Myristoylated α subunits of guanine nucleotide-binding regulatory proteins

(myristic acid/G proteins/adenylyl cyclase/immunoprecipitation)

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ABSTRACT Antisera directed against specific subunits of guanine nucleotide-binding regulatory proteins (G proteins) were used to immunoprecipitate these polypeptides from metabolically labeled cells. This technique detects, in extracts of a human astrocytoma cell line, the α subunits of G_s (stimulatory) (α_{45} and α_{52}), a 41-kDa subunit of G_i (inhibitory) (α_{41}), a 40-kDa protein (α_{40}), and the 36-kDa β subunit. No protein that comigrated with the α subunit of G_o (unknown function) (α_{39}) was detected. In cells grown in the presence of [³H]myristic acid, α_{41} and α_{40} contained ³H label, while the β subunit did not. Chemical analysis of lipids attached covalently to purified α_{41} and α_{39} from bovine brain also revealed myristic acid. Similar analysis of brain G protein β and γ subunits and of G_t (transducin) subunits (α, β, and γ) failed to reveal fatty acids. The fatty acid associated with α_{41}, α_{40}, and α_{39} was stable to treatment with base, suggesting that the lipid is linked to the polypeptide via an amide bond. These GTP binding proteins are thus identified as members of a select group of proteins that contains myristic acid covalently attached to the peptide backbone. Myristate may play an important role in stabilizing interactions of G proteins with phospholipid or with membrane-bound proteins.

A family of guanine nucleotide-binding regulatory proteins (G proteins) transduces signals across cellular membranes by sequential interactions with surface receptors for various ligands and with the appropriate effector proteins (e.g., enzymes and ion channels) (reviewed in ref. 1). G_s and G_i transduce messages between receptors and adenylyl cyclase, resulting in the stimulation or inhibition of the enzyme. Transducin (G_t), localized in the disc membranes of retinal rod outer segments, interacts with photolyzed rhodopsin and stimulates a cyclic GMP-specific phosphodiesterase. G_o, originally purified from brain, is a fourth member of the family (2, 3); although its function has not been definitively established, it may be involved in regulation of Ca^{2+} channels (4). G proteins are heterotrimers: distinct α subunits bind and hydrolyze GTP and interact specifically with particular effector proteins; a complex of β and γ subunits, with apparent molecular masses in the range of 36 and 8 kDa, respectively, appears to be shared among several G protein α subunits.

A large number of cDNA clones that encode G protein subunits have now been characterized. This has led to the realization that there are multiple forms of several of these polypeptides. Given this level of complexity, we have adopted the format of using the apparent molecular mass of each subunit to distinguish the various proteins. For example, two forms of the α subunit of G_s are designated α_{45} and α_{52}; very

similar G_i-like proteins are designated α_{40} and α_{41}; two forms of the β subunit are referred to as β_{36} and β_{35}.

The mechanism of interaction between G proteins and the plasma membrane is largely unknown. The deduced amino acid sequences of α subunits show few hydrophobic regions that might promote such interactions. The predicted sequences of the β and γ subunits also lack a clear hydrophobic domain; however, the β–γ subunit complex from brain readily associates with phospholipid vesicles (5).

Attachment of a lipid to the polypeptide backbone is a newly recognized mechanism that has the potential to stabilize the binding of a protein to the lipid bilayer (6). There are at least three types of such modification: a complex glycophospholipid can be attached to the processed carboxyl terminus of a protein, as reported for Thy-1 (7); a palmitic acid molecule may be added to a cysteine residue within the body of the protein chain, as is found with p21[v-ras] (8) and the transferrin receptor (9); or a myristic acid can be attached to a glycine residue at the amino terminus of the polypeptide, as in p60[v-src] (10). The lipid moieties of Thy-1, p21[v-ras], and p60[v-src], although structurally distinct and attached to different sites within each protein, are required to enable these proteins to interact stably with membranes.

The development of antipeptide antisera that are specific for individual G protein subunits (11) permits us to study the biosynthesis of G proteins and to search for potential posttranslational modifications of their structures. We have been able to immunoprecipitate four α subunits (α_{52}, α_{45}, α_{41}, α_{40}) and one β subunit (β_{36}) from extracts of a human astrocytoma cell line, 1321N1. Two of these proteins, α_{41} and α_{40}, contain amide-linked myristate. Chemical analysis of α_{39} and α_{41}, purified from bovine brain, indicates that these polypeptides also contain amide-linked myristic acid. This unusual modification is likely to play an important role in interactions between these proteins and cellular membranes.

MATERIALS AND METHODS

Cells and Antisera. The 1321N1 line of human astrocytoma cells (12) was grown in Dulbecco–Vogt modified Eagle's medium, supplemented with 5% fetal bovine serum. Polyclonal rabbit antisera were generated to peptides synthesized according to the amino acid sequences of G protein subunits (11). The antisera used here were U-49, specific for β_{36}; A-572, specific for $G_{s\alpha}$ (α_{45} and α_{52}); and A-569, which

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Abbreviations: G proteins, guanine nucleotide-binding regulatory proteins; G_s and G_i, G proteins that mediate stimulation and inhibition, respectively, of adenylyl cyclase; G_o, G protein of unknown function, originally isolated from bovine brain; G_t, G protein (transducin) that mediates stimulation of cGMP phosphodiesterase in visual transduction; α_{52}, α_{45}, α_{41}, α_{40}, and α_{39}, G protein α subunits of apparent molecular mass 52, 45, 41, 40, and 39 kDa, respectively; β_{35} and β_{36}, G protein β subunits of apparent molecular mass 35 and 36 kDa.

recognizes all known α subunits. The monoclonal antibody Y13-259 was used to precipitate p21$^{c\text{-}ras}$ from the astrocytoma cells (13). Monoclonal antibody B325 to the human transferrin receptor was provided by Ian Trowbridge (The Salk Institute).

Biosynthetic Labeling. Cells were labeled overnight with [^{35}S]methionine (100 μCi/ml; >1000 Ci/mmol; 1 Ci = 37 GBq) in Dulbecco–Vogt modified Eagle's medium containing 0.02 mM methionine and 5% dialyzed fetal bovine serum. For fatty acid labeling, [9,10-^{3}H]palmitic acid (22 Ci/mmol) or [9,10-^{3}H]myristic acid (12 Ci/mmol; New England Nuclear) was dried, dissolved in dimethyl sulfoxide as described (14), diluted to 1 mCi/ml with Dulbecco–Vogt modified Eagle's medium and 5% fetal bovine serum, and incubated with the cells overnight.

Immunoprecipitation and Peptide Blocking. For successful immunoprecipitation, we found it was necessary to lyse cells and form precipitates in a detergent-buffer mixture containing 0.15 M NaCl, 10 mM sodium phosphate (pH 7.2), 1% Trasylol (Mobay), 1% sodium deoxycholate, 1% Nonidet P-40, 0.5% NaDodSO$_4$, and 1 mM dithiothreitol. The efficiency of immunoprecipitation could be further increased if cells were lysed directly in 0.5% NaDodSO$_4$/50 mM sodium phosphate, pH 7.2/1 mM dithiothreitol and then boiled for 3 min to denature proteins. Samples were then diluted with 4 vol of 1.25-fold concentrated detergent buffer mixture. All samples were next incubated with normal rabbit serum and fixed *Staphylococcus aureus* (Pansorbin, Calbiochem–Behring) to reduce the number of proteins bound nonspecifically. Specific antisera were added to the cleared supernatants, and samples were processed as described by Sefton *et al.* (15). To distinguish nonspecific from specific interactions with the antisera, 2 μl of affinity-purified antiserum was preabsorbed by overnight incubation at 4°C with 10 μl of the peptide (50 μg/ml) used for immunization or an unrelated peptide. Cell lysates that had been cleared with normal serum were incubated with the peptide–antibody mixtures and processed as usual.

Polyacrylamide Gel Electrophoresis and Hydroxylamine Treatment. Samples were analyzed on 15% polyacrylamide gels with a low bisacrylamide content (16). Labeled proteins were detected by fluorography with presensitized film and were quantified by densitometry of the fluorograms. The stability of the fatty acid to hydroxylamine was tested by analysis of duplicate samples on a polyacrylamide gel; one set of samples was then treated overnight at room temperature with two changes of 1 M hydroxylamine (pH 6.7). The hydroxylamine was removed by soaking the gel in 10% acetic acid/10% isopropanol. Both sets were then processed for fluorography as usual.

Purification of G Proteins. Membranes from bovine brain (2) and bovine retinal rod outer segments (17) were prepared as described. The two major G proteins from bovine brain (G$_i$ and G$_o$) were purified by the method of Sternweis and Robishaw (2). Further chromatography of these purified proteins on heptylamine-Sepharose in the presence of Al^{3+}, Mg^{2+}, and F$^-$ (18, 19) yielded preparations of pure G$_o$ α subunit (α_{39}) and G$_i$ oligomer (α_{41} β–γ). G$_t$ was purified from bovine rod outer segments by the method of Fung (20). The α subunit of G$_t$ was resolved from the purified oligomer by chromatography on heptylamine-Sepharose (11).

Chemical Analysis of Fatty Acids. The purified proteins (50–100 μg of α subunit or 100–200 μg of oligomeric α–β–γ complex) were resolved by electrophoresis through 1.5-mm thick NaDodSO$_4$/polyacrylamide gels (12% crosslinking) as described by Laemmli (21). The proteins were located in the gel by staining for 5 min with 0.25% Coomassie blue in 30% isopropanol/10% acetic acid. The gel was washed for 16 hr with three changes of 30% methanol/10% acetic acid and then for 4 hr with two changes of 30% methanol. The protein

bands were excised from the gel, placed in tubes (13 × 100 mm), and washed for 16 hr with three changes of 50% methanol. The solvent was then removed and the gel slices were dried under a stream of nitrogen, suspended in 0.6 ml of 1.5 M NaOH, and incubated for 3 hr at 30°C to hydrolyze ester- or thioester-linked fatty acids. The solution was acidified to pH 1–2 with 6 M HCl, and any released fatty acids were extracted with chloroform/methanol (22). The chloroform-soluble material was then derivatized and analyzed as described below. The gel slices in residual chloroform/methanol were dried under a stream of nitrogen, suspended in 0.7 ml of 6 M HCl, and incubated at 100°C for 4 hr to hydrolyze amide-linked fatty acids from the protein. Released fatty acids were again extracted from this mixture with chloroform/methanol.

Extracted fatty acids were derivatized with dibromoacetophenone in the presence of a crown ether catalyst in acetonitrile (23). Immediately prior to the derivatization, 1.0 nmol of heptadecanoic acid was added to each sample as an internal standard to monitor efficiency of derivatization and recovery during chromatography. The derivatized fatty acids were filtered through a C$_{18}$ Sep-Pak cartridge (Waters Associates), dried under nitrogen, and dissolved in a solution of methanol/water (88:12). The derivatives were separated by reverse-phase HPLC on an Altex Ultrasphere-octyl 5-μm column using methanol/water (88:12) as the mobile phase. The flow rate was 1 ml/min. The elution positions of fatty acids of interest were determined by chromatography of derivatized standards under the same conditions as the experimental samples. The stoichiometry of the fatty acids released from G proteins was estimated by comparing the appropriate peak areas with that of the heptadecanoic acid standard in the same sample.

RESULTS

Immunoprecipitation of G Proteins from Metabolically Radiolabeled Cells. A panel of antisera of designed specificity toward G proteins has been described (11). Certain of these antisera were used to immunoprecipitate proteins from cells labeled with either [^{35}S]methionine, [^{3}H]myristic acid, or [^{3}H]palmitic acid. Precipitated proteins with apparent molecular masses of 35–55 kDa were examined by three criteria to determine whether they were authentic G proteins or unrelated contaminating proteins. Specificity was assessed by use of nonspecific sera, comigration with purified proteins, and competition with synthetic peptides or purified proteins. All G protein subunits discussed below meet these criteria. G$_s$-like α_{52} and α_{45} subunits, G$_i$-like α_{41} protein, and β_{36} were identified in several cell lines labeled with [^{35}S]methionine (S49 murine lymphoma, B49 rat neuroblastoma, and NIH 3T3 cells, data not shown). A human astrocytoma cell line, 1321N1, in which the labeled proteins were particularly abundant, was used for the majority of the experiments.

A ^{35}S-labeled 36-kDa protein, β_{36}, was precipitated specifically from 1321N1 cell extracts by antiserum U-49; this antiserum was generated by immunization with a peptide synthesized according to the sequence of the β subunit that is apparently shared by G$_i$, G$_s$, G$_o$, and G$_t$ (Fig. 1, lane 1). β-subunit protein preparations from most tissues contain both a 35-kDa and a 36-kDa protein. Only the 36-kDa (β_{36}) protein was detected in immunoprecipitates. The same specificity was observed when immunoblotting was performed with this antiserum (11). β_{36} failed to incorporate either labeled myristate or palmitate (lanes 2 and 3), implying that β_{36} is not a fatty acyl protein. The lack of labeling of this relatively abundant protein also indicates that ^{3}H from myristate or palmitate was not incorporated significantly into amino acids during the long incubation period used.
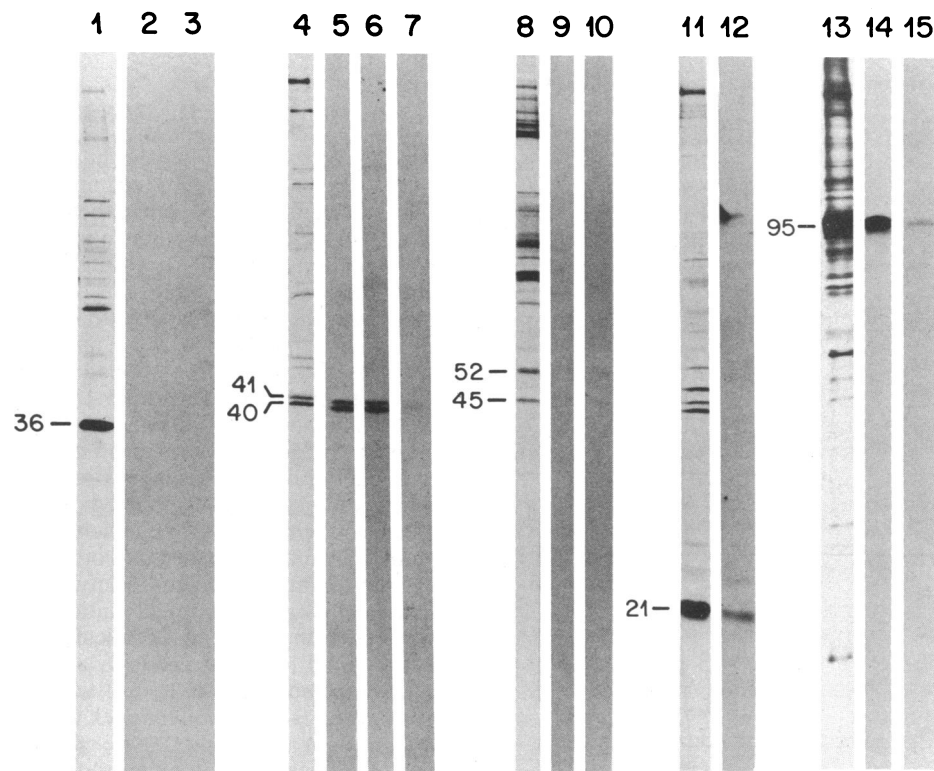
FIG. 1.   Immunoprecipitation of radiolabeled G proteins. Astrocytoma cells were incubated overnight with [^{35}S]methionine, [^{3}H]myristic acid, or [^{3}H]palmitic acid, and immunoprecipitates were formed with subunit-specific antisera. Samples were analyzed by NaDodSO$_4$/PAGE, followed by fluorography for 2 days for [^{35}S]methionine or 21 days for ^{3}H fatty acid-labeled samples. Numbers indicate position and apparent size in kDa of relevant proteins precipitated by each antiserum. Lanes: 1–3, β_{36}-subunit antiserum U-49; [^{35}S]methionine (lane 1), [^{3}H]myristic acid (lane 2), and [^{3}H]palmitic acid (lane 3); 4–7, common α-peptide antiserum A-569; [^{35}S]methionine (lane 4), [^{3}H]myristic acid (lane 5), [^{3}H]myristic acid after treatment with hydroxylamine (lane 6), and [^{3}H]palmitic acid (lane 7); 8–10, G$_s$ α peptide antiserum A-572; [^{35}S]methionine (lane 8), [^{3}H]myristic acid (lane 9), and [^{3}H]palmitic acid (lane 10); 11 and 12, anti-p21^{ras} monoclonal antibody Y13-259; [^{35}S]methionine (lane 11) and [^{3}H]palmitic acid (lane 12); 13–15, anti-transferrin receptor monoclonal antibody; [^{35}S]methionine (lane 13), [^{3}H]palmitic acid (lane 14), and [^{3}H]palmitic acid after treatment with hydroxylamine (lane 15).

Antiserum A-569, raised against a peptide common to the α subunits of G$_s$, G$_i$, G$_o$, and G$_t$, precipitated two ^{35}S-labeled proteins of interest (Fig. 1, lane 4); these comigrated with α_{40} and α_{41} purified from bovine brain (S.M.M., A.G.G., I. Pang, and P. C. Sternweis, unpublished data). The 40-kDa protein was found in immunoprecipitates from lysates prepared by boiling cells directly in NaDodSO$_4$-containing buffer, indicating that the 40-kDa protein is unlikely to be a proteolytic artifact of sample preparation. α_{41} and α_{40} proteins immunoprecipitated from cells incubated with [^{3}H]myristic acid contained ^{3}H label (lane 5). However, in immunoprecipitates formed from [^{3}H]palmitic acid-labeled cells, α_{41} and α_{40} incorporated barely detectable amounts of radioactivity (lane 7). The poor incorporation of [^{3}H]palmitic acid into α_{41} and α_{40} was not due to inefficient labeling of appropriate proteins with [^{3}H]palmitic acid, since two proteins known to be palmitoylated, p21^{c-ras} and the 95-kDa transferrin receptor, contained amounts of [^{3}H]palmitic acid that could be visualized easily (lanes 12 and 14).

The efficient incorporation of myristic acid into α_{41} and α_{40} and their poor labeling with palmitic acid is reminiscent of the labeling pattern seen with another acyl protein, p60^{v-src}. Although this protein contains only myristic acid, p60^{src} can be labeled metabolically with [^{3}H]palmitic acid. This radioactivity is the result of limited conversion of the [^{3}H]palmitic acid to [^{3}H]myristic acid and incorporation of the shortened fatty acid chain (24). Similar metabolic conversion could also explain the limited incorporation of radioactivity into α_{41} and α_{40} in [^{3}H]palmitic acid-labeled cells.

To determine the nature of the bond between [^{3}H]myristic acid and the α_{41} and α_{40} proteins, the sensitivity of the

^{3}H-labeled proteins to hydroxylamine was examined. After the gel containing the labeled proteins was soaked overnight in 1 M hydroxylamine (pH 6.7), 93% of the ^{3}H remained attached to α_{41} and α_{40} (Fig. 1, lane 6). In contrast, only 3% of the thioester-linked palmitic acid remained bound to the transferrin receptor (lane 15) after hydroxylamine treatment. This indicated that fatty acid was linked to α_{41} and α_{40} primarily via an amide bond.

Although the 45-kDa and 52-kDa α subunits of G$_s$ could be visualized by prolonged exposure of the fluorogram of immunoprecipitates formed with A-569 (data not shown), more satisfactory results were obtained with a G$_{s\alpha}$-specific antiserum, A-572. Immunoprecipitates of [^{35}S]methionine-labeled cells revealed bands that correspond to α_{52} and α_{45} (Fig. 1, lane 8). Of interest, there was no significant level of incorporation of [^{3}H]myristate or [^{3}H]palmitate into either of these subunits (lanes 9 and 10).

We were unable to detect the α subunit of G$_o$ (α_{39}) from the astrocytoma cells in immunoprecipitates formed with antiserum A-569 or with α_{39}-specific antisera S-214 and U-46 (data not shown) (11).

Chemical Determination of Fatty Acids on G Proteins. The fatty acids attached to bovine brain α_{39} and α_{41} were identified chemically by HPLC analysis of derivatized fatty acids cleaved from the purified proteins by base or acid (Fig. 2). For comparison, an equivalent gel slice, taken from a lane loaded with only the buffer in which the proteins were stored, was processed by the same procedure. Acid hydrolysates of both α_{39} (*Upper*) and α_{41} (*Middle*) yielded prominent peaks that comigrated with the derivatized myristate standard, while the control gel slice (*Lower*) yielded only a very small
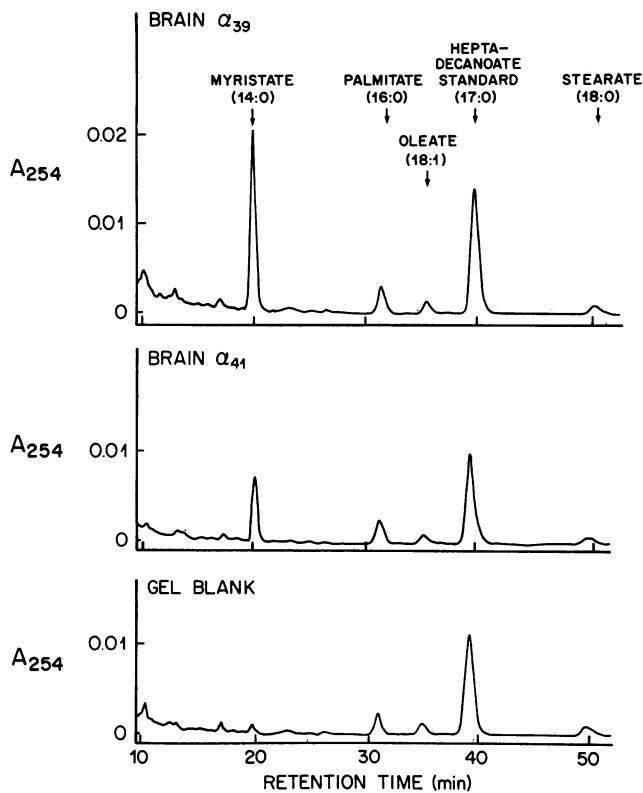
FIG. 2. Chemical analysis of acid-labile fatty acids on G protein α subunits. The chloroform/methanol extracts of gel slices containing α_{39} (*Upper*), α_{41} (*Middle*), or the buffer lane (gel blank; *Lower*) were subjected to derivatization and chromatography as described. The initial quantities of each subunit loaded on the gel were 90 μg of α_{39} and 60 μg of α_{41}. Arrows identify elution positions of the appropriate fatty acid standards derivatized by the same procedure as the samples. The experiment shown is representative of three separate experiments using two different preparations of each protein.

peak at that position. In addition, small peaks of absorbance that comigrated with derivatized standards of palmitate, oleate, and stearate were observed in all hydrolysates analyzed, including control gel slices. These peaks were apparently due to trace contamination by these fatty acids of the materials used during the processing of the samples. Base-stable myristate was also observed from α_{41} purified from rabbit liver (data not shown).

By comparing the area of the myristate peak with that of the heptadecanoate added as an internal standard, the minimum stoichiometry of myristate to α subunit was determined to be 0.43 ± 0.03 mol/mol for α_{39} ($n = 3$) and 0.34 ± 0.04 mol/mol for α_{41} ($n = 3$). This calculation was based on the amount of protein loaded on to the gel and thus does not account for any losses of protein during processing. We believe it quite likely that the α subunits of G_o and G_i are myristoylated stoichiometrically. It is possible, however, that these purified proteins are heterogeneous and that only certain species are acylated. In contrast to such results, only minor amounts of myristate were released from the α subunit of purified bovine transducin (≤0.10 mol per mol of protein) in three separate experiments (data not shown).

No detectable fatty acids were released from α_{39} or α_{41} by treatment of the gel slices containing these proteins with 1.5 M NaOH. This suggested, as did the hydroxylamine treatment of radiolabeled α_{41} and α_{40} from the astrocytoma cells, that the linkage of the myristate to these proteins is of the amide type. In addition, no acid- or base-labile fatty acids were observed on β or γ subunits derived from brain G

proteins or on any of the three subunits of G_t (data not shown).

DISCUSSION

G proteins expressed by the human astrocytoma cell line 1321N1 were detected by specific immunoprecipitation of proteins metabolically radiolabeled with [^{35}S]methionine. Two ^{35}S-labeled proteins that comigrate with bovine brain α_{40} and α_{41} during NaDodSO$_4$/PAGE were immunoprecipitated with the common α peptide antiserum A-569. We believe that these are the human astrocytoma forms of α_{40} and α_{41}. Immunoprecipitation with the antiserum specific for the α subunits of G_s yielded ^{35}S-labeled α_{45} and α_{52}. ^{35}S-labeled β_{36} was precipitated by antiserum U-49. No α_{39} was detected with antiserum A-569 (Fig. 1) or with two α_{39}-reactive antisera, S-214 and U-46 (11) (data not shown).

Acylation of G proteins was assayed by immunoprecipitation of metabolically labeled protein and by chemical analysis of G proteins. We conclude that myristate is covalently linked to α_{41}, based on both immunoprecipitation and chemical data. Association of myristate with α_{40} was determined only by immunoprecipitation. Myristate bound to α_{39} was determined only by chemical analysis. Immunoprecipitation failed to detect myristoylation of α_{45} and α_{52}. Fatty acids were not found in hydrolysates of any of the subunits of G_t or the β–γ subunit complex purified from bovine brain.

G protein α subunits share short regions of sequence homology with the *ras* gene product p21 (25, 26), including (except $G_{s\alpha}$) a cysteine four residues from the carboxyl terminus. Acylation with palmitate occurs via a thioester linkage at this cysteine residue in p21 (8). However, no palmitate was found associated with any of the G-protein subunits tested.

The myristate associated with the α subunits from purified proteins or immunoprecipitates of metabolically labeled cells was found to be stable to treatment with base. An ester or thioester linkage should be hydrolyzed under such conditions. Since treatment with acid was necessary to hydrolyze the myristate from purified G proteins for chemical analysis, it is likely that the myristate is linked through an amide bond.

Published analyses of the location of amide-linked myristate in proteins have thus far revealed acylation of the N-terminal glycine of each protein (6). With only one exception (27), all published sequences for G protein α subunits encode an N-terminal glycine following the initiator methionine. Proof that this glycine residue is myristoylated will require analysis of N-terminal fragments of the α subunits. However, an N-terminal glycine does not appear to be sufficient to direct G protein acylation at this site, since α_{45} and α_{52} from the human astrocytoma cells and the α subunit of G_t from bovine rod outer segments do not appear to be myristoylated. Variations found in other amino acid residues near the N terminus of α subunits might influence the occurrence of myristoylation at this site. Alternatively, acylation may not occur at the N-terminal glycine but at another site found only in a subset of α subunits.

The function of the myristate bound to α subunits is not known. Myristate is attached during or immediately after proteins are synthesized and becomes a permanent part of the molecule (6, 28). Of the myristoylated proteins that have been analyzed, the modification of the protein is stoichiometric. If myristate is an unchanging component of an α subunit, the myristate may not modulate the activity of the G protein but rather may facilitate interaction between the α subunit and hydrophobic domains of other proteins or phospholipids in the membrane. This may be particularly relevant to the functions of G protein α subunits. It has been hypothesized that the primary mechanism of association of α with the membrane is via β–γ (5), but that α dissociates from β–γ when

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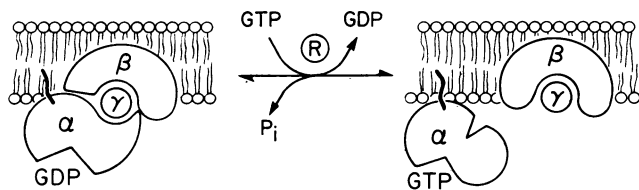
Proc. Natl. Acad. Sci. USA 84 (1987)   7497

FIG. 3. A model for the interactions of G protein subunits with the plasma membrane. Receptor with ligand bound (circled R) promotes exchange of GDP bound to α for GTP. This causes dissociation of α from β–γ and concomitant conformational changes. The covalently attached myristate (heavy line) on α may contribute to the continued association of α with the membrane.

activated by receptor and GTP (1) (Fig. 3). Myristoylation might provide a mechanism for continued association of α with the bilayer following subunit dissociation. Lack of myristoylation of the α subunits of transducin and G_s may contribute to their ability to be released from membranes by GTP and cholera toxin, respectively (17, 29). Such release has not been reported for α_{39}, α_{40}, or α_{41}. However, the myristoylation and primary sequence of α_{39} do not appear to be sufficient for reconstitution of the purified protein into phospholipid vesicles, since a requirement for β–γ has been demonstrated (5). Alternatively, fatty acylation may play a role in the interaction of α subunits with β–γ, receptors, or effector proteins required for signal transduction. When the site for myristoylation of α subunits is determined, site-directed mutagenesis can be undertaken as an approach to investigate these questions.

Note Added in Proof. The absence of fatty acid in $G_{s\alpha}$ subunits has been verified by chemical analysis of G_s purified from bovine brain.

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