Characterization of \textit{Saccharomyces cerevisiae} Acyl-protein Thioesterase 1, the Enzyme Responsible for G Protein \(\alpha\) Subunit Deacylation \textit{in Vivo}*

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Thioacylation is a reversible lipid modification of proteins that plays a role in the regulation of signal transduction. Acyl-protein thioesterase 1 (APT1) was identified as an enzyme capable of deacylating some thioacylated proteins \textit{in vitro}. \textit{Saccharomyces cerevisiae} open reading frame YLR118c encodes an enzyme homologous to \textit{Rattus norvegicus} APT1. We demonstrate that the catalytic activity of the protein encoded by the yeast open reading frame is similar to that of rat APT1, and we designate the protein \textit{S. cerevisiae} APT1p. Yeasts bearing a disruption of the \textit{APT1} gene lack significant biochemically detectable acyl-protein thioesterase activity. They also fail to deacylate Gpa1p, the yeast G\(\alpha\) subunit, in metabolic radiolabeling studies. We conclude that native APT1 is the enzyme responsible for G\(\alpha\) subunit deacylation in \textit{S. cerevisiae} and presumably other eukaryotes as well.

The covalent attachment of lipid moieties to polypeptide backbones is required for the biological activity of many eukaryotic proteins. Lipid modification of proteins has been found to play roles in protein stability, catalytic activity, protein-protein interactions, and subcellular localization. Isoprenylation, N-myristoylation, and thioacylation are the best characterized forms of lipid modifications of intracellular proteins. Of these, only thioacylation (also called palmitoylation or S-acylation) is metabolically reversible. Thioacylated proteins cycle between acylated and deacylated states, allowing thioacylation (like other, better characterized, reversible covalent modifications) to act as a regulated switch of protein activity.

Proteins involved in many signal transduction pathways are thioacylated, including Ras proteins, nonreceptor tyrosine kinases, and nitric-oxide synthase. Heterotrimeric G protein-regulated signaling systems have a relative abundance of thioacylated proteins, including many G protein-coupled receptors, G\(\alpha\) subunits, regulator of G protein signaling (RGS) proteins, and G protein-coupled receptor kinases (1–4). Some thioacylated G\(\alpha\) proteins have a higher affinity for G\(\beta\gamma\) complexes and are relatively resistant to the GTPase-accelerating properties of RGS proteins when compared with their nonacylated counterparts \textit{in vitro} (5, 6). These alterations in the biochemical properties of G\(\alpha\) proteins suggest that thioacylation has an effect on G protein-coupled signaling \textit{in vivo}. Additionally, mutant G\(\alpha\) proteins that lack thioacylated cysteine residue(s) are localized to internal rather than plasma membranes, indicating that thioacylation probably plays a role in the subcellular localization of signaling events (7, 8).

The deacylation of G\(\alpha\) proteins (and other thioacylated proteins) is the regulated step of the thioacylation cycle. In the basal state, G\(\alpha\) proteins are thought to be thioacylated stoichiometrically and associated with G\(\beta\gamma\). Upon activation by ligand-bound receptor, G\(\alpha\) proteins dissociate (at least partially) from G\(\beta\gamma\), and the rate of deacylation (determined by metabolic radiolabeling) increases dramatically (9, 10). Similar increases in thioester-bound palmitate turnover have been noted for other thioacylated proteins involved in signal transduction pathways, including several G protein-coupled receptors and endothelial cell nitric-oxide synthase (11–13). The role that the regulated deacylation of G\(\alpha\) plays in the signal transduction process has yet to be determined.

The regulated deacylation of G\(\alpha\) and other proteins is presumed to be carried out by an intracellular thioesterase. An enzyme, designated acyl-protein thioesterase 1 or APT1, that performs regulated deacylation of G\(\alpha\)a and other thioacylated proteins \textit{in vitro} was isolated from rat liver (14). Although this enzyme had previously been identified as a source of lysophospholipase activity, palmitoyl-G\(\alpha\)a is a better substrate for APT1 than lysophospholipids by several hundred-fold (14, 15). Furthermore, overexpression of APT1 in cultured cells accelerates palmitate turnover on G\(\alpha\)a and endothelial cell nitric-oxide synthase. Although overexpressed APT1 is capable of deacylating proteins \textit{in vivo}, proof of the involvement of the native enzyme in this process has been lacking until now.

Putative APT1 orthologs exist in many eukaryotic organisms, including the budding yeast \textit{Saccharomyces cerevisiae}. We now have demonstrated that YLR118c, a \textit{S. cerevisiae} open reading frame, encodes an enzyme with both lysophospholipase and acyl-protein thioesterase activity \textit{in vitro}. Targeted disruption of the gene encoding APT1p (YLR118c) reveals that \textit{S. cerevisiae} APT1p appears to be responsible for virtually all of the acyl-G\(\alpha\) thioesterase activity detectable in yeast extracts. Furthermore, the turnover of palmitate associated with the yeast G\(\alpha\), Gpa1p, is dramatically reduced in yeast lacking the \textit{APT1} gene, demonstrating that APT1p is actually involved in deacylation of G\(\alpha\) subunits \textit{in vivo}.

**EXPERIMENTAL PROCEDURES**

\textit{Materials and Miscellaneous Procedures}—Ni\(^{2+}\)–NTA-agarose was purchased from Qiagen. All other chromatographic resins were acquired from Amersham Biosciences. Isotopes were from PerkinElmer Life Sciences. All chemicals were supplied by Sigma or Calbiochem,

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unless otherwise noted. Media for growth of S. cerevisiae were obtained from either CLONTECH or Bio 101, when available; other media were prepared as described by Guthrie and Fink (16). Yeast strain YPH501 was obtained from Dr. Henrik Dohlam (Yale University School of Medicine, New Haven, CT). The plasmid pRS303 was obtained from Dr. Joel Goodman (University of Texas Southwestern Medical Center, Dallas, TX). The plasmid pKMI1062-2 was from Carol Manahan (Washington University School of Medicine, St. Louis, MO). The FUS1-galactosidase pheromone-responsive reporter plasmid, pBS234, was prepared as described by Guthrie and Fink (16). Yeast strain YPH501 was isolated from YPH501 Matα/leu2–Δ1 ure3–Δ2 his3–Δ200 ade2–101 i/ade2–101 i; trp1–Δ63/tra1–Δ63 harbored the plasmid pQE60–6H–eAPT1 or pQE60–6H–A PI T1 (encoding the rat enzyme) was grown in 6 liters of LB medium containing 50 mg/liter ampicillin. Recombinant protein expression was induced as previously described for the expression of Goα, (19). The bacterial pellet was harvested and frozen in liquid N2. The cells were thawed in 250 ml of buffer TP (50 mM Tris–HCl (pH 8.0) and proteinase inhibitors (leupeptin, 6 μg/ml; lima bean trypsin inhibitor, 6 μg/ml; t-1-tosyl-amino-2-phenylethyl chloromethyl ketone, 32 μg/ml; 1-chloro-3- tosylamido-2-aminopeptane, 32 μg/ml; and aprotinin, 2 μg/ml)) and lysed by treatment with lysozyme (50 mg), followed by 500 ng of DNase I. A soluble lysate was generated by centrifugation of the lysed cells at 100,000 × g for 30 min. Recombinant APT1 was purified by application of the soluble lysate to a column containing 20 ml of Ni2+-NTA-agarose. The resin was washed by adding 50 ml of buffer B (50 mM Tris–HCl (pH 8.0), 100 mM imidazole, and 100 mM NaCl) and eluted with 50 ml of buffer C (50 mM Tris–HCl (pH 8.0) with 100 mM imidazole). The eluted protein was concentrated in a Centricon 30 (Amicon) and dialyzed against two changes of HME (20 mM Na-HEPES (pH 8.0), 2 mM MgCl2, and 1 mM EDTA) buffer (2 liters each) over 16 h at 4 °C. Aliquots of this dialyzed protein (~19 mg/ml) were frozen in liquid N2 and stored at −80 °C. Roughly 3 g of each recombinant APT1 was recovered using this protocol.

Preparation of Acyl-protein Substrates—Palmitoylated Ha-Ras was prepared essentially as described by Camp and Hofmann; however, CHAPS detergent (1% (w/v) for extraction, 0.1% elsewhere) was substituted for n-octyl glucoside (23). Palmitoyl-Goα1 and palmitoyl-GRS4 were both prepared using hexahistidine-tagged, bacterially expressed recombinant proteins. Each protein was diluted to ~50 μM in HEME (HME supplemented with 10 mM CHAPS) buffer containing 50 μM [3H]palmitoyl-CoA (200–2000 cpm/pmol) and incubated at 30 °C for 20 min. Incorporation of palmitate into these proteins was assessed by precipitation with trichloroacetic acid and found to be ~0.7 mol of palmitate/moL of Goα1 and ~1.3 mol of palmitate/moL of RGS4 (24). The reaction mixtures were diluted to 5 ml in TC buffer (50 mM Tris–HCl (pH 8.0) and 10 mM CHAPS) and applied to 300 μl of Ni2+-NTA-agarose equilibrated in the same buffer. The columns were washed five times with 3 ml of TC buffer and eluted with 1.5 ml of HEME buffer supplemented with 100 mM EDTA. The eluted palmitoylated proteins were diluted 1:20 in HEME buffer and concentrated in a Centricon 30 (Amicon) to ~50 μM palmitoyl-protein (assessed by liquid scintillation spectrometry). The concentrated acyl-protein substrates were frozen in liquid N2 and stored at −80 °C until use.

Construction of APT1 Targeting Plasmid—The following DNA fragments were amplified using the polymerase chain reaction with primer sequences listed in Table II and template DNA indicated in parentheses: the 5′-flanking region of YLR118c (5′Frag fragment, genomic DNA isolated from YPH501), the 3′-flanking region of YLR118c (3′Frag fragment, genomic DNA isolated from YPH501), and the entire HIS3 gene (HIS3 fragment, plasmid pRS303). The oligonucleotide primers were

### Table I

| Strain | Genotype | Source |
|--------|----------|--------|
| YPH501 | Matα/a; ura3–52/ura3–52; his3–Δ200/his3–Δ200; ade2–101 i/ade2–101 i; leu2–Δ1/leu2–Δ; trp1–Δ63/tra1–Δ63; lys2–801mm/lys2–801mm; APT1/APT1 | Ref. 32 |
| YAD100 | Matα/a; ura3–52/ura3–52; his3–Δ200/his3–Δ200; ade2–101 i/ade2–101 i; leu2–Δ1/leu2–Δ; trp1–Δ63/tra1–Δ63; lys2–801mm/lys2–801mm; APT1/APT1 Δ1: HIS3 | This work |
| YAD101 | Mata; ura3–52; his3–Δ200; ade2–101 i; leu2–Δ1; trp1–Δ63; lys2–801mm; APT1 | This work |
| YAD102 | Mata; ura3–52; his3–Δ200; ade2–101 i; leu2–Δ1; trp1–Δ63; lys2–801mm; APT1 | This work |
| YAD103 | Mata; ura3–52; his3–Δ200; ade2–101 i; leu2–Δ1; trp1–Δ63; lys2–801mm; trp1–Δ1; HIS3 | This work |
| YAD104 | Mata; ura3–52; his3–Δ200; ade2–101 i; leu2–Δ1; trp1–Δ63; lys2–801mm; apt1 Δ1: HIS3 | This work |

### Table II

| Amplified fragment | Sense/Antisense | Sequence |
|--------------------|-----------------|----------|
| YLR118c 5′-flanking region | Sense | 5′-GGCCTTCAGCTTGTTGGTTGATGATGTC3′ |
| YLR118c 5′-flanking region | Antisense | 5′-GGAGCTCAAATTGATAGCCTAAATAGG3′ |
| YLR118c 3′-flanking region | Sense | 5′-GGGATCCCAATGGATAGCCTGAGCC3′ |
| YLR118c 3′-flanking region | Antisense | 5′-CGAGCAATTCTTCTTCTCTTCTTCTCTTCTTCTTTG3′ |
| HIS3 gene | Sense | 5′-GGGATCCATAATTCCTTTTTAAGGG3′ |
| HIS3 gene | Antisense | 5′-GGGATCCATGGCGTTATTTTCCACCCG3′ |
designed to produce restriction endonuclease sites on each end of the DNA fragments to facilitate cloning. The 5′FR fragment was digested with PstI and BamHI and ligated into the plasmid pGEM-4Z (Promega), which had been digested with the same enzymes. The resulting plasmid, pGEM-5FR, was cut with EcoRI and BamHI and ligated with the similarly digested 3′FR fragment. The product of this ligation, pGEM-5FR-3FR, was opened at the BamHI site lying between 5FR and 3FR, and the HIS3 fragment was inserted, producing the complete targeting plasmid, pGEM-5FR-HIS3-3FR. The linear targeting fragment, used for transformation of S. cerevisiae strains, could then be excised from pGEM-5FR-HIS3-3FR using EcoRI and PstI.

Preparation of S. cerevisiae Extract— Cultures of S. cerevisiae, typically 50–250 ml, were grown in either YPD medium (or synthetic dropout medium for maintenance of the indicated plasmids) at 30 °C to stationary phase (culture A$_{600}$ of $>$2.5). The cells were then pelleted by centrifugation for 30 min at 2,500 × g. The cell pellet was washed with H$_2$O, collected by centrifugation, and frozen in liquid N$_2$. Frozen yeast were suspended in five packed cell volumes of HEC buffer (50 mM Na-HEPES (pH 8.0), 10 mM EDTA, and 10 mM CHAPS) supplemented with protease inhibitors (see above). The cell suspension was frozen in liquid N$_2$ and thawed at 37 °C three times. The extraction procedure was repeated by rocking the suspension at 4 °C for 30 min. The CHAPS-solubilized cell extract was finally isolated by centrifugation of the cell suspension for 45 min at 35,000 × g and collection of the supernatant fraction.

Metabolic Labeling with [3H]Palmitate and Analysis of Yeast Lipids— Indicated strains of S. cerevisiae were grown in complete synthetic medium. When the cultures reached an A$_{600}$ of 0.5, [3H]palmitate in ethanol (50 μCi/ml, 1% ethanol final) was added to the culture. At the indicated times, 25 μl of each culture was removed and frozen in liquid N$_2$. These samples were thawed by the addition of 25 μl of PDI buffer (100 mM NaPO$_4$ (pH 7.4), 10 mM dithiothreitol, and 5 mg/ml yeast lytic enzyme) and incubated for 1 min at 37 °C. The total lipids were extracted by the addition of 500 μl of chloroform/methanol (1:1), vigorous vortexing, and incubation for 5 min at 45 °C. The samples were centrifuged at 13,000 × g for 10 min, and the lipid-containing supernatants were analyzed by thin layer chromatography (24).

Metabolic Labeling with [14C]Acetate or [32P]Orthophosphate and Analysis of Yeast Lipids—S. cerevisiae were grown to an A$_{600}$ of 0.5 in complete synthetic medium. The yeast were then transferred into synthetic minimal medium and grown at 30 °C for 45 min. Metabolic labeling reagents ([14C]acetate (10 μCi/ml) or [32P]orthophosphate (10 μCi/ml)) were added to the appropriate cultures, and the yeast were grown for an additional 2 h. The cells were collected by centrifugation at 4,000 × g and resuspended in 100 μl of PDL buffer. The lipid extraction proceeded as described above, except 1 ml of chloroform/methanol was used. The lipid extracts were then dried under N$_2$ and suspended in 50 μl of chloroform/methanol (2:1). The resuspended lipids (10 μl) were subjected to thin layer chromatography using PE Sil G thin layer chromatography plates (Whatman) developed with chloroform/methanol/ammonium hydroxide/H$_2$O (65:25:2:2) mobile phase. The migration of radiolabeled lipids was detected using an MP1000 phosphorimaging screen and BAS1500 scanner (Fuji Medical Systems).

RESULTS

Isolation of Recombinant Proteins—AP1 from R. norvegicus and the open reading frame YLR118C from S. cerevisiae were expressed as hexahistidine-tagged fusion proteins in E. coli. These enzymes were synthesized efficiently in E. coli, accumu-
FIG. 2. The palmitoyl-G\(\alpha\)i1 substrate preference is conserved between \textit{R. norvegicus} APT1 and \textit{S. cerevisiae} Apt1p. A, the initial rate of lysophospholipase activity was measured with 10 ng of recombinant \textit{R. norvegicus} APT1 (left) or \textit{S. cerevisiae} Apt1p (right). B, the initial rate of palmitoyl-CoA hydrolase activity was measured with 10 ng of recombinant \textit{R. norvegicus} APT1 (left) or \textit{S. cerevisiae} Apt1p (right). C, the initial rate of palmitoyl-G\(\alpha\)i1 thioesterase activity was measured with 100 pg of recombinant \textit{R. norvegicus} APT1 (left) or \textit{S. cerevisiae} Apt1p (right). Rates are derived from linear regression analysis of palmitate released at four or more time points. Data shown are representative of experiments performed twice. Curves shown are fit to the Michaelis-Menten rate equation for each data set.
lating to levels of 25–40% of the soluble cellular protein (data not shown). Each protein was purified essentially to homogeneity using a single step of Ni²⁺-NTA-agarose affinity chromatography (Fig. 1). Both enzymes migrated as a single peak of ~29 kDa upon Superdex 75 gel filtration chromatography (data not shown). These enzymes also demonstrated chromatographic properties very similar to the native rat APT1 isolated from liver (data not shown). They bound tightly to phenyl-Sepharose, eluting between 35 and 50% ethylene glycol. Even at pH 6.0, both failed to bind to cation exchange resins (SP-Sepharose and mono S). Despite only 33% amino acid sequence identity, these recombinant proteins exhibit remarkably similar chromatographic profiles.

Acyl-protein Substrate Preference Is Conserved between Rat and Yeast APT1—The native rat APT1 exhibits both acyl-thioesterase activity (toward acyl-proteins and acyl-CoA) and acyl-esterase activity (toward lysophospholipids). Under the in vitro assay conditions utilized, APT1 has a marked preference for the acyl-protein substrates (14). The recombinant rat and yeast enzymes were assayed using varying concentrations of three substrates: lysophosphatidylcholine, palmitoyl-CoA, and palmitoyl-Gαi1 (Fig. 2). Like APT1 isolated from rat liver, re-
combinant rat APT1 preferred palmitoyl-Gαi1 substrate over either lysophosphatidylcholine or palmitoyl-CoA. For the yeast enzyme, this preference was even more pronounced, with a 2,000-fold difference in catalytic efficiency (Vmax/Km) between palmitoyl-Gαi1 and lysophosphatidylcholine. Having established that the enzyme encoded by S. cerevisiae open reading frame YLR118c has both biochemical activity and amino acid sequence similar to rat APT1, we will refer to YLR118c as the S. cerevisiae APT1 gene in the further description of our work.

*Rat and Yeast APT1 Orthologs Differ in Acyl-protein Substrate Preference*—The recombinant APT1 enzymes were assayed for acyl-protein thioesterase activity using a panel of palmitoyl-protein substrates: palmitoyl-Gαi1, in which the thioacylated cysteine is adjacent to a myristoylated, amino-terminal glycine (26); palmitoyl-RGS4, in which multiple thioacylated cysteines are found several residues away from the amino terminus of the protein (27); and palmitoyl-Ras, in which myristoylated amino-terminus glycine (26); palmitoyl-RGS4, in which multiple thio
cysteines are located near a farnesylated carboxyl-terminal cysteine (28). These assays were performed at substrate concentrations substantially below the enzymes' Ks value for each, demonstrated by a linear relationship between substrate concentration and initial reaction rate (data not shown). Under these conditions, the relative catalytic efficiency of the enzyme for each substrate can be assessed by comparing the t1/2 to the completion of each reaction. Both rat APT1 and yeast APT1 depalmitoylated these substrates in *vitro*. The recombinant rat APT1 showed a 10-fold variance in catalytic efficiency between the best (palmitoyl-Gαi1) and worst (palmitoyl-Ras) acyl-protein substrates (Fig. 3A), roughly comparable with the values observed previously with native rat APT1 (24). Surprisingly, the yeast APT1 demonstrated more specificity among acyl-protein substrates. The catalytic efficiency of yeast APT1 for palmitoyl-Gαi1 was at least 35- and 70-fold higher than that for palmitoyl-RGS4 and palmitoyl-Ras, respectively (Fig. 3A). Both recombinant enzymes were also able to discriminate between heterotrimeric and free palmitoyl-Gαi1 (Fig. 3B). Again, as observed with the native enzyme, free palmitoyl-Gαi1 was used as a substrate more efficiently than heterotrimeric palmitoyl-Gαi1 by recombinant APT1 in this assay (14). The difference in the activity of yeast APT1 toward heterotrimeric and free palmitoyl-Gαi1 was greater than observed for the rat enzyme. We note, however, that assays of the yeast enzyme were performed only with mammalian proteins as substrates.

*Yeast Strains Bearing a Deletion of the APT1 Gene*—Having established that the protein encoded by YLR118c did indeed have acyl-protein thioesterase activity, we proceeded to create a yeast strain that lacked the APT1 gene to assess further the *in vivo* function of APT1. An APT1 targeting vector was created by adding ~350 base pairs of DNA from the 3′ and 5′ regions flanking the S. cerevisiae APT1 open reading frame to the ends of the HIS3 gene. The integration of this DNA fragment into the S. cerevisiae genome results in the removal of the entire open reading frame encoding APT1 (Fig. 4A). The diploid S. cerevisiae strain, YPH501, was transformed with the APT1 targeting construct and selected for growth on medium lacking histidine. The resulting yeast colonies were confirmed as APT1Δ::HIS3 (referred to as the APT1Δ allele) heterozygotes by performing the polymerase chain reaction on their genomic DNA with primer sets specific for either the wild type or disrupted allele. (Note that these primers cannot amplify the nonintegrated targeting vector; Fig. 4, A and B.) To obtain haploid yeast bearing the APT1Δ allele, the diploid APT1Δ yeast were induced to sporulate, and the resulting meiotic
tetrad survival was assessed by plating dilutions of each sample on YPD plates and counting the yeast colonies that grew.

**Growth of Haploid Yeast Carrying the APT1 or apt1 Δ Allele**—Because thioacylated proteins are involved in a variety of cellular processes, the apt1Δ S. cerevisiae strain was tested for defects in normal growth. There were no obvious abnormalities observed in yeast strains that lacked the APT1 gene, including normal budding morphology (data not shown). The growth rates in complete synthetic medium of APT1 and apt1Δ strains were essentially identical at three temperatures (25, 30, and 37 °C; Fig. 5, A and B). There was also no difference in the growth rates of the APT1 and apt1Δ strains in YPD, an enriched medium (data not shown). Normal haploid S. cerevisiae, cultured in the laboratory, exhibit three phases of growth. Initially, they expand exponentially, rapidly exhausting the dextrose included in the medium. As the dextrose in the culture is depleted, the growth of the yeast slows to a more linear rate. This slowing of growth is accompanied by an alteration in metabolism known as the diauxic shift. During growth after the diauxic shift, the glycolytic products present from nonoxidative utilization of dextrose are metabolized through an oxidative pathway. When the carbon source is exhausted, the yeast enter a dormant but viable state known as stationary phase, from which they emerge when a nutrient-rich environment is restored. The apt1Δ yeast strains maintained their viability for at least 1 month after entering the stationary phase, similar to the wild type strains (Fig. 5C). Thus, it appears that APT1 does not play an important role in the normal growth stages of the haploid S. cerevisiae organism, including spore germination (observed during the isolation of apt1Δ haploid yeast), normal growth, diauxic shift, or poststationary phase survival.

**Apparently Normal Lipid Metabolism in apt1Δ Yeast Strains**—In addition to acyl-protein thioesterase activity, recombinant Apt1p has esterase activity (although less efficient) toward a number of other substrates. Several assays unrelated to acyl-protein metabolism were performed to determine whether Apt1p might be involved in some form of lipid metabolism. There was no detectable difference in the levels of lysophospholipase activity in cell extracts derived from either APT1 or apt1 Δ strains of yeast (data not shown). Application of exogenous lysophospholipids to the medium of either APT1 or apt1Δ yeast resulted in inhibition of growth (data not shown), a toxicity presumably attributable to the detergent like characteristics of the lysophospholipids. APT1 and apt1Δ yeast had identical sensitivity to the growth-inhibitory effects of exogenous lysophospholipids (data not shown). To test for gross perturbations in acyl-CoA metabolism, the incorporation of radiolabeled fatty acids into cellular lipid pools was measured. No differences were observed between wild type and apt1Δ yeast strains in the

Δ; squares) in minimal medium at three separate temperatures (filled black, 37 °C; filled gray, 30 °C; unfilled, 25 °C) was measured as described under “Experimental Procedures.” Growth from 0 to 14 h is plotted in A, showing the exponential phase of growth for these strains. Growth from 0 to 72 h is plotted in B, showing the diauxic shift and final culture densities. C, S. cerevisiae strains, YAD101 (APT1; circles), YAD102 (APT1; squares), YAD103 (apt1Δ; diamonds), and YAD104 (apt1Δ; triangles) were grown to stationary phase in YPD medium. The yeast were isolated and suspended in H2O. After various times at 30 °C, the number of surviving yeasts was assessed by plating dilutions of each sample on YPD plates and counting the yeast colonies that grew.
rate or the identity of lipids into which [3H]palmitate was incorporated (Fig. 6A). An aberrant accumulation of a particular lipid species in the steady state would suggest an alteration of the metabolism of a specific lipid or group of lipids. However, no difference was detected between lipids extracted from either strain following metabolic labeling for 2 h with [14C]acetate or [32P]orthophosphate (Fig. 6B).

**Fig. 6.** apt1Δ yeast exhibit apparently normal lipid metabolism. A, yeast strains YAD101 (APT1) and YAD103 (apt1Δ) were incubated with [3H]palmitate. At the indicated times, lipids from the cultures were extracted and subjected to thin layer chromatography as described under “Experimental Procedures.” A fluorograph (3-day exposure) of the TLC plate is shown with the migration of a palmitate standard indicated by the letter P. B, Yeast strains from A were grown in the presence of either [14C]acetate or [32P]orthophosphate for 2 h. Lipids were extracted and subjected to analysis by thin layer chromatography. Images of the plates were obtained by two-week (14C) or one-day (32P) exposure to an MP1000 phosphorimaging screen. The origin (O) and migrations of phosphatidylserine (PS) and free fatty acid (FA) standards are indicated.

**Acyl-Protein Thioesterase Activity in Haploid S. cerevisiae—** Although *S. cerevisiae* Apt1p clearly exhibits acyl-protein thioesterase activity in vitro, it is possible that other acyl-protein thioesterase activities exist within *S. cerevisiae*. These activities may normally (or in the absence of Apt1p) be able to carry out deacylation reactions essential to the survival of the yeast, resulting in no obvious phenotype following deletion of the APT1 gene. Alternatively, these activities could be increased in apt1Δ yeast, allowing them to carry out reactions that would normally be the responsibility of Apt1p. Extracts from both APT1 and apt1Δ yeast strains were prepared and assayed for acyl-protein thioesterase activity. Although the activity present in the extracts was low when compared with mammalian cell extracts, the extracts from wild type yeast could clearly deacylate palmitoyl-Gαι₁ (Fig. 7A). Extracts from apt1Δ strains exhibited dramatically lower, nearly undetectable activity (Fig. 7A). This biochemical phenotype was observed in several extracts prepared from apt1Δ strains derived from two separate laboratory yeast strains (YPH501 and MMY030; data not shown). The apt1Δ extracts had between 2 and 10% of the acyl-protein thioesterase activity detected in the wild type (APT1) extracts. However, because of the small percentage of substrate utilized in the reaction, we have not been able to confirm that the organic solvent-soluble, palmitate-containing material released from palmitoyl-Gαι₁ by extracts from the apt1Δ yeast represents free fatty acid (rather than an organic solvent-soluble proteolysis product). Because differences in *S. cerevisiae* Apt1p activity toward various acyl-protein substrates had been observed in vitro, these cell extracts were also tested for their ability to deacylate palmitoyl-Ras. The palmitoyl-Ras thioesterase activity in wild type extracts was very low (0.36 pmol min⁻¹ mg⁻¹ extract protein) and could only be detected reliably when the concentration of palmitoyl-Ras substrate was roughly 6-fold higher than in similar assays utilizing palmitoyl-Gαι₁ substrate. Interestingly, the extracts from apt1Δ yeast exhibited a low level of activity toward palmitoyl-Ras substrate that was virtually indistinguishable from APT1 strain extracts (Fig. 7; different extract preparations ranged from 75 to 110% of that detected in wild type extracts). The specific activity of both extracts toward palmitoyl-Ras, when corrected for the different concentrations of substrates, was very similar to that for the palmitoyl-Gαι₁ thioesterase activity observed in apt1Δ yeast strain extracts. These results indicate that the product of the APT1 gene is responsible for almost all of the palmitoyl-Gαι₁ thioesterase activity detected in the
S. cerevisiae strains examined but contributes very little, if any, to the detectable palmitoyl-Ras thioesterase activity.

Thioacylation of Gpa1p in apt1Δ S. cerevisiae—To determine whether Apt1p plays a role in thioacyl-protein metabolism in vivo, we examined metabolic labeling of the protein Gpa1p with [3H]palmitate. Wild type and apt1Δ yeast strains were transformed with plasmids that direct overexpression of the yeast heterotrimeric G protein α subunit: Gpa1p (with a carboxy-terminal HA tag) from a copper-inducible promoter (25). This protein, when expressed in yeast, can complement the growth-arrested phenotype of gpa1Δ yeast strains. It has also been shown to incorporate [3H]palmitate (as well as a metabolic product, myristate) through a thioester bond in metabolic labeling studies (29). Overexpressed Gpa1p was metabolically labeled with [3H]palmitate as a substrate. B, palmitoyl-Gα, thioesterase activity (60 nM substrate), palmitoyl-Ras thioesterase activity (340 nM substrate), and lysophospholipase activity (3 μM substrate) of extracts described in A (solid, APT1; striped, apt1Δ) were assayed as described under “Experimental Procedures.” Initial reaction rates were determined by linear regression analysis of palmitate release at four or more time points.

S. cerevisiae strains bearing a deletion of the APT1 gene appear to be unable to deacylate heterotrimeric G protein α-subunits in vivo.

We note that the rate of loss of radiolabeled palmitate from Gpa1p in apt1Δ yeast appeared to be less than the rate of turnover of the protein in these cells. We suspect that this is caused by the slow chase of the high concentration of [3H]palmitate used in labeling, coupled with continued synthesis...
methionine and cysteine. Subsequently, the Gpa1p was pulse-labeled with [35S]methionine/cysteine for 20 min, followed by a chase with unlabeled}

of the G protein Gpa1p, since the protein was overexpressed in the absence type cells.) It is also possible that there are two populations of discrepancy. over, nonacylated population of protein would explain this

proteins (Gpa1p and Ste18p) involved in the yeast mating type counterparts) was tested using two assays: the "pheromone response pathway in signal transduction cascade are known to be thioacylated. The proteins (Gpa1p and Ste18p) involved in the yeast mating process. To assay for this transcriptional activation, wild type and apt1Δ yeast were transformed with a plasmid carrying the E. coli β-galactosidase cDNA transcribed from the FUS1 promoter. The level of β-galactosidase activity in the yeast was assayed after challenge with pheromone. In this assay, the apt1Δ yeast consistently showed modestly elevated β-galactosidase activity compared with the wild type strains (Fig. 9B). However, when the β-galactosidase activity is plotted as a percentage of maximal activity attained in each strain, it becomes apparent that the elevation was seen at all concentrations of pheromone tested (including no pheromone; Fig. 9C). The most straightforward explanation of these results is that APT1 is not exerting an effect on the hormone responsiveness of the reporter in this assay.

DISCUSSION

In mammalian tissues, the bulk (if not all) of biochemically detectable acyl-protein thioesterase activity can be attributed

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to two enzymes, PPT1 and APT1 (14). PPT1 is a lysosomal enzyme and appears to act on thioacylated lipopeptide substrates, which may be derived from the breakdown of intracellular thioacylated proteins (30). Unlike PPT1, where a mutant (essentially null) allele exists in humans, our knowledge about the substrate(s) and physiologic role of APT1 are primarily inferred from biochemical studies of the enzyme. Because the entire *S. cerevisiae* genome has been sequenced, it is known that the organism contains no protein homologous to PPT1 and only a single open reading frame, YLR118C, encoding a protein homologous to APT1.

We felt that *S. cerevisiae* was an attractive system for studies of APT1 for at least three reasons: 1) methods for genetic manipulation of the organism are well established and easy to utilize; 2) heterotrimeric G protein signaling occurs in the organism (however, unlike mammalian systems, there are only two receptor-G protein combinations (31)); and 3) thioacylation of heterotrimeric G proteins and other proteins has been reported in the organism (7). The first step in establishing *S. cerevisiae* as a model organism for the study of APT1 was to demonstrate that the protein encoded by the YLR118c open reading frame had biochemical properties similar to mammalian APT1, as suggested by their homologous primary amino acid sequence.

Using recombinant enzymes, we have shown that the acyl-protein thioesterase activity of APT1 is conserved between *R. norvegicus* and *S. cerevisiae* and that the palmitoyl-G/H9251 thio-esterase activity is more highly conserved than the other catalytic activities of the enzymes from these species. The activities of both recombinant and native rat APT1 toward lysophospholipids are comparable with those observed by other groups (*V* max between 2 and 4 μmol/min/1 mg), suggesting that this is indeed the maximum rate at which APT can hydrolyze acyl-ester bonds (15, 33). In all cases, the *V* max toward acyl-thioester substrates appears to be at least 10 times greater than this number. Camp and Hofmann (23) found no acyl-protein thioesterase activity in several known esterases (including a *B. cereus* phospholipase C, *Crotalus adamanteus*...
phospholipase A$_2$, Candida cylindracea lipase, and Oryctolagus cuniculus carboxylesterase); thus, thioesterase activity is not a general property of esterase enzymes. Instead, the data suggest that the active sites of at least two APT1 enzymes are better suited to catalyzing a thioesterase reaction than an esterase reaction.

Comparisons of the $K_m$ values for all of the substrates described in the literature are confounded by varying critical micellar concentrations of each substrate and differences in type and concentration of detergent used. In the work described herein, the concentration and type of detergent was constant for all substrates. Differences in the observed $K_m$ toward any substrate should then reflect a variance in the affinity of APT1 for each substrate. Again, APT1 enzymes from Rattus norvegicus and S. cerevisiae had at least a 10-fold (and as high as several hundredfold) higher apparent affinity for palmitoyl-G$_{a_i}$ substrate than for small molecule substrates (either palmitoyl-CoA or lyso-PC).

In contrast to mammalian APT1, the yeast enzyme did exhibit a marked preference for palmitoyl-G$_{a_i}$ over other acyl-protein substrates. The physiological relevance of this substrate preference is unclear. The in vivo rate of thioacylation turnover for most thioacylated proteins in S. cerevisiae has not been reported. It is possible that these rates may be proportional to the catalytic efficiency of S. cerevisiae APT1 toward each particular substrate. Alternatively, other acyl-protein thioesterase activities or nonenzymatic mechanisms for protein deacylation may exist in S. cerevisiae.

To define further the function of APT1 in vivo, we generated a strain of S. cerevisiae lacking the APT1 gene. We sought evidence that Apt1p, either through protein deacylation or some other esterase activity, was important in some aspect of the growth or survival of S. cerevisiae. The phenotype of apt1$^+/_{	ext{H9004}}$ heterozygote diploids appears normal with regard to growth and sporulation when compared with the wild type strain (data not shown). Both the phenotype and levels of acyl-protein thioesterase activity of wild type and apt1$^+/_{	ext{H9004}}$ diploid yeast will be the subject of future investigation. Haploid apt1$^+/_{	ext{H9004}}$ yeast appeared to behave identically to their wild type counterparts during all phases of growth under a variety of conditions (Fig. 5).

Apt1p is unlikely to function as a lysophospholipase in S. cerevisiae. The lysophospholipase activity of S. cerevisiae APT1 detected in vitro can be described as poor at best. Additionally, S. cerevisiae carrying the apt1$^+/_{	ext{H9004}}$ allele show no alteration in biochemically detectable lysophospholipase activity. Previous studies have shown that yeast that lack the PLB1 gene exhibit almost no detectable lysophospholipase activity in vitro and do not metabolize lysophospholipids in vivo, suggesting that Plb1p is the primary lysophospholipase activity in S. cerevisiae (34).

Other roles for Apt1p in lipid metabolism also seem unlikely, since no gross alterations in palmitate metabolism or lipid accumulation were detected in the apt1$^+/_{	ext{H9004}}$ strain (Fig. 6).

Biochemical analyses indicate that Apt1p is the primary enzyme in S. cerevisiae responsible for palmitoyl-G$_{a_i}$ thioesterase activity. Extracts prepared from strains of haploid yeast carrying the apt1$^+/_{	ext{H9004}}$ allele had a dramatic reduction in biochemically detectable palmitoyl-G$_{a_i}$ thioesterase activity (Fig. 7). Interestingly, our data suggest that a low level of thioesterase activity (no more than 10% of total) that cannot discriminate between palmitoyl-Ras and palmitoyl-G$_{a_i}$ substrates appears to exist in S. cerevisiae. When compared with mammalian cells, even wild type haploid S. cerevisiae exhibit very low levels of acyl-protein thioesterase activity. In light of the normal growth characteristics of the apt1$^+/_{	ext{H9004}}$ yeast, it is possible that the lower level of thioesterase activity in these yeast is sufficient for the protein deacylation needs of a haploid S. cerevisiae grown under laboratory conditions.

We sought to determine whether Apt1p was involved in the in vivo deacylation of Gpa1p. In metabolic labeling studies utilizing [3H]palmitate, apt1$^+/_{	ext{H9004}}$ yeast accumulated less Gpa1p-bound tritium than wild type counterparts. This finding is consistent with the incorporation of label into newly synthesized Gpa1p in the apt1$^+/_{	ext{H9004}}$ strain and the incorporation of label into newly synthesized and previously existing Gpa1p in the wild type strain. In wild type yeast, the incorporated label turned over when the yeast were incubated with unlabeled fatty acids. When apt1$^+/_{	ext{H9004}}$ yeasts were incubated with unlabeled fatty acids, the Gpa1p-associated tritium remained stable. These in vivo experiments demonstrate that apt1$^+/_{	ext{H9004}}$ yeasts are unable to deacylate Gpa1p effectively and strongly correlate with our findings that extracts from these yeast lack significant acyl-protein thioesterase activity in vitro.

Mutations in the palmitoylation sites of three thioacylated proteins from S. cerevisiae are known to produce distinguishable phenotypes in behaviors regulated by those proteins. Mutation of Cys$^{318}$ in Ras2p produces a deleterious growth phenotype similar to but milder than that observed in the ras2 null strain (35). When the thioacylation site of S. cerevisiae Gy, STE18, is removed, yeasts carrying the mutant gene are nearly sterile, matting with 100-fold less efficiency than wild type yeast. Finally, S. cerevisiae carrying the spa1 C34 or C38 allele exhibit supersensitivity to mating factor (7, 29). These phenotypes are presumed to be the result of loss of thioacylation of the protein in question, rather than the loss of the cysteine residue per se. The APT1 gene of S. cerevisiae represents the first identified yeast protein involved in the metabolism of thioacylated proteins. Although we have shown that yeast carrying a disrupted allele of this gene have a defect in the turnover of Gpa1p-associated thioacyl groups, the yeast have no obvious phenotypic alterations, either in general growth characteristics or response to pheromone (Figs. 5 and 9). Thioacylation has been shown to affect interactions between Ga subunits and regulatory proteins in vitro. Consequently, we hope the future use of apt1$^+/_{	ext{H9004}}$ yeast in combination with the wide array of mutations in the pheromone response pathway that already exist will serve to shed further light on the in vivo role of the enigmatic process of thioacylation in heterotrimeric G protein signaling.

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