Purification and Biochemical Characterization of a Protein-palmitoyl Acyltransferase from Human Erythrocytes*

Amit K. Das‡, Biplab Dasgupta, Raja Bhattacharya§, and Joyoti Basu¶

From the Department of Chemistry, Bose Institute, 93/1 Acharya Prafulla Chandra Road, Calcutta 700 009, India

Protein palmitoylation involves the post-translational attachment of palmitate in thioester linkage to cysteine residues of proteins. The labile nature of the thioester linkage makes possible the palmitoylation-depalmitoylation cycles that have emerged in recent times as additions to the repertoire of cellular control mechanisms. However, detailed understanding of these cycles has been limited by the lack of knowledge of the transferases and thioesterases likely to be involved. Here, we describe the purification of a protein-palmitoyl acyltransferase (PAT) from human erythrocytes. PAT behaved as a peripheral membrane protein and catalyzed the attachment of palmitate in thioester linkage to the β-subunit of spectrin. On SDS-polyacrylamide gel electrophoresis, PAT appeared as a 70-kDa polypeptide. Antibody against this polypeptide could immunodeplete PAT activity from the crude extract, confirming the assignment of the 70-kDa polypeptide as PAT. PAT-mediated spectrin palmitoylation could be inhibited by nonradioactive palmitoyl-, myristoyl-, or stearoyl-CoA. The apparent K_m for palmitoyl-CoA was 16 μM.

A large number of proteins in cells are modified by the covalent attachment of long chain saturated fatty acid residues (1–3). The two most common modifications involve acylation with myristate and palmitate. Myristate is usually attached to an N-terminal glycine via an amide bond in a relatively stable linkage. Palmitate is usually attached via a thioester bond to cysteine residues of proteins. Palmitoylation occurs in membrane proteins with hydrophobic transmembrane segments as well as in hydrophilic proteins such as Ras (1–4). These lipid modifications have often been implicated in membrane association of the modified proteins (5). Palmitoylation occurs post-translationally. Moreover, rapid turnover of the protein-bound fatty acid has been shown for several proteins (6–8). The recent surge of interest in studying protein palmitoylation is largely due to the fact that a number of proteins involved in intracellular signaling are palmitoylated, and this appears to be regulated by the activation status of these proteins (9–12). Palmitoylation occurs in, among others, the families of (i) G protein-linked receptors, (ii) the subunits of heterotrimeric G proteins, and (iii) the non-receptor tyrosine kinases. Palmitate plays a role in the membrane association of these polypeptides.

Moreover, recent evidence suggests that this may regulate protein-protein interactions such as those between growth cone-associated protein 43 and $G_{o\alpha}$ in neurons (13). Although evidence that the interactions of G proteins with effectors are regulated by palmitoylation is becoming compelling, very little is known about the enzymes involved in the catalysis of the palmitoylation and depalmitoylation of these or other proteins.

A clear understanding of the palmitoylation-depalmitoylation cycle as a cellular control mechanism obviously entails characterization of the protein palmitoyltransferase(s) and thioesterase(s). A thioesterase has recently been purified (14), and a corresponding cDNA has been cloned (15). However, since this thioesterase is a secreted protein, whereas Ras and G_α subunits are located on the cytoplasmic layer of the plasma membrane, these proteins may not be its physiologic substrates. The protein-palmitoyl acyltransferase (PAT)[1] has remained, until lately, refractory to purification. PAT activity has been demonstrated in several eukaryotic membranes (16–20) and partially purified from bovine brain (21) and from rat liver (22).

In our laboratory and that of others, palmitate associated with erythrocyte membrane cytoskeletal proteins has been demonstrated to undergo turnover (23), and palmitoylation has been found to modulate association of erythrocyte protein 4.2 with the membrane (24). These findings necessitated understanding the role of palmitoylation-depalmitoylation cycles in modulating interactions among erythrocyte cytoskeletal proteins, and efforts were made to characterize the PAT from human erythrocytes. This study reports the purification and biochemical characterization of a protein-palmitoyl acyltransferase from human erythrocytes.

MATERIALS AND METHODS

Reagents—[^{14}C]Palmitoyl-CoA and [9,10-^{3}H]palmitic acid were from Amersham International (Buckinghamshire, United Kingdom). Aprotinin, leupeptin, pepstatin, benzamidine, phenylmethylsulfonyl fluoride, dithiothreitol, nonradioactive fatty acyl-CoAs, and Formalin-fixed *Staphylococcus aureus* bearing protein A were from Sigma. Staphylococcal V8 protease was from Pierce. All other reagents were of analytical grade.

Purification of PAT—Fresh blood was collected from normal healthy volunteers and washed in phosphate-buffered saline, and erythrocytes were packed and lysed in lysis buffer (7.5 mM sodium phosphate, 1 mM Na$_2$EDTA, pH 7.5, containing 30 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml benzamidine, and 20 μg/ml phenylmethylsulfonyl fluoride). Ghosts were pelleted, washed, and extracted with 10 volumes of 0.2 mM Na$_2$EDTA, pH 7.5 (*i.e.* low ionic strength extraction buffer), containing 20 μg/ml phenylmethylsulfonyl fluoride at 37 °C for 30 min. After centrifugation at 19,000 rpm (SS-34) for 30 min, the pellet was further extracted with an equal volume of KCl extraction buffer (7.5 mM sodium phosphate, 1 mM Na$_2$EDTA, 1 mM dithiothreitol, 1 M KCl, pH 7.5) on ice for 30 min. After centrifugation at 20,000 rpm (SS-34) for 30 min, the supernatant (KCl extract) was dialyzed extensively against 20 mM piperazine buffer, pH 9.5. The dialysate was

[1] The abbreviations used are: PAT, protein-palmitoyl acyltransferase; PAGE, polyacrylamide gel electrophoresis.

Protein-palmitoyl Acyltransferase from Human Erythrocytes
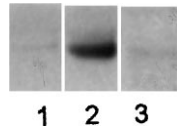
FIG. 1. **Palmitoylation of spectrin catalyzed by the 1 M KCl extract from human erythrocytes.** Spectrin (100 μg/ml) was incubated in a volume of 20 μl for 40 min at 37 °C with 40 μM [^{14}C]palmitoyl-CoA (50 mCi/mmol) in imidazole buffer without the KCl extract (*lane 1*), with the KCl extract (150 μg/ml) (*lane 2*), or with the KCl extract after it had been boiled for 5 min at 100 °C (*lane 3*), followed by SDS-PAGE and fluorography (exposure time: 15 days).

loaded on a Mono Q HR5/5 column (Pharmacia Biotech Inc.) equilibrated against the same buffer and fitted to a Pharmacia FPLC system. After washing the column, proteins were eluted with an increasing salt gradient (0–1 M NaCl). Fractions were assayed for PAT activity by the immunoprecipitation technique and analyzed by SDS-PAGE. Fractions that appeared homogeneous on SDS-PAGE and showed PAT activity were pooled, neutralized, and processed further.

Fast Desalting Chromatography—Mono Q fractions containing PAT activity were chromatographed on a Pharmacia Fast desalting column (HR10/10) equilibrated in 100 mM imidazole buffer, pH 7.5, to bring the purified PAT in this buffer. Fractions were finally stored at −70 °C. Under these conditions, the enzyme gradually lost activity over a period of 2 weeks.

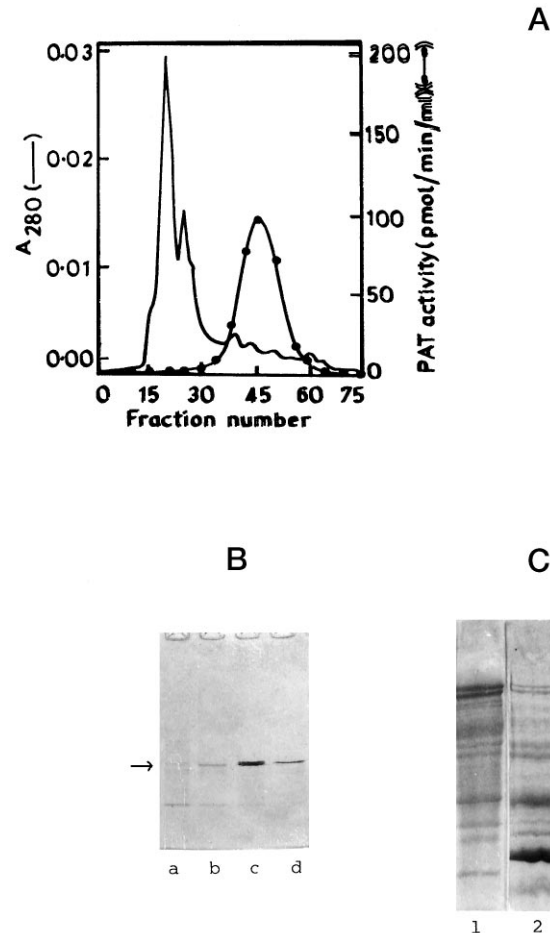*Purification of Spectrin*—Spectrin was purified as described by Bennett (25), stored at −70 °C, and used within 2 weeks.

Raising of Antibodies against PAT and against Spectrin—Purified PAT or spectrin (75 μg) was injected subcutaneously with complete Freund's adjuvant into one male rabbit each. Successive injections of 40 μg each with incomplete Freund's adjuvant were given at the end of the 2nd, 3rd, and 4th weeks, followed by bleeding at the end of the 5th week.

SDS-PAGE—SDS-PAGE was performed using the discontinuous buffer system of Laemmli (26). Samples were dissolved in 62.5 mM Tris-HCl containing 2% (w/v) SDS, 10% (v/v) glycerol, 1% (v/v) 2-mercaptoethanol, and 0.01% bromphenol blue. Gels were impregnated with 2,5-diphenyloxazole for autoradiography as described by Laskey and Mills (27).

PAT Assay and Analysis by Fluorography—Routinely, spectrin was incubated with PAT and [^{14}C]palmitoyl-CoA in 100 mM imidazole buffer, pH 7.5, at 37 °C as described in the figure legends. Following reaction, samples were boiled in denaturing SDS gel buffer and separated on 7.5% SDS gels, followed by fluorography. To study the nature of PAT-mediated palmitoylation of spectrin, gels containing ^{14}C-palmitoylated spectrin were treated (*a*) with CHCl$_3$/CH$_3$OH or (*b*) with 1 M neutral hydroxylamine prior to fluorography. Competition with nonradioactive octanoyl-, decanoyl-, lauroyl-, myristoyl-, palmitoyl-, and stearoyl-CoAs was performed by assaying PAT activity using 40 μM [^{14}C]palmitoyl-CoA and a 2-fold excess of nonradioactive acyl-CoA as described above.

PAT Assay by Immunoprecipitation of Spectrin—PAT assay was carried out using 8 μg of spectrin and 40 μM [^{14}C]palmitoyl-CoA (50 mCi/mmol) in 100 mM imidazole buffer, pH 7.5, in a final volume of 30 μl. Incubations were usually carried out for 40 min at 37 °C unless otherwise stated. Following reaction, 20 μl of anti-spectrin antibody was added in a final volume of 100 μl containing 120 mM KCl, 5 mM sodium phosphate, pH 8, 0.5 mM EGTA, 0.5 mM dithiothreitol, 0.1% Triton X-100, and 1 mg/ml gelatin and incubated for 30 min at 25 °C. The amount of antibody and the time of incubation were found to be adequate for immunoprecipitating spectrin in all the assays reported here. This was followed by the addition of 100 μl of 10% Formalin-fixed *S. aureus* bearing protein A (washed with Buffer A (120 mM KCl, 5 mM sodium phosphate, pH 8, 0.5 mM EGTA, 0.5 mM dithiothreitol, 1 mM EDTA, 0.1% Triton X-100, 1 mg/ml gelatin)) and a further incubation for 1 h at 25 °C.

The *S. aureus* protein A with the bound antigen-antibody complex was sedimented at 10,000 × *g* for 3 min in a microcentrifuge and washed three times with 1-ml aliquots of Buffer A. The pellet with bound spectrin was counted in a liquid scintillation counter. For each experiment, a control sample lacking PAT was prepared and treated exactly as described above to correct for any nonspecific acylation of spectrin and any adherence of [^{14}C]palmitoyl-CoA to the *S. aureus* protein A beads. The counts associated with the beads in these control tubes were subtracted from the counts of the corresponding PAT-containing tubes. The control values did not exceed 10–15% of those ob-

FIG. 2. **Purification of erythrocyte PAT.** *A*, chromatographic elution profile of PAT activity on Mono Q HR5/5 at pH 9.5. PAT activity (pmol of [^{14}C]palmitoyl-CoA transferred per min/ml of fraction) was assayed by immunoprecipitation of spectrin as described under "Materials and Methods." Assays were performed using 15 μl of the Mono Q fractions after neutralization. *B*, SDS-PAGE analysis of proteins present in fractions 41 (*lane a*), 43 (*lane b*), 45 (*lane c*), and 47 (*lane d*) of the Mono Q column. 100 μl of each fraction was freeze-dried and loaded on the gel. PAT activity has been assigned to the 70-kDa protein (indicated by the *arrow*). *C*, sequentially purified fractions of erythrocyte ghosts after Coomassie Brilliant Blue staining. *Lane 1*, erythrocyte ghost; *lane 2*, KCl extract; *lane 3*, purified PAT after chromatography on Mono Q (fraction 45, 1 μg)

tained in the presence of PAT. The stoichiometry of spectrin palmitoylation was determined using varying amounts of spectrin (2–6 μg), 40 μM [^{14}C]palmitoyl-CoA, and 10 μg/ml PAT. The reaction was allowed to proceed for 60 min, followed by immunoprecipitation of spectrin as described above.

Immunoprecipitation of PAT—This was performed essentially as described by Firestone and Winguth (28). Fresh KCl extract (25 μg) was incubated with various amounts of rabbit anti-PAT IgG or preimmune IgG in 30 μl for 1 h on ice in plastic tubes. Then 10 μl of a 10% suspension of prewashed *S. aureus* protein A was added and incubated for 1 h on ice with occasional shaking. The tubes were centrifuged, and the supernatants (15 μl) were assayed for PAT activity.

Analysis of the Spectrin-bound Labeled Fatty Acid—^{14}C-Palmitoylated spectrin was excised from SDS gels. The gel slices were incubated with 1 ml of 1.5 N NaOH at 30 °C for 3 h. The pH was then adjusted to 1–2 with 6 M HCl. The hydrolysate was extracted with CHCl$_3$/CH$_3$OH, and the organic phase was dried under nitrogen. The extracted lipid was analyzed by ascending chromatography on a reverse-phase C$_{18}$ TLC plate using acetonitrile/acetic acid (1:1, v/v) as the mobile phase. [^{3}H]Palmitic acid and [^{3}H]myristic acid were used as standards. The plate was scraped, and the radioactivity was determined in parts to determine the relative migration of radiolabeled species in the samples.

Labeling of Erythrocytes with [^{3}H]Palmitic Acid and Extraction of Labeled Spectrin—Human erythrocytes were labeled with [^{3}H]palmitic acid, and tightly membrane-associated spectrin was extracted as de-

TABLE I
Summary of purification of erythrocyte PAT

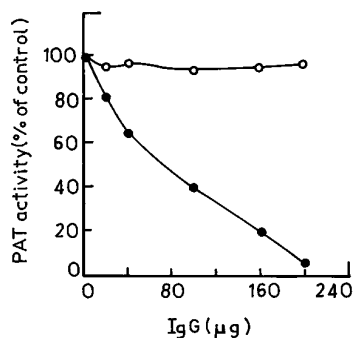| Step	Protein	Activity	Specific activity	Purification	Yield
	mg	*pmol CoA/min*	*pmol CoA/min/mg*	*-fold*	*%*
KCl extract	14	840	60		100
Mono Q chromatography	0.08	330	4700	78	39
Fast desalting chromatography	0.06	300	5200	86	36

FIG. 3. **Immunoprecipitation of PAT activity from the KCl extract.** The KCl extract was incubated with varying amounts of preimmune (○) or immune (●) IgG, and the antigen-antibody complexes were precipitated. PAT activity was quantitated in the supernatants by spectrin immunoprecipitation as described under "Materials and Methods."

scribed by Mariani *et al.* (4) with slight modifications. After washing the cells three times with phosphate-buffered saline/glucose (10 mM phosphate, 140 mM NaCl, 5 mM KCl, 0.5 mM EDTA, 5 mM glucose, pH 7.4), the cells were incubated twice for 15 min at room temperature with 10 volumes of Buffer B (40 mM imidazole, 90 mM NaCl, 5 mM KCl, 5 mM MgCl$_2$, 15 mM glucose, 0.5 mM EGTA, 30 mM sucrose, 0.3 mM phenylmethylsulfonyl fluoride, 200 units of penicillin G, pH 7.6) containing 0.2% fatty acid-free bovine serum albumin to lower the endogenous pool of fatty acids. The bovine serum albumin-treated cells were washed once with Buffer B containing 5 μM coenzyme A and 5 mM pyruvate (Buffer C). Packed erythrocytes (2 ml) were labeled with 0.5 mCi of [9,10-^{3}H]palmitic acid (54 Ci/mmol) at a hematocrit of 0.3 in Buffer C for 12 h at 37 °C. The labeling was stopped by adding cold phosphate-buffered saline/glucose and washing twice with the same buffer containing 0.2% fatty acid-free bovine serum albumin. Membranes were prepared by hypotonic lysis as described above. KCl-stripped inside-out vesicles were prepared as described by Mariani *et al.* (4), and tightly membrane-associated spectrin was extracted in the presence of 5 M urea (4) and concentrated with a Millipore Ultrafree microcentrifuge filter (M_r 100,000 cutoff) to 1–2 mg/ml.

Peptide Analyses of Spectrin—The method of Cleveland *et al.* (29) was followed. Tightly membrane-associated spectrin (representing the *in vivo* palmitoylated form of spectrin) as well as spectrin palmitoylated *in vitro* using PAT were electrophoretically separated on a 5% SDS gel. Gel pieces containing the β-subunit of spectrin were excised, rehydrated in Buffer D (0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA), and placed in the sample well of a second SDS gel (15% acrylamide). The spaces of the wells were filled with Buffer D containing 20% glycerol and *S. aureus* V8 protease (at an enzyme/substrate ratio of 1:2). Digestion was allowed to proceed in the stacking gel for 30 min. Electrophoresis was then continued, and the gel was treated for fluorography.

RESULTS

Purification of PAT from Human Erythrocytes and Use of Spectrin as a Substrate of PAT—Human erythrocyte ghosts have previously been shown to have PAT activity (20). In this study, ghosts were subjected to sequential extraction with buffers of increasing ionic strength. PAT activity was assessed by studying the palmitoylation of spectrin. After extraction with low ionic strength extraction buffer, PAT activity remained associated with the membrane vesicles, and no activity was detectable in the supernatant. PAT activity could be extracted in buffer containing 1 M KCl. The 1 M KCl extract catalyzed the palmitoylation of predominantly the β-subunit of spectrin (Fig.

1). This extract was dialyzed against 20 mM piperazine buffer, pH 9.5, and fractionated on a Mono Q column as described under "Materials and Methods." PAT activity was associated with a 70-kDa polypeptide that eluted between 0.7 and 0.9 M NaCl (Fig. 2). Active fractions that appeared homogeneous on SDS-PAGE and showed an activity ≥80 pmol/min/ml were neutralized, chromatographed on a Pharmacia Fast desalting column, and brought to 100 mM imidazole buffer, pH 7.5. The purification is summarized in Table I. Both erythrocyte ghosts and vesicles remaining after low ionic strength extraction were sources of PAT as assessed by fluorography using spectrin as substrate. However, these could not be included in the purification table. Quantitative measurements of PAT activity could not be made with ghosts or vesicles remaining after low ionic strength extraction since this would necessitate solubilization of the PAT from the membranes. However, we and others (20) have observed that detergents such as Triton X-100 used at concentrations usually necessary for solubilization of membrane proteins (≈1%) were inhibitory for PAT activity. Besides this, ghosts contained substantial amounts of spectrin, the substrate used in these studies to assess PAT activity. Therefore, this was an additional impediment in the quantitation of PAT activity. PAT obtained after Fast desalting chromatography was purified 86-fold over the KCl extract. However, an assessment of the -fold purification over the ghosts could not be made for the reasons stated above.

The assignment of the 70-kDa protein as PAT was confirmed by immunoprecipitation using antibody raised against this protein. This antibody precipitated PAT activity from the KCl extract in a dose-dependent manner (Fig. 3), while preimmune IgG showed no such effect.

The PAT-catalyzed incorporation of radioactivity into spectrin from [^{14}C]palmitoyl-CoA was studied as a function of time. PAT palmitoylated spectrin in a time-dependent manner (Fig. 4A), reaching a plateau after 40 min of incubation. Palmitoylation of spectrin increased with increasing amounts of the enzyme (Fig. 5), confirming that the transfer of the palmitoyl moiety to spectrin is an enzymatic process. PAT activity in the KCl extract was abolished on heating the enzyme for 5 min at 100 °C (Fig. 1, *lane 3*). The apparent K_m for palmitoyl-CoA was found to be 16 μM (Fig. 4B). It was observed that the inhibition of PAT activity by nonradioactive fatty acyl-CoAs was dependent on the chain length of the fatty acyl-CoAs. Octanoyl-, decanoyl-, and lauroyl-CoAs were poor inhibitors of PAT activity (<10% inhibition). Among the longer chain fatty acyl-CoAs, when used in 2-fold excess over radioactive palmitoyl-CoA, the ability to compete with [^{14}C]palmitoyl-CoA was as follows: palmitoyl-CoA > myristoyl-CoA > stearoyl-CoA (Fig. 6A). In this connection, it may be mentioned that myristate has been demonstrated to be present in thioester linkage in platelet proteins (30).

Palmitoylation of Spectrin Occurs via a Thioester Linkage—The chemical nature of PAT-mediated spectrin palmitoylation was investigated by soaking the polyacrylamide gel containing palmitoylated spectrin in CHCl$_3$/CH$_3$OH (Fig. 6B) or in neutral hydroxylamine. While soaking the gel in CHCl$_3$/CH$_3$OH had no effect, the bound radioactive palmitate could be removed by treatment with neutral hydroxylamine (Fig. 6B), confirming
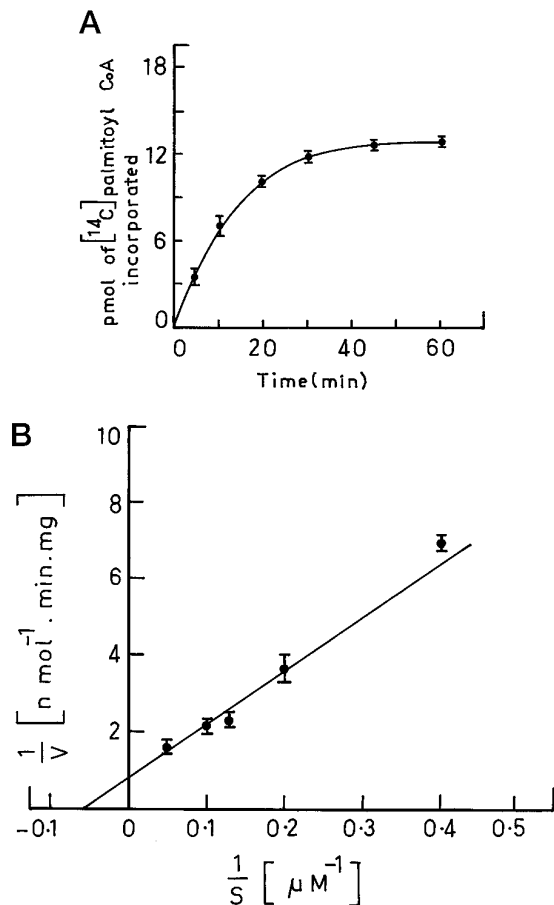
FIG. 4. *In vitro* **assay of erythrocyte PAT activity.** *A*, the effect of varying incubation time on PAT activity. PAT activity was assessed using the immunoprecipitation technique described under "Materials and Methods." Assays were performed using 64 μg of spectrin, 40 μM [^{14}C]palmitoyl-CoA (50 mCi/mmol), and 1.2 μg of enzyme in a final volume of 200 μl. Aliquots of 25 μl were withdrawn at different time points and heated at 100 °C for 5 min in order to inactivate PAT. Immunoprecipitation of spectrin was performed as described under "Materials and Methods." *B*, determination of the K_m for [^{14}C]palmitoyl-CoA. Spectrin (8 μg) was incubated with increasing concentrations of [^{14}C]palmitoyl-CoA (50 mCi/mmol) at 37 °C and 0.15 μg of PAT in 25 μl. PAT activity was quantified by immunoprecipitation of spectrin, and the K_m was determined from the Lineweaver-Burk plot. The results shown represent the means ± S.D. from three different experiments.

FIG. 5. **Effect of increasing concentrations of erythrocyte PAT on spectrin palmitoylation.** PAT activity was assessed by fluorography as described under "Materials and Methods." Assays were performed using 2 μg of spectrin and 40 μM [^{14}C]palmitoyl-CoA. *Lanes 1–4* represent assays performed using 1, 2, 3, and 6 μg/ml PAT, respectively.

FIG. 6. **Fatty acyl-CoA specificity of spectrin palmitoylation.** *A*, spectrin was acylated using 40 μM [^{14}C]palmitoyl-CoA in the absence (*lane a*) and presence (80 μM) of nonradioactive lauroyl-CoA (*lane b*), stearoyl-CoA (*lane c*), myristoyl-CoA (*lane d*), and palmitoyl-CoA (*lane e*). *B*, gels of palmitoylated spectrin were treated with 1 M Tris-HCl, pH 7.5 (*lane 1*); CHCl$_3$/CH$_3$OH (7:3, v/v) (*lane 2*); and 1 M neutral hydroxylamine (*lane 3*) for 4 h. *C*, radioactivity released after hydrolysis of palmitoylated spectrin was analyzed by reverse-phase TLC with [^{3}H]palmitic acid as a standard. After ascending chromatography, samples 1 cm in height and 2 cm in width were scraped from the plate, and radioactivity was determined. ○, standard [^{3}H]palmitic acid; ●, radioactive fatty acid released from palmitoylated spectrin.

that palmitoylation of spectrin occurs via a thioester linkage. In a parallel experiment, palmitoylated spectrin was excised from the gel and hydrolyzed. The hydrolysate was extracted with organic solvents and analyzed by TLC. The R_F value of the sample was identical to that of standard radioactive palmitic acid, identifying the fatty acid incorporated into spectrin as palmitic acid (Fig. 6*C*). The stoichiometry of palmitoylation was determined by immunoprecipitation of spectrin after labeling with [^{14}C]palmitoyl-CoA. Incorporation of the palmitoyl moiety into spectrin occurred in a 1:1 molar ratio of fatty acid to spectrin ($\alpha + \beta$) (data not shown).

Cleveland Mapping of in Vitro and in Vivo Palmitoylated β-Spectrin—To investigate whether the fatty acid bound to spectrin palmitoylated *in vitro* using PAT is incorporated in the same site(s) as *in vivo*, peptide maps of *in vivo* and *in vitro* labeled spectrin (β-subunit) were compared using the Cleveland V8 protease digestion technique of gel slices, followed by fluorography (as described under "Materials and Methods"). The V8 protease digestion gave one major palmitoylated peptide in the second dimension SDS-PAGE after fluorography. This was present in the case of both the *in vivo* and *in vitro*
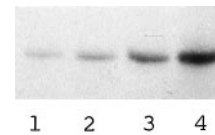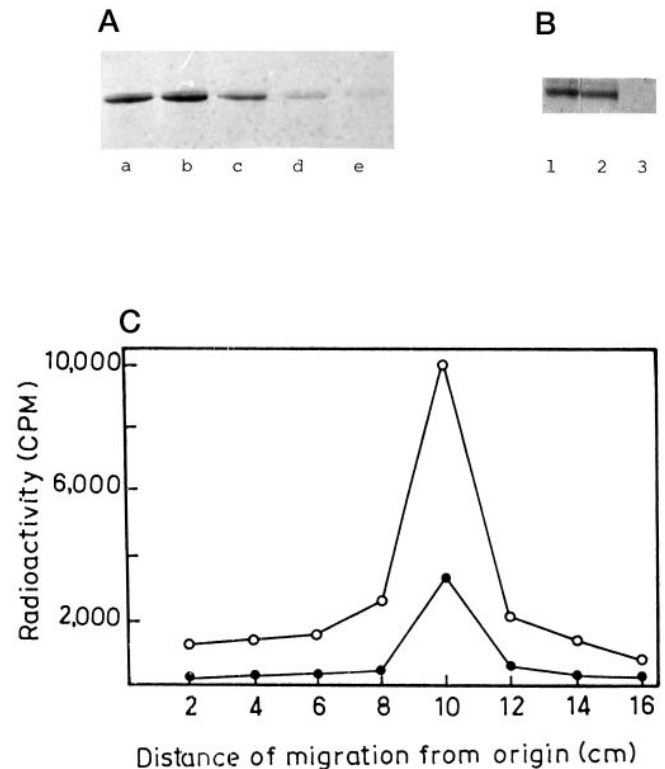
labeled spectrin (migrating with an apparent molecular mass of 45 kDa) (Fig. 7), suggesting that the same site is labeled on the protein in both cases.

DISCUSSION

Our understanding of dynamic protein palmitoylation as a cellular control mechanism has been limited by the lack of detailed knowledge about the enzymology of palmitoylation and the fact that no PAT has been isolated to date. Progress toward the purification of PAT has been hampered by the lack of a rapid and sensitive method of assay, by the absence of an apparent consensus sequence at sites of palmitoylation, and by the relative instability of the enzymes identified so far. Our search for the PAT from human erythrocytes was based on our own observations and that of other laboratories that protein-bound palmitate associated with erythrocyte membrane proteins turns over (8, 23). Moreover, palmitoylating activity has
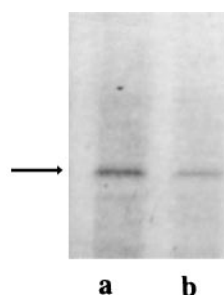
FIG. 7. **Peptide mapping after *in vitro* and *in vivo* palmitoylation of spectrin.** Spectrin was labeled with [^{14}C]palmitoyl-CoA and PAT (for *in vitro* acylation) as described under "Materials and Methods." *In vivo* acylation was carried out on erythrocytes using radioactive palmitic acid as described under "Materials and Methods." Tightly membrane-associated spectrin labeled *in vivo* or spectrin labeled *in vitro* was electrophoresed on a 5% SDS gel, and the bands corresponding to the β-subunit were excised (5 μg of protein) and subjected to V8 protease digestion during the second dimension electrophoresis on a 15% SDS gel. The gels were treated for fluorography. *Lane a*, *in vitro* acylation; *lane b*, *in vivo* acylation. The position of the major palmitoylated peptide is indicated with an *arrow*.

recently been demonstrated in human erythrocyte ghosts (20). Our choice of spectrin as substrate was based on the observations of Mariani *et al.* (4) that a tightly membrane-associated fraction of spectrin is palmitoylated when human erythrocytes are metabolically labeled with [^{3}H]palmitic acid. Spectrin is an abundant cytoskeletal protein of the erythrocyte that can be easily purified in substantial quantities. In the absence of knowledge on the defined substrate requirement of erythrocyte PAT, spectrin appeared to be a feasible alternative to a peptide substrate that could be used for monitoring the purification of PAT. PAT activity could be extracted from erythrocyte ghosts in buffer containing 1 M KCl. Erythrocyte PAT therefore appeared to behave like a peripheral membrane protein, unlike the PAT identified in bovine brain (21). On the other hand, the rat liver palmitoyltransferase is solubilized from membranes using 150 mM KCl, suggesting that this enzyme, like erythrocyte PAT, may not be tightly bound to membranes. The increase in specific activity of purified PAT in comparison with the KCl extract was 86-fold. PAT was extremely labile and susceptible to proteolysis, as reported earlier by others (21). A 67-kDa band reactive with anti-PAT antibody appeared below the 70-kDa polypeptide in the KCl extract on storage (data not shown). To avoid proteolysis, the purification steps were performed as quickly as possible.

Purified PAT appeared as a 70-kDa polypeptide on SDS-PAGE. The identity of this polypeptide as PAT was confirmed by immunodepletion of PAT activity using antibody against the 70-kDa polypeptide. Nonradioactive palmitoyl-, myristoyl-, and stearoyl-CoAs were competitors of [^{14}C]palmitoyl-CoA, a feature also observed in the case of bovine brain PAT (21).

Dynamic protein palmitoylation represents a recent addition to the repertoire of cellular control mechanisms. Understanding the control of palmitoylation-depalmitoylation cycles necessitates characterization of the enzymes involved in these processes. Purification of the PAT from human erythrocyte membranes has opened up exciting avenues. Whether the plasma membrane-associated PAT from erythrocytes is identical to or different from PAT activities associated with other membranes (21, 22) needs to be evaluated. Dissection of the palmitoylation site of spectrin will provide necessary knowledge of the likely substrate requirements of the PAT purified here. Partially purified bovine brain PAT recognizes the N-terminal "myristoyl-Gly-Cys" sequence of the Src family protein-tyrosine kinases like Fyn (21), while rat liver PAT recognizes a farnesylated and methylated Ha-Ras peptide. Since spectrin lacks these motifs, erythrocyte PAT is likely to represent one of a family of palmitoyltransferases of different substrate specificity and probably of different subcellular localization.

REFERENCES

1. Schmidt, M. F. G. (1989) *Biochim. Biophys. Acta* **988,** 411–426
2. James, G., and Olsen, E. N. (1990) *Biochemistry* **29,** 2623–2634
3. Schlesinger, M. J., Veit, M., and Schmidt, M. F. G. (1993) in *Lipid Modification of Proteins* (Schlesinger, M. J., ed) pp. 1–19, CRC Press, Inc., Boca Raton, FL
4. Mariani, M., Maretzki, D., and Lutz, H. U. (1993) *J. Biol. Chem.* **268,** 12996–13001
5. Galbiati, F., Guzzi, F., Magee, A. I., Milligan, G., and Parenti, M. (1996) *Biochem. J.* **313,** 717–720
6. Omary, M. B., and Trowbridge, I. S. (1981) *J. Biol. Chem.* **256,** 12888–12892
7. Magee, A. I., Gutierrez, L., McKay, I. A., Marshall, C. J., and Hall, A. (1987) *EMBO J.* **6,** 3353–3357
8. Staufenbiel, M. (1988) *J. Biol. Chem.* **263,** 13615–13622
9. Milligan, G., Parenti, M., and Magee, A. I. (1995) *Trends Biochem. Sci.* **20,** 181–186
10. Ross, E. M. (1995) *Curr. Biol.* **5,** 107–109
11. Mumby, S. M., Kleuss, C., and Gilman, A. G. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 2800–2804
12. Casey, P. J. (1995) *Science* **268,** 221–224
13. Skene, J. H. P., and Virag, I. (1989) *J. Cell Biol.* **126,** 127–138
14. Camp, L. A., and Hofmann, S. L. (1993) *J. Biol. Chem.* **268,** 22566–22574
15. Camp, L. A., Verkruyse, L. A., Afendis, S. J., Slaughter, C. A., and Hofmann, S. L. (1994) *J. Biol. Chem.* **269,** 23212–23219
16. Gutierrez, L., and Magee, A. I. (1991) *Biochim. Biophys. Acta* **1078,** 147–154
17. Adam, M., Rodriguez, A., Turbide, C., Larrick, J., Meighen, E., and Johnstone, R. M. (1984) *J. Biol. Chem.* **259,** 15460–15463
18. Berger, M., and Schmidt, M. F. G. (1985) *FEBS Lett.* **187,** 289–294
19. Slomiany, A., Liau, Y. H., Takagi, A., Laszewicz, W., and Slomiany, B. L. (1984) *J. Biol. Chem.* **259,** 13304–13308
20. Schmidt, M. F. G., McIllhinney, R. A. J., and Burns, G. R. (1995) *Biochim. Biophys. Acta* **1257,** 205–213
21. Berthiaume, L., and Resh, M. D. (1995) *J. Biol. Chem.* **270,** 22399–22405
22. Liu, L., Dudler, T., and Gelb, M. H. (1996) *J. Biol. Chem.* **271,** 23269–23276
23. Das, A. K., Kundu, M., Chakrabarti, P., and Basu, J. (1992) *Biochim. Biophys. Acta* **1108,** 128–132
24. Das, A. K., Bhattacharya, R., Kundu, M., Chakrabarti, P., and Basu, J. (1994) *Eur. J. Biochem.* **224,** 575–580
25. Bennett, V. (1983) *Methods Enzymol.* **96,** 316–317
26. Laemmli, U. K. (1970) *Nature* **227,** 680–685
27. Laskey, R. A., and Mills, A. D. (1975) *Eur. J. Biochem.* **56,** 335–341
28. Firestone, G. L., and Winguth, S. D. (1990) *Methods Enzymol.* **182,** 688–718
29. Cleveland, D. W., Fischer, S. G., Kirschner, M. W., and Laemmli, U. K. (1977) *J. Biol. Chem.* **252,** 1102–1106
30. Muszbek, L., and Laposata, M. (1993) *J. Biol. Chem.* **268,** 8251–8255