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The G Protein α_s Subunit Incorporates [^{3}H]Palmitic Acid and Mutation of Cysteine-3 Prevents This Modification

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ABSTRACT: We investigated whether α_s could be acylated by palmitate by transfecting COS cells with the cDNA for the wild-type, long form of α_s and metabolically labeling with [^{3}H]palmitate or [^{35}S]methionine. Cells were separated into particulate and soluble fractions and immunoprecipitated with a specific peptide antibody. [^{3}H]Palmitate was incorporated into both endogenous and transfected α_s. Inhibition of protein synthesis with cycloheximide did not block the radiolabeling of α_s with [^{3}H]palmitate. Hydroxylamine treatment caused a release of the tritium radiolabel, demonstrating that the incorporation was through a thioester bond. The tritium radiolabel was base-labile and comigrated with [^{3}H]palmitate on thin-layer chromatography. The third residue of the wild-type α_s was mutated from a cysteine to an alanine by site-directed mutagenesis. This mutant was expressed in COS cells and localized to the particulate fraction as determined by immunoprecipitation of the [^{35}S]methionine-labeled cells. The cysteine-3 mutant did not undergo radiolabeling with [^{3}H]palmitate, indicating that this residue is crucial for the modification.

Heterotrimeric guanine nucleotide binding proteins are located on the cytoplasmic face of membranes where they couple receptors to intracellular effectors (Lochrie & Simon, 1988; Spiegel et al., 1992). When the G protein[1] α subunit binds GDP, it forms a heterotrimer with the tightly bound β–γ dimer. Upon activation by an agonist-bound receptor, the α subunit releases GDP, binds GTP, and probably dissociates from the β–γ dimer.

The α subunit is relatively hydrophilic and does not reconstitute into phospholipid vesicles unless β–γ subunits are present (Sternweis, 1986). This observation has led to the suggestion that the β–γ dimer is responsible for anchoring the α subunit to the membrane. If the activated α subunit in fact dissociates from the β–γ dimer, another mechanism for membrane attachment may also be present. Certain α subunits undergo myristoylation, the addition of a 14-carbon fatty acid to amino-terminal glycine, which is important in their membrane attachment (Buss et al., 1987; Jones et al., 1990; Mumby et al., 1990). The mechanism of membrane attachment and localization of α_s, the α subunit which stimulates adenylyl cyclase, is not known since it does not undergo myristoylation (Buss et al., 1987; Jones et al., 1990).

Fatty acid modification of proteins can also occur by the covalent addition of palmitate to cysteine through a thioester bond (Towler et al., 1988). In contrast to myristoylation, which in most cases is cotranslational and irreversible, this modification is reversible and independent of protein synthesis (Hancock et al., 1989). This modification occurs on many proteins including the β and α2A adrenergic receptors, rhodopsin, and several low molecular weight GTP binding proteins (O'Dowd et al., 1989; Kennedy & Limbird, 1993;

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[1] Abbreviations: G protein, guanine nucleotide binding regulatory protein; α_s and α_{i1}, G protein α subunits associated with adenylyl cyclase stimulation and inhibition, respectively; α_s*, a mutant of α_s with a substitution of glutamate for arginine at position 201; DMEM, Dulbecco's modified Eagle's media; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; IgG, immunoglobulin G; TLC, thin-layer chromatography.

Karnik et al., 1993; Hancock et al., 1989). While a previous study reported that α_s did not undergo palmitoylation (Buss et al., 1987), we decided to reinvestigate whether α_s is modified by palmitate because the low level of expression of endogenous α_s and the reversibility of the modification may have made detection of this modification difficult. In a recent study with myristoylated and nonmyristoylated α_i/α_s chimeras, we found that increased membrane avidity correlated with increased α subunit adenylyl cyclase stimulating activity (unpublished observations). This suggested that the wild-type α_s might undergo a hydrophobic modification other than myristoylation. Reduced activity of α_s expressed in *Escherichia coli* (and not undergoing putative posttranslational modification) compared to native α_s has also suggested the possibility of some form of posttranslational modification of α_s (Graziano et al., 1989).

In the present study, the α_s subunit was overexpressed in COS cells, and transfected cells were incubated with [^{3}H]-palmitate. Immunoprecipitation with an antibody specific for α_s showed incorporation of the [^{3}H]palmitate radiolabel into the endogenous and transfected α_s. This incorporation showed several biochemical properties characteristic of palmitoylation. Since palmitoylation occurs on cysteines through a thioester bond, we performed site-directed mutagenesis to change the cysteine at position 3 to an alanine to test the location of this modification within the protein. This C3A α_s mutant did not incorporate [^{3}H]palmitate though it was still primarily localized to the particulate fraction.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis and Vector Construction. The cDNAs for the rat wild-type α_s and α_{i1} and the α_s mutants were cloned into the PCD expression vector and purified as previously described (Jones et al., 1990; Juhnn et al., 1992). The cDNA of the constitutively active mutant, α_s*, was created by site-directed mutagenesis to code for a glutamate instead of an arginine at position 201. Site-directed mutagenesis was performed using the polymerase chain reaction and the oligonucleotide TAG ATA GCC CGG CCG CGC CCC GCC GCC GAT GGG CGC CCT CGG CAA CAG to change the α_s and α_s* cDNAs to code for an alanine instead of a cysteine at residue 3 (C3A and C3A*) as previously described (Juhnn et al., 1992).

COS Cell Transfection and Incorporation of Radioisotopes. COS cells were maintained in culture and transfected using the DEAE-dextran method as described previously (Butrynski et al., 1992). Forty-eight hours after transfection the cells were prepared for metabolic labeling. For cycloheximide treatment, the cells were exposed to 50 μg/mL cycloheximide (Calbiochem) for 60 min prior to incubation with the radiolabels. For [^{35}S]methionine incorporation the cells were incubated for 5 h in DMEM with 20 μM methionine supplemented with 10% (v/v) fetal bovine serum with or without cycloheximide and 250 μCi of [^{35}S]methionine/mL (specific activity 1200 Ci/mmol, Du Pont). For [^{3}H]palmitate incorporation the cells were incubated for 5 h in DMEM supplemented with 5% (v/v) dialyzed fetal bovine serum, 5 mM sodium pyruvate, 25 μg/mL cerulenin (Calbiochem), 1% (v/v) dimethyl sulfoxide, and 1 mCi of [9,10-^{3}H]palmitate/mL (specific activity 40–60 Ci/mmol, Du Pont). For [^{3}H]-myristate incorporation the cells were incubated in the same media as for [^{3}H]palmitate incorporation except with 300 μCi of [9,10-^{3}H]myristic acid/mL (specific activity 33.5 Ci/mmol, Du Pont). After the incubation, the cells were washed once in warm phosphate-buffered saline, scraped, and centrifuged at 2000*g* for 10 min.

Cell Fractionation. The cell pellets were homogenized using a 25-gauge needle and syringe in approximately 4 volumes of a homogenization buffer composed of 5 mM Hepes, pH 7.4, 50 mM mannitol, 100 μg/mL soybean trypsin inhibitor, 0.5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 mM EDTA, 0.7 μg/mL pepstatin, and 10 milliunits/mL α2 macroglobulin (Boehringer-Mannheim). The cell lysate was first centrifuged at 3000*g* for 5 min in an Eppendorf 5415 microcentrifuge, and then the supernatant was centrifuged at 125 000*g* for 30 min at 4 °C in a Beckman TLA45 rotor. The supernatant (soluble fraction) was separated and the pellet (particulate fraction) resuspended in the buffer, and both fractions were recentrifuged to minimize cross-contamination. The washed pellet was resuspended in the original volume of homogenization buffer.

Immunoprecipitation and Hydroxylamine Treatment. Immunoprecipitation was performed as previously described (Jones et al., 1990) on equivalent amounts of total protein in each cell fraction except that the proteins were solubilized in 50 mM Tris-HCl, pH 7.5 (25 °C), 150 mM NaCl, 0.8% (w/v) Triton X-100, 0.2% (w/v) SDS, and 1 mM EDTA during incubation overnight at 4 °C with the antibodies. The immunoprecipitates were recovered by incubating with protein A–Sepharose CL-4B (Pharmacia LKB), washed, solubilized, separated by 12% SDS–PAGE, and prepared for fluorography as previously described (Jones et al., 1990) or underwent hydroxylamine treatment. Replicate lanes from fresh gels were incubated either in 1 M Tris-HCl, pH 7.5, or 1 M hydroxylamine, pH 7.5, for 60 min at room temperature with one change of solutions after 30 min and then prepared for fluorography.

Thin-Layer Chromatography. Gel slices from [^{3}H]palmitate immunoprecipitations were washed overnight in 50% methanol. The gel slices were treated with 1.5 M NaOH for 3 h at 30 °C followed by 6 M HCl at 100 °C with extraction of the released lipids in chloroform/methanol after each treatment, as previously described (Buss et al., 1987). The samples and standards of [^{3}H]myristate and [^{3}H]palmitate, in chloroform/methanol (2:1) with 2 mg/mL myristic acid, were separated on RP-18 TLC plates (Merck) in acetonitrile/acetic acid (9:1). The plates were sprayed with ENHANCE (Du Pont) for fluorography. Areas of the plates were identified and scraped, and the radioactivity was determined in a scintillation counter.

Miscellaneous Procedures. Immunoblotting was performed with the affinity-purified RM antibody specific for the carboxy-terminal decapeptide of α_s and peroxidase-labeled second antibodies (Kirkegaard & Perry Laboratories) as previously described (Gierschik et al., 1985). Protein concentration was determined by the Bio-Rad protein assay dye kit with IgG as the standard (Bio-Rad Laboratories). Densitometry of the fluorographs and immunoblots was performed with a LKB 2202 UltroScan laser densitometer (LKB). Gel bands were counted using Solvable gel solubilizer (Du Pont) according to the manufacturer's instructions.

RESULTS

Metabolic Labeling of α_s. To determine whether α_s could incorporate [^{3}H]palmitate, COS cells transfected with the PCD vector alone or with the cDNA for the long form of α_s (α_s1) were incubated with [^{35}S]methionine or [^{3}H]palmitate and separated into particulate and soluble fractions by centrifugation. After immunoprecipitation of the [^{35}S]-methionine-labeled cells with the RM antibody and separation by SDS–PAGE, the two endogenous forms of α_s were

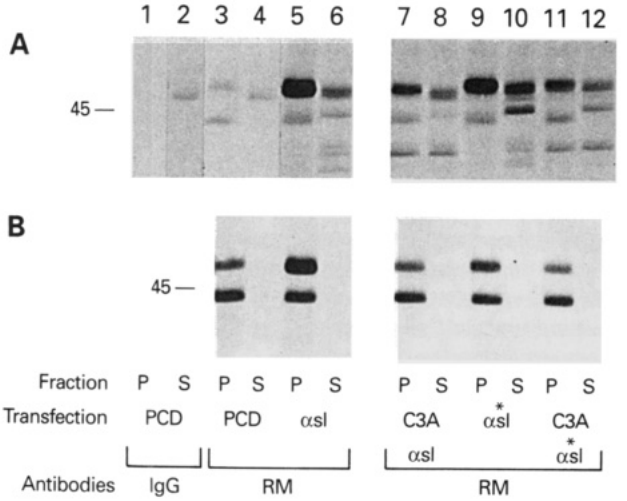

FIGURE 1: Subcellular distribution and incorporation of metabolic labels in α_s and its mutants. COS cells were transfected either with vector alone (PCD) or with the cDNA inserts of the wild-type or mutant α_s subunits (α_s1, C3A α_s1, α_s*1, C3A α_s*1). The cells were radiolabeled with [^{35}S]methionine (panel A) or with [^{3}H]palmitic acid (panel B) for 5 h. The cells were harvested and separated into particulate (P) and soluble (S) fractions by centrifugation. Then 100 μg of total radiolabeled protein from each fraction from [^{35}S]-methionine and 760 μg from [^{3}H]palmitic acid labeled cells were immunoprecipitated with the affinity-purified RM antibodies specific for α_s or with rabbit IgG. The immunoprecipitates were analyzed by SDS–PAGE and fluorography. Fluorograms were exposed for 3 (A) or 9 (B) days at −70 °C.

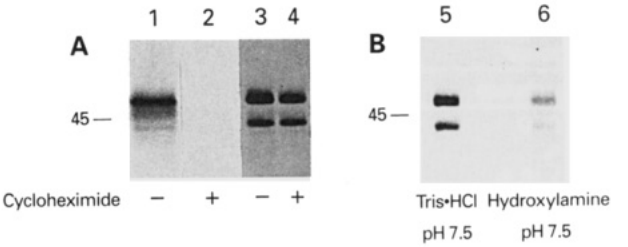

FIGURE 2: Incorporation of [^{3}H]palmitate into the α_s subunit is posttranslational and hydroxylamine sensitive. (A) Forty-eight hours after transfection, COS cells overexpressing α_s1 were incubated for 1 h without metabolic labels and then for 5 h with metabolic labels in the presence of 50 μg/mL cycloheximide (lanes 2 and 4) or in its absence (lanes 1 and 3). After cell fractionation, 127 μg of the particulate fraction protein from [^{35}S]methionine-labeled cells (lanes 1 and 2) and 400 μg from [^{3}H]palmitate-labeled cells (lanes 3 and 4) were immunoprecipitated with RM antibodies, separated by SDS–PAGE, and analyzed by fluorography. (B) Particulate fractions (600 μg) of COS cells overexpressing α_s1 and radiolabeled with [^{3}H]-palmitate were immunoprecipitated with RM antibodies. The immunoprecipitate was divided into two parts and separated by SDS–PAGE. The gel strips were incubated for 1 h in either 1 M Tris-HCl, pH 7.5 (lane 5), or 1 M hydroxylamine, pH 7.5 (lane 6). The gel strips were fixed and analyzed by fluorography.

seen as bands at approximately 44 and 48 kDa in the particulate fractions (Figure 1A, lane 3). Nonspecific IgG did not immunoprecipitate α_s (Figure 1A, lane 1). In the cells transfected with the α_s plasmid, an increased amount of protein is seen in the 48-kDa band in the particulate fraction and a small amount in the soluble fraction (Figure 1A, lanes 5 and 6). A band is seen at 47 kDa in the soluble fractions after immunoprecipitation with both the nonspecific IgG and RM antibody (Figure 1A, lanes 2 and 4).

Immunoprecipitation of the COS cell fractions incubated with [^{3}H]palmitate shows radiolabel incorporation in the endogenous and transfected α_s protein in the particulate fractions (Figure 1B, lanes 3 and 5). The pattern of radiolabel incorporation in the particulate fractions is similar to that of the [^{35}S]methionine-radiolabeled proteins and immunoblots of the cell fractions with an increased amount in the short form (44 kDa) compared to the long form (48 kDa) of the endogenous proteins and an increase in the long form after its overexpression. No tritium-radiolabeled bands are seen in the soluble fraction, even after longer exposures of the fluorograms, though a 48-kDa band was detected in the soluble fraction of the α_s-transfected cells by immunoprecipitation of [^{35}S]methionine-labeled cells and immunoblot (data not shown).

Characterization of the [^{3}H]Palmitate Incorporation. Metabolic labeling was performed in the presence of cycloheximide to test if α_s could incorporate [^{3}H]palmitate after inhibition of protein synthesis. COS cells transfected with α_s were pretreated with and without cycloheximide, incubated with [^{35}S]methionine or [^{3}H]palmitate, separated into cell fractions, and immunoprecipitated. Cycloheximide blocked incorporation of [^{35}S]methionine into α_s (Figure 2A, lanes 1 and 2) but did not significantly change incorporation of [^{3}H]-palmitate (Figure 2A, lanes 3 and 4).

Hydroxylamine at neutral pH can break thioester bonds and has been used to distinguish different types of protein acylation (Masterson & Magee, 1992). To investigate the chemical linkage of [^{3}H]palmitate to α_s, SDS–PAGE gel strips of immunoprecipitates of [^{3}H]palmitate-radiolabeled particulate fractions were treated at pH 7.5 with hydroxylamine or Tris-HCl. Figure 2B shows the significant decrement in the intensity of the protein bands with hydroxylamine treatment (lanes 5 and 6). The residual radiolabeled band is most likely due to incomplete cleavage with hydroxylamine which is seen with these conditions for proteins which have been proven to undergo palmitoylation (Alvarez et al., 1990). As a control for the specificity of hydroxylamine treatment, the myristoylated α_{i1} protein was expressed in COS cells, incubated with [^{3}H]myristic acid, and immunoprecipitated with a specific antibody. Hydroxylamine treatment caused no decrease in the intensity of the α_i-radiolabeled band as expected since myristoylation occurs through an amide bond (Towler et al., 1988) (data not shown).

Since [^{3}H]palmitate can be readily converted in the cell to other fatty acids, especially myristic acid (Christgau et al., 1992, Masterson & Magee, 1992), we analyzed the tritium radiolabel by subjecting the radiolabeled protein in gel slices to treatment with base and acid. The base treatment and to a lesser extent the acid treatment released compounds which comigrated with the [^{3}H]palmitate standard on thin-layer chromatography (data not shown). No radioactivity comigrated with the [^{3}H]myristate standard, which is consistent with the lack of myristoylation of α_s. While palmitoylation is a base-labile modification, the release of some radioactivity with acid may have been due to the harsher conditions which could degrade the gel matrix leading to release from the gel.

Expression of the C3A α_s Mutants. The metabolic labeling experiments suggested that α_s underwent palmitoylation through a thioester bond on a cysteine. We tested whether the cysteine, residue 3 from the amino terminus, was important for the modification by replacing it with an alanine in both the wild-type, long form of α_s and a constitutively active α_s*. Figure 1A shows the overexpression for each of the transfected α_s proteins as an increased density of the 48-kDa band in the particulate fraction after immunoprecipitation of [^{35}S]methionine-labeled cells. A fluorogram of the immunoprecipitation of fractions from cells labeled with [^{3}H]palmitate shows the incorporation into the endogenous α_s proteins and the expressed wild-type α_s and mutant α_s* (Figure 1B, lanes 3,

Table I: Quantitation of α Subunit Expression and [^{3}H]Palmitate Incorporation[a]

transfection	immunoblot	^{3}H incorpn
PCD	0.5[b]	0.6[c]
α_s	3.8	2.4
C3A α_s	2.5	0.6

[a] COS cells transfected with the vector alone (PCD) or with the cDNAs for the wild-type, long form of α_s or the C3A α_s mutant were either prepared as described in Figure 1B or separated into particulate and soluble fractions followed by immunoblotting with an antibody specific for α_s. The optical densities of the 48- and 44-kDa bands (long and short forms of α_s) in the particulate fraction of the immunoblot were determined to measure the relative amount of protein expressed. The amount of radioactivity in the 48- and 44-kDa bands after palmitate labeling was determined by counting the gel slices. The results from one representative experiment are shown. The values for the 44-kDa band were roughly equivalent within each preparation. [b] The ratio of the density of the 48-kDa band to the 44-kDa band. [c] The ratio of the counts per minute of the 48-kDa band to the 44-kDa band.

5, and 9). The immunoprecipitations of both the C3A mutants are essentially the same as the PCD control with incorporation only seen in the endogenous α_s proteins (Figure 1B, compare lanes 7 and 11 with lane 3). The ratios of the ^{3}H incorporation of the 48-kDa band to the 44-kDa band for this immunoprecipitation are compared to the ratios of densities of an immunoblot from the transfected cell fractions (Table I). [^{3}H]-Palmitate incorporation into α_s subunits is proportional to the amount of protein except for the C3A mutant, which did not show the increased incorporation. Since the [^{3}H]palmitate incorporation was independent of protein synthesis (Figure 2A), the level of tritium incorporation is best compared to the total amount of protein rather than the newly synthesized protein as seen with [^{35}S]methionine labeling (Figure 1A).

Both the immunoprecipitation of the [^{35}S]methionine-labeled cells (Figure 1A) and immunoblotting (data not shown) show that the C3A mutant is primarily localized to the particulate fraction. A 48-kDa band is seen in the soluble fraction of the transfected cells, with the three mutants having a relatively higher portion in the soluble fraction, which may be due to decreased affinity for the membrane. Since approximately 60% of the total lysate protein is in the particulate fraction and 40% in the soluble fraction, most of the expressed protein is still found in the particulate fraction.

DISCUSSION

This study showed that α_s and a constitutively active α_s mutant can incorporate [^{3}H]palmitate, but mutation of a cysteine at residue 3 prevented this incorporation. These mutants were still primarily localized to the particulate fraction.

Two recent reports show that α_s and other α subunits undergo palmitoylation (Linder et al., 1993; Parenti et al., 1993). Linder and colleagues (1993) showed by high-performance liquid chromatography analysis that incorporation of [^{3}H]palmitate into α_s was due to palmitoylation. The radiolabeling with [^{3}H]palmitate in our studies is consistent with palmitoylation—independence of protein synthesis, hydroxylamine sensitivity, and comigration with [^{3}H]palmitate on TLC. While a novel acylation of α_s other than palmitoylation is unlikely, our results and even the direct chemical analysis by Linder et al. (1993) cannot exclude such a possibility since a definitive study on the chemical identity of the modification may require native protein purified from tissue. For example, transducin expressed in COS cells could be radiolabeled with myristic acid (Mumby et al., 1990), but mass spectrometry of transducin purified from retinas showed modifications with four different fatty acids including myristic acid (Neubert et al., 1992).

α_s has eight cysteine residues, four of which are conserved among several α subunits (Lochrie & Simon, 1988). The amino termini of α subunits are important for their membrane attachment through both modification with myristoylation and β–γ dimer interaction (Navon & Fung, 1988; Eide et al., 1987). In addition, structural models of the α subunit place the amino terminus facing the membrane (Berlot & Bourne, 1992). The cysteine at the third position from the amino terminus therefore appeared as a likely candidate for the site of palmitoylation and when mutated led to a loss of [^{3}H]-palmitate incorporation. However, this mutation could have changed the protein conformation or the recognition site for the palmitoyl transferase, thus preventing palmitoylation at another site. Site-directed mutagenesis of the cysteine residue fourth from the carboxy terminus of *ras* led to a loss of palmitoylation not because of the direct palmitoylation of that residue but because this mutation prevented isoprenylation which is required for subsequent palmitoylation (Hancock et al., 1989). Since a recently published tryptic cleavage study of α_s labeled with [^{3}H]palmitate showed that palmitoylation occurred at the amino terminus (Linder et al., 1993) the most likely explanation of our findings is that palmitoylation occurs through a thioester bond to cysteine-3.

Our evidence for palmitoylation of α_s raises a series of questions about the possible localization, regulation, and function(s) of this modification. Palmitoylation of a cysteine at the amino-terminal end of the protein may function like myristoylation to increase membrane avidity directly or indirectly through a tighter interaction with β–γ subunits. An increased membrane avidity can explain the requirement of detergents to release α_s from the membrane (Sternweis, 1986). Christgua et al. (1992) have studied the posttranslational processing of the enzyme glutamic acid decarboxylase and found that only the palmitoylated form required detergents for release from the membrane. If palmitoylation is involved in β–γ interaction, the reversibility of the modification could lead to two different types of α_s, acylated and deacylated, with different affinities for the β–γ subunits. The presence or absence of this modification could be the explanation for the variable affinity of α_s subunits to a β–γ agarose matrix (Pang & Sternweis, 1989). A large fraction of α_s could bind to a β–γ affinity matrix and be eluted with activation, but approximately 30% either did not bind or was not released with activation.

Another difference between myristoylation and palmitoylation is that myristoylation generally occurs cotranslationally whereas palmitoylation occurs posttranslationally on membranes. In this study as well as others, [^{3}H]palmitate incorporation was only seen in the particulate fractions. The loss of myristoylation of α_{i1} led to a loss of membrane attachment (Jones et al., 1990; Mumby et al., 1990) whereas the loss of [^{3}H]palmitate incorporation in the C3A mutants did not significantly change their targeting to the membrane. This result is consistent with our previous finding that deletion of a 26 amino acid region from the amino terminus of α_s which includes the cysteine at position 3 did not significantly change the membrane localization (Juhnn et al., 1992).

The reversibility of palmitoylation makes it a target for regulation of protein function besides membrane attachment. For example, palmitoylation of the transferrin receptor inhibits its endocytosis (Alvarez et al., 1990), and palmitoylation of the β-adrenergic receptor is important for coupling to α_s (O'Dowd et al., 1989). One function of the α subunit is to

exchange GDP for GTP upon activation. Since wild-type α subunits have a basal level of activation, we could not determine whether [^{3}H]palmitate incorporation into the endogenous, wild-type α_s occurred in the GDP or GTP conformation. However, the constitutively active mutant, α_s*, which has decreased GTPase activity and is predominantly in the GTP-bound form, could incorporate [^{3}H]palmitate. More studies will be needed to test the relationship between palmitoylation and the GTPase cycle.

A recent report that α_s or an α_s-like protein is involved in the regulation of protein transport to the apical surface of polarized epithelial cells suggests another possible function of palmitoylation of α_s (Pimplikar & Simons, 1993). Since palmitoylation of secretory proteins has been localized to occur to an intermediate compartment in the post-ER/cis-Golgi network (Bonatti et al., 1989), α_s may undergo palmitoylation at this site. Deacylation of α_s at the plasma membrane could lead to release to the cytosol and then acylation at the post-ER/cis-Golgi site. The acylated α_s could function in transport of not only the content of the vesicles but also the acylated α_s back to the plasma membrane. In *Saccharomyces cerevisae*, cycles of protein acylation/deacylation have been shown to occur during the transport of vesicles through the secretory pathway (Simon & Aderem, 1992). Levis and Bourne (1992) have shown release of α_s upon activation, with reassociation to the membranes much slower than expected for the intrinsic GTPase activity of the protein.

One may speculate concerning several other potential roles for palmitoylation of α_s, including altering protein turnover and receptor/effector interactions. Comparison with other proteins which undergo this modification may even suggest new functions of α_s. Conversely, understanding the role of palmitoylation of α subunits may explain the function of palmitoylation of other proteins.

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