Biochemical Characterization of a Palmitoyl Aciytransferase Activity That Palmitoylates Myristoylated Proteins*

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Dynamic regulation of signal transduction by reversible palmitoylation-depalmitoylation cycles has been recently described. However, further understanding of fatty acylation reactions has been hampered by our lack of knowledge about the specific transferases and thioesterases involved. Here, we describe an assay for the palmitoyl acyltransferase (PAT) that palmitoylates "myrGlyCys" containing members of the Src family of protein tyrosine kinases (PTKs). Since N-myristoylation of Fyn PTK, a member of the Src family, has been shown to be a prerequisite for palmitoylation, a new single plasmid vector that allows overexpression of myristoylated Fyn substrate in Escherichia coli was developed. Purified myristoylated protein substrates were incubated with [125I]iodopalmitoyl CoA, a palmitoyl coenzyme analog, in the presence of bovine brain lysates. Transfer of radiolabel to the Fyn substrate was detected by SDS-polyacrylamide gel electrophoresis and autoradiography. This assay was used to partially purify and characterize PAT activity from bovine brain. Here, we demonstrate that PAT is a membrane-bound enzyme, which palmitoylates myristoylated Fyn substrates containing a cysteine residue in position three. The PAT activity attached palmitate to Fyn proteins via a thioester linkage and exhibited a fatty acyl CoA preference for long chain fatty acids. It is likely that palmitoylation of Fyn and other Src family members by PAT regulates PTK localization and signaling functions.

Protein fatty acylation can be divided into two categories: myristoylation and palmitoylation. N-Myristoylation involves the cotranslational attachment of the 14-carbon fatty acid myristate onto an N-terminal glycine residue of a protein via an amide linkage. Due to the high stability of this amide bond, myristoylation is irreversible, with some exceptions (7). The enzymology of N-myristoylation has been well characterized (Ref. 8 and references therein). A methionyl aminopeptidase first removes the initiator methionine. N-Myristoyl transferase (NMT) then catalyzes the transfer of myristate to the glycine residue in position two. This glycine residue is an essential element of the substrate recognition sequence, since its substitution by any other amino acid within a protein prevents myristoylation.

Many proteins contain the 16-carbon fatty acid palmitate attached to specific cysteine residues. In contrast to myristoylation, palmitoylation occurs post-translationally and is readily reversible. The enzymology of palmitoylation is not well characterized, and a palmitoyl acyltransferase (PAT) has not yet been isolated. No apparent consensus sequence has been found at the palmitoylation site, suggesting the presence of more than one type of PAT. However, the N termini of 7 of 9 members of the Src family of PTKs and several of the α subunits of the heterotrimeric G proteins contain the sequence myrGlyCys, where Cys-3 is palmitoylated (2, 3). In most of these cases, myristoylation has been shown to be a prerequisite for palmitoylation to occur (9, 10).

Many signal-transducing proteins translocate reversibly between plasma membrane and cytosol (1, 2). Several have been shown to be reversibly palmitoylated, such as α subunits of G proteins and nitric oxide synthase. These proteins undergo agonist-stimulated palmitate turnover (11, 12), suggesting that dynamic palmitoylation of proteins can regulate signal transduction.

Little is known about the enzymes that specifically remove or transfer palmitate onto signal-transducing proteins. Recently, a palmitoyl thioesterase, which deacylates Ras proteins and α subunits of G proteins in vitro, has been purified and the corresponding cDNA cloned (13, 14). Upon further analysis, this palmitoyl thioesterase was shown to be a secreted protein. Ras proteins and Gα subunits, which are located on the cytoplasmic layer of the plasma membrane, are therefore unlikely to be physiologic substrates for a secreted palmitoyl thioesterase.

Progress toward the identification of a PAT has been limited.

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The abbreviations used are: NMT, N-myristoyl transferase; FynSH432Hiso, Fyn protein-tyrosine kinase truncated after the SH2 domain to which a hexahistidine tag was appended; IC16, iodohexadecanoic acid (a palmitate analog); myr, myristate; pal, palmitate; PAT, palmitoyl acyltransferase; PTK, protein-tyrosine kinase; SH4, SH3, and SH2, Src homology domains 4, 3, and 2, respectively; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; WT, wild type.
by lack of a rapid, sensitive, and specific assay. Here, we report development of such an assay for the PAT that palmitoylates the Tyr kinase PTKs containing the myrmcolysin (MYR) conserved sequence. Since prior myristoylation of these proteins has been shown to be a prerequisite for palmitoylation, a source of myristoylated protein to be used as substrate in the assay was required. An Escherichia coli overexpression vector for protein myristoylation was used to produce large amounts of myristoylated truncated Fyn PTK proteins. To circumvent long exposure times required to detect incorporation of tritiated palmitate into proteins, a [125I]iodopalmitoyl CoA analog was used (15, 16). Analysis of the products of the PAT assay by SDS-PAGE allowed visualization of incorporated iodopalmitate in as little as 30 min using phosphorimager technology. This assay was used to partially purify and characterize a PAT activity from bovine brain that palmitoylates myristoylated proteins.

MATERIALS AND METHODS

Reagents—Fatty acyl CoAs and acyl CoA ligase were from Sigma. Radioisotopes [α-32P]dATP (Sequenase grade), [3H]myristate, and [14C]NaI were from Amersham and DuPont NEN. Radioiodination of the [14C]NaI palmitate analog with [32P]NaI and synthesis of the 125I-labeled IC16 CoA derivative using acyl CoA ligase were carried out as previously reported (15-17). Nickel chelating arograse and Q-Sepharose Fast Flow were from Qiagen and Pharmacia Biotech Inc., respectively. RPS-F TLC plates used for fatty acid analysis were from Analtech. Bacterial Strains, Plasmids, and Cloning—E. coli strain DH5α was originally purchased from Life Technologies, Inc. Plasmid pETSh432 and pETSh432hNMT plasmid. Second, an intercistronic region including a polyhistidine tag, a ribosome binding site (Shine-Dalgarno sequence), and a stop codon was appended after the SH2 domain of the Fyn PTK allowing tandem expression of a truncated version of the Fyn protein tyrosine kinase and human NMT. pETFyn432hNMT was constructed taking advantage of the polycistronic nature of prokaryotic mRNA and the development of such an assay for the PAT that palmitoylates proteins.

Radioisotopes [3H]myristate was used to partially purify and characterize a PAT activity from bovine brain that palmitoylates myristoylated proteins.

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for 30 min at 25 °C. The reaction was buffered with 100 mM imidazole-HCl, pH 7.0, and 0.3% Triton X-100 was added to keep PAT soluble. The FynSH432His6 substrate was from a 1.0-3.2.0 mg/ml stock in 8 M urea, pH 5.7. The products of the reaction were separated by SDS-PAGE on a 12.5% polyacrylamide gel (27). Incorporation of the labeled palmitate analog into the 32-kDa FynSH432His6 protein was visualized by phosphorimager analysis for 1 h or after a 36-h exposure by autoradiography on x-ray film.

To study substrate specificity, 2.5 μg of partially purified PAT from the Q-Sepharose pool was assayed as above in the presence of 1.0 μg of the purified FynSH432His6 mutants. Alternatively, PAT activity was assayed with the WT FynSH432His6 in the presence of myristoylated dodecapeptides (100 μM) corresponding to the N termini of Src-related protein tyrosine kinases (19).

To study temperature sensitivity of PAT activity, 2.5 μg of partially purified PAT (Q pool) were preincubated for 5 min at 25, 37, 45, 55, 75, and 100 °C, chilled for 5 min on ice, and assayed for activity. The fatty acyl CoA specificity was investigated by assaying PAT activity in the presence of fatty acyl CoAs (10 μM) of increasing chain length.

In Situ Hydrolysis and TLC Analysis of Fatty Acid Bound to FynSH432—PAT activity was assayed in the presence of WT and C3,6S FynSH432His6 proteins. Polyacrylamide gels were soaked in neutral 1 M hydroxylamine or 1 M Tris-HCl, pH 7.0, for 16 h at 25 °C. The gels were washed with water three times for 10 min, and assayed for activity. The fatty acyl CoA specificity was investigated by assaying PAT activity in the presence of fatty acyl CoAs (10 μM) of increasing chain length.

RESULTS

Design and Construction of an Artificial Operon for Tandem Expression of Truncated Fyn Protein-Tyrosine Kinase Constructs and Human N-Myristoyl Transferase in E. coli—A. The pETFyn432hNMT plasmid is shown with the sequence of the intercistronic region containing the Shine-Dalgarno (SD) sequence. B. N-terminal sequences of WT and mutant Fyn engineered in the FynSH432His6 protein. The substituted amino acids are underlined.

FIG. 1. Design of an artificial operon for tandem expression of truncated Fyn protein-tyrosine kinase constructs and human N-myristoyl transferase in E. coli. A. The pETFyn432hNMT plasmid is shown with the sequence of the intercistronic region containing the Shine-Dalgarno (SD) sequence. B. N-terminal sequences of WT and mutant Fyn engineered in the FynSH432His6 protein. The substituted amino acids are underlined.

Single Plasmid—Previous work by this laboratory established that the Src family member Fyn is palmitoylated and that N-myristoylation is required for efficient palmitoylation in vivo (9). To study the palmitoylation reaction in vitro, an expression system was designed to generate a myristoylated Fyn substrate. Milligram amounts of myristoylated protein can be produced in E. coli provided that the NMT is coexpressed with the target protein (17). Initial experiments, in which Fyn and NMT were maintained on two separate plasmids, resulted in low yields of myristoylated Fyn product. We therefore constructed an artificial operon with four important characteristics. First, the genes for Fyn and NMT were maintained on one plasmid under control of the T7lac promoter (Fig. 1). Second, human NMT cDNA was used instead of NMT from the yeast Saccharomyces cerevisiae. Yeast NMT exhibits a high Km for peptides containing a cysteine at position six (28) and was inefficient at myristoylating Fyn. Third, a truncated version of Fyn was employed, which terminated after the SH2 domain. We found that the expression level of full-length Fyn in E. coli was very low and that removal of the C-terminal catalytic domain enhanced Fyn expression dramatically. Finally, a hexahistidine tag was appended to the C-terminal end of the truncated Fyn to facilitate purification.

To ensure that the palmitoylation assay was specific for the N-terminal region of the FynSH432His6 protein, a series of mutations was engineered at the FynN terminus. As shown in Fig. 1B, cysteine residues were substituted for serine residues individually (C3S and C6S) and together (C3,6S). In addition, the myristoylation signal was abolished by substituting the essential glycine residue with an alanine residue (G2A). A SrcFyn chimera containing the first 10 amino acids of Src replacing the corresponding Fyn N-terminal region was also engineered as a negative control, since the Src N

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pressed myristoylated FynSH432His6 proteins were found exclusively in the insoluble fraction (membranes and bacterial debris), and the FynSH432His6 proteins were only partially solubilized using detergent or high salt conditions (data not shown). FynSH432His6 proteins were therefore solubilized in 8 M urea, purified under denaturing conditions, and diluted in reaction buffer. Urea at low concentrations did not interfere with the palmitoylation assay. Upon induction with isopropyl-β-D-galactopyranoside, all the FynSH432His6 proteins readily incorporated the [1H] myristate label with the exception of the G2A mutant, which lacks the myristoylation signal (Fig. 2B). All the myristoylated FynSH432His6 constructs were overexpressed to equivalent levels and were myristoylated to a similar extent.

Development of a PAT Assay and Partial Purification of PAT—The substrates for the PAT assay consisted of highly purified FynSH432His6 proteins and [125I]iodopalmitoyl CoA, a palmitoyl CoA analog previously described by our laboratory (15). The use of the [125I]iodopalmitate analog allows rapid detection of PAT activity, requiring as little as 30–60 min of exposure when using phosphorimager technology. Incubation of myristoylated FynSH432His6, CoA and crude homogenates of various mouse organs resulted in incorporation of radiolabel into FynSH432His6 protein. The amount of label incorporated was linear up to 30 min at 25 °C, and the assay exhibited specificity with respect to protein and fatty acyl CoA substrates (see below). Brain homogenates had the most PAT activity (data not shown).

To scale up the purification of PAT and to determine its intracellular localization, a bovine brain homogenate was fractionated into membrane-bound and soluble compartments. Membrane fractions contained the most PAT activity, while the cytosolic fraction was devoid of detectable activity (Fig. 3). The 100,000 × g pellet (P100) was washed with carbonate (pH 11) to remove peripheral membrane proteins. PAT activity was clearly detected in the carbonate-washed membranes and could readily be extracted with detergent (Fig. 3). Solubilization of PAT from the low speed, P10 membrane fraction was inefficient.

Solubilized PAT was fractionated on a Q-Sepharose Fast Flow column at pH 8.5 and eluted between 175 and 300 mM NaCl (Fig. 4). This PAT pool was used in all subsequent assays. The PAT activity was very labile, thereby complicating its purification. Indeed, enzymatic activity could not be measured after storage for more than 10 days at 4 °C, and the enzyme did not tolerate multiple freeze-thaw cycles.

Substrate Sequence Requirements—The substrate specificity of the Q-Sepharose-purified PAT activity was investigated using the six FynSH432His6 substrates. When equal amounts of PAT and FynSH432His6 proteins were incubated in the presence of the [125I]iodopalmitoyl CoA analog, the labeled iodo-palmitate was readily transferred to the WT and C65 mutant FynSH432His6 substrates (Fig. 5A). The C35 and C3,65 mutant substrates were not palmitoylated, nor was the SrcFyn

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In this manuscript, we report a rapid and sensitive assay for the PAT that palmitoylates myrGlyCys containing proteins of Src-related PTKs and its use in the purification and characterization of a PAT activity from bovine brain. The assay employs a high energy radiolabeled [125I]iodopalmitoyl CoA analog and a variety of highly purified myristoylated FynSH432His6 protein substrates. Detection of PAT activity was accomplished with less than 1 h of exposure of dried SDS-PAGE gels on a phosphorimager screen using 2.5 μg of protein or less. This
represents a significant improvement over previous palmitoylation assays, which required a minimum of 48 h of exposure using tritiated palmitate and 50 μg of PAT extract to visualize palmitate incorporation into viral glycoproteins (31).

A series of myristoylated Fyn substrates was overexpressed in E. coli with a single plasmid myristoylation system and used to delineate the substrate specificity of PAT. All FynSH432His6 substrates were shown to be expressed and myristoylated to equivalent levels (except G2A, which lacks the myristoylation signal) (Fig. 2). N-Myristoylation was shown to be essential for palmitoylation in vitro and is likely to be part of the recognition signal for PAT within the Fyn sequence. Two lines of evidence support this conclusion: 1) the G2A mutant FynSH432His6 substrate, which is not myristoylated, is also not palmitoylated (Fig. 5A) and 2) only the myristoylated form of the Yes dodecapeptide was an inhibitor of the PAT reaction (Fig. 5B). These results confirm previous observations made in vivo, suggesting that myristoylation of the MetGlyCys sequence of Src-related PTKs and Gα subunits is a prerequisite for palmitoylation (9, 10). It is not known whether other lipid moieties can substitute for myristate.

Recently, Degtyarev et al. (32) reported that membrane association, rather than myristoylation, was required for palmitoylation of Gαs in vivo. Likewise, it is important to note that Gαs, which is not myristoylated, is still palmitoylated at Cys-3 (33, 34). Although our in vitro assays are performed in cell-free lysates, in the absence of membranes, the assay buffer does contain 0.3% Triton X-100, a detergent concentration above the critical micelle concentration. The presence of a myristoyl moiety could enhance the ability of the Fyn substrate to productively interact with PAT in a detergent micelle. Alternatively, Fyn and Gα proteins may be palmitoylated by different PATs in vivo with different substrate specificities.

Using the cell-free assay and the various FynSH432His6 substrates, we delineated the substrate requirements of the partially purified PAT (Q-Sepharose pool). The presence of Cys-3 was required for palmitoylation of Fyn in vitro, since the C3S substitution within the FynSH432His6 substrate completely abolished palmitoylation (Fig. 5). Likewise, the SrcFyn chimera, which lacks Cys-3, was not palmitoylated, and myrSrc peptide failed to inhibit the PAT activity. It is not known whether sequences containing cysteine at positions 4 or 5 can serve as PAT substrates.

Mutation of Cys-6 within Fyn did not inhibit palmitoylation in vitro. In contrast, C6S mutants of p59^Fyn expressed in vivo exhibit reduced levels of palmitoylation (9). It is possible that additional PATs are responsible for palmitoylating Cys-6 in vivo or that the absence of Cys-6 in the C6S mutant enhances palmitoylation of Cys-3 in vitro. Alternatively, differences observed in palmitoylation of C6S mutants may reflect the use of monkey cells (COS-1) versus bovine brain.

The PAT activity also transferred iodopalmitate onto two other chimeric substrates: YesFynSH432His6 and a GαoFynSH432His6. Since no homology could be found among the N termini (10-amino acid sequence) of Fyn, Yes, and Gαo, other than that encoding for the myristoylation signal and the presence of the cysteine in position three, the GαoFynSH432His6 sequence is likely to be necessary and sufficient to direct palmitoylation.

The PAT activity exhibited selectivity for long chain fatty acyl CoAs, as demonstrated by the inhibitory properties of the various acyl CoAs in the assay (Fig. 6). Only fatty acyl CoAs containing 14 carbons or more were significant inhibitors of the PAT, palmitoyl CoA being the best inhibitor and the likely natural substrate of the enzyme. Whether PAT activity can

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transfer shorter or longer fatty acids onto proteins in vivo remains to be tested. The transfer of \(^{125}\text{I}\)iodopalmitate to FynSH432His\(_{6}\) occurred via the formation of an alkali and hydroxylamine-sensitive linkage, indicative of a thioester bond. Taken together, these data support the conclusion that PAT catalyzes attachment of palmitate to Cys-3 in the Src family members.

The PAT activity was shown to be membrane bound upon subcellular fractionation of bovine brain (Fig. 3), consistent with the membrane localization of Src-related PTKs and that of G-proteins. Other PAT activities have also been found in membrane fractions, e.g. the PAT that palmitoylates viral glycoproteins and H-Ras (31, 35). Whether these PAT activities correspond to identical or different enzymatic activities is not known at the present. The identity of the particular membrane subtype containing the PAT activity is also not known. PAT and its substrates could potentially interact at specific membrane sites. Upon palmitate transfer, the palmitoylated protein would remain stably anchored in the membrane. Identification of membranes enriched for PAT might shed light on how these myrGlyCys-containing proteins are targeted to specific intracellular sites. Upon palmitate transfer, the palmitoylated protein remains to be tested. The transfer of \(^{125}\text{I}\)iodopalmitate to Cys-3 in the Src family members.

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