Palmitoylation of a G Protein α subunit Requires Membrane Localization Not Myristoylation

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Palmitoylation is a dynamic, post-translational modification of the amino terminus of heterotrimeric G protein α subunits. Since myristoylation, βγ interactions, and membrane attachment also involve the amino terminus of the G protein α subunit, we studied the relationships between palmitoylation and these events. Using COS cell transfection, the turnover of palmitate was slower on α1 subunits co-expressed with β and γ subunits than on the α1 subunit expressed alone. Mutation of cysteine 3 of α1 prevented [3H]palmitate but not [3H]myristate incorporation and decreased the membrane localization of this protein. The nonpalmitoylated mutant could form a heterotrimer with co-expressed βγ subunits which restored its membrane localization. A nonmyristoylated α1 mutant (glycine 2 to alanine) could incorporate [3H]palmitate when co-expressed with βγ subunits and localized to the membrane. The [3H]palmitate turnover of this nonmyristoylated mutant was more rapid than seen with the wild-type α1 subunit. While myristoylation is not required for palmitoylation, both myristoylation and βγ association can slow the turnover of palmitate on α1. These results suggest that palmitoylation maintains the membrane attachment of the free α subunit and changes in βγ association could modulate palmitoylation during signaling.

Heterotrimeric G proteins function at membranes to relay signals from cell surface receptors to intracellular effectors (1, 2). Each heterotrimer consists of an α subunit, which exchanges GDP for GTP upon activation by a receptor, and β and γ proteins, which are tightly bound together. The formation of the heterotrimer is necessary for interaction with the receptor, but after activation and GTP binding, the heterotrimer probably dissociates into free α and βγ subunits which are both capable of modulating effectors. The localization of G proteins to membranes markedly increases their effective concentration for interactions with membrane-bound receptors and effectors. The membrane attachment of the α subunit (especially in the GDP-bound state) is facilitated by binding to the relatively hydrophobic βγ complex (3). The post-translational lipid modification of myristoylation, which occurs on the amino-terminal glycine of a subset of α subunits (α2, α5, α6, and α7) (4–7), is also critical for membrane attachment of these subunits (7–9).

Palmitoylation, the post-translational thioesterification of cysteine residues with palmitate, occurs on many α subunits: α1, α4, α5, α6, α7, α9, and α13 (7, 10–14). Residues at the amino terminus, cysteine 3 for α1 and α4, and cysteines 9 and 10 for α2 are critical for the modification (10, 12, 13). Mutation of these residues to prevent palmitoylation leads to some loss of membrane attachment, the extent of which depends on the α subunit and expression system used (10, 12, 13, 15, 16). Reduced membrane affinity may explain the decreased effector activation of the nonpalmitoylated α1 and α4 (13).

Palmitoylation is a reversible modification. Activation of α2 by agonist stimulation or cholera toxin leads to increased turnover of palmitate (15, 17, 18). Changes in the degree of palmitoylation may lead to changes in α subunit membrane affinity which is observed during the GTPase cycle and allow modulation of signaling (19). Our previous finding that increased palmitate incorporation with agonist stimulation did not occur on the 549 cell H21a mutant of α2, which dissociates slowly if at all from βγ, suggests that βγ dissociation may affect palmitoylation (17). The amino terminus of α subunits is the site for both palmitoylation and βγ interactions (11, 20).

Myristoylation and palmitoylation share the common feature of increasing protein hydrophobicity. They both occur at the amino terminus of several G protein α subunits and Src family tyrosine kinases including p56λc and p59fcr (21, 22). Some studies have suggested that myristoylation is critical for palmitoylation and may be part of a general consensus sequence for the modification (7, 15, 22). The function of palmitoylation in membrane attachment could be similar to myristoylation which markedly increases the affinity of the α subunit for the βγ complex (8, 23). Alternatively, its increased hydrophobicity (16 carbon palmitate versus 14 carbon myristate) could cause a strong direct interaction with the lipid bilayer.

The relationships between palmitoylation, myristoylation, and βγ association of an α subunit and their relevance to membrane localization were investigated in this study. We found that the modifications occurred independently; palmitoylation did not require myristoylation. Either palmitoylation or binding to the βγ complex was sufficient to localize a functional α subunit at the membrane. Association with βγ did not require a palmitoylated α subunit, but it did slow the turnover of palmitate on the α subunit.

EXPERIMENTAL PROCEDURES

Vector Constructs and Site-directed Mutagenesis—The cDNAs for the rat wild-type and mutant α1 subunits were cloned into the pCD-PS eukaryotic expression vector as described previously (8, 24). The construction of the GA–α13 mutant with the substitution of alanine for glycine 2 was described earlier (8). Site-directed mutagenesis was performed using the polymerase chain reaction and oligonucleotide TAG ATA CGG CGG CGG AGG TCG GCC ACC ATG GCC GCG ACA CTCG AAC GC to change the α1 CDNA to code for an alanine instead of cysteine 3 (C3A) using reaction conditions as described (24). The part of
the 5'-untranslated region and the coding sequence between the unique restriction sites for KasI and BstXI was amplified and introduced by ligation into the α subunit cDNA in pCD-PS vector using the same unique pair of restriction enzymes. To create the GA-α, mutant, the mutation of glycine to alanine was introduced by polymerase chain reaction and the amplified cDNA was ligated into the unique cloning ACC GCT GTC GGC ACC ACC AGT GCA GTC CTC GGC. The amplified cDNA region with the mutation was ligated into the corresponding region of α subunit cDNA in pCD-PS vector using a unique pair of restriction enzymes, EagI and MluI. The mutations were confirmed by nucleotide sequencing and restriction analysis. The construction of WT, α, and the C68S mutant of y2 cDNAs in pCDM8.1 expression vector was described previously (25).

Transfection and Radio labeling—COS-7 monkey kidney cells were maintained and transfected using the DEAE-dextran method as described previously (26). 48 h after transfection the cells were prepared for metabolic labeling by incubation in serum-free DMEM for 2 h. Since [3H]palmitate can be metabolized to [3H]myristate, metabolic labeling with [3H]palmitate was performed in the presence of 50 μg/ml of cycloheximide (Calbiochem) in serum-free media for 30 min prior to and during the incubation with [3H]palmitate to prevent co-translational myristoylation. The cells were labeled for 30 min with 500 μCi of [3H]-palmitate/ml (American Radiolabeled Chemicals; specific activity 30 Ci/mmol) or 300 μCi of [3H]-myristate/ml (DuPont NEN, specific activity 16 Ci/mmol) in 5 ml of serum-free media supplemented with 1% (v/v) dimethyl sulfoxide/75-ml flask. For the pulse-chase experiments, the cells were incubated for 20 min with [3H]palmitate, washed once with serum-free DMEM, and then incubated in complete DMEM containing 10% (v/v) fetal bovine serum. The cells were scraped in cold phosphate-buffered saline and centrifuged at 2000 × g for 10 min. The pellets were resuspended in homogenization buffer and frozen at −70 °C.

Cell Fractionation—The cell pellets were homogenized by passing 25 times through a 25-gauge needle in homogenization buffer composed of 5 mM HEPES, pH 7.4, 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 μl/milliliter α catalytic subunit (Boehringer Mannheim). The cell lysate was centrifuged at 1000 × g for 3 min in an Eppendorf 5415 microcentrifuge to pellet nuclei and unbroken cells. The supernatant was centrifuged at 125,000 × g for 30 min at 4 °C in a Beckman TLS45 rotor. The supernatant (soluble fraction) was separated and the pellet (particulate fraction) resuspended in the buffer and recentrifuged. The washed pellet was resuspended in the original volume of homogenization buffer. The amount of protein in the particulate and soluble fractions was approximately equal.

Immunoprecipitation and Immunoblotting—The affinity-purified antibodies specific for the carboxy-terminal decapeptide of α subunit (AS) and α (RM) were used for immunoblotting and immunoprecipitation (27, 28). Anti sera SW (29) and EDPL were used for detection of β1 and β2 subunits, respectively. EDPL was raised against the carboxy-terminal peptide of β2, corresponding to residues 47-64. Immunoblotting with detection of the primary antibodies with [125I]-protein A, and autoradiography was performed as described previously (24, 30). Immunoprecipitation was performed on equivalent amounts of protein in a solubilization buffer of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.8% (w/v) Triton X-100, 0.2% (w/v) SDS, and 1 mM EDTA with an incubation overnight at 4 °C as described previously (8). The immunoprecipitates were recovered by incubating with protein A-Sepharose CL-4B (Pharmacia Biotech Inc.), washed, solubilized, separated by SDS-PAGE on 10% Tris-glycine gels (Novex), and prepared for fluorography.

Detergent Extraction—The particulate fractions from transfected COS cells were incubated for 1 h on ice with (or without) Triton X-100, 120 mM NaCl, 0.1 mM GTP, and 10 mM MgCl2. The final protein concentration was 1 mg/ml. The samples were separated by centrifugation at 125,000 × g for 30 min at 4 °C.

Trypsin Cleavage—The COS cell fractions were treated with 30 min at 4 °C with or without 1 mM GTP-S and then incubated with L-1-1-tosylamido-2-phenylbenzamidohemolysin ketone-treated trypsin (Sigma) (trypsin/cell protein ratio was 1:15) for 30 min at 4 °C in a buffer containing NaCl, pH 7.5, 10 mM MgCl2, 1 mM EDTA. Activation of the trypsin was done by adding 1 mM ATP. Proteolysis was stopped by stopping trypsin inhibitor (Sigma) at 4 molar excess over trypsin. The samples were analyzed by SDS-PAGE and immunoblotting.

Miscellaneous Procedures—Pertussis toxin-catalyzed ADP-ribosylation was performed with activated toxin (List Biologicals), [3H]NAD (DuPont NEN, specific activity 800 Ci/mmol) and purified bovine retinal β subunits (31) as described previously (8). In some experiments, the particulate fractions were first incubated with 1 mM GTP-S or GTP for 15 min at 37 °C. Protein concentration was determined by the Bio-Rad protein assay dye kit with IgG as the standard (Bio-Rad). Thin layer chromatography and hydroxylamine treatment were performed as described earlier (12).

Quantitation—Densitometry of the fluorographs was performed with a LKB 2202 UltroScan laser densitometer. Statistical data analysis was performed with commercial software, Sigmaplot (Jandel Scientific) and Statview (Abacus Concepts). The quantitative analysis of the gels with [3P]ADP-ribose-labeled material and the nitrocellulose membranes treated with [3P]protein-A was done using a PhosphorImager (Molecular Dynamics).

RESULTS

Mutation of Cysteine 3 Prevents [3H]Palmitate Incorporation—COS cells were transfected with plasmids containing the cDNA for the wild-type α (WT) or an α mutant, in which the coding sequence was changed to replace cysteine 3 with an alanine (C3A) (Table I). Immunoprecipitation of fractions from cells metabolically labeled with [3H]myristate showed incorporation into both the WT and C3A proteins in both the particulate and soluble fractions (Fig. 1). The distribution of the proteins will be discussed. Incorporation of [3H]myristate, which is a co-translational event, was prevented with the protein synthesis inhibitor, cycloheximide. The C3A mutant did not incorporate [3H]palmitate (Fig. 1). The WT protein incorporated the radiolabel only in the particulate fraction. Hydroxylamine treatment and thin layer chromatography of the radiolabel after release by alkalai treatment confirmed the thioester linkage and [3H]palmitate incorporation, respectively (data not shown).

Intracellular Localization of the C3A Mutant—To determine the role of palmitoylation in membrane attachment, we compared the subcellular distribution of the WT, nonpalmitoylated C3A mutant and a mutant α, in which the glycine 2 was changed to alanine (GA, Table I). The WT is predominantly in the particulate fraction and the GA mutant which does not undergo myristoylation (8) is predominantly in the soluble fraction (Fig. 2). The localization of the C3A mutant is intermediate with approximately two-thirds of the expressed protein in the soluble fraction.

Since overexpression leads to more α subunits than β subunits (32), we evaluated the intracellular localization of the nonpalmitoylated C3A protein with co-transfection of β subunits. With co-expression of β1 and β2 subunits, approximately two-thirds of the C3A protein was now in the particulate fraction (Fig. 3).

Association of the Nonpalmitoylated C3A Mutant with β Subunits—We tested whether the nonpalmitoylated mutant could form a heterotrimer with β subunit by performing PTX-catalyzed ADP-ribosylation. The α subunit is a substrate for ADP-ribosylation only when it is bound to β (33, 34). The soluble fractions of transfected COS cells were incubated with purified β subunits. Both the WT (not shown) and C3A proteins underwent maximal ADP-ribosylation at a much lower concentration of β than needed for the GA mutant which has a decreased affinity for β (8) (Fig. 4).

For the particulate fraction, PTX ADP-ribosylation was significantly increased in the cells co-transfected with both the
encoding the wild-type noprecipitation with a1-specific AS antibody from 500 pg of total protein, cycloheximide pretreatment. The cells were harvested and separated into particulate (Part.) and soluble (Sol.) fractions followed by immunoprecipitation with a1-specific AS antibody from 500 µg of total protein from the fractions. The immunoprecipitates were resolved by SDS-PAGE and analyzed by fluorography. Exposure time was 3 days.

Wild-type cells were transfected with vector alone (Vector) or with cDNAs for the a1 subunit co-expressed with the nonmyristoylated (Vector), wild-type (WT), and C3A mutant a1 subunits compared to the same particulate fraction pretreated with GTPyS, but there was no further increase in ADP-ribosylation compared to the vector transfected cells (Fig. 4). Pertussis toxin-catalyzed [32P]ADP-ribosylation of soluble mutant a1, subunits. Soluble fractions from COS cells transfected with the C3A and GA mutants of a1 subunit were subjected to ADP-ribosylation with PTX. To equalize the amount of a1 subunits in the reaction 14 µg of total protein of the C3A-soluble fraction and 8 µg of total protein of the GA-soluble fraction were used. Increasing amounts of purified retinal transfducin b subunits (b subunits) were added to the soluble fractions for the ADP-ribosylation reaction. The samples were analyzed by SDS-PAGE and autoradiography.

Subcellular localization of the wild-type and mutant forms of a1, subunit after transient expression in COS cells. The cells were transfected with vector alone (Vector) or with cDNAs for the wild-type (WT), cysteine 3 to alanine (C3A), and glycine 2 to alanine (GA) mutants of a1, 48 h later the cells were separated into particulate (P) and soluble (S) fractions 40 µg of total protein from each fraction were resolved with SDS-PAGE and analyzed by immunoblotting with the AS antibody and 125I-protein A, followed by autoradiography. The film was exposed overnight.

Subcellular localization of the wild-type and mutant forms of a1, subunit co-expressed with b1y2 subunits. COS cells in 75-cm² flasks were transfected with 7 µg of DNA of the vector alone (Vector), wild-type (WT), C3A, or GA mutant a1, subunits with (+) or without (−) co-transfection with 10 µg of DNA of b1 and y2 vectors. 48 h later the cells were harvested and separated into particulate (P) and soluble (S) fractions 40 µg of the total protein from each fraction were analyzed by SDS-PAGE and immunoblotting with the a1-specific antibody and detected with 125I-protein A. The film exposure time was overnight. The GA protein migrates slightly slower than the endogenous a1 proteins.

Nonpalmitoylated C3A mutant and b subunits compared to the vector transfected cells (Fig. 5). When this particulate fraction was pretreated with GTP-γS to cause dissociation of the subunits, the PTX ADP-ribosylation was markedly decreased compared to the same particulate fraction pretreated with GTP (data not shown).

The particulate fraction of the cells transfected with the nonpalmitoylated C3A mutant alone showed a small increase in PTX ADP-ribosylation compared to the vector transfected cells (Fig. 5, open bars), but there was no further increase in ADP-ribosylation in the presence of purified b subunits as was seen for the WTo1, protein (Fig. 5, filled bars). To test whether this lack of effect with purified b subunits was due to improper folding of the transfected a subunit, detergent solubilization and trypsin cleavage in the presence of GTP-γS was performed. This nonpalmitoylated protein could not be solubilized with the detergent Triton X-100. The endogenous a1, transfected WT a1, and the nonpalmitoylated C3A protein in cells co-transfected with b subunits were solubilized by the detergent (data not shown). GTP-γS binding decreases the trypsin proteolysis of a subunits and leads to a partial proteolytic product of approximately 39 kDa (35, 36) which was seen for the cells overexpressing the WTo1, protein. The nonpalmitoylated C3A protein in the particulate fraction of cells without co-transfection of b subunits was completely degraded by trypsin in the presence of GTP-γS (data not shown).

Palmitylation of the Nonmyristoylated GA Protein: Co-expression of the b and y subunits with the nonmyristoylated GA mutant led to a redistribution of some of the protein to the
Fig. 6. Membrane localization and palmitoylation of the non-myristoylated GA mutant α3 subunit after co-expression with \( \beta\gamma \) subunits. A, COS cells were transfected with 7 μg of the vector (V), wild-type (WT), or GA mutant α3 cDNAs alone (–) or co-transfected with 7 μg of the cDNAs encoding β1 and γ2 subunits (+). B and C, the GA mutant α3 subunit cDNA 5 μg was co-transfected into COS cells with increasing concentrations of the cDNAs encoding β1 and γ2 subunits (indicated is the amount for each subunit/75-cm\(^2\) flask). Vector DNA was added to keep the total amount of transfected DNA constant. DNA was added to keep the total amount of transfected DNA constant. Vector DNA was added to keep the total amount of transfected DNA constant. D, COS cells were transfected with 5 μg of vector (V) or GA mutant cDNA in the absence or presence of 30 pg each of β1 and γ2 subunits. 48 h later the cells were treated with cycloheximide, incubated with \[^{[\text{H}]}	ext{palmitic acid}\], harvested, and fractionated. 300 μg of protein (A), 800 μg of protein (B), and 450 μg of protein (D) from the particulate fractions were immunoprecipitated with the AS antibody and analyzed by SDS-PAGE and fluorography. The exposure time was 16 h. The GA protein migrates slightly slower than the endogenous α3 subunits.

Fig. 7. Pulse-chase \[^{[\text{H}]}	ext{palmitic acid}\] labeling of the wild-type and nonmyristoylated GA mutant α3 subunits co-expressed with \( \beta\gamma \) subunits. COS cells were transfected with the wild-type α3 subunit cDNA (7 μg of DNA/75-cm\(^2\) flask) with (filled squares) or without (open squares) the cDNAs encoding β1 and γ2 subunits (7 μg for each subunit). GA mutant α3 subunit cDNA (5 μg) was transcribed together with the β1 and γ2 subunits cDNAs (8 μg for each subunit) (triangles). 48 h after transfection the cells were incubated with \[^{[\text{H}]}	ext{palmitic acid}\] for 20 min, the media changed, and the cells harvested at the indicated times. The particulate fractions of the cells (800 μg of the total protein) were immunoprecipitated with AS antibody and analyzed by fluorography. The exposure time was 2 days. The \[^{[\text{H}]}	ext{palmitate incorporation was determined by densitometry of the exposed films.}

DISCUSSION

The interplay of acylation with membrane attachment and protein interactions has biologic significance for many signal transduction proteins. For the heterotrimeric G protein α3,
myristoylation and palmitoylation occur in a region important for both protein interaction (βγ) and membrane attachment. We found that mutation of cysteine 3 prevented palmitoylation and led to loss of membrane attachment. This nonpalmitoylated mutant could form a heterotrimer with the βγ subunits which restored its membrane localization. Since palmitoylation is reversible, we investigated the regulation of this modification. Membrane localization and cysteine 3 were crucial for palmitoylation. Myristoylation was not required for palmitoylation, but myristoylation and βγ association could slow the α subunit palmitate turnover.

**Palmitoylation and Membrane Attachment**—Palmitoylation, myristoylation, and βγ association all contributed to the membrane attachment of the α subunit; membrane attachment being the greatest with the occurrence of all three but possible with any two. After translation, the 14 carbon myristoyl group can provide a marginal affinity for membranes as tested with acylated peptides and evidenced by the distribution of myristoylated proteins to both the membrane and cytosol. The myristoylated α and C3A proteins are possibly localized at membranes by the presence of endogenous βγ subunits or weakly attach through the myristate group. Palmitoylation may stabilize the membrane localization of the α subunit, whereas the nonpalmitoylated C3A mutant, when it is free of βγ, may either dissociate from the membrane or form aggregates. The amount of the nonpalmitoylated C3A mutant we found in the particulate fraction without βγ co-transfection did not reflect true membrane affinity because most of the protein was nonfunctional and possibly aggregated.

Palmitoylation may also be important in stabilizing the α subunit at its proper intracellular location. Cadwallader and colleagues showed that palmitoylation can lead to nonspecific membrane attachment of Ras mutants whereas palmitoylation was needed for plasma membrane attachment. The role of palmitoylation in membrane attachment may be different for the myristoylated and nonpalmitoylated α subunits. Mutation of the third cysteine in α, or α(10, 15, 16) led to a significantly greater decrease in membrane attachment than the same mutation in α(12, 15). The proximity of the myristate and palmitate groups on the α and α subunits suggests that they may be part of the same membrane attachment domain. Alternatively, palmitoylation on α and α may act independently of another membrane attachment domain. Since this domain has not been identified, it is possible that the epitope tagging of α and α by Wedegaertner et al. (13) could have altered this domain and explain the marked decrease in membrane attachment of the nonpalmitoylated α and α in their study.

**Palmitoylation, βγ Association, and Myristoylation**—Unlike palmitoylation which markedly increases the affinity of α and α for βγ(8, 23), palmitoylation was not crucial for heterotrimer formation. Consequently, the decreased βγ affinity of a Gaα, mutant described by van der Neut and colleagues (40) is unlikely to be associated with altered palmitoylation. In any case, we found that this mutant could undergo palmitoylation.

The importance of palmitoylation may be to primarily affect the structure of the α subunit enhancing βγ affinity, whereas the importance of palmitoylation may be to cause binding of the α subunit to the lipid bilayer. While this study did not address whether palmitoylated α subunits can form a heterotrimer, indirect evidence suggests that they do. The H21a mutant of α, which is inactive and does not dissociate from βγ has a basal incorporation of [3H]palmitate similar to the wild-type α(17).

The C3A mutation in α did not prevent myristoylation of glycine 2. Myristoylation also occurs on the nonpalmitoylated C3A or C3S mutants of α(10, 15). These results are consistent with myristoylation being a co-translational event in the cytosol and palmitoylation occurring later at membranes (38, 41). The third residue is not a critical component of the amino-terminal consensus sequence for N-myristoyl transferase activity (38).

**Regulation of Palmitoylation**—In this study, the crucial determinants of α palmitoylation appeared to be the third cysteine residue and membrane localization. This third residue is probably the actual site of palmitoylation since it is the sole cysteine within the first 21 amino acids shown to have the site of palmitoylation (11). Previous reports have indicated that myristoylation may be required for palmitoylation because myristoylation defective GA2 mutants of α and α did not undergo palmitoylation (7, 15). However, in this study a nonmyristoylated mutant could incorporate [3H]palmitate when it was localized at the membrane after co-expression with βγ. The frequent occurrence of palmitoylation with either myristoylation at the amino terminus (22) or with isoprenylation at the carboxyl terminus (42) may be due to the myristate and isoprenoid groups first allowing membrane attachment and then positioning the cysteine close to the membrane.

Regulation of the availability of the cysteine thiol group to a palmitoyl CoA could occur from both the local and global protein conformation. The adjacent amino acids may facilitate the reaction by creating a favorable secondary structure and increasing membrane interactions. Dynamic regulation of palmitoylation can occur from global conformational changes which change the distance of the cysteine from the membrane or change the closeness of the βγ subunits. The rapid exchange of the palmitate on the nonmyristoylated GA protein is an example of both levels of regulation. The loss of the myristate on the adjacent glycine could provide more exposure of the cysteine. The decreased βγ affinity, probably resulting from a change in amino-terminal conformation secondary to a lack of myristoylation, could also contribute to rapid turnover of palmitate.

For α subunits, the βγ subunits may be the primary regulator of palmitoylation. The increased palmitate turnover of α upon activation could be the result of decreased βγ affinity due to the conformational changes of the α subunit upon binding of GTP. However, more studies are needed for a full understanding of the regulation and function of α subunit palmitoylation. Its reversibility and effects on membrane affinity suggest it has a prominent role in the regulation of G protein signal transduction.

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