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Biochemistry

Myristoylation of an inhibitory GTP-binding protein α subunit is essential for its membrane attachment

(site-directed mutagenesis/immunoprecipitation/signal transduction/β-γ subunits)

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ABSTRACT **We transfected COS cells with cDNAs for the α subunits of stimulatory and inhibitory GTP-binding proteins, α_s and α_{i1}, respectively, and immunoprecipitated the metabolically labeled products with specific peptide antibodies. Cells were separated into particulate and soluble fractions before immunoprecipitation; [^{35}S]methionine-labeled α_s and α_i were both found primarily in the particulate fraction. [^{3}H]Myristate was incorporated into endogenous and transfected α_i but could not be detected in α_s even when it was overexpressed. We converted the second residue, glycine, of α_{i1} into alanine by site-directed mutagenesis. Upon transfection of the mutant α_{i1} into COS cells, the [^{35}S]methionine-labeled product was localized primarily to the soluble fraction, and, also unlike normal α_{i1}, the mutant failed to incorporate [^{3}H]myristate. The unmyristoylated mutant α_{i1} could still interact with the β–γ complex, since purified βγ subunits promoted pertussis toxin-catalyzed ADP-ribosylation of both the normal and mutant α_{i1} subunits. These results indicate that myristoylation is critical for membrane attachment of α_i but not α_s subunits.**

Heterotrimeric guanine nucleotide-binding proteins (G proteins) act as signal transducers by coupling receptors for hormones and neurotransmitters to effector enzymes and ion channels (1–3). Members of the G-protein family include G_s and G_i, the stimulatory and inhibitory G proteins, respectively, associated with adenylyl cyclase. G proteins are distinguished by their α (GTP-binding) subunits, which are believed to confer specificity in receptor–effector coupling. Molecular cloning of multiple cDNAs encoding α subunits indicates considerable diversity in the G-protein family, including at least three separate genes for G_i, arbitrarily termed G_{i1}, G_{i2}, and G_{i3}, as well as a closely related gene encoding a protein termed G_o (1–4).

Of the three G-protein subunits, β and γ remain tightly associated as a complex, except upon denaturation, whereas α subunits dissociate from the β–γ complex upon activation by GTP analogs such as guanosine 5′-[γ-thio]triphosphate (GTP[γS]) (1–3). G-protein heterotrimers are known to be associated with the cytoplasmic surface of plasma membranes, but the basis for membrane association has not been defined. Purified α subunits do not require detergent to prevent aggregation in aqueous solution (5), consistent with the relatively hydrophilic structure predicted by α subunit cDNAs (1–4). Purified α subunits also fail to associate with phospholipid vesicles, unless such vesicles contain β–γ complexes (5). This has led to the suggestion that β–γ complexes serve as membrane anchors for α subunits. Nonetheless, the failure of α subunits to be released from the membrane in non-detergent-containing buffers, even after activation by GTP[γS] (6), suggests that α subunits are tightly attached to cell membranes through a mechanism that may be independent of interaction with the β–γ complex.

Recently, α_i and α_o (7, 8) have been shown to be myristoylated. Covalent attachment of this 14-carbon fatty acid is a cotranslational modification that may be important for membrane association (9, 10). To assess the role of myristoylation in the membrane association of G-protein α subunits, we performed immunoprecipitation studies on metabolically labeled COS cells, expressing endogenous and transfected α subunits. Although both α_s and α_i were primarily membrane associated, only the latter was found to be myristoylated. By site-directed mutagenesis, we created a G_i α subunit that could not undergo myristoylation, and we found that it was predominantly localized to the cell cytosol. Thus, myristoylation appears to be critical for membrane association of α_i but not α_s subunits.

MATERIALS AND METHODS

Transfection and Radiolabeling. Rat olfactory cDNAs for α_s and α_{i1} (4) (kindly provided by R. Reed and D. Jones, Johns Hopkins University) were inserted into the pCD-PS plasmid (11) derived from the Okayama–Berg expression vector (12). COS-7 cells (13) maintained in Dulbecco's modified Eagle's medium (Biofluids, Rockville, MD) supplemented with 10% (vol/vol) fetal calf serum were transfected when subconfluent with the pCD-PS plasmids with and without a cDNA insert or sham-transfected using the diethylaminoethyl-dextran method (14). After 48 hr, protein radiolabeling was achieved by incubating the cells for 4 hr with either 200 μCi of [^{35}S]methionine/cysteine per ml (Tran^{35}S-label; ICN Radiochemicals; specific activity, ≈1100 Ci/mmol; 1 Ci = 37 GBq) in Dulbecco's modified Eagle's medium containing 10% the normal concentration of methionine and 10% fetal calf serum or 200 μCi of [^{3}H]myristic acid per ml (DuPont-New England Nuclear; specific activity, ≈39 Ci/mmol) in Dulbecco's modified Eagle's medium with 5% (vol/vol) fetal calf serum and 1% dimethyl sulfoxide. [^{3}H]Myristic acid was concentrated 5:1 by evaporating the ethanol solvent before adding it to the medium. Cell pellets were obtained by scraping and by centrifugation (500 × *g* for 10 min) in cold phosphate-buffered saline with storage at −70°C.

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Abbreviations: G protein, guanine nucleotide-binding regulatory protein; G_s and G_i, the stimulatory and inhibitory G proteins, respectively, associated with adenylyl cyclase; α_s and α_i, the α subunits of the corresponding G proteins; GTP[γS], guanosine 5′-[γ-thio]triphosphate.

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Fractionation. The cell pellets were lysed for 20 min on ice with 4 vol of homogenization buffer [20 mM Tris·HCl, pH 7.4/1 μM leupeptin/0.3 μM aprotinin/100 microunits of α_2-macroglobulin per ml/1 μM pepstatin (Boehringer Mannheim)/1 mM EDTA/100 μg of soybean trypsin inhibitor per ml (Sigma)]. The cells were then homogenized by trituration through a 20-gauge needle and centrifuged at 1000 × *g* for 3 min to remove the nuclear pellet. The supernatant was transferred to polyallomer Quick-Seal tubes (Beckman) and centrifuged at 400,000 × *g* for 30 min in a Beckman 70.1 Ti rotor. The pellet (particulate fraction) was resuspended in the homogenization buffer by using a polypropylene homogenizer (Kontes). The supernatant was recentrifuged at 400,000 × *g* for 30 min. The final supernatant (soluble fraction) was concentrated with a Centricon-10 microconcentrator. The protein concentration was determined by the Bradford method (15) with IgG (Bio-Rad) used as a standard.

Immunoprecipitation. The preparation and characterization of the antisera to the synthetic peptides corresponding to the carboxyl-terminal decapeptides of α subunits of G_s and transducin, respectively, have been described (16, 17). For immunoprecipitation, the affinity-purified antibodies from antisera RM and AS, specific for α_s and α_{i1}/α_{i2}, respectively, were used. Purified rabbit IgG (Pel-Freez Biologicals) was used as a control for nonspecific immunoglobulin binding. Equivalent amounts of protein were solubilized in 150 mM NaCl/50 mM Tris·HCl, pH 7.4/SDS to give a detergent/protein (wt/wt) ratio of >10:1. After 1 hr, the samples were centrifuged at 11,600 × *g* for 10 min. Triton X-100 [final Triton X-100/SDS (wt/wt) ratio, 5:1] and the affinity-purified antibody or IgG was added to the supernatant and incubated at 4°C overnight on a rotator. Protein A-Sepharose (Pharmacia) was added and incubated for 2 hr at 4°C. The immune complexes were pelleted by centrifugation at 11,600 × *g* for 10 min. The pellet was washed twice in 600 mM NaCl/50 mM Tris·HCl, pH 8.3/SDS/Triton X-100 (the same detergent concentration as used in solubilization). The pellet was washed once in 150 mM NaCl/50 mM Tris·HCl, pH 7.4/5 mM EDTA. The sample was solubilized in denaturing buffer [0.125 M Tris·HCl, pH 6.8/4% (wt/vol) SDS/20% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol], heated at 90°C for 5 min, and centrifuged at 11,600 × *g* for 10 min.

PAGE and Autoradiography. Samples and molecular weight markers (Bethesda Research Laboratories) were analyzed by SDS/PAGE on 10% polyacrylamide gels. The gels were fixed in 30% (vol/vol) methanol/10% (vol/vol) acetic acid, treated with En^{3}Hance (DuPont-New England Nuclear), dried, and exposed to XAR-2 film (Kodak) at −70°C. Autoradiographs from multiple exposures of three separate experiments were analyzed with an LKB 2202 ultrascan laser densitometer and LKB 2220 recording integrator. The density of the bands corresponding to the transfected α_i subunits was used to calculate the relative amounts of the subunit in the particulate and soluble fractions by correcting for the yield of total protein in each fraction.

Hydroxylamine Treatment. Duplicate polyacrylamide gels of [^{3}H]myristic acid-labeled proteins were fixed in 25% (vol/vol) isopropyl alcohol/10% (vol/vol) acetic acid and exposed to either 1 M hydroxlyamine (pH 7.0) or 1 M Tris·HCl (pH 7.0) for 12 hr followed by analysis by fluorography as described above.

Site-Directed Mutagenesis. A 21-base oligonucleotide changing the codon for glycine-2 to alanine (see Fig. 2) was prepared on the Applied Biosystems DNA synthesizer model 380B and the salts were removed by using a Sep-Pak C18 cartridge (Millipore). The cDNA for α_{i1} was removed from the pCD-PS by using the restriction enzyme *Eco*RI and was inserted into the polylinker of M13mp19 expression vector (Bethesda Research Laboratories) for the mutagenesis reaction. The mutagenesis was performed by the Amersham system, which is based on the method of Eckstein and co-workers (18). The mutant cDNA, screened for the presence of the new *Sph* I restriction site (see Fig. 2), was removed from the M13mp19 vector by digestion with the restriction enzyme *Eco*RI, purified from a low-melting-point agarose gel (NuSieve GTG FMC) with an Elutip filter (Schleicher & Schuell), and then ligated with a linear, dephosphorylated pCD-PS vector using T4 DNA ligase (New England Biolabs) as described (19). XL-Blue *Escherichia coli* (Stratagene) were transformed with the ligation mixture by the $CaCl_2$ method (20). Transformed bacteria were selected by growth on ampicillin-agar plates. A small-scale plasmid purification preparation (20) followed by restriction analysis of the plasmids with the enzymes *Eco*RI, *Sma* I, and *Sph* I (International Biotechnologies) was performed to select colonies that had been transformed with pCD-PS vector containing the mutant α_{i1} cDNA in the correct orientation. The plasmid from the selected colony was purified by the Triton-lysosome method as described on a chloramphenicol-treated culture (21). Sequence analysis of the mutant after alkali denaturation was performed by the GemSeq K/RT sequencing system (Promega Biotec) with [α^{32}P]dATP (DuPont-New England Nuclear; specific activity, 400 Ci/mmol).

[^{32}P]ADP-Ribosylation with Pertussis Toxin. The method of Ribeiro-Neto *et al.* (22) was used with minor modifications. Briefly, samples of the soluble fractions from the transfected COS cells (control and wild-type α_{i1}, total protein, 10 μg; mutant α_{i1}, total protein, 4 μg) and purified transducin βγ subunits [isolated from bovine holotransducin by chromatography on Blue-Sepharose as described (23, 24)] were incubated in 50 μl (final volume) with a reaction mixture containing 5 μCi of [^{32}P]NAD (DuPont—New England Nuclear; specific activity, 800 Ci/mmol), 10 mM thymidine, 1 mM ATP, 0.1 mM GTP, 10 mM arginine, bovine serum albumin (0.2 mg/ml), 100 mM Tris·HCl (pH 7.5), 0.1% Lubrol with 0.5 μg of preactivated pertussis toxin (List Biological Laboratories, Campbell, CA) at 37°C for 60 min. Then, 50 μl of quenching solution [0.5% sodium deoxycholate/bovine serum albumin (0.2 mg/ml)/500 μM NAD] was added, followed by 1 ml of ice-cold 20% trichloroacetic acid. Samples were kept on ice for 15 min and then centrifuged at 16,000 × *g* for 10 min in an Eppendorf 5415 microcentrifuge. The supernatants were discarded and the pellets were washed with 1 ml of cold acetone. After recentrifugation and drying of the pellets, the samples were dissolved directly in 150 μl of denaturing buffer with 50 μl used for analysis on SDS/PAGE. Recoveries were highly uniform among different samples as estimated by Coomassie blue staining of the bovine serum albumin present in each washed precipitate (22). Autoradiography was performed on the dried gels with XAR-2 film (Kodak) in Kodak X-Omatic cassettes with regular intensifying screens for 12–48 hr at −70°C.

When studying the effects of GTP[γS], the samples were prepared as described above except that the samples from the soluble fractions were first incubated in a reaction mixture with either 0.5 mM GTP or 0.5 mM GTP[γS] and 5 μCi [^{32}P]NAD, 10 mM thymidine, 1 mM ATP, 10 mM arginine, bovine serum albumin (0.2 mg/ml), 100 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, and 0.1% Lubrol for 15 min at 37°C. Then, preactivated pertussis toxin (0.5 μg) and purified βγ subunits (50 μg/ml) were added, followed by incubation at 37°C for 60 min and processing as described above.

RESULTS

Transient Expression and Immunoprecipitation of G Protein α Subunits. Immunoprecipitation of [^{35}S]methionine-labeled proteins in the particulate fraction of control (sham transfected) COS cells (Fig. 1*A*, lanes 3–5) with the affinity-purified antibody RM, specific for α_s, shows faint protein

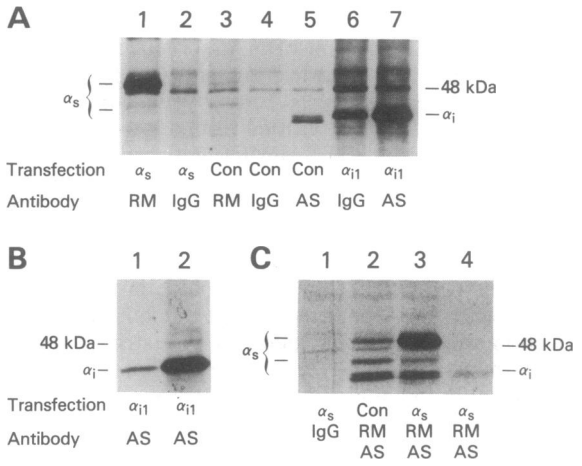

FIG. 1. Immunoprecipitation of G-protein α subunits in transfected COS cells. COS cells were transfected by using the diethylaminoethyl-dextran method with the cDNA for α_s or α_{i1} inserted into the pCD-PS expression vector or were sham transfected (Con) and radiolabeled after 48 hr with [^{35}S]methionine or [^{3}H]myristic acid. A particulate fraction was prepared after cell lysis, homogenization, low-speed centrifugation (1000 × *g* for 3 min) and ultracentrifugation of the supernatant (400,000 × *g* for 30 min). Equivalent amounts of protein were immunoprecipitated with rabbit IgG, affinity-purified RM antibodies specific for the α_S subunit, or affinity-purified AS antibodies specific for α_{i1} and α_{i2}. Samples were analyzed by SDS/PAGE, fixed, treated with En^{3}Hance, and exposed to XAR-2 film at −70°C for 7–21 days. An endogenous [^{35}S]methionine-radiolabeled protein of 48 kDa, determined by molecular mass markers, is labeled for reference. (*A*) [^{35}S]Methionine-labeled proteins (125 μg of protein). (*B*) [^{3}H]Myristic acid (lane 1)- and [^{35}S]methionine (lane 2)-labeled proteins (13 μg of protein). (*C*) [^{35}S]Methionine (lanes 1–3)- and [^{3}H]myristic acid (lane 4)-labeled proteins (13 μg of protein).

bands at 52 and 45 kDa, representing the long and short forms of α_s (25). Immunoprecipitation of the same fraction with the affinity-purified antibody AS, specific for α_{i1}/α_{i2} (weak cross-reactivity with α_{i3}) (26), shows protein bands at 41 kDa (α_{i3} and/or α_{i1}) and 40 kDa (α_{i2}). Immunoprecipitation with IgG used as a control for nonspecific binding of protein to immune complexes shows only a 48-kDa band representing an endogenous protein with a high level of [^{35}S]methionine incorporation.

Transfection of COS cells with cDNA for α_{i1} or the long form of α_s in the pCD-PS expression vector resulted in a marked increase in the expression of these subunits (Fig. 1*A*, lanes 1, 2, 6, and 7). The expression of the α_{i1} subunit, in particular, was greater than most of the native cellular proteins and therefore was seen even after immunoprecipitation with control IgG. Immunoprecipitation with the RM and AS antibodies of the soluble fractions of cells transfected with the cDNA for α_s or α_{i1} was also performed (data not shown). While a small amount of α_s and α_{i1} in the cells transfected with the cDNA for those subunits was seen in the soluble fraction, the majority was found in the particulate fraction. For the control COS cells, immunoprecipitations with the RM and AS antibodies did not show either α_s or α_i subunits in the soluble fraction.

Myristoylation. When COS cells were radiolabeled with [^{3}H]myristic acid after sham transfection or transfection with the cDNA for α_{i1} and immunoprecipitated with the AS antibody, autoradiography showed incorporation of [^{3}H]-myristic acid into the endogenous α_i subunits (Fig. 1*C*, lane 4) and into transfected α_{i1} subunits (Fig. 1*B*). ^{3}H labeling of the 40- to 41-kDa band corresponding to α_i was not diminished by hydroxylamine treatment (data not shown). This excludes conversion of [^{3}H]myristic to [^{3}H]palmitic acid, since the thioester bond that binds palmitic acid to proteins is sensitive to hydroxylamine (10). Data below (see Fig. 3) will show that the ^{3}H-labeled band also does not reflect conversion of [^{3}H]myristic acid to form tritiated amino acids.

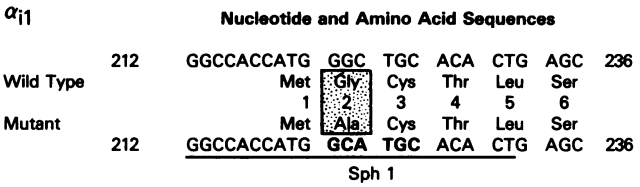

FIG. 2. Nucleotide and amino acid sequence of the wild-type and the mutant α_{i1}. The numbers before and after the nucleotide sequences correspond to nucleotide residues of the coding sequences of rat α_{i1} (4). The 21-base oligonucleotide from residues 212–232 (underlined) was used for site-directed mutagenesis. The base sequence GCATGC (boldface) present in the mutant is a restriction site for the enzyme *Sph* I. The amino acid change is noted in the stippled box.

Immunoprecipitation of the particulate fraction of COS cells transfected with the long form of α_s and radiolabeled with [^{35}S]methionine or [^{3}H]myristic acid was performed by using the RM antibody alone (data not shown) or by using both the RM and AS antibodies simultaneously (Fig. 1*C*). Immunoprecipitation of the [^{35}S]methionine-labeled proteins shows the relative amounts of the transfected and endogenous α_s subunits as compared to the endogenous α_i subunits (lane 3). [^{3}H]Myristic acid incorporation into α_s was not seen even under conditions in which incorporation of [^{3}H]myristic acid into the less abundant endogenous α_i subunits was seen (lane 4).

Site-Directed Mutagenesis. Since an amino-terminal glycine is essential for myristoylation (10), site-directed mutagenesis was used to generate an α_{i1} cDNA in which the amino-terminal glycine is converted to alanine. Fig. 2 shows the amino-terminal nucleotide and amino acid sequence of the wild-type and mutant α_{i1}. This change was confirmed by restriction digests with the enzyme *Sph* I and sequence analysis of the mutant α_{i1} (data not shown).

Subcellular Localization of the Mutant α_{i1}. COS cells transfected with the cDNA for either the wild-type or mutant α_{i1} were radiolabeled with either [^{35}S]methionine or [^{3}H]myristic acid and were separated into particulate and soluble fractions; immunoprecipitation was performed with the AS antibody (Fig. 3). Both the wild-type and mutant α_{i1} were highly expressed, as shown by immunoprecipitation of the [^{35}S]methionine-labeled proteins (Fig. 3*A*), as well as being readily

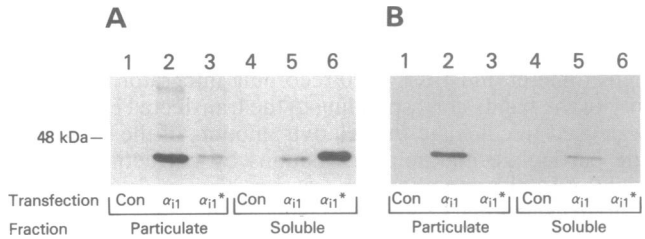

FIG. 3. Immunoprecipitation of cellular fractions after transfection with the mutant α_{i1}. COS cells were transfected with the pCD-PS vector without inserts (Con) or with cDNA for the wild-type α_{i1} (α_{i1}) or the mutant α_{i1} (α_{i1}*) and were radiolabeled after 48 hr with [^{35}S]methionine or [^{3}H]myristic acid. The cells were lysed, homogenized, and centrifuged at low speed (1000 × *g* for 3 min). The supernatant was centrifuged at 400,000 × *g* for 30 min. The pellet was resuspended (particulate fraction) and the supernatant was recentrifuged at 400,000 × *g*. The final supernatant was concentrated with a Centricon-10 microcentrator (soluble fraction). Equivalent amounts of protein were immunoprecipitated with affinity-purified AS antibodies and analyzed by SDS/PAGE and fluorography as described in Fig. 1. (*A*) [^{35}S]Methionine-labeled proteins (40 μg of protein). (*B*) [^{3}H]Myristic acid-labeled proteins (40 μg of protein).

visualized by SDS/PAGE and fluorography of total (nonimmunoprecipitated) proteins of the particulate and soluble fractions (data not shown). The wild-type α_{i1} was primarily seen in the particulate fraction (lanes 2 and 5), while the mutant α_{i1} was primarily in the soluble fraction (lanes 3 and 6). Densitometric analysis of the autoradiographs and calculations based on the yield of total protein in each fraction showed that 11.1% ± 1.7% (mean ± SEM; $n = 6$) of the transfected wild-type α_{i1} subunit was in the soluble fraction compared to 81.1% ± 4.1% of the transfected mutant α_{i1} subunit. The mobility of the mutant protein was slightly retarded on SDS/PAGE by comparison with the normal form.

Myristic acid incorporation into the wild-type α_{i1} was evident in both the particulate fraction (see Fig. 1*B* and Fig. 3*B*, lane 2) and in the soluble fraction (Fig. 3*B*, lane 5). The mutant α_{i1} did not show [^{3}H]myristic acid incorporation in either the particulate or supernatant fraction (Fig. 3*B*, lanes 3 and 6).

ADP-Ribosylation with Pertussis Toxin. ADP-ribosylation of α subunits with pertussis toxin requires the heterotrimeric G protein (1, 2) and therefore can test whether the mutant α_{i1} subunit is capable of binding $\beta\gamma$ subunits. Soluble fractions from control, wild-type α_{i1}, and mutant α_{i1} transfected cells were incubated with pertussis toxin and [^{32}P]NAD without addition of $\beta\gamma$ subunits (Fig. 4*A*, lanes 1, 6, and 11). Virtually no ADP-ribosylation was seen for any of the three soluble fractions because the endogenous $\beta\gamma$ subunits are not soluble (5) and therefore are not present in the soluble fraction to form heterotrimeric proteins with the α subunits.

Addition of $\beta\gamma$ subunits to the soluble fraction of the control transfected cells led to a very small increase in ADP-ribosylation (Fig. 4*A*, lane 12), reflecting either a low concentration of endogenous α_i subunits in the soluble fraction or slight contamination of the $\beta\gamma$ preparation with α subunits. In contrast, the addition of $\beta\gamma$ subunits to the soluble fraction of the mutant α_{i1} transfected cells showed a major increase in ADP-ribosylation (lanes 7–10), thereby showing that the mutant α_{i1} can effectively combine with $\beta\gamma$ subunits. Addition of various concentrations of $\beta\gamma$ subunits to the soluble fractions of the mutant α_{i1} and wild-type α_{i1} transfected cells showed that ADP-ribosylation of the wild-type α_{i1} was promoted by a lower concentration of $\beta\gamma$ subunits compared to the mutant α_{i1} soluble fraction.

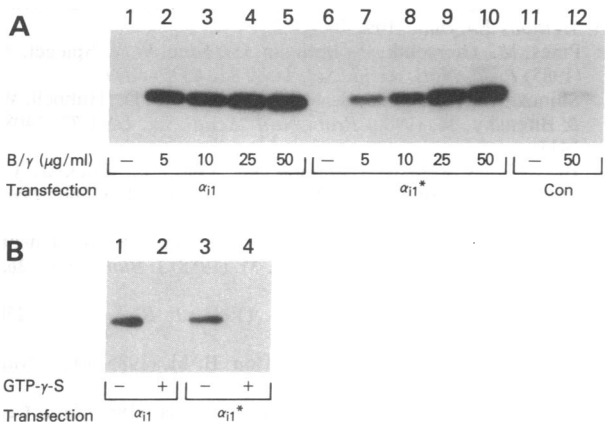

FIG. 4. [^{32}P]ADP-ribosylation with pertussis toxin. The soluble fractions of COS cells transfected with the pCD-PS vector alone (Con) or with the cDNA for the wild-type (α_{i1}) or mutant α_{i1} (α_{i1}*) was prepared as described in Fig. 3. To approximate equivalent amounts of α_i subunits in the soluble fractions of COS cells transfected with the cDNA for the wild-type and mutant α_{i1}, 10 μg of total protein of the wild-type α_{i1} soluble fraction and 4 μg of total protein of the mutant α_{i1} soluble fraction was used. Ten micrograms of total protein from the soluble control transfected cells was also used. ADP-ribosylation of the α subunits was performed in the presence of pertussis toxin in a reaction mixture as described in *Materials and Methods*. After quenching and precipitation with trichloroacetic acid, samples were dissolved in denaturing buffer and aliquots were analyzed by SDS/PAGE. The gels were dried and exposed to XAR-2 film in cassettes with intensifying screens at −70°C. (*A*) Increasing amounts of purified $\beta\gamma$ subunits were added to samples of the soluble fraction for the ADP-ribosylation reaction. (*B*) Samples of the soluble fractions were incubated with 0.5 mM GTP (lanes 1 and 3) or 0.5 mM GTP[γS] (lanes 2 and 4) for 15 min at 37°C before addition of purified $\beta\gamma$ subunits (50 μg/ml) and pertussis toxin.

Binding of GTP[γS], nonhydrolyzable GTP analog, to soluble heterotrimeric G proteins activates α subunits and causes irreversible dissociation of the α from $\beta\gamma$ subunits (1, 2). Since ADP-ribosylation by pertussis toxin requires the heterotrimer, we could test the ability of the mutant α_{i1} subunit to bind and be activated by GTP[γS] by performing pertussis toxin-catalyzed ADP-ribosylation on α subunits incubated with and without GTP[γS] prior to addition of $\beta\gamma$ subunits. As noted above (Fig. 4*A*), addition of $\beta\gamma$ to the mutant and wild-type α_{i1} supported pertussis toxin-catalyzed ADP-ribosylation (Fig. 4*B*, lanes 1 and 3). Incubation with GTP[γS] prevented ADP-ribosylation of both the wild-type and mutant α_{i1} subunits, indicating that mutant α_{i1} could bind GTP[γS] and presumably GTP.

DISCUSSION

The present study addresses the basis for membrane attachment of G-protein α subunits. Although their primary sequence suggests that they are hydrophilic proteins, α subunits ordinarily require detergents to extract them from membranes. Trypsin digestion of brain or neutrophil membranes cleaves α_i and α_o subunits near the amino terminus and releases the large, carboxyl-terminal fragment from the membrane (6). This suggested that α subunits are essentially cytosolic proteins tethered to the membrane via an amino-terminal 1- to 2-kDa stalk. The amino terminus could be involved in membrane anchoring in two ways. First, the amino terminal 1- to 2-kDa tryptic fragment is required for α-subunit interaction with the β–γ complex (27). The latter has been suggested as a membrane anchor for α subunits (5). Second, recent evidence suggests that α_i and α_o subunits are myristoylated (7, 8), a cotranslational modification exclusively found on amino-terminal glycine residues (9, 10). Myristoylated proteins are often, but not invariably, membrane associated (9, 10).

In agreement with an earlier study (7), the present results indicate that α_i, but not α_s, subunits are myristoylated. The negative results for α_s are particularly noteworthy, since relatively high levels of α_s expression, reflected by [^{35}S]-methionine incorporation, were achieved by transfection of COS cells. Under these conditions, [^{3}H]myristic acid incorporation should have been readily detected. α_s may be less susceptible to myristoylation, since it lacks a consensus hydroxyamino acid in the sixth position (asparagine rather than serine for all other G-protein α subunits) that facilitates this modification (10). Since the multiple forms of α_s arising from alternative splicing of a single gene all have an identical amino-terminal sequence (25), our results for transfection of the long form of α_s cDNA likely apply to shorter forms as well. The present results do not reveal the mechanism for α_s membrane attachment but tend to exclude a role for myristoylation in this process. Previous evidence (5) suggests that α_s has higher affinity for $\beta\gamma$ subunits than either α_i or α_o; thus, anchoring by $\beta\gamma$ subunits may be the basis for α_s membrane attachment. Other, as yet undefined, posttranslational modifications are also possible.

By converting the glycine in the second position that is obligatory for myristoylation into an alanine residue, we created a mutant form of α_{i1} that fails to incorporate myristate

and is localized primarily in the cytosol. A similar mutation of $p60^{src}$ prevents myristoylation and membrane association of this protein (28, 29). The mechanism whereby myristate promotes membrane association of proteins such as $p60^{src}$ and α_{i1} is unclear, but it could relate to the intrinsic hydrophobicity of this fatty acid and/or to its interaction with membrane-bound proteins that serve as myristate "receptors" (9, 10). It is possible that the β-γ complex acts as such a receptor for myristoylated α subunits. Unmyristoylated, mutant α_{i1} are still capable of interaction with $\beta\gamma$ subunits, but their affinity for $\beta\gamma$ subunits may be reduced (Fig. 4*A*). Reduction in affinity for $\beta\gamma$ subunits may compromise membrane attachment. After transfection of COS cells with α_i cDNAs, however, α_i subunits can be expressed at >10-fold excess over $\beta\gamma$ subunits but still are primarily membrane associated (30). Thus, myristoylation *per se* or interaction with a component other than $\beta\gamma$ subunits may be sufficient for membrane attachment of α_i subunits.

As has been shown for $p60^{src}$ (29), it may be possible to dissociate α subunit sequence requirements for myristoylation and membrane association from those required for other functional properties of the protein. Although the amino-terminal 1- to 2-kDa segment of α_i may be critical for both myristoylation and interaction with $\beta\gamma$ subunits, the present data indicate that a discrete change in this sequence can abolish myristoylation without totally abolishing interaction with $\beta\gamma$ subunits. It will be interesting to determine whether other sequence modifications can disrupt interaction with $\beta\gamma$ subunits while preserving the ability to become myristoylated and to assess the cellular localization (membrane vs. cytosol) of such a modified α subunit.

The carboxyl terminus of α_i subunits shows sequence similarity to that of $p21^{ras}$ proteins (1–3). This has led to the suggestion that as for $p21^{ras}$, lipid modification (possibly farnesylation) on a carboxyl-terminal cysteine of α_i subunits may be critical for membrane attachment (31). Failure of the mutant α_{i1} to associate with the membrane tends to exclude this possibility. Myristoylation is essential for membrane attachment of α_i subunits, but the present data do not reveal whether this is critical for membrane targeting or for stable association—e.g., upon G-protein activation. The conversion of glycine to alanine in the second residue of the mutant α_{i1} is unlikely to cause gross conformational changes. The mutant subunit can still interact with $\beta\gamma$ subunits and can be activated by guanine nucleotides. The mutant protein should prove useful in further studies on the importance of membrane localization for various aspects of G-protein function.

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