G protein γ subunits contain a 20-carbon isoprenoid

(covalent modification/prenylation/geranylgeranyl group/mevalonate/PC-12 cells)

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ABSTRACT A small subset of cellular proteins are covalently modified by the addition of isoprenoid groups. These include p21ras, fungal mating factors, and nuclear lamins, which are isoprenylated at carboxyl-terminal cysteine residues with a 15-carbon farnesyl group. The similarity of the carboxyl-terminal sequences of these proteins with the α and γ subunits of signal-transducing guanine nucleotide-binding regulatory proteins (G proteins) prompted examination of isoprenylation of G protein subunits. PC-12 cells were incubated with the isoprenoid precursor [3 H]mevalonolactone. The β and γ subunits were isolated by specific association with an affinity column of immobilized α subunits. The γ subunits were radiolabeled, and the tritiated lipid released from them by treatment with methyl iodide comigrated chromatographically with the 20-carbon isoprenoid geranylgeraniol. Label was not detected in G protein α or β subunits. Isoprenylation of γ subunits by the geranylgeranyl group is presumed to contribute to the association of G proteins with membranes.

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins) are associated with the inner face of the plasma membrane, where they are positioned to couple membrane-spanning receptors with intracellular effectors (1). Little is known about the molecular basis of the interaction of G proteins with membranes. The deduced amino acid sequences of their subunit polypeptides (designated α , β , and γ) lack clear hydrophobic domains that might promote such interactions. However, with the exception of transducin (G_t , the major G protein in retina), detergent is required to extract G proteins from membranes and to maintain them in solution. The tightly associated β and γ subunits appear to be largely responsible for this requirement for detergent (2). Furthermore, purified $\beta\gamma$ subunits can be efficiently incorporated into phospholipid vesicles; the presence of $\beta\gamma$ then allows efficient association of α subunits with the vesicles (2).

Most types of α subunits are covalently modified by the 14-carbon saturated fatty acid, myristic acid (3–5); fatty acylation is involved in the association of α subunits with the plasma membrane (5, 6). Myristate may facilitate interactions of α subunits with phospholipid and/or other proteins in the membrane, such as $\beta\gamma$. Fatty acids have not been found in covalent association with β or γ (3).

Members of another class of guanine nucleotide-binding proteins, exemplified by the protooncogene product p21ras, are not oligomeric. The ras proteins have recently been shown to be modified by isoprenylation, a process that contributes to their interaction with the plasma membrane (7, 8). A carboxyl-terminal CAAX motif (where C = cysteine, A = an aliphatic amino acid, and X = any amino acid) is a feature common to p21ras and to other known isoprenylated proteins (9). This CAAX motif is also found in the deduced amino acid sequences of G protein γ subunits (10–12). A

number of α subunits (G_t , G_i , and G_o) contain a carboxyl-terminal sequence CGA ϕ (where C = cysteine, G = glycine, A = an aliphatic amino acid, and ϕ = an aromatic amino acid). The presence of the CAAX motif in γ subunits and the similar sequence in some α subunits prompted us to test the possibility of their isoprenylation.

We assessed the incorporation of radioactivity from tritiated mevalonolactone, (*R,S*)-[5- 3 H]mevalonolactone ([3 H]-MVA), a precursor of isoprenoids, into G protein subunits from PC-12 cells. The β and γ subunits were copurified by affinity chromatography on a support containing immobilized α subunits. The affinity chromatographic procedure takes advantage of the G protein subunit dissociation cycle. G proteins are activated when GTP is bound and dissociate into α -GTP and $\beta\gamma$ (13). Subsequent hydrolysis of GTP results in the inactivation of the protein and reassociation of α -GDP with $\beta\gamma$. Dissociation can also be induced by Al^{3+} , Mg^{2+} , and F^- [30 μ M $AlCl_3$ /6 mM $MgCl_2$ /10 mM NaF (AMF)] (14)—the technique used herein. A cycle of activation and deactivation facilitates exchange of cellular $\beta\gamma$ subunits with the matrix of immobilized α . Washing and subsequent elution with AMF yields isolated $\beta\gamma$. The isolation of α subunits by use of immobilized $\beta\gamma$ has been reported (15). Here, we demonstrate the utility of immobilized α subunit affinity chromatography for the isolation of $\beta\gamma$ subunit complexes and show that the γ subunits are modified by isoprenylation.

MATERIALS AND METHODS

Cell Culture and Metabolic Radiolabeling. Rat pheochromocytoma PC-12 cells were grown in high-glucose Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, 5% horse serum, penicillin at 10 units/ml, and streptomycin at 10 μ g/ml. Cells for metabolic radiolabeling were cultured on poly(L-lysine)-coated dishes to 70–100% confluency. For radiolabeling, medium was supplemented with 5% dialyzed fetal calf serum (without horse serum) and incubated with the cells for 16–20 hr. For radiolabeling of isoprenylated proteins, the medium contained [3 H]MVA at 2 mCi/ml (1 Ci = 37 GBq), 40 μ M compactin, 0.35 mM sodium pyruvate, and nonessential amino acids. The [3 H]MVA labeling medium was reused for three to four consecutive overnight incubations because uptake of radioactivity by the cells was low and the mevalonate and compactin were stable. [3 H]MVA (40 Ci/mmol) was purchased from DuPont/NEN, and the compactin was provided by M. Brown and J. Goldstein (of this institution).

Cell and Tissue Fractionation. PC-12 cells were first scraped into a phosphate-buffered saline solution and centrifuged. The cell pellet was resuspended in a hypotonic buffer composed of 20 mM 4-(2-hydroxyethyl)-1-piper-

zineethanesulfonic acid (Hepes), 2 mM EDTA, and 1 mM dithiothreitol (pH 8). The cells were disrupted by passage through a 25-gauge needle five times or by homogenization in a Potter-Elvehjem tissue grinder. The homogenate was fractionated into soluble and particulate fractions by centrifugation in a Beckman 100.3 rotor at 75,000 rpm ($200,000 \times g$) at 2°C for 20 min. The supernatant was removed, and the particulate fraction was suspended in the hypotonic Hepes buffer. Both the soluble and particulate fractions were frozen and stored at -70°C. Crude membrane fractions from bovine brain were prepared as described (16).

Synthesis of α_0 -Agarose. Purified $G_0 \alpha$ subunit (α_0) was prepared as described (16). Before synthesis of the affinity matrix, the protein was diluted with 20 mM NaHepes (pH 8), 100 mM NaCl, and 0.25% Lubrol PX (Sigma) and applied to hydroxylapatite (≈ 1 mg of protein/ml of gel) at 4°C. The matrix was washed with the same solution and eluted with 200 mM potassium phosphate (pH 8)/200 mM NaCl/0.25% Lubrol. GDP was added to each fraction to a concentration of 5 μ M. This procedure removed dithiothreitol and exchanged the buffer and detergent in which the α_0 was stored.

Synthesis of the α_0 -affinity matrix followed essentially the procedure used for immobilization of $\beta\gamma$ subunits (15). ω -Aminobutylagarose (Sigma) was washed with 50 mM sodium phosphate (pH 7) and mixed with an equal volume of the same buffer containing 0.25 mM *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester. The mixture was purged with N_2 , incubated for 30 min at room temperature, and washed with 10 ml of phosphate buffer/ml of resin. Purified α_0 (1–2 mg/ml of resin) was added in an equal volume containing 200 mM potassium phosphate (pH 8), 200 mM NaCl, 0.25% Lubrol, and 5 μ M GDP. The mixture was stirred gently for 1 hr at 4°C. The resin was collected and washed with 5 vol of 50 mM sodium phosphate (pH 7)/1 mM EDTA/300 mM NaCl/2 μ M GDP/0.2% Lubrol, followed by an equal volume of the same solution containing 50 mM 2-mercaptoethanol. The matrix was stored in 50 mM NaHepes (pH 8)/1 mM EDTA/1 mM dithiothreitol/100 mM NaCl/0.1% Lubrol/5 μ M GDP/30% glycerol (vol/vol) at 4°C.

The amount of α subunit attached to the matrix was estimated by subtraction of the protein that did not react from total protein. The number of binding sites for guanosine 5'-[γ -thio]triphosphate was determined by direct binding to aliquots of the gel (16). The number of $\beta\gamma$ binding sites was determined by applying a saturating amount of purified $\beta\gamma$, washing, and then eluting with a solution containing AMF (see below). The amount of $\beta\gamma$ eluted with AMF was estimated from staining intensity after electrophoretic analysis or, more directly, by assay of protein (17) when sample size was sufficient.

Isolation of $\beta\gamma$ Subunits with α_0 -Agarose. Membranes (2–6 mg/ml of protein) were extracted at 4°C with solution A (20 mM NaHepes, pH 8/1 mM EDTA/3 mM dithiothreitol/5 μ M GDP) containing 1% Lubrol and were centrifuged at $100,000 \times g$ for 40 min. Extracts (supernatant) were adjusted to 200 mM NaCl/10 mM NaF/6 mM $MgCl_2$ /30 μ M $AlCl_3$; this treatment dissociates α from $\beta\gamma$. The treated extract was combined with an approximately equal volume of α_0 -agarose that had been equilibrated with solution A containing 400 mM NaCl, 10 mM NaF, 6 mM $MgCl_2$, 30 μ M $AlCl_3$, and 0.5% Lubrol. After mixing at 4°C for 50 min, 0.1 M EDTA was added to a final concentration of 17 mM, and the incubation was continued for 50 min to allow association of $\beta\gamma$ subunits in the extract with the immobilized α_0 . The mixture was then packed into a small column, and the effluent was passed through the matrix twice and collected as fraction 1. The matrix was then washed several times at 4°C with equal volumes of solution A containing 400 mM NaCl and 0.5% Lubrol, followed by several washes of solution A containing 400 mM NaCl and 0.05% Lubrol. Elution was accomplished

with solution A containing 200 mM NaCl, 10 mM NaF, 50 mM $MgCl_2$, 30 μ M $AlCl_3$, and 0.05% Lubrol.

Gel Electrophoresis. PAGE was performed with the discontinuous buffer system described by Laemmli (18). Gradient gels of 10–20% acrylamide (acrylamide/bisacrylamide 26:1) containing 4 M urea were used to resolve γ subunits. Samples were concentrated by precipitation with 15% trichloroacetic acid. The pellets were washed twice with ice-cold acetone, dissolved in 25 mM NaHepes (pH 8)/1 mM EDTA/1% SDS, alkylated with *N*-ethylmaleimide (16), and heated with standard sample solutions. Gels were stained with either silver (19) or Coomassie blue. Radioactive samples on Coomassie-blue stained gels were detected by fluorography by processing gels with EN³HANCE according to manufacturer's instructions (DuPont/NEN).

Chemical Analysis of Isoprenoids. Two methods were used. (i) The first is a modification of a reported method (8), using trypsin digestion on nitrocellulose (20) to prepare samples for analysis. Samples (*i.e.*, α_0 -agarose flowthrough or AMF eluates) were supplemented with 10% SDS to a final concentration of 2% SDS and then diluted to 0.25 ml with a solution of 2% SDS. After 5 min at 20°C, 0.25 ml of 30% trichloroacetic acid was added. The samples were allowed to precipitate for 10 min at 20°C and were then filtered through 7-mm nitrocellulose disks. The filters were washed with a total of 1 ml of 6% trichloroacetic acid in 5 aliquots and then placed in a 1.5-ml microcentrifuge tube and rinsed with 0.2 ml of H_2O . In preparation for trypsin treatment, the filters were treated with polyvinylpyrrolidone and washed with H_2O (20). After the last H_2O wash, filters were suspended in 0.2 ml of 100 mM Tris-HCl, pH 7.7, containing 5% acetonitrile. Trypsin (1.6 μ g in 2 μ l of 50 mM Hepes, pH 8/1 mM EDTA/1 mM dithiothreitol/10 mM $MgSO_4$) was added to each tube. After overnight incubation at 37°C, the trypsin-containing solution was removed, and 0.2 ml of 25 mM Tris-HCl, pH 7.7, containing 80% (vol/vol) acetonitrile was added to each tube. The filters were incubated in this solution for 3 hr at 20°C; the solution was removed and combined with the trypsin solution from the overnight incubation. The filters were finally rinsed with an additional 50 μ l of Tris/80% acetonitrile, and this rinse was combined with the previous elutions. To each sample was added 0.8 ml of 3% (vol/vol) formic acid, and the cleavage reaction was initiated by the addition of 0.1 ml of methyl iodide. After an overnight-stirred incubation in the dark, the methyl iodide was removed by evaporation under reduced pressure. The reaction was quenched by adding 0.15 ml of 35% (wt/vol) Na_2CO_3 , and the preparation was incubated in the dark for an additional 12 hr. The resulting mixture was extracted twice with 1.2 ml each of $CHCl_3$ /MeOH (9:1), and the organic phases were combined and dried under a stream of N_2 . The sample was dissolved in 0.4 ml of 50% (vol/vol) CH_3CN /25 mM H_3PO_4 (solvent A), spiked with 6 nmol each of geraniol, farnesol, and geranylgeraniol, and an aliquot (typically 60% of the total) was injected onto a 0.5 $\times$ 25 cm ODS reverse-phase HPLC column (Phenomenex). The column was developed with a 40-ml linear gradient of solvent A to 100% CH_3CN /25 mM H_3PO_4 (solvent B), followed by 20 ml of solvent B at a flow rate of 1 ml/min. Fractions of 1 ml were collected, and the radioactivity in each was determined by scintillation counting. Control samples were processed identically except that the methyl iodide was omitted from the reactions. The isoprenoid standards were purchased from Aldrich with the exception of geranylgeraniol, which was from R. Coates (University of Illinois).

(ii) In one experiment, AMF eluate from the α column was processed directly without precipitation or filtration. An aliquot (0.2 ml) of the peak fraction pool was incubated with trypsin (8 μ g in 10 μ l of 50 mM NaHepes, pH 8/1 mM EDTA/1 mM dithiothreitol/10 mM $MgSO_4$) at 37°C for 3 hr, whereupon 0.25 ml of 25 mM Tris-HCl, pH 7.7, containing

80% acetonitrile was added. The sample was then subjected to cleavage and analysis as described above.

RESULTS

Three preparations of α_0 -agarose were used for these experiments. The crosslinking efficiency of α_0 to the agarose ranged from 42–67%, resulting in matrices containing 0.6–1.0 mg/ml of the α subunit. The matrices bound guanosine 5'-[γ -thio]triphosphate and $\beta\gamma$ subunits with specific activities of 10–20 nmol/mg of protein and 3–4 nmol/mg, respectively. Although the capacity to bind guanosine 5'-[γ -thio]triphosphate varied considerably, the capacity to bind $\beta\gamma$ was more constant and indicated that ≈ 10 –15% of the immobilized α_0 was functionally accessible to this subunit complex.

Examples of the isolation of $\beta\gamma$ subunits from an extract of bovine brain membranes and of PC-12 membranes are shown in Fig. 1. Most of the proteins flowed through the matrix. When a solution containing AMF was applied, two major groups of polypeptides were eluted specifically; they comigrated on a polyacrylamide gel with the β and γ subunits purified from brain by conventional procedures (16). The identity of the eluted β subunits from the brain membrane extract was confirmed by immunoblot analysis using antisera U-49 and J-887, which are specific for β_1 and β_2 , respectively (data not shown) (21). Immunoblot analysis of flowthrough and AMF-eluted fractions from the brain extract indicated that binding and retention of the two forms of β subunit exceeded 50% (data not shown). The retention and elution of at least two β and several γ subunits by association with α_0 confirm the relative lack of specificity of the interactions of this α subunit with various forms of $\beta\gamma$ (22).

PC-12 cells were incubated with [3 H]MVA and compactin, an inhibitor of 3-hydroxy-3-methylglutaryl CoA reductase, to radiolabel isoprenylated proteins. Particulate fractions derived from these cells were extracted with Lubrol to solubilize proteins before chromatographic analysis on the α_0 -agarose matrix. Chromatographic fractions were analyzed by liquid scintillation counting and PAGE, followed by fluorography. The results of one of three individual experiments are shown in Fig. 2. Approximately 1% of the applied radioactivity was eluted from the α_0 -agarose column by AMF in each experiment. Fluorography indicated that the radiolabeled proteins eluted by AMF migrated with mobilities similar to G protein γ subunits purified from bovine brain (Fig. 2). β subunits did not contain significant radioactivity. Affinity chromatography of the extracts with $\beta\gamma$ -agarose (15) did not reveal radioactivity associated with α subunits after elution with AMF (data not shown).

The radiolabeled proteins resolved on α_0 -agarose were subjected to a sulfonium salt cleavage reaction to release thioether-linked isoprenoids. The tritiated isoprenoids were then identified by reverse-phase HPLC with unlabeled isoprenoid alcohols as standards. Greater than 80% of the tritium from the trichloroacetic acid-precipitated proteins from each fraction tested was released by methyl iodide cleavage. These results indicate that the tritium was incorporated as a thioether, presumably through the thiol of a cysteine residue. Essentially all of the released radioactivity could be extracted with chloroform/methanol, as would be expected for a hydrophobic isoprenoid moiety. Fig. 3 shows three representative chromatograms. Flowthrough and AMF-eluted fractions from the α_0 -agarose yielded similar results. The major peak of radioactivity resolved by reverse-phase chromatography comigrated with the 20-carbon diterpenoid, geranylgeraniol. A sample of the material eluted with AMF that was then precipitated with trichloroacetic acid and filtered (Fig. 3B) yielded a more complex chromatogram than did the sample that had not been precipitated (Fig. 3C).

FIG. 1. Isolation of β and γ subunits from extracts of membranes from bovine brain and PC-12 cells. (A) Extract from brain (2 ml, 1.2 mg of protein) was subjected to affinity purification with 2.5 ml of α_0 -agarose as described. Fraction 1 is the flowthrough; fractions 2–5 were individual washes of 2.5 ml with the 0.5% Lubrol wash; fractions 6–8 and 9–15 were washes of 2.5 ml and 1 ml, respectively, of the 0.05% Lubrol wash; fractions 16–28 were washes of 1 ml at 4°C with the AMF solution. The quantity of each fraction subjected to electrophoresis was as follows: fraction 1, 87 μ l; fractions 3–17, 174 μ l; fractions 19–27, 87 μ l. (B) Extract from PC-12 cells (5 ml, 7 mg of protein) was processed with 5 ml of α_0 -agarose. Washes and elutions were the same as in A, but the volumes were doubled. The quantity of each fraction analyzed was as follows: fraction 1, 217 μ l; fractions 3–28, 543 μ l. The gels were stained with silver. Fraction numbers and identity of the G protein standards are indicated at bottom of each panel. Numbers to left of the gels indicate the apparent M_r of standards ($\times 10^{-3}$). Positions of the purified G protein α , β , and γ standards from brain are indicated at right.

DISCUSSION

We have demonstrated that G protein γ subunits are isoprenylated by analyzing the proteins after biosynthetic radiolabeling with [3 H]MVA, the precursor of cellular isoprenoids. The ability to purify the $\beta\gamma$ subunit complex by affinity chromatography on a matrix of immobilized α subunits made such analysis possible. We have not detected isoprenylation of α or β subunits, either by affinity chromatography or by immunoprecipitation from PC-12 or COS cells (not all data

shown). The deduced amino acid sequences of a number of α subunits (G_o , G_i , G_t) include a cysteine four residues from the carboxyl terminus, similar to that found in isoprenylated proteins. However, in these α subunits this cysteine residue is followed by a glycine, which is not found in isoprenylated proteins; furthermore, this cysteine is the site for ADP-ribosylation catalyzed by pertussis toxin and, thus, it presumably cannot be isoprenylated (23).

Previously identified isoprenylated proteins (including fungal mating peptides, lamin B, and p21ras) contain the 15-carbon farnesyl group (9). In contrast, G protein γ subunits are derivatized with what appears to be the more predominant isoprenoid in cellular proteins, a 20-carbon geranylger-

anyl moiety. Two research groups identified the diterpenoid geranylgeranyl (C_{20}) group as the major isoprenoid modification of total protein from HeLa and Chinese hamster ovary cells (24, 25). An exception to these findings was reported by Maltese and Erdman (26). They identified the radiolabeled isoprenoids bound to total protein from murine erythroleukemia cells as 15-carbon farnesyl-related compounds. However, the chromatographic methods utilized may not have been suitable to distinguish between 15- and 20-carbon isoprenoids. It seems unlikely that protein isoprenyl structure is cell-type specific, because both geranylgeranyl and farnesyl isoprenoids are associated with different proteins in HeLa cells (24, 27).

We speculate that the biosynthetic processing of G protein γ subunits is similar to that of other identified isoprenylated proteins such as p21ras. If true, the process would then include isoprenylation, proteolytic cleavage of the three carboxyl-terminal amino acids, and carboxyl methylation of the resulting terminal cysteine residue. Preliminary evidence for the carboxyl methylation of γ subunits has been reported (28, 29). Because the carboxyl-terminal processing appears

FIG. 2. Affinity chromatography of detergent extracts from [3 H]MVA-labeled PC-12 cells on α_0 -agarose. Particulate fractions (3 mg of protein) were extracted (1 ml) as described. The extract (0.85 ml, 0.8 mg of protein) was purified with 1.5 ml of α_0 -agarose. Fractions 1–10 were individual washes (1.5 ml) at 4°C; fractions 11–25 were elutions (0.5 ml) with AMF at 20°C. (A) Radioactivity associated with each fraction was determined by liquid scintillation counting. Fractions 1–3 contained 3×10^7 cpm (data not shown). (B) Thirty-three microliters of the indicated fraction was subjected to PAGE and fluorography as described. Numbers at left indicate the migration of prestained M_r standards (apparent $M_r \times 10^{-3}$). Migration of β and γ subunits purified from bovine brain is indicated at right of the fluorogram. The gel was exposed to film for 8 days at -70°C .

FIG. 3. HPLC analysis of [3 H]MVA-derived label in fractions from α_0 -agarose chromatography. The elution positions of authentic standards for geraniol (C_{10}), *trans,trans*-farnesol (C_{15}), and all-*trans*-geranylgeraniol (C_{20}) are indicated by their respective labeled arrows. Flowthrough (fraction 2) (A) and AMF eluate (pooled fractions) (B), from α_0 -agarose chromatography processed by trichloroacetic acid precipitation and filtration through nitrocellulose. (C) AMF eluate processed directly without precipitation.

necessary for association of p21ras and nuclear lamins with membranes (7, 30, 31), isoprenylation of G protein γ subunits probably contributes to the association of the $\beta\gamma$ subunit complex with the plasma membrane.

Based on the requirements for chemical cleavage of the isoprenoid from the protein and the similarity of the amino acid sequence between γ and other isoprenylated proteins (the carboxyl-terminal CAAX box), we suggest that γ subunit isoprenylation occurs on the cysteine four residues from the carboxyl terminus. It is unclear what specifies modification by a 20-carbon rather than a 15-carbon isoprenoid. Different enzymes are probably required for the transfer of 15-carbon and 20-carbon isoprenoids to proteins. Reiss *et al.* (32) have characterized a farnesyl:protein transferase purified from rat brain. They have found that synthetic peptides corresponding to the carboxyl-terminal amino acid sequences of all known p21ras proteins and of nuclear lamins A and B competitively inhibit farnesylation of recombinant p21H-ras. In contrast, a peptide corresponding to the carboxyl-terminal sequence of brain γ_2 subunit (11, 12) did not inhibit the enzyme (32). The γ subunits may, instead, be substrates for an enzyme with geranylgeranyl-specific transferase activity.

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