A Cytoplasmic Acyl-Protein Thioesterase That Removes Palmitate from G Protein α Subunits and p21RAS*

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Thioacylation is one of a handful of reversible covalent protein modifications, but the enzymes responsible for addition and removal of long chain fatty acids from protein cysteine residues in vivo have not yet been identified. The α subunits of some heterotrimeric G proteins cycle between thioacylated and deacylated states in a receptor-regulated fashion. We have identified, purified, and characterized an enzyme acyl-protein thioesterase that deacylates Ga proteins and at least some other thioacyl protein substrates, including Ha-RAS. The action of this enzyme on thioacylated heterotrimeric Gα is regulated by activation of the G protein. Although native and recombinant acyl-protein thioesterases act as both acyl-protein thioesterases and lysophospholipases in vitro, we demonstrate by transfection that the enzyme can accelerate the turnover of thioacyl groups on Gα in vivo.

Protein function is regulated by dynamic covalent modifications of polypeptide backbones. Although phosphorylation of serine, threonine, or tyrosine residues is the most studied of these modifications, the importance of other alterations is increasingly appreciated; included are carboxymethylation of prenylcysteine residues, acylation of lysine residues, and thioacylation of cysteine residues (1–3).

Thioacylation (palmitoylation or S-acylation) is the addition of long chain fatty acids to specific cysteine residues within a protein by formation of a thioester bond. Almost all heterotrimeric G protein (Ga)1 subunits, as well as a broad range of other membrane-associated proteins of diverse biological function, are so modified (4, 5). Thioacylation and other lipid modifications introduce significant hydrophobicity to a protein, which can influence both protein-lipid and protein-protein interactions. Unlike myristoylation, isoprenylation, or glypiaction, thioacylation is a dynamic process, and thioacylated proteins cycle between acylated and deacylated states many times during their existence within a cell (6). Regulation of thioacylation of proteins involved in signal transduction is apparently widespread and has been formally demonstrated in at least four cases as follows: endothelial nitric oxide synthase, the β-adrenergic receptor, the m2 muscarinic receptor, and the α subunit of the heterotrimeric G protein Ga3 (7–10).

Reversible thioacylation of Gα has been well documented in vivo. In an unstimulated cell, Gα and presumably other Ga proteins exist primarily as thioacylated (predominantly palmitoylated) GDP-bound heterotrimers. In this state Gα-bound thioacyl groups turn over with a t1/2 of 20–90 min. Activation of Gα by β-adrenergic agonists, cholera toxin, or mutation of residues critical for GTP hydrolysis causes a dramatic (>10-fold) increase in the rate of palmitate turnover (10–12). However, the deacylated state is transient, because receptor stimulation apparently does not cause a significant alteration in the stoichiometry of thioacylation of Gα (13).

Thioacylation is probably involved in several aspects of G protein-mediated transmembrane signaling, although the interpretation of some functional studies performed in vivo is confounded by the fact that mutation of certain relevant cysteine residues per se can cause substantial functional deficits not due to loss of thioacylation (14). In transfected cells, mutation of thioacylated cysteine residues in Gα, Gα, and Gα causes incomplete localization to the plasma membrane (15, 16). Such mutations in Gα, GPA1p (the Gα protein in Saccharomyces cerevisiae), endothelial nitric oxide synthase, and Fyn kinase result in accumulation of the protein in internal membranes2 (17–19). Experiments performed in vitro have been hampered by difficulties in determining the stoichiometry of thioacylation. This obstacle has recently been surmounted in two studies. Iiri and colleagues (20) developed a chromatographic method to separate unmodified Gα from its thioacylated counterpart. The affinity of thioacylated Gα for Gβγ was higher than that of the nonacylated protein. Taking advantage of the ability of some Ga proteins to autoacylate in vitro (21), Tu et al. (22) demonstrated that thioacylated α subunits of some Gβ subfamily members were resistant to the GTPase accelerating activity of regulators of G protein signaling proteins.

Despite intensive investigation, the molecular machinery responsible for the thioacylation cycle of intracellular proteins has not been identified. Many thioacylated proteins, including G protein α subunits, spontaneously incorporate thioacyl groups at the relevant cysteine residues when incubated in vitro with an appropriate acyl donor (e.g. palmitoyl-CoA) (21, 23, 24). Putative enzymatic activities capable of thioacylating Ga proteins and Fyn kinase have also been characterized, although neither has been purified to homogeneity (25, 26). A protein acyltransferase that acts on farnesylated RAS was purified (27); however, this activity is due to a recently characterized form of thiolase, called thiolase A, and further studies will be needed to see if thiolase A palmitoylates RAS in vivo.3 Determination of the respective roles of autoacylation and enzymatic thioacylation in vivo awaits identification of the relevant enzymes.

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1 The abbreviations used are: Ga, heterotrimeric G protein α subunit; Gβγ, heterotrimeric G protein β and γ subunit complex; PPT1, palmitoyl protein thioesterase 1; APT1, acyl-protein thioesterase 1; GTP-S, guanosine 5′-O-(thiotriphosphate); lyso-PC, lyso-(1-acylphosphatidylcholine; HA, influenza hemagglutinin; HSV, herpes simplex virus; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid, MES, 4-morpholineethanesulfonic acid.

2 Dr. Susanne Mumby, personal communication.

3 Dr. Michael Gelb, personal communication.
Sorting impostors from the relevant players has also been a challenge to those studying protein deacetylation. Camp and Hofmann (28) purified an enzyme (palmitoyl protein thioesterase; PPT1) capable of deacetylating both thioacylated RAS and Ga proteins. However, subsequent work revealed that the enzyme is a lysosomal resident (29, 30), and mutations in the PPT1 gene cause a fatal lysosomal storage disease, infantile neuronal ceroid lipofuscinosis (31, 32). Affected individuals accumulate unidentified cysteine-containing lipid moieties, presumably the endogenous substrates of PPT1, within the cells of brain and other tissues (33).

We report herein the isolation of a second acyl-protein thioesterase (APT1). Identification of the gene encoding this enzyme revealed that the protein had been purified previously as a lysophospholipase (34). We demonstrate that thioacylated proteins are preferred substrates for this enzyme in vitro and that it can act in vivo to deacetylate Gα. We propose that APT1 represents the first bona fide player in the regulated thioacylation of intracellular proteins.

EXPERIMENTAL PROCEDURES

Materials—All chromatographic resins were purchased from Amer sham Pharmacia Biotech; isopropyl alcohol was from Sigma or Calbiochem, unless otherwise noted. Antiserum P054, directed against whole PPT1, was a gift from Dr. Sandra Hofmann. Polyclonal antiserum 584, directed against Gα (35), and monoclonal ascites 12CA5, directed against the HA epitope, were supplied by Dr. Susanne Mumbly.

Methods—Molecular biological procedures were carried out using standard methods (36). Qiagen products were used for DNA preparation. The polymerase chain reaction was performed using VENT ther moplastic polymerase (New England Biolabs) under conditions recommended by the manufacturer. Peptide sequencing of APT1 was carried out by Carolynn Moomaw and Steve Afendis in the Biopolymer Core Facility of The University of Texas Southwestern Medical Center.

Palmiotly-CoA α-Glucuronidase—Myristoylated Gα1, synthesized in Escherichia coli and prepared as described by Duncan and Gilman (21), was incubated with [3H]palmitoyl-CoA (700 cpm/pmol) in HMEC buffer supplemented with 200 ml of 3m (NH4)2SO4, and, after 10 min, the mixture was again centrifuged at 100,000 g for 30 min. The supernatant was centrifuged at 100,000 g for 30 min, and the pellets were frozen in liquid N2. The cells were packed in a 26/10 FPLC column. This column was subsequently eluted with 200 ml of TE buffer containing 1 m NaCl. The flow-through fractions containing thioesterase were brought to 1 m (NH4)2SO4 by slow addition of 3 m (NH4)2SO4. After stirring for 15 min, the sample was centrifuged at 100,000 g for 30 min, and the supernatant was applied to 40 ml of phenyl-Sepharose HP packed in a 26/10 FPLC column. This column was washed with 150 ml of TE buffer and then eluted with a 300-ml gradient of ethylene glycol from 0 to 75% in TE buffer. Thioesterase-containing fractions were dialyzed for 16 h against ME buffer (75 m NaMES (pH 6.0), 1 m EDTA) prior to application to 12 ml of SP-Sepharose FF equilibrated in ME buffer; the flow-through was collected. The column was eluted with a single 100-ml application of ME buffer containing 1 m NaCl. Thioesterase activity was found in both the flow-through and eluate. However, immunoblots indicated that PPT1 was in the eluate (consistent with its original purification); the flow-through was devoid of PPT1. The flow-through was brought to 1 m (NH4)2SO4 and then applied to 10 ml of phenyl-Sepharose HP packed in a 16/10 column. The column was washed with 40 ml of TE buffer and then eluted with a 75-ml gradient of ethylene glycol (0–75%). Fractions containing thioesterase were pooled and run on a Superdex-200 16/60 preparative gel filtration column equilibrated in HMEC buffer (with no protease inhibitors). Fractions containing thioesterase were concentrated to 100 ng/ml in a Centricon 10 (Amicon), frozen in liquid N2, and stored at −80 °C.

Palmiotly-CoA α-Hydrolase Assay—[3H]Palmitoyl-CoA (15 μl, 200–1000 cpm/pmol) in HMEC buffer was added to 10 μl of enzyme, also in HMEC buffer; reactions were incubated at 30 °C for 2–15 min and stopped by addition of 200 μl of 6% phosphoric acid/CH3CN (1:9). Free palmitate was separated from palmitoyl-CoA by addition of 100 μl of toluene. After centrifugation for 1 min in a microfuge, 100 μl of the organic (upper) phase was processed for liquid scintillation counting. Data shown are averages of duplicate reactions from representative experiments.

Lysophospholipase Assay—[3H]Lysophospholine (lyso-PC) substrate was dried in a speed vac and suspended in HMEC buffer. This substrate was added to enzyme in the same buffer. After incubation at 30 °C for 10–30 min, free palmitate was separated from lyso-PC substrate as described by Lee et al. (37).

APT1 Expression in E. coli—APT1 cDNA was amplified from rat brain cDNA (CLONTECH) using the polymerase chain reaction with the following primers: 5′-GCCCATGGGCCGCAACAGATGCCC-3′ (sense) and 5′-GCAAGCTTCTCATGGGAAAGTTGTATACCC-3′ (antisense); note that Cys2 of APT1 was mutated to Gly to facilitate cloning. The amplified cDNA was digested with NcoI and HindIII and ligated into the bacterial expression vector pQE60-6H (38), which had been modified with the same restriction enzymes. E. coli carrying pQE60-6HAPT1 can pQE60-6Hβ-galactosidase were grown in 50 ml of LB containing 50 μg/ml ampicillin. When cultures reached an absorbance of 0.6, protein synthesis was induced by addition of isopropylthiogalactoside to 200 μM. After 16 h at 30 °C, cells were harvested by centrifugation, and the pellets were frozen in liquid N2. The cells were thawed in 5 ml of TE buffer with protease inhibitors and lysed with lysozyme (1 mg). Soluble lysate was generated by treatment of the lysed cells with 100 ng of DNase, followed by centrifugation at 100,000 g for 30 min.

Stable Expression of APT1 in HEK293 Cells—A vector for expression of APT1 carrying two tandem copies of HSV-Tag at the amino terminus was generated as follows. The polymerase chain reaction was performed using 5′-GCCGTTACCTTCGCGCACACATCGTCCG-3′ (sense) and 5′-GCCCTGAGTCATGGGAAAGTTGTATACCC-3′ (antisense) primers. The product was digested with KpnI and XhoI and ligated into the vector pCD3.1-HSV (provided by Elizabeth Duncan, University of Texas Southwestern), such that the amino-terminal sequence, to Cys9, of the HSV-tagged APT1 was as follows: MQPPEAPEDPDQPELADYRRFF. HEK293 cells were infected with either pCDNA3.1-HSV-APT1 or pCDNA3.1-isi8-β-galactosidase (Clontech) using the CaPPhos Maximizer Transfection kit (CLONTECH) according to the manufacturer’s instructions. The transfected cells were switched to and maintained in selection media (Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, 5 units/ml penicillin, 5 μg/ml streptomycin, 4 μg/ml G418 (Life Technologies, Inc.)) after 30 h. Clonal cell lines were isolated using standard techniques.
Whole cell homogenates were prepared from a single 100-mm plate of HEK293 APT1 or HEK293 β-galactosidase. The cells were scraped into 5 ml of phosphate-buffered saline, pelleted by centrifugation at 1500 × g, and washed with 5 ml of phosphate-buffered saline. The washed cell pellet was frozen in liquid N2 and lysed by addition of 250 μl of HMEC with proteinase inhibitors and five passages through a 26-gauge hypodermic needle. Debris was removed by centrifugation at 16,000 × g for 30 min, and the supernatant was used for designated enzyme assays.

**Metabolic Labeling of Gα1r—** Fifty 100-mm dishes of HEK293 β-galactosidase and HEK293-AP1 cells were transfected with HA-αs-pCDNAI (15) using the Cal-Phos Maximizer Transfection Kit (CLONTECH). After 36 h, the cells were harvested by the plates by washing with Puck’s saline G with 2 mM EDTA and pelleted by centrifugation for 2 min at 500 × g. The pellets were then suspended (106 cells/ml) in 10 ml of labeling media containing Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, and 250 μCi/ml [3H]palmitate (50 Ci/mmol). For labeling time courses, 1 ml of suspended cells was removed at each time point and pelleted by centrifugation for 15 s in a microcentrifuge; the labeling medium was aspirated, and the cell pellet was frozen immediately in liquid N2. For pulse-chase experiments, cells were labeled for 30 min and centrifuged for 2 min at 500 × g. The labeled cell pellet was then suspended in chase medium (Dulbecco’s modified Eagle’s medium and 10% fetal calf serum, supplemented with 50 μM unlabeled palmitate), and at indicated times, the cells were harvested as described above.

**Immunoprecipitation of HA-Gα1r—** Pellets from 1 ml of cell culture were suspended in 1 ml of RIPA buffer (50 mM NaHES (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) with proteinase inhibitors and solubilized by five passages through a 251/2 gauge needle. Debris was removed by centrifugation in a microcentrifuge for 30 min at 4 °C. Monoclonal antibody 12CA5 ascites (1 μl) was added to each solubilized extract. The extracts were incubated with rocking at 4 °C for 1 h. HA-Gα12CA5 complexes were precipitated by the addition of 25 μl of protein G-Sepharose and washed three times with 500 μl of RIPA buffer. HA-Gα was eluted by addition of SDS-PAGE sample buffer (with 5 mM dithiothreitol) and incubation for 3 min at 95 °C. The immunoprecipitates were subjected to SDS-PAGE and transferred to nitrocellulose. These filters were exposed to phosphorimaging analysis (TR2040S imaging plates and BAS1500 scanner; Fuji Medical Systems) after a 1-month exposure. The amount of Gα was quantitated using NIH Image (version 1.61) software on a scanned image of the film.

**Metabolic Labeling and Separation of Cellular Lipids—** Cells were incubated with [3H]palmitate as described for metabolic labeling of Gα1r. To identify the relevant thioesterase activity, we sought to separate it from the lysosomal protein exhibiting the same biochemical activity, PPT1. We chose rat liver as a source of PPT1 mRNA in this tissue (29). The majority of the thioesterase activity was in the soluble fraction (S100) of rat liver homogenate (Fig. 1A), a centrifugation of rat liver homogenate produced P100 (100,000 × g pellet) and S100 (100,000 × g supernatant) fractions. The S100 fraction (1 mg of total protein) was applied to 200 μl of concanavalin A-Sepharose, and protein bound to this matrix was eluted with 200 mM methyl α-D-mannopyranoside. The fractions were assayed for palmitoyl-Gα1r thioesterase activity. The activity in each fraction is expressed as a percentage of total homogenate activity (left panel) or total soluble activity (right panel). B, equal amounts of thioesterase activity (10 pmol/min) from fractions described in A were subjected to SDS-PAGE and analyzed by immunoblotting using antisemur P054, directed against PPT1.

**RESULTS**

Purified myristoylated Gα1r was palmitoylated in vitro with [3H]palmitoyl-CoA to provide a source of radiolabeled, thioacetylated Gα1r, (21), a suitable substrate for palmitoyl protein thioesterase assays similar to those previously described for palmitoyl-RAS (28). To identify the relevant thioesterase activity, we sought to separate it from the lysosomal protein exhibiting the same biochemical activity, PPT1. We chose rat liver as a source of thioesterase activity because of the relatively low abundance of PPT1 mRNA in this tissue (29). The majority of the thioesterase activity was in the soluble fraction (S100) of rat liver homogenate (Fig. 1A). However, immunoblot analysis of aliquots containing equal amounts of activity from the particulate (P100) and S100 fractions with an antiserum directed against PPT1 revealed that the membrane fraction contained approximately four times more PPT1 than did the soluble fraction (Fig. 1B). The P100 fraction was further fractionated on a concanavalin A-Sepharose chromatography. Approximately 70% of the soluble thioesterase activity flowed through the column, and this fraction was devoid of immunologically detectable PPT1 (Fig. 1, A and B). The enzyme(s) present in this flow-through fraction removed thioester-bound palmitate from Cys3 of Gα1r, but failed to remove amide-linked [3H]myristate from Gly2 of the protein (data not shown). Separation of reaction products by thin layer chromatography demonstrated that palmitate was the isopropyl alcohol-soluble radiolabeled product measured in the thioesterase assay (data not shown).

The acyl-protein thioesterase activity not attributable to PPT1 (i.e. APT1) was purified from rat liver S100 by ammonium sulfate precipitation and six chromatographic steps (Table 1). During the final step, Superdex-200 gel filtration chromatography, a single peak of thioesterase activity comigrated with a single protein peak, measured by absorbance at 280 nm, with an apparent molecular mass of 29 kDa (Fig. 2A). A single silver-staining band, with a mobility corresponding to a molecular mass of 27 kDa, was detected by SDS-PAGE in fractions containing this activity (Fig. 2B). All thioesterase activity (PPT1 and APT1) behaved homogeneously at every step of this procedure with two exceptions. First, PPT1 bound to SP-Sepharose, permitting separation of PPT1 and APT1 (Fig. 2C). Second, a minor peak of thioesterase activity (approximately 10% of that recovered) eluted between 30 and 55% ethylene glycol on the first phenyl-Sepharose column; the bulk of the activity (including PPT1 and APT1) eluted between 40 and 55% ethylene glycol (data not shown). The activity in the minor peak was purified by subsequent chromatographic steps identical to those described for the major peak. Although this protein exhibited a lower specific thioesterase activity, it was indistinguishable by SDS-PAGE and silver staining from the purified APT1 contained in the major peak (data not shown). Sequenc-
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TABLE I
Purification of APT1

| Step                  | Total protein (mg) | Specific activity (nmol/min/mg) | Activity (%) | Purification (fold) |
|-----------------------|--------------------|---------------------------------|--------------|--------------------|
| S100                  | 10,000             | 0.19                            | 100          | 1                  |
| (NH₄)₂SO₄ supernatant | 7100               | 0.27                            | 100          | 1.5                |
| Butyl-Sepharose pool  | 500                | 1.9                             | 51           | 10                 |
| Q-Sepharose pool      | 170                | 7.1                             | 64           | 38                 |
| Phenyl-Sepharose pool | 2.3                | 240                             | 29           | 1300               |
| SP-Sepharose pool     | 0.15               | 2800                            | 21           | 14,000             |
| Phenyl-Sepharose (b) pool | 0.14           | 1200                             | 8.6          | 6500               |
| Superdex-200 pool     | 0.03               | 3400                            | 5.5          | 18,000             |

Purification of APT1 using autoacetylated Gαi, and [3H]palmitoyl-RAS (isolated from S9 cells as described by Camp and Hofmann (28)). Purified APT1 removed palmitate from both proteins (Fig. 3A). Because the stoichiometry of palmitoylation of Ha-RAS isolated in this manner is unknown, detailed comparison of kinetic parameters for the two substrates is not possible. However, the assays were performed at substrate concentrations well below the Km of the enzyme, demonstrated by linear increases in initial reaction rates with increasing substrate concentration (data not shown). Under these conditions, the catalytic efficiencies (Vmax/Km) of the enzyme for each substrate should be inversely proportional to the t1/2 of substrate in the reaction. The catalytic efficiency of APT1 for palmitoyl-Gαi was roughly 3-fold higher than the value for palmitoyl-RAS.

Activation of a heterotrimeric G protein in vitro by an appropriately coupled receptor causes increased turnover of palmitate on the G protein α subunit. Wedegaertner and Bourne (12) found that addition of AlF₄⁻, which activates heterotrimeric G proteins, increased the rate of palmitate loss from Gα in broken cell preparations. We sought to confirm the activation-dependent regulation of deacylation using purified proteins. Gα heterotrimer was autoacetylated as described previously (21). When this palmitoylated protein was used as a substrate for APT1 in the presence or absence of AlF₄⁻, the activated, deacetylated α subunit was deacylated more rapidly than the inactive heterotrimer (Fig. 3B). Similar results were obtained with Gα (data not shown). Palmitoyl-Gα was never fully deacylated in these reactions; during substrate preparation some palmitoyl-Gα was denatured, and this protein is no longer a substrate for APT1. Since the rate of deacylation of Gα proteins by APT1 is not affected by the identity of the bound nucleotide (GDP or GTPγS; data not shown), the enhanced rate of deacylation in vitro following activation is explained by the fact that the free α subunit is a better substrate for APT1 than is the heterotrimer. Thus, the cycle of G protein subunit dissociation and association can apparently account for regulation of Gα deacylation in vitro.

To identify the gene encoding APT1, the purified protein was subjected to tryptic digestion and peptide sequencing. Three peptide sequences were found in the deduced amino acid sequence of a recently purified 25-kDa rat lysophospholipase (Fig. 4) (34). The mouse ortholog of this protein has also been cloned, again based on purification of a lysophospholipase activity (40). Data from various genome sequencing projects allowed us to identify putative orthologs of this protein in many species, including Schistosoma mansoni, Saccharomyces cerevisiae, Caenorhabditis elegans, and Homo sapiens (Fig. 4). These orthologs contain several highly conserved regions, including one between residues 113 and 131 of the rat amino acid sequence. This region contains a Gly-X-Ser-X-Gly motif found in a wide variety of proteins that exhibit esterase activity. APT1 lacks the sequence Gly-Asp-His near its carboxyl terminus; this sequence is found in PPT1 and other enzymes with thioesterase activity (29). The predicted molecular weight of 25,000 is consistent with the observed migration of purified APT1 by SDS-PAGE and gel filtration chromatography. Although there is similarity between lysophospholipid and palmitoyl-Gα substrates (an oxyester-bound fatty acid versus a thioester-bound fatty acid), we wished to confirm the notion that APT1 has both lysophospholipase and palmitoyl-Gα thioesterase activity. The rat lysophospholipase gene was expressed in E. coli as a hexahistidine-tagged fusion protein. Lysates from bacteria expressing rat lysophospholipase contained substantially higher activities of both lysophospholipase and palmitoyl-Gα thioesterase than did their β-galactosidase-expressing counterparts (Fig. 5). Purification of recombinant APT1 using Ni-NTA agarose and gel filtration chromatography indicated that this protein was indeed responsible for the observed activities (data not shown).

Because of the broad substrate specificity of APT1 in vitro, we were unsure of its physiological substrate. To address this issue biochemically, we assayed the activity of the enzyme while titrating three different substrates presented in mixed detergent micelles (10 mM CHAPS) under otherwise identical conditions (Fig. 6). The apparent Km of APT1 for the acyl protein substrate was 25- and 250-fold lower than the values observed for palmitoyl-CoA or lysophosphocholine (lyso-PC), respectively. In addition, the catalytic efficiency (Vmax/Km) for the acyl thioester substrates (palmitoyl-CoA and palmitoyl-Gα) was at least 200-fold higher than that for the acyl ester containing lyso-PC. Although the acyl moiety of all three substrates presumably projects into the hydrophobic core of the detergent micelles, the relative accessibility of each substrate under these or physiological conditions (with substrates present on the surface of a phospholipid bilayer) is not known. Thus, under these assay conditions, acyl proteins are the preferred substrate for APT1 in vitro.

To demonstrate that palmitoyl-Gα proteins could be substrates for this enzyme in vivo, we stably transfected 293 cells with plasmids carrying the rat APT1 cDNA fused to the HSV Tag, an epitope derived from the herpes simplex glycoprotein.

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4 Partially purified hexahistidine-tagged APT1, expressed in E. coli, exhibited substrate specificity (Vmax/Km for palmitoyl-Gα > palmitoyl-CoA >> lyso-PC) and specific activities in vitro similar to the purified, nonrecombinant enzyme (data not shown).
D, or the E. coli β-galactosidase cDNA. Whole cell homogenates from these cells were assayed for their ability to hydrolyze lyso-PC, palmitoyl-α,1, and palmitoyl-CoA. The cells transfected with APT1 showed roughly 2-fold elevations in APT and lysophospholipase activity (Fig. 7A). However, there was no detectable increase in palmitoyl-CoA thioesterase activity, presumably because of the large number of palmitoyl-CoA hydro-lase activities within normal cells (41). The expression of APT1 was confirmed by immunoblot analysis using a monoclonal antibody specific for the HSV Tag epitope (data not shown). We used these cell lines to determine if APT1 could act on thioacylated proteins in vivo. The thioacylation of Gαs is well characterized; deacylation of the protein is the rate-limiting step for incorporation of exogenously added palmitate (10). These cell lines were transfected with a cDNA encoding Gαs with an HA tag (15). After incubation with [3H]palmitate, cells expressing APT1 exhibited a moderate increase in the rate of incorporation of label into Gαs compared with cells expressing β-galactosidase (Fig. 7B), consistent with the level of expression of APT1. A similar increase in rate was observed when loss of radioactivity from Gαs was monitored in four separate pulse-chase experiments (Fig. 7C). We were concerned that the

**Fig. 2. Purification of APT1, the non-PPT1 acyl-protein thioesterase.** A, APT1 (pooled from the second phenyl-Sepharose column described in Table I) was eluted from a preparative Superdex-200 gel filtration column. The thioesterase activity of fractions (circles) and absorbance at 280 nm (dotted line) is plotted against the volume of elution. The volume of elution of molecular weight standards are indicated by arrowheads. B, proteins in fractions collected from the Superdex 200 column were resolved by SDS-PAGE and visualized by silver staining. Fractions containing thioesterase activity are indicated by the bar below the fraction numbers. C, protein pools from each step documented in Table I were analyzed by SDS-PAGE and immunoblotting with antiserum reactive with PPT1. Lanes 1–4 contain 2 μg of protein from the S100, (NH₄)₂SO₄ supernatant, butyl-Sepharose pool, and Q-Sepharose pool, respectively. Lanes 4–8 contain 0.4 μg of protein from the phenyl-Sepharose (a) pool, SP-Sepharose pool, phenyl-Sepharose (b) pool, and Superdex-200 pool, respectively. Longer exposure reveals PPT1 in the starting material (lane 1).

**Fig. 3. Analysis of palmitoyl protein substrates for APT1.** A, [3H]palmitoyl-Gαs (50 nM, circles) and [3H]palmitoyl-RAS (15 nM, squares) were used separately as substrates for APT1. Reactions were initiated by addition of APT1 purified from rat liver (0.5 ng). Released palmitate was measured as described under "Experimental Procedures" and plotted as a percentage of the maximum amount of palmitate released versus time. B, Gs heterotrimer was autoacylated in the presence of 50 μM [3H]palmitoyl-CoA at room temperature for 1 h. Palmitoyl-Gαs heterotrimer (100 nM final concentration) was diluted with reaction buffer with (circles) or without (squares) AlF₄⁻, and depalmitoylation was initiated by the addition of 0.25 ng of APT1. Data are plotted as a percentage of the initial [3H]palmitate bound to Gαs.
palmitoyl-CoA hydrolase activity of APT1 might increase the rate of turnover of palmitoyl-CoA and cause an apparent increase in the rate of G\textsubscript{a}\textsubscript{deacylation}. To be incorporated into cellular lipids, exogenously added palmitate must first be incorporated into the palmitoyl-CoA pool. We observed no change in the rate of incorporation of [\textsuperscript{3}H]palmitate into cellular lipids in cells overexpressing APT1 (Fig. 7D). This set of experiments demonstrates that APT1 is able to remove thioacyl groups from G\textsubscript{a}\textsubscript{1} in vivo.

**DISCUSSION**

Isolation of enzymes based on biochemical activities can lead to the identification of molecules whose function is tangential to the activity in hand. The search for an enzyme responsible for deacylation of intracellular thioacylated proteins highlights two such tales. The first palmitoyl protein thioesterase activity to be purified and cloned was PPT1. A variety of data indicate that the role of this lysosomal enzyme is to hydrolyze cysteine-containing lipid products rather than to deacylate intracellular proteins (33). We have now purified and characterized a second palmitoyl protein thioesterase activity; APT1 had already been purified as a lysophospholipase (34).

Several lines of evidence suggest that intracellular deacylation of at least some thioacylated proteins is the actual function of this enzyme. During the purification of APT1, the palmitoyl-G\textsubscript{i}\textsubscript{1} thioesterase activity was purified at least 18,000-fold, and only one other species of activity was observed (PPT1). During the purification of lysophospholipase (the same polypeptide as APT1), lysophospholipase activity was enriched only 500-fold over the starting material because only one of many characterized lysophospholipase activities was purified (34). We have shown that thioacylated proteins are the preferred substrate of APT1 in vitro. APT1 has both a substantially lower \( K_M \) and a higher catalytic efficiency (\( V_{\text{max}}/K_M \)) for palmitoyl-G\textsubscript{i}\textsubscript{1} substrate when compared with lyso-PC.

In addition to these suggestive arguments, two other pieces of evidence support our hypothesis that APT1 is an acyl-protein thioesterase in vivo. First, the product of the phospholipase B gene (PLB1) of \textit{S. cerevisiae} has both phospholipase B activity and lysophospholipase activity. Disruption of PLB1 eliminates more than 99% of lysophospholipase activity, revealed by both biochemical assay and metabolic labeling studies (37). These data suggest that the putative lysophospholipase activity of \textit{S. cerevisiae} APT1 contributes insignificantly to the total lysophospholipase activity within this species. Finally, we have also demonstrated that overexpression of APT1 leads to an increase in the basal rate of turnover of palmitate in G\textsubscript{a}\textsubscript{1}. Studies on the effects of such overexpression on the turnover of APT1...
thioacyl moieties bound to other proteins are in progress. When appropriate G protein-coupled receptors are activated, they trigger at least a 10-fold increase in the rate of deacylation of G\textsubscript{s}α; however, such stimulation has been shown to not alter the stoichiometry of thioacylation of G\textsubscript{s}α (10, 13). It is thus unlikely that the 2-fold overexpression of APT1 that we achieved by stable transfection causes a decrease in the steady-state stoichiometry of thioacylation of G\textsubscript{protein} α subunits. Dramatic overexpression of APT1 might lower the stoichiometry of Gα thioacylation in vivo. However, it is important to note that interpretation of results from studies of G protein signaling under these conditions would be complicated by thioacylation of at least five components of the G protein signaling

**FIG. 6.** Palmitoyl-G\textsubscript{s}α is the preferred substrate for lysophospholipase/APT1. A, the initial rate of lysophospholipase activity was measured with 10 ng of APT1 purified from rat liver. B, the initial rate of palmitoyl-CoA hydrolysis activity was measured with 10 ng of APT1 purified from rat liver. Data shown are averages of duplicate reactions for representative experiments performed in triplicate. Curves shown are fit to the Michaelis-Menten rate equation for each data set.

**FIG. 7.** APT1 removes palmitate from G\textsubscript{s}α in vivo. A, extracts from HEK293 cells stably transfected with cDNAs encoding β-galactosidase (control, open bars) or APT1 (hatched bars) were assayed for lysophospholipase, palmitoyl-G\textsubscript{s}α thioesterase, and palmitoyl-CoA hydrolase activities as described under “Experimental Procedures.” B, HEK293 cells expressing β-galactosidase (control, circles) or APT1 (squares) were transiently transfected with cDNA encoding an HA epitope-tagged form of G\textsubscript{s}α (HA-G\textsubscript{s}α). The cells were incubated with \textsuperscript{3}H-palmitate, and at the indicated times, tritium specifically incorporated into HA-G\textsubscript{s}α was quantified as described under “Experimental Procedures.” Data plotted are arbitrary units of specific incorporation of tritium into G\textsubscript{s}α, determined by dividing the tritium counts (phosphorimager data) by the relative amounts of G\textsubscript{s}α protein (immunoblot data). The two panels demonstrate two independent experiments. The data are fit to the equation for a single exponential rise (f = a(1 - e\textsuperscript{-kt})). C, cells transfected with HA-G\textsubscript{s}α cDNA were incubated with \textsuperscript{3}H-palmitate and, at the indicated times, tritium specifically incorporated into HA-G\textsubscript{s}α was quantified as described under “Experimental Procedures.” Data plotted are arbitrary units of specific incorporation of tritium into G\textsubscript{s}α, determined by dividing the tritium counts (phosphorimager data) by the relative amounts of G\textsubscript{s}α protein (immunoblot data). The two panels demonstrate two independent experiments. The data are fit to the equation for a single exponential decay (f = a(e\textsuperscript{-kt}) + c). D, cells expressing β-galactosidase (control) and APT1 were incubated with \textsuperscript{3}H-palmitate as described in B. At the indicated times, cellular lipids were extracted with chloroform/methanol (1:1), separated by thin layer chromatography, and visualized by fluorography (2-day exposure). The position of palmitate is indicated by the arrow.
machinery, including Gα proteins themselves, seven transmembrane receptors, G protein-coupled receptor kinases, adenylyl cyclases, and certain regulator of G protein signaling proteins (42–45). Despite repeated attempts to isolate 293 cell clones with higher levels of expression of APT1, enzyme activity was never increased more than 3-fold and HSV-tagged protein, assayed by immunoblotting, never varied by more than 2-fold among clones (data not shown). These observations are consistent with toxicity resulting from high levels of APT1 or protein, assayed by immunoblotting, never varied by more than 3-fold and HSV-tagged APT1 to specific protein complexes (34) while studying APT1 as a lysophospholipase. Dramatic overexpression of APT1 (e.g. with recombinant adenoviral vectors) or targeting of overexpressed APT1 to specific protein complexes (e.g. with APT1 fusion proteins) may provide a tool for specifically probing the role of thioacyl cysteine residues in cellular processes.

Genetic or pharmacological inactivation of APT1 will ultimately demonstrate whether it is the only enzyme within the cell responsible for deacylating Gα subunits or other thioacylated proteins. To this end, putative orthologs of APT1 have been identified in mouse and S. cerevisiae, systems commonly used for genetic manipulations. We have used targeted disruption to create S. cerevisiae strains lacking APT1. These yeast lack detectable acyl-protein thioesterase activity, and we are currently characterizing their phenotype.

We believe the identification of APT1 will allow many studies of the function of thioacyl cysteine residues in cellular processes.

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Acyl-Protein Thioesterase

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