100, pH 7.4) was added. After five minutes at 0°C, samples were centrifuged at 10,000 g for five minutes (0°C). The Triton-insoluble pellets were washed with a modified lysis buffer (145 mmol/L NaCl, 0.1 mmol/L MgCl2, 15 mmol/L HEPES, 5 mmol/L EGTA, 0.5 mmol/L PMSF, 400 μg/mL leupeptin, and 10 g/L Triton X-100, pH 7.4) and finally dissolved in SDS-PAGE sample buffer (without reducing agent).

Preparation of the membrane fraction from 3H-palmitate-labeled platelets. The membrane fraction from sonicated radiolabeled platelets was separated on a continuous 1.0 to 3.5 mol/L sorbitol gradient using the method of Hack and Crawford.38 The final membrane pellet was dissolved in SDS-PAGE sample buffer, and the protein content and radioactivity was determined.

Extraction of noncovalently bound lipids. Acetone-precipitated proteins from 1 mL platelet suspension were extracted with 6 mL chloroform/methanol (2:1). The mixture was vortexed for 60 seconds and then incubated at room temperature for 30 minutes. The protein precipitate, pelleted by centrifugation (4,500 g, room temperature, ten minutes), was extracted twice more with 6 mL of the same chloroform/methanol (2:1) solvent, and then sequentially extracted with 6 mL of chloroform/methanol (1:2), 6 mL chloroform/methanol/water (1:1:0.3), and 6 mL acetone. After each extraction step, the samples were left at room temperature for five minutes and centrifuged as described. The final delipidated protein residue was dried completely under nitrogen. It contained no radioactivity removable by lipid-extracting solvents, and, as determined in separate experiments, the recovery of protein after multiple extractions was consistently >90%. Certain samples were dissolved in SDS-PAGE sample buffer (without β-mercaptoethanol), and the protein content and radioactivity were determined. Other samples were used for identification and quantitation of fatty acids bound to proteins.

Release of protein-bound fatty acids and identification of released fatty acids by HPLC. The dripped delipidated protein preparations were first subjected to alkaline methanolysis to disrupt O-ester and S-ester linkages. In this process, the delipidated protein was treated with 2 mL 0.2 mol/L potassium hydroxide (KOH) in
methanol for 30 minutes at 37°C and then centrifuged (4,500 g, 10 minutes, room temperature). The supernatant was removed, acidified with 2 mol/L HCl, and then extracted three times with 1 mL hexane. Approximately 95% of the radioactivity released by methanolic KOH was recovered in the combined hexane extract, which was then dried completely under nitrogen and resuspended in 0.2 mL methanol. Of the 0.2-mL vol, 50 μL was removed for determination of extracted radioactivity before HPLC. To the remainder of the sample, 150 μg of each unlabeled fatty acid (myristate, palmitate, stearate) and corresponding unlabeled fatty acid methyl ester was added as standards. Separation of the three fatty acids and three fatty acid methyl esters as six separate peaks was achieved by reversed-phase HPLC using two interconnected Microsorb C18 columns (4.6 mm x 10 cm, Rainin, Woburn, MA). The fatty acids were eluted with 66% vol/vol acetonitrile/H2O at a flow rate of 1 mL/min. Fractions of 1 mL were collected and counted in 4 mL Ecoscint scintillation mixture. The absorbance profile of eluted compounds was monitored at 205 nm, and retention times of radioactive alkaline methanolysis products were determined by coelution with unlabeled fatty acid and fatty acid methyl ester standards.

The protein pellet that remained after alkaline methanolysis and removal of alkaline methanol was washed with 2 mL methanol, and then subjected to acid methanolysis, to release amide-bound fatty acid. The samples were treated with 2 mL methanolic HCl (3 mol/L) at 110°C for 24 hours under nitrogen. The reaction solutions were extracted three times with 1.5 mL hexane, and the combined hexane fractions were processed for HPLC as described for alkaline methanolysis products. The nonextractable radioactivity in the methanolic phase was also determined.

Isolation of platelet GPIIb/IIIa complex by immunoadsorption. Typically, 5 mL final 3H-palmitate–labeled platelet suspension (1.5 x 10^9 platelets) was used for isolation of the GPIIb/IIa complex. Nonactivated platelets or platelets activated with 0.2 U/mL thrombin for five minutes were pelleted and extracted by occasional vortexing in 1 mL solubilizing buffer (2 mmol/L Tris, 10 g/L Triton X-100, 1 g/L SDS, 1 mmol/L PMSF, 1 mmol/L carbamamepine (CBZ)-glutamyl-tyrosine, pH 7.5) for 30 minutes at 4°C. After centrifugation, the supernatant was incubated with 20 μL nonimmune rabbit serum on a rotary mixer for 15 minutes at 4°C. The supernatant was then added to 0.15 mL Protein A-Sepharose for 15 minutes, at which time the Sepharose was removed by centrifugation and 2 mL immunoprecipitation buffer (2 mmol/L Tris, 150 mmol/L NaCl, 1 g/L Triton X-100, 1 g/L SDS, 1 mmol/L PMSF, 1 mmol/L CBZ-glutamyl-tyrosine), and 100 μg A2A9 MoAb was added for one hour. The antigen–antibody complex was adsorbed by 0.2 mL Protein A-Sepharose for one hour. The Sepharose was pelleted, and the pellet was washed five times with immunoprecipitation buffer before being dissolved in 0.2 mL 2 x concentrated SDS-PAGE sample buffer. Unreduced and reduced samples (100 μL) were analyzed by SDS-PAGE and fluorography. On the Coomassie-stained gels, two highly intense bands appeared, representing GPIIb and GPIIIa without any contaminating platelet protein. In control experiments, radiolabeled platelet extracts in solubilizing buffer were incubated for the same time under the same conditions as in the immunoprecipitation studies. The fluorograms obtained from these samples were the same as those derived from freshly solubilized radiolabeled platelets. These control experiments demonstrated that the radiolabeled fatty acid that had been covalently linked to protein was not removed under the experimental conditions used for immunoprecipitation.

The samples in the solubilizing buffer for SDS-PAGE were either treated (reduced samples) or not treated (nonreduced samples) with 50 g/L β-mercaptoethanol. SDS-PAGE was performed on a 5% to 20% gradient gel according to the method of Laemmlii, with the exception that after electrophoresis the gels were first fixed in a 50% ethanol/5% acetic acid solution for 45 minutes, stained with two changes of 40 mg/L Coomassie blue in 20% ethanol/5% acetic acid overnight, and destained in 20% ethanol/5% acetic acid. This modification of staining and destaining techniques resulted in a more intense staining of lower mol wt proteins and a more complete destaining of the gel than the original procedure. After fixation, selected gels were washed three times for ten minutes (each wash) in distilled H2O and treated with 1 mol/L hydroxylamine, pH 7.0 (to disrupt S-ester linkages), or with 1 mol/L Tris-HCl, pH 7.0 (as a control) for six hours at room temperature. Hydroxylamine or Tris was removed by rinsing the gel three times for ten minutes (each wash) in distilled H2O. The gels were then stained and destained as described. Destained gels were soaked in Amplify solution supplemented with 1% glycerol for one hour, briefly rinsed with distilled H2O, and dried at 60°C. The dried gels were exposed to Kodak X-OMAT AR film (Rochester, NY) at ~70°C for 14 days. As determined in separate experiments, staining and destaining did not decrease the intensity of radiolabeled bands on the fluorograms to any significant extent; treatment with Tris-HCl or hydroxylamine did not result in any change in the protein patterns on the gel, and Tris-HCl had no effect on the fluorograms. Reduced and unreduced samples were either run on separate gels prepared from the same acrylamide gradient or if on the same gel, they were separated from each other by at least eight empty lanes to prevent even minimal contamination of unreduced samples with β-mercaptoethanol from wells with reduced samples.

Other methods. Protein concentration was measured with a BCA protein assay kit (Pierce). Radioactivity was counted in a Beckman (Fullerton, CA) LS 6800 liquid scintillation counter.

RESULTS

Table 1 shows that a substantial amount of 3H-palmitate was incorporated into platelets during the 45-minute labeling period, and ~99% of it was extractable in organic solvents. This indicates that, as expected, most of the fatty acid incorporated into the platelet was esterified into phospholipids and neutral lipids or remained as free fatty acid, and only a relatively small portion (1.37%) became covalently bound to proteins. From the experiments in which fatty acid was hydrolyzed from protein (Table 2), we determined that essentially all radioactivity covalently bound to protein could be recovered as fatty acid. Thus, even if a portion of radiolabeled palmitate had been catabolized by β-oxidation and converted into 3H-labeled amino acids, the extremely low level of protein synthesis in platelets prevented the possible incorporation of significant amounts of fatty acid radioactivity into proteins.

Table 1. Incorporation and Covalent Attachment of 3H-Palmitate to Proteins in Platelets

<table>
<thead>
<tr>
<th>Protein</th>
<th>Radioactivity (dpm/ng)</th>
<th>Incorporation into Cells (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total dpm</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incorporated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>into cells</td>
<td>7</td>
<td>146 ± 7</td>
</tr>
<tr>
<td>Covalently bound</td>
<td>5</td>
<td>2.02 ± 0.14</td>
</tr>
</tbody>
</table>

Values are mean ± SEM.

*Radioactivity incorporated into cells was arbitrarily set at 100%.
Table 2. Analysis of Fatty Acids Covalently Bound to Proteins in 3H-Palmitate-Labeled Platelets

<table>
<thead>
<tr>
<th>Treatment/Released Fatty Acid</th>
<th>Total Radioactivity Released by Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline methanolysis</td>
<td>95.1 ± 2.0</td>
</tr>
<tr>
<td>Myristate</td>
<td>1.3</td>
</tr>
<tr>
<td>Palmitate</td>
<td>92.7</td>
</tr>
<tr>
<td>Stearate</td>
<td>1.1</td>
</tr>
<tr>
<td>Acid methanolysis</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td>Myristate</td>
<td>0</td>
</tr>
<tr>
<td>Palmitate</td>
<td>2.1</td>
</tr>
<tr>
<td>Stearate</td>
<td>0</td>
</tr>
<tr>
<td>Nonreleasable</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Total</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Values are mean ± SEM of three experiments.

In our experiments with platelets, >95% of protein-linked radiolabeled fatty acid was released by alkaline methanolysis and, as determined by fluorography, by neutral hydroxylamine treatment as well. Acid methanolysis after alkaline methanolysis resulted in release of only a small amount (2.1%) of additional radiolabeled material (Table 2). Alkaline methanolysis cleaves all types of ester linkages but leaves amide bonds intact. Hydroxylamine at neutral or slightly acidic pH disrupts only thioester linkages (and perhaps very labile O-ester linkages) but, as was verified with model compounds and with the glycosyl phosphatidylinositol membrane anchor, does not cleave the usual hydroxyester linkage. Thus, our results indicate that in 3H-palmitate-labeled platelets, the protein-bound radiolabeled fatty acid was almost exclusively thioester-linked palmitate.

Reverse-phase HPLC clearly separated palmitic acid and methyl palmitate from myristic acid, stearic acid, and their corresponding methyl esters, thereby permitting a clear identification of radiolabeled fatty acids released by alkaline or acid methanolysis (Fig 1). Although limited β-oxidation and chain elongation of fatty acids occurs in platelets, resulting in transformation of a portion of incorporated 3H-palmitate into 3H-myristate and 3H-stearate, respectively, during the incubation period, 3H-palmitate was identified as the single major radiolabeled fatty acid covalently bound to protein. 3H-myristate and 3H-stearate were linked to protein in negligible amounts (Fig 1, Table 2).

To determine the mol wt of the individual fatty acid acylated platelet proteins, whole platelets (WPs) were solubilized in SDS-PAGE sample buffer and the proteins were analyzed by SDS-PAGE and fluorography. Lanes r and WP in Fig 2A and B show resting platelets on Coomassie-stained gels and the respective fluorograms, both with reduced and unreduced platelet samples. As shown in Fig 2B, a surprisingly large number of platelet proteins were covalently labeled with 3H-palmitate. In WP samples reduced with β-mercaptoethanol (Fig 2B, WP), the mol wt of major radiolabeled polypeptides was as follows: 134 Kd, a triplet between 87 and 96 Kd, 61 Kd, a doublet in the vicinity of 41 Kd (probably moved from its original position by the very large unlabeled actin band just above it), 34 Kd, a triplet near 29 Kd, 23 Kd (the most intense band on the fluorogram), and 18 Kd. Most of the corresponding polypeptides in unreduced samples migrated somewhat faster (ie, slightly lower mol wt values were obtained when estimated with reduced mol wt marker proteins). The major differences between reduced and unreduced samples were that in the unreduced samples a new mol wt 154 Kd band appeared, and the low mol wt band at 18 Kd became more prominent than its counterpart in the reduced sample.

We next determined that during platelet activation, although there was apparently no palmitoylation of additional proteins or removal of 3H-fatty acid from protein, palmitoylated proteins were incorporated into platelet cytoskeleton. Radiolabeled platelets were activated by thrombin for 0.5, 1.5, or five minutes and analyzed by SDS-PAGE and fluorography. In separate experiments, we showed that ATP release occurred within one minute and platelet aggregation proceeded to nearly full extent in two minutes. As thrombin-induced activation proceeded, the amount of protein assembled into the cytoskeleton increased sharply, from 2.6% of the total platelet protein mass to 22%, 25%, and 27% in the cytoskeleton of platelets activated for 0.5, 1.5, and five minutes with thrombin. Figure 2A shows the protein composition of cytoskeleton prepared from an equal number of
activated platelets, no radiolabel was observed on the fluorogram of resting cytoskeleton (Fig 2B lane r, CSK). However, some \(^{3}H\)-palmitoylated proteins appeared on the fluorogram of cytoskeletons obtained after addition of thrombin (Fig 2B, CSK lanes t0.5, t5). Bands of \(^{3}H\)-palmitoylated proteins of specific mol wt incorporated into the platelet cytoskeleton at 0.5 minutes of thrombin activation increased in intensity as thrombin activation proceeded. In the unactivated platelets, because of the low protein content, the amount of radiolabeled proteins was below the limit of detection (described below). Among the \(^{3}H\)-palmitoylated platelet proteins, those at 134 Kd, 91 Kd, and 40 Kd (Fig 2, reduced samples, lane CSK t0.5; Fig 3, reduced lane t,F) were largely recovered in the cytoskeleton, as was a 154-Kd band detectable only in unreduced samples. The 61 Kd and 18 Kd bands were significantly better detected in unreduced cytoskeleton samples. The 22-Kd radiolabeled polypeptide, the most prominent band in whole platelet samples, appeared in the cytoskeleton in an insignificant amount and became detectable only at 1.5 (not shown) and five minutes after thrombin activation. Evidently none of the major cytoskeletal proteins were \(^{3}H\)-palmitoylated.

To discover if there were palmitoylated proteins in the resting cytoskeleton and how they might relate to the proteins in the cytoskeleton of thrombin activated platelets, we compared the same amount of cytoskeletal protein from resting and thrombin-activated platelets by SDS-PAGE and fluorography (Fig 3). To obtain the same amount of cytoskeletal protein from resting platelets as from thrombin-activated cells, a tenfold higher number of unactivated platelets than activated platelets was needed for the preparation. The Coomassie-staining patterns of the two cytoskeletons (lanes C, r, and t) were similar. However, differences were noted at mol wt ranges of 45 to 66 Kd and <29 Kd. On the fluorograms (unreduced lanes F, r, and t), the most obvious differences were the nearly complete absence of the 134-Kd and 23-Kd bands and the decreased intensity of the 18 Kd band in the cytoskeleton of resting platelets.

\(\beta\)-Mercaptoethanol present in reduced samples can influence the electrophoretic patterns in two different ways: (a) by disrupting interchain disulfide bonds and causing disassembly of disulfide-linked dimeric or multimeric protein structures into separate subunits, and (b) by altering the electrophoretic mobility of single polypeptide chains. The mobility of several polypeptides, including actin (42 Kd), the most abundant platelet protein, is slightly enhanced when \(\beta\)-mercaptoethanol is omitted from the sample buffer. Paradoxically, by cleaving intrachain disulfide bonds and consequently changing the compactness of the protein structure, \(\beta\)-mercaptoethanol treatment results in an increased mobility of certain monomeric proteins with multiple disulfide linkages. \(\beta\)-mercaptoethanol can also alter the fluorographic pattern by releasing a portion of radiolabeled fatty acids linked to proteins by thioester bonds. This is reflected in the decreased intensity of the fluorograms obtained with reduced samples.

The bands on the fluorograms represent radiolabeled fatty acids covalently bound to protein. The possibility that they represent noncovalently associated fatty acids or radiolabel incorporated into the polypeptide backbone is ruled out by...
several lines of evidence. First, thoroughly delipidated samples produced exactly the same fluorographic patterns (not shown) as platelets treated only with acetone or dissolved directly in SDS-PAGE buffer. Noncovalently associated fatty acids are separated from proteins in the sample buffer with high SDS concentration and migrate well in front of the polypeptide with the lowest mol wt (detectable as an intense diffuse black spot at the very bottom of the fluorogram and not shown in Figs 1 through 4). Second, as previously shown, the protein-linked radiolabel was completely released by alkaline and acid methanolysis and recovered as 3H-fatty acid. Finally, after hydroxylamine treatment of fixed gels at neutral pH, no radioactivity could be detected by fluorography (F). Since an equal amount of cytoskeletal protein (32 µg) was applied to all lanes, the fluorogram provides both a reliable quantitative and qualitative comparison of the 3H-fatty acid-acylated proteins incorporated into the cytoskeleton in resting and thrombin-activated platelets.

DISCUSSION

Structural studies involving posttranslational modification of proteins with fatty acids have shown, in general, that a few selected fatty acids (myristate, palmitate, and stearate) bind in specific linkages (amide, O-ester, or thioester) to a limited number of cellular proteins. The functional significance of covalent fatty acid binding to protein has lagged behind the structural studies of this phenomenon. It has been shown for two myristoylated proteins, p60GPIIb and Pr65GPIIa, and 1 palmitoylated protein, p21GPIIb, that fatty acid is necessary to anchor
which prevents myristoylation abolishes not only its membrane binding ability, but also its transforming activity. For the dozens of other proteins modified with myristate and palmitate, several possible roles for the fatty acid have been proposed without experimental support. In addition to anchoring a protein to a lipid bilayer, a fatty acid moiety could modify the local conformation of a polypeptide and establish new protein-protein interactions. Such interactions may be within or outside the bilayer. For example, a fatty acid-acylated cytoplasmic polypeptide tail segment may act as a "receptor" for certain cytoplasmic proteins. In this regard, Fox showed that the GPIb/IX complex in the platelet membrane functions as the attachment site for the underlying cytoskeleton.32,43

The results of the present investigation show not only that these proteins to cellular membranes.4,5 A mutation of p60 
which prevents myristoylation abolishes not only its membrane binding ability, but also its transforming activity.7 For the dozens of other proteins modified with myristate and palmitate, several possible roles for the fatty acid have been proposed without experimental support. In addition to anchoring a protein to a lipid bilayer, a fatty acid moiety could modify the local conformation of a polypeptide and establish new protein-protein interactions. Such interactions may be within or outside the bilayer. For example, a fatty acid-acylated cytoplasmic polypeptide tail segment may act as a "receptor" for certain cytoplasmic proteins. In this regard, Fox showed that the GPIb/IX complex in the platelet membrane functions as the attachment site for the underlying cytoskeleton.32,43

The results of the present investigation show not only that some platelet proteins contain thioester-linked palmitate, but also that a prominent component of platelet activation by thrombin is the incorporation of palmitate-modified proteins into whole cell cytoskeleton. Since covalently bound palmitate turns over independent of protein synthesis, it was expected that during the metabolic changes that occur with thrombin-induced platelet activation unmodified platelet proteins would become palmitoylated or the palmitate label would be removed from palmitate-modified proteins. However, with few exceptions, the prominent radiolabeled bands in the cytoskeleton of stimulated platelets appear to represent corresponding bands in unstimulated cells, detectable only when large numbers of 3H-palmitate-labeled platelets are used to prepare cytoskeleton. Thus, thrombin stimulation of platelets increases incorporation of specific palmitoylated proteins already present in the cytoskeleton in low amounts. In an earlier study,44 we showed that platelet lipids are noncovalently associated with the contractile-cytoskeletal apparatus, and recent reports31,32,45 confirm this finding.

In the present experiments, we demonstrated that in addition to these noncovalent associations, the fatty acid palmitate is also covalently linked to proteins of the cytoskeleton. Livne et al recently demonstrated in rabbit and human platelets prelabeled with 3H-palmitate (5 μCi/mL, 15 minutes, room temperature) that stimulation with thrombin or ADP induced an aggregation-related association of 3H radiolabel with the platelet cytoskeleton.31 In light of our present findings, Livne's observation may represent a small amount of incorporation of palmitate-modified proteins into platelet cytoskeleton along with a much larger amount of noncovalently associated palmitate. Palmitoylated proteins in the cytoskeleton could be specific cytoskeletal-contractile proteins, linkage proteins involved in association of cytoskeletal filaments to membrane domains, or membrane proteins attached through linkage complexes to the contractile filaments.

The results of our experiments with platelet membranes apparently exclude the possibility that GPIIb and IIIa (the fibrinogen receptor30,46) are palmitoylated proteins. These proteins showed characteristic differences in their electrophoretic mobilities under reducing and nonreducing conditions47 and are readily apparent on Coomassie-stained gels after SDS-PAGE of the membrane fraction. On the fluorograms from our platelet membrane experiments, no polypeptide was detected that corresponded to the mol wt of GPIIb or IIIa and demonstrates the electrophoretic behavior of these two GPs on reduction with ß-mercaptoethanol. Furthermore, when the GPIIb/IIIa complex was immunoprecipitated from resting and thrombin-activated 3H-palmitate-labeled platelets and the components were separated by SDS-PAGE, no radiolabeled bands were detected by fluorography. The results of this initial investigation into fatty acid acylation of platelet proteins will, we hope, lead to identification of the individual palmitoylated proteins in platelet membranes.
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Covalent modification of platelet proteins by palmitate

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