Influence of γ Subunit Prenylation on Association of Guanine Nucleotide-Binding Regulatory Proteins with Membranes

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Two approaches were taken to address the possible role of γ -subunit prenylation in dictating the cellular distribution of guanine nucleotide-binding regulatory proteins. Prenylation of γ subunits was prevented by site-directed mutagenesis or by inhibiting the synthesis of mevalonate, the precursor of cellular isoprenoids. When β or γ subunits were transiently expressed in COS-M6 simian kidney cells (COS) cells, the proteins were found in the membrane fraction by immunoblotting. Immunofluorescence experiments indicated that the proteins were distributed to intracellular structures in addition to plasma membranes. Replacement of Cys⁶⁸ of γ with Ser prevented prenylation of the mutant protein and association of the protein with the membrane fraction of COS cells. Immunoblotting results demonstrated that some of the β subunits were found in the cytoplasm when coexpressed with the nonprenylated mutant γ subunit. When Neuro 2A cells were treated with compactin to inhibit protein prenylation, a fraction of endogenous β and γ was distributed in the cytoplasm. It is concluded that prenylation facilitates association of γ subunits with membranes, that the cellular location of γ influences the distribution of β , and that prenylation is not an absolute requirement for interaction of β and γ .

INTRODUCTION

Heterotrimeric guanine nucleotide-binding regulatory proteins (G proteins)¹ are associated with the inner face of the plasma membrane where they are positioned to interact with both membrane-spanning receptors and effectors (Gilman, 1987). However, the molecular basis for the interaction of G proteins with membranes is only partially understood. The deduced amino acid sequences of the G protein subunit polypeptides (designated α , β , and γ) lack clear hydrophobic domains that might promote interactions of the proteins with the lipid bilayer. Nevertheless, detergent is required to extract G

proteins (with the exception of transducin [G_t]) 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 (Sternweis, 1986). Furthermore, $\beta\gamma$ -subunit complexes (but not α subunits) can be incorporated into phospholipid vesicles; the presence of $\beta\gamma$ then allows efficient association of α subunits with the vesicles (Sternweis, 1986).

Covalent attachment of lipid to a polypeptide has the potential to stabilize its binding to the lipid bilayer or to proteins that are associated with the bilayer. Many G protein α subunits contain myristic acid in amide linkage at the amino terminus (Buss *et al.*, 1987; Schultz *et al.*, 1987; Mumby *et al.*, 1990a), whereas γ subunits are modified by isoprenoids (Fukada *et al.*, 1990; Lai *et al.*, 1990; Maltese and Robishaw, 1990; Mumby *et al.*, 1990b; Yamane *et al.*, 1990). Myristoylation facilitates the interaction of α subunits with the $\beta\gamma$ -subunit complex (Linder *et al.*, 1991) and with cellular membranes (Jones *et al.*, 1990; Mumby *et al.*, 1990a). No covalent modifications of β subunits have been identified.

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¹ Abbreviations used: γ_2 C68S, mutant γ_2 isoform with cysteine at position 68 replaced by serine; COS cells, COS-M6 simian kidney cells; pCMV1 and pCMV5, cytomegalovirus promoter based mammalian cell expression vectors; G protein, guanine nucleotide-binding regulatory protein; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

We have taken two approaches to address the possible role of γ -subunit prenylation in dictating the cellular distribution of G proteins. We prevented prenylation of γ subunits by site-directed mutagenesis or by inhibiting the synthesis of mevalonate, the precursor of cellular isoprenoids. G protein subunit distribution was determined both by western immunoblotting of fractionated cells and by immunocytochemical studies of whole cells and isolated plasma membranes. A description of similar site-directed mutagenesis experiments was published during the preparation of this manuscript (Simonds *et al.*, 1991).

MATERIALS AND METHODS

Cell Culture and Fractionation

Simian kidney COS-M6 (COS) cells and murine neuroblastoma Neuro 2A cells (from American Type Culture Collection, Rockville, MD) were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. They were maintained at 37°C in an atmosphere of 10% CO₂. For experiments designed to determine the effect of inhibition of protein prenylation on the distribution of endogenous G proteins, nearly confluent cultures of Neuro 2A cells were incubated for 1 or 2 d with medium containing 2% dialyzed fetal calf serum in the presence or absence of 20 μ M compactin (provided by J. Goldstein, University of Texas Southwestern Medical Center, Dallas). To radiolabel prenylated proteins, COS cells were incubated overnight in medium supplemented with 700 μ Ci/ml RS-[5-³H]mevalonolactone, 5% dialyzed fetal calf serum, 20 μ M compactin, nonessential amino acids, and sodium pyruvate (Mumby and Buss, 1990). Cells were harvested and fractionated as previously described (Mumby *et al.*, 1990a), except that the nuclear and unbroken cell pellet from the 1000 $\times$ g centrifugation was resuspended and subjected to a second 1000 $\times$ g centrifugation. The two supernatant fractions were combined to increase the yield of membranes from the final 200 000 $\times$ g centrifugation.

Antisera

Production and characterization of the β_1 -specific peptide antiserum U-49 have been described previously (Mumby *et al.*, 1986; Gao *et al.*, 1987). The antibodies specifically recognize endogenous and overexpressed β_1 in COS cells by western immunoblotting (Figure 1A). Although overexpressed β_1 is detected in transfected COS cells with U-49 antibodies by immunofluorescence, the endogenous β_1 in untransfected or mock-transfected cells is not detected. The production and characterization of the common α antiserum A-569 has been described previously (Mumby *et al.*, 1986).

An antiserum reactive with G protein γ subunits was generated with a synthetic peptide as immunogen (Mumby and Gilman, 1991). The amino acid sequence for the peptide, CNNTASIAQAR-KLVEQLKM, was chosen from the published sequence deduced from a bovine brain cDNA clone encoding γ_2 (Gautam *et al.*, 1989). The amino terminal cysteine residue (not part of the γ_2 sequence) was added to facilitate coupling of the peptide to a carrier protein, hemocyanin. Antiserum X-263 was generated against the peptide-hemocyanin conjugate by an established procedure (Green *et al.*, 1982). The antibodies react with γ in bovine brain membranes and in preparations of $\beta\gamma$ purified from the membranes, as determined by western immunoblotting (Figure 1B). Although X-263 antibodies do not detect endogenous γ subunits in COS cells, they do react with overexpressed γ_2 in transfected COS cells as judged by both western immunoblotting and immunofluorescence. The X-263 antibodies react with γ in Neuro 2A cells, but the subtypes of γ in these cells are not known. The X-263 antiserum is expected to react with γ_3 in addition to γ_2 because

the deduced amino acid sequences of γ_2 and γ_3 are similar in the region spanned by the peptide immunogen (Gautam *et al.*, 1990).

Expression Vectors and Mutagenesis

Standard methods were used to construct plasmid vectors capable of directing the synthesis of β_1 (β_1 pCMV5) and γ_2 (γ_2 pCMV5) G protein subunits. Bluescript-based plasmids encoding rat β_1 and bovine γ_2 (Gautam *et al.*, 1989) were generously provided by R. Reed (The Johns Hopkins University, Baltimore, MD) and M. Simon (California Institute of Technology, Pasadena, CA), respectively. Each of the G protein subunit DNA inserts was present in the Bluescript vector at the *Eco*RI site and was subcloned into the *Eco*RI site of pCMV5, a cytomegalovirus promoter-based expression vector (kindly provided by D. Russell, University of Texas Southwestern Medical Center) (Andersson *et al.*, 1989; Mumby *et al.*, 1990a).

In an effort to prevent isoprenylation of γ_2 , site-directed mutagenesis was used to replace the cysteine at position 68 with a serine residue (γ_2 C68S) (Kunkel *et al.*, 1987). The oligonucleotide primer AGGATAGCGCTGAAAACTT was annealed to single stranded γ_2 Bluescript DNA isolated from bacteria infected with helper phage R408 (Russell *et al.*, 1986). The primer sequence was chosen to introduce a site for digestion by the restriction enzyme *Hae* II as well as to change Cys to Ser. The fidelity of the mutagenesis was confirmed by digestion with *Hae* II and by DNA sequencing. The mutant insert was subcloned into pCMV5 and designated γ_2 C68SpCMV5.

Transfections and Immunocytochemistry

Cultures of COS cells were transfected by either the DEAE dextran method or by lipofection. A published procedure for the DEAE dextran method was followed (Adams and Rose, 1985), except for the addition of a glycerol shock. After the chloroquine step, cells were rinsed twice with tris(hydroxymethyl)aminomethane (Tris) saline (Adams and Rose, 1985) and were then incubated with 20% glycerol (vol/vol) and 20 mM Na N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.1, in Dulbecco's modified Eagle's medium for 4 min at room temperature. The cells were then rinsed twice with Tris saline before incubation for 40 h in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 10 mM NaHepes, pH 7.4. The lipofection method was used for cells that were grown in 35-mm dishes or wells and incubated with [³H]mevalonolactone. This method resulted in the loss of fewer cells from the plate during transfection and radiolabeling than was experienced with the DEAE dextran method. Lipofectin was purchased from Bethesda Research Laboratories (Gaithersburg, MD) and was used according to the manufacturer's instructions. Nearly confluent cultures of cells were transfected for cell fractionation experiments, and 10–20% confluent cultures on poly-L-lysine-coated coverslips (in 6-well dishes) were transfected for immunofluorescence experiments.

Two days after transfection, cells on coverslips were fixed on ice for 20 min in either 3% or 10% formalin in phosphate-buffered saline (pH 7.4) containing 1 mM MgCl₂. Coverslips were then washed with the same buffer containing 0.1% saponin, and saponin was included in all subsequent incubations until the final rinse. Cells were first incubated in 5% bovine serum albumin in phosphate-buffered saline for 15 min at 37°C and then were incubated for 1 h at 37°C in a 1:100 dilution of affinity-purified γ -subunit specific X-263, β_1 -specific U-49, or α -specific A-569 antibodies. After washing, cells were incubated in normal goat serum (1:20 dilution) followed by incubation in fluorescein-conjugated goat anti-rabbit IgG (50 μ g/ml, Vector Laboratories, Burlingame, CA) for 1 h at 37°C. The cells were washed and mounted in Fluoromount G (Fisher, Pittsburgh, PA) with 1% 1,4-diazabicyclo[2.2.2]-octaine to prevent photobleaching. In some experiments, cells overexpressing β_1 and γ_2 subunits were labeled with the β_1 -specific primary antibodies conjugated to Texas Red to visualize γ_2 and β_1 subunits independently. The specificities of the antibody reactions were confirmed with blocking experiments. Undiluted an-

tibody was incubated with the peptide (or protein) used as immunogen or an irrelevant peptide (or protein) at 1 μ M in phosphate-buffered saline overnight in the cold. The mixture was diluted to the appropriate antibody concentration immediately before use. Whole-cell labeling was observed using a Zeiss Universal fluorescence microscope (Zeiss, Thornwood, NY) with a mercury vapor lamp and the appropriate filters for visualizing fluorescein and Texas Red. Intracellular labeling was visualized using a Bio-Rad confocal microscope (Bio-Rad, Richmond, CA) with an argon laser and filters appropriate for visualizing fluorescein.

To visualize plasma membrane labeling, transfected cells were sonicated by a modification of a published technique (Moore *et al.*, 1987). During sonication the external surface of the plasma membrane remains attached to the coverslip, the internal surface of the plasma membrane is exposed, and cytosolic components of the cell are washed away. Cells on coverslips were washed in cold phosphate-buffered saline with 1 mM MgCl₂ and placed on ice for 15 min. The cells were then rinsed twice with cold sonication buffer (25 mM NaHepes, 25 mM KCl, and 2.5 mM Mg acetate, pH 7.0) and sonicated with a Vibracell Sonicator (model VC60 from Sonics and Materials, Danbury, CT). The sonication was performed at 12 J (output 100%, 475 W). A $\frac{1}{2}$ " probe was positioned 12 mm above the coverslip, which was present in a well of a 6-well plate containing 18 ml of sonication buffer. Immediately after sonication the cells were washed and fixed with 10% formalin in phosphate-buffered saline for 20 min on ice and stained as for whole cells, except that 1:50 dilutions of affinity purified X-263 and U-49 antibodies were utilized. After examination of immunofluorescence, the cells were incubated for 20 min at room temperature in wheat germ agglutinin conjugated to Texas Red (160 μ g/ml, Vector) for visualization of the membranes, as they cannot be visualized by phase contrast microscopy. Scanning microscopy of the membranes indicated that there was little debris remaining on the internal surface of the membrane. To quantify the number of labeled membranes, 30 randomly selected fields (25 $\times$ objective) were examined from each coverslip and the number of labeled membranes was counted.

Gel Electrophoresis and Immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a discontinuous buffer system (Laemmli, 1970). Gradient gels of 10–20% acrylamide (acrylamide:bisacrylamide 26:1) containing 4 M urea were used to resolve γ subunits. α and β subunits were resolved on standard 9% acrylamide gels (acrylamide:bisacrylamide 30:0.8). Where indicated in the figure legends, samples were precipitated with trichloroacetic acid and treated with *N*-ethylmaleimide as described previously (Mumby *et al.*, 1990b). Gels analyzed by fluorography were treated with En³Hance according to the manufacturer's instructions (Dupont New England Nuclear, Boston, MA). Prestained molecular weight standards were purchased from Bio-Rad. Most immunoblotting experiments were conducted as previously described utilizing [¹²⁵I]goat anti-rabbit IgG F(ab')₂ (Mumby *et al.*, 1988). In some experiments donkey anti-rabbit IgG F(ab')₂ conjugated to horseradish peroxidase was utilized with the enhanced chemiluminescence western blotting detection system from Amersham (Arlington Heights, IL) according to the manufacturer's instructions.

Purification of G Proteins

G_i was purified from bovine rod outer segments as described (Fung, 1983). Preparations of $\beta\gamma$ -subunit complexes that were enriched in four different γ polypeptides were obtained from bovine brain (Sternweis and Robishaw, 1984). Fractions from the first step of purification of G proteins (DEAE-Sephacel) that follow the main peak of guanosine 5'-(3-*O*-thio)triphosphate binding activity were enriched for $\beta\gamma$ complexes of various compositions. These fractions were pooled and purified by chromatography on AcA34 (LKB, Piscataway, NJ) and he-

tylamine-Sepharose (Sternweis and Robishaw, 1984). Al³⁺ and F⁻ were included in the latter column to facilitate separation of $\beta\gamma$ from α subunits (Northup *et al.*, 1983). The preparations were then subjected to combinations of chromatography on DEAE-Sephacel in 20 mM TrisCl (pH 8), 1 mM dithiothreitol, 1.5% (wt/vol) Lubrol PX (Sigma, St. Louis, MO), and a gradient of NaCl from 0 to 250 mM and on hydroxylapatite (Bio-Rad) in 20 mM NaHepes (pH 8), 100 mM NaCl, 1.5% (wt/vol) sodium cholate, and a gradient of potassium phosphate, pH 8, from 0 to 80 mM. Fractions enriched for various γ subunits were pooled, concentrated, and stored frozen at -80°C.

RESULTS

Subunit-specific antibodies were utilized to determine the subcellular localization of G protein polypeptides. U-49 antibodies react with the β_1 isoform of this subunit but not with β_2 (Mumby *et al.*, 1986; Gao *et al.*, 1987). The antibody preparation detects only endogenous β (Figure 1A, lane 1) and overexpressed β_1 (lane 2) in western immunoblots of COS cell membranes. Diversity among γ subunits has been demonstrated by electrophoretic, immunological, and protein sequencing techniques. Four distinct isotypes have been isolated as cDNA clones. Information on the correspondence between the cDNA clones and electrophoretically resolved forms of γ is limited (Hurley *et al.*, 1984; Robishaw *et al.*, 1989). In experiments reported here, γ subunits were detected with a γ_2 peptide antiserum, X-263 (Figure 1B). These antibodies react with γ subunits purified from brain (lane 4) but not from retina (lane 3). G_t (transducin) from retina is composed of $\alpha_t\beta_1\gamma_1$. The marked sequence difference between γ_1 from retina (Hurley *et al.*, 1984) and γ_2 from brain (Gautam *et al.*, 1989) accounts for the lack of reactivity of the γ_2 peptide antibodies with γ_1 from retina. A protein with the electrophoretic mobility of purified brain γ is detected with the γ_2 peptide antibodies in membranes from COS cells transfected with γ_2 pCMV5 (Figure 1B, lane 6) but not in membranes from cells transfected with pCMV without a G protein subunit DNA insert (lane 5). Other unidentified proteins migrate above the 16-kDa protein standard and react with the affinity-purified γ antibody preparation (Figure 1B, lanes 5 and 6).

Different fractions of brain $\beta\gamma$ purified by chromatography on hydroxylapatite and DEAE-Sephacel vary in their composition of γ subunits. We are able to resolve four putative γ subunits by SDS-PAGE and have designated the silver-staining proteins as a, b, c, and d in order of increasing mobility on gels (Figure 1C). An example of a pool of $\beta\gamma$ that clearly exhibits all four bands is shown in lane 11. It is not known if each band represents an independent gene product. In addition, any of the four bands could be composed of more than one protein. A protein (or proteins) in each of the four bands reacts with the γ_2 peptide antiserum X-263 by western immunoblotting (Figure 1, D and E). Based on the similarity of the deduced amino acid sequences of γ_2 and γ_3 , we anticipate that the X-263 antibodies will

Figure 1. Specificity of immunoblotting. Purified G_i ($\alpha\beta\gamma$) from retina (150 ng, lane 3), $\beta\gamma$ from brain (75 ng, lane 4 or 200 ng, lanes 7–16, 17, and 19), and COS cell membrane proteins (10 μ g, lanes 1 and 2; 40 μ g, lanes 5, 6, and 18) were resolved on 9% acrylamide gels (A) or 10–20% gradient gels (B–E). Proteins were precipitated with trichloroacetic acid and were treated with *N*-ethylmaleimide. Numbers at the margins of the figure indicate the migration position of prestained molecular weight standards expressed in kDa. COS cells had been transfected with vector DNA without insert (lanes 1 and 5), β_1 cDNA (lane 2), γ_2 cDNA (lane 6), or both β_1 and γ_2 cDNAs (lane 18). Proteins were either stained with silver (C) or transferred to nitrocellulose, and the β_1 subunits (A) or the γ_2 subunits (B–E) were detected with affinity purified U-49 (1:1000 dilution) or X-263 (1:100 dilution) antibodies, respectively. Lanes 7–11 are silver-stained γ subunits from preparations of $\beta\gamma$ purified and further resolved by DEAE-Sephacel or hydroxylapatite chromatography. The same preparations were transferred to nitrocellulose (lanes 12–16) and processed with the γ -subunit antibodies. The migration of overexpressed γ_2 in COS membranes (lane 18) is compared with two of the purified $\beta\gamma$ preparations (lanes 17 and 19) to determine which of the purified γ subunits is likely to be γ_2 . Blots were processed with [125 I]goat anti-rabbit IgG F(ab')₂. The blot in A was exposed to film for 3 d, B for 6 d, and E for 10 d without intensifying screens. The blot in D was exposed to film for 3 d with an intensifying screen. The entire length of the blots in A and B is shown but only the area of the blots exhibiting immunoreactivity is shown in D and E.

react with both proteins (Gautam *et al.*, 1990). The complete sequence of the γ_4 subunit is not yet available, but γ_4 may also react with the γ_2 peptide antiserum (Gautam *et al.*, 1990). The X-263 antibodies do not react with α or β subunits. The antibodies showed greatest reactivity with the γ subunit designated c (Figure 1D). When membranes from COS cells overexpressing γ_2 were analyzed by western immunoblotting with antiserum X-263, the reactive protein comigrated with the purified brain γ subunit designated c (Figure 1E, lane 18). The migration of purified γ subunits was not influenced by mixture with COS membrane proteins. Thus, the stained protein designated c appears to correspond to the product of the γ_2 cDNA and to the 6-kDa γ subunit (γ_6) that has been purified and sequenced by Robishaw and coworkers (1989).

To examine the possible role of prenylation in facilitating association of endogenous γ subunits with membranes, cells were treated with compactin to inhibit

production of mevalonate and protein prenylation. Eleven cell types were screened for γ immunoreactivity with affinity-purified X-263 antibodies. Murine Neuro 2A cells gave the greatest signal by western immunoblotting and were chosen for further study. After incubation of Neuro 2A cells with compactin for 2 d, the cells were fractionated into crude membrane and cytoplasmic fractions. Distribution of G protein subunits was examined by western immunoblotting (Figure 2). A portion of both β_1 and γ subunits was found in the cytoplasmic fraction after treatment with compactin. α subunits remained exclusively membrane-bound even after 2 d of exposure to compactin. β and γ subunits remained associated with the membrane fraction of compactin-treated cells when the medium was supplemented with 2 mM mevalonate.

To examine further the role of prenylation in facilitating association of G proteins with membranes, we mutagenized a γ_2 cDNA to eliminate the protein's putative site of covalent modification. A carboxyl-terminal

Figure 2. Effect of compactin on the distribution of G protein subunits in Neuro 2A cells. Cells cultured in the presence (+) or absence (–) of 20 μ M compactin were harvested and fractionated after 1 or 2 d (indicated at the bottom of the figure). Proteins in the cytoplasmic and membrane fractions (50 μ g, trichloroacetic acid-precipitated and *N*-ethylmaleimide treated) were resolved on 9% acrylamide (top) or 10–20% acrylamide (bottom) gels and transferred to nitrocellulose for immunoblot analysis. Endogenous α and β subunits (top) were detected with A-569 and U-49 antibody preparations (each at 1:1000 dilutions) and with [125 I]goat anti-rabbit IgG F(ab')₂. Film was exposed to the radioactive blots for 3 d without an intensifying screen. Only the area of the blot that exhibited immunoreactivity is shown in the upper panels. Endogenous γ_2 subunits (bottom) were detected with X-263 antibodies at a 1:500 dilution and donkey anti-rabbit antibodies conjugated to horseradish peroxidase. The chemiluminescent blot was exposed to film for 30 min. Only the region of the film corresponding to the location of γ subunits is shown.

CAAX motif (where C = Cys, A = an aliphatic amino acid, and X = Ser or Met for farnesylated proteins and Leu for geranylgeranylated proteins) is a feature that is characteristic of many prenylated proteins (Glomset *et al.*, 1990; Seabra *et al.*, 1991), including γ subunits (Gautam *et al.*, 1990). The cysteine of the CAAX sequence (position 68) was changed to serine (γ_2 C68S) in an effort to produce a protein that could not be prenylated. The mutant and wild-type γ subunit cDNAs were ligated into the pCMV vector for transient expression in COS cells. In some cases a cDNA that encodes the β_1 subunit was expressed concomitantly. The cellular distribution of G protein subunits was determined by western immunoblotting of fractionated cells and by examination of immunofluorescence of whole cells and of isolated plasma membranes.

The results of western immunoblotting of crude membrane and cytoplasmic fractions from transfected COS cells are shown in Figure 3. The bulk of the transiently expressed wild-type γ_2 subunit or of the β_1 subunit is found in the membrane fraction (β_1 , lane 2; γ_2 , lane 3). This distribution is similar to what we have observed previously for transiently expressed α subunits in COS cells (Mumby *et al.*, 1990a). In contrast, the γ_2 C68S mutant protein is present in the cytoplasmic fraction (lane 11). Of interest is the apparent influence of the γ_2 C68S mutant protein on the distribution of the overexpressed β_1 subunit. Coexpression of the two proteins consistently led to an increase in the amount of β_1 protein found in the cytoplasmic fraction (Figure 3, upper panel, lane 12 compared with lanes 8 and 10). The results suggest that the prenylation of γ influences not only the cellular distribution of γ itself but also the

distribution of β_1 . Cotransfection of γ_2 or γ_2 C68S cDNA with the β_1 cDNA consistently resulted in increased expression of the γ proteins (lane 4 compared with 3 and lane 12 compared with 11). We have not observed any changes in expression of endogenous α subunits as a result of overexpression of β_1 or γ_2 nor any consistent changes in β_1 expression as a result of overexpression of either α or γ subunits. In the experiment shown in Figure 3, the level of expression of β_1 subunits appears to be reduced when coexpressed with the γ proteins (lane 2 compared with lanes 4 and 6). However, in three other similar experiments that we have performed, the expression of β_1 was not influenced by coexpression with either wild-type or mutant γ subunits. Simonds and coworkers (1991) detected little or no expression of β_1 , γ_2 , or the γ_2 C68S mutant proteins unless COS cells were cotransfected with the β_1 cDNA and either of the γ cDNAs. The basis for this minor discrepancy between their results and ours is not known. However, we are in agreement concerning the distribution of the coexpressed proteins in fractionated cells.

The interaction of both endogenous and overexpressed β subunits with γ subunits was assessed by limited proteolysis with trypsin. Treatment of native $\beta\gamma$ with trypsin produces two stable fragments of β , estimated to be 23–27 kDa and 12–14 kDa (Fung and Nash,

Figure 3. Distribution of β_1 , γ_2 , and γ_2 C68S proteins in fractions from transfected COS cells. COS cells (150-mm plates) were transfected with 5 μ g of vector DNA without insert (lanes 1 and 7), γ_2 cDNA (lanes 3, 4, 9, and 10), or γ_2 C68S cDNA (lanes 5, 6, 11, and 12) in the presence (lanes 2, 4, 6, 8, 10, and 12) or absence (lanes 1, 3, 5, 7, 9, and 11) of an additional 5 μ g of β_1 cDNA. Trichloroacetic acid-precipitated and *N*-ethylmaleimide-treated proteins from membrane and cytoplasmic fractions of COS cells were resolved by 9% acrylamide SDS-PAGE (top) or on 10–20% acrylamide gradient gels (bottom). (Top) 40 μ g of membrane and cytoplasmic proteins were loaded. (Bottom) 25 μ g of protein was loaded in lanes 1–4 and 7–10. Because γ_2 C68S protein expression is always lower than wild-type γ_2 , 50 μ g was loaded in lanes 5, 6, 11, and 12. The greater signal for β_1 in lane 2, relative to lanes 4 and 6 (cotransfection with γ cDNAs), is not a common result (see text). Nitrocellulose blots of the gels were processed with β_1 -specific U-49 antibodies and [125 I]goat anti-rabbit F(ab')₂ (top) or γ_2 -specific X-263 antibodies and the enhanced chemiluminescence detection system (bottom). Film was exposed to the blots for 3 d (top, no intensifying screen to detect 125 I) or for 1 min (bottom to detect chemiluminescence). Only those sections of the blots corresponding to the location of β and γ subunits are shown.

1983; Winslow *et al.*, 1986). Two such fragments of β are also observed after tryptic digestion of $\beta_1\gamma_2$ synthesized by translation in vitro (Schmidt and Neer, 1991). In contrast, when β_1 is synthesized alone and subjected to tryptic digestion, only a 19-kDa fragment is detected (Schmidt and Neer, 1991). Thus, we have assessed the interaction of β with γ in COS cell fractions by tryptic proteolysis and quantitation of the 23-kDa fragment that we presume is a reflection of subunit interaction. The β_1 -specific U-49 antibodies react with the 23-kDa fragment of trypsinized $\beta\gamma$ but not the 12-kDa fragment. We did not detect a 19-kDa fragment in any of our experiments. Either the fragment was not formed or the peptide antibodies did not recognize it. The results of western immunoblotting are shown in Figure 4. Proteolysis of endogenous β_1 in brain membranes (lane O) and mock-transfected COS cell membranes (lane M) resulted in the formation of the stable 23-kDa fragment (lanes P and N, respectively). The radioactivity corresponding to the 23-kDa β_1 fragment from the proteolyzed samples and the intact β_1 protein in the unproteolyzed samples was quantitated and the ratio of the

two determined. This value was 0.41 and 0.42 in brain membranes and mock-transfected COS cell membranes, respectively. We have obtained similar results for purified $\beta\gamma$ from brain. In contrast, proteolysis of overexpressed β_1 subunits in COS cell membranes did not give rise to a proportional increase in the 23-kDa β_1 fragment (ratio of fragment to intact β_1 of only 0.07, lanes G and H). We presume that this is due to expression of β_1 to a concentration above that of endogenous γ . However, when β_1 and γ_2 were coexpressed, the β_1 in the membranes was more stable to tryptic digestion and the ratio of fragment to intact β_1 increased to 0.22 (lanes I and J). This increase is likely due to interaction between the overexpressed subunits. If, instead, β_1 was coexpressed with the γ_2 C68S mutant, there was no effect on subsequent generation of the β_1 fragment in the membranes (lanes K and L). This is consistent with the fact that the mutant γ was not found in the membranes after cell fractionation (Figure 3). When β_1 was overexpressed in COS cells, a small but detectable portion was found in the cytoplasmic fraction (Figure 4, lane A). The amount of overexpressed β_1 detected in the cy-

Figure 4. Trypsin sensitivity of endogenous and overexpressed β subunit; an assay for interaction of β with γ . Cytoplasmic or membrane fractions (indicated at top of the figure) containing detectable β subunit were incubated at 37°C in the presence (+) or on ice in the absence (-) of trypsin for 60 min. The ratio of trypsin to cell fraction protein was 1/20. The incubations also contained 20 mM NaHepes (pH 8.0), 4 mM MgCl_2 , 1 mM EDTA, and 0.8% (wt/vol) Lubrol PX. The amount of protein in each incubation is indicated at the bottom of the figure. COS cells were transfected with β_1 cDNA alone (β , lanes A, B, G, and H), β_1 cDNA in combination with γ_2 cDNA ($\beta + \gamma$, lanes C, D, I, and J), β_1 cDNA with mutant γ_2 C68S cDNA ($\beta + \gamma_{\text{mut}}$, lanes E, F, K, and L), or vector DNA without insert (vector mock, lanes M and N). Bovine brain membranes (brain, lanes O and P) were also digested. The incubations were stopped by the addition of 1/3 volume of 4× concentrated electrophoresis sample buffer containing SDS and heating at 90–100°C for 3 min. Proteins were resolved on a 9% acrylamide gel and transferred to nitrocellulose for immunoblotting with the U-49 β_1 -specific antibodies and [^{125}I]goat anti-rabbit IgG F(ab') $_2$. Film was exposed to the blot for 10 d without an intensifying screen. Only the portion of the blot exhibiting immunoreactivity is shown. The positions of the β subunit and the 23-kDa fragment are indicated at the right margin of the blot. Radioactivity on the blot corresponding to the bands on the film was quantitated by radioanalytic imaging. The ratio of radioactivity in the β_1 fragments divided by the radioactivity in the corresponding unproteolyzed β_1 samples (+ trypsin lanes/- trypsin lanes) is shown above the pairs of samples.

Figure 5. Fluorogram of [^3H]mevalonate-labeled proteins from COS cells expressing γ_2 and $\gamma_2\text{C68S}$. COS cells (35-mm dishes) were transfected with 5 μg of γ_2 cDNA (+, lanes 2 and 5) or $\gamma_2\text{C68S}$ cDNA (mut, lanes 3 and 6) together with 5 μg of β_1 cDNA (to increase expression of γ_2 and γ_2 mutant proteins). Control cells were transfected with 1 μg of β_1 cDNA alone (-, lanes 1 and 4). After an overnight incubation with 700 $\mu\text{Ci}/\text{ml}$ of [^3H]mevalonolactone and 20 μM compactin (to increase the specific activity of [^3H]mevalonate in the cells), the cells were harvested and fractionated. One-half of each fraction (equivalent to 35 μg of membrane protein and 55 μg of cytoplasmic protein) was precipitated with trichloroacetic acid and treated with *N*-ethylmaleimide before loading on the gel. Film was exposed to the En^3Hance -treated and dried gel for 2 d. The migration position for γ_2 is indicated to the right of the figure. The entire length of the fluorogram is shown.

toplasm was increased by coexpression with γ_2 but more so with the $\gamma_2\text{C68S}$ mutant. Trypsinization of these cytoplasmic fractions resulted in ratios of β_1 fragment to intact β_1 of 0.35–0.58—values similar to those obtained for endogenous β_1 in COS cell and brain membranes. The similarity of the ratios suggests that the small amount of overexpressed β_1 that was present in the cytoplasmic fraction was associated with endogenous γ (Figure 4, lanes A and B) and/or overexpressed γ_2 proteins (lanes C–F). Furthermore, the results indicate that prenylation of γ is not absolutely required for interaction with β_1 (lanes E and F).

Modification of γ_2 by prenylation was assessed by labeling COS cell proteins with the isoprenoid precursor, [^3H]mevalonate. Cells were transfected with β_1 cDNA in addition to γ_2 or $\gamma_2\text{C68S}$ cDNA to increase the expression of γ subunits. The incorporation of [^3H]mevalonate into proteins from the crude membrane and cytoplasmic fractions was examined by SDS-PAGE and fluorography. Tritium-labeled, wild-type γ_2 protein was found largely in the membrane fraction of cells transfected with γ_2 cDNA (Figure 5, lane 5), consistent with results obtained by immunoblotting (Figure 3, lane 4). A trace of tritiated wild-type γ_2 protein was detected in the cytoplasm (Figure 5, lane 2). As anticipated, there was no signal in the region of the fluorogram corre-

sponding to overexpressed γ_2 for cells transfected with the mutant $\gamma_2\text{C68S}$ cDNA. The results indicate that Cys^{68} is required for prenylation of γ_2 .

The location of overexpressed γ subunits was defined in greater detail by examination of indirect immunofluorescence of whole COS cells. Ten to 20% of transfected cells were positively labeled by our antibody preparations. It is likely that this fraction represents the transfection efficiency of the COS cells. We judged the immunofluorescence signals as specific because no labeling was observed without transfection of the cells with a γ cDNA and there was no labeling if the antibody preparation was first incubated with the peptide used as immunogen to produce the antiserum (Figure 6, C and D). Incubation of the antibodies with an irrelevant peptide of the same length did not affect labeling (Figure 6, A and B). The patterns of immunofluorescence staining and the fixation conditions necessary to achieve staining were markedly different for the expressed wild-type γ_2 and mutant $\gamma_2\text{C68S}$ proteins. Both conventional and confocal microscopic methods were utilized to observe the immunofluorescence (Figures 6 and 7). The reticular pattern of perinuclear staining was consistent with the association of a substantial fraction of expressed γ_2 with endoplasmic reticulum (Figure 6A). The identity of the punctate structures is unknown, but confocal analysis clearly demonstrated that most of the γ_2 protein was not reaching the plasma membrane (Figure 7A). The localization of γ_2 to the perinuclear region may well be a consequence of overexpression of the protein in transfected COS cells and may not accurately reflect the proper localization of γ subunits. In contrast, cells expressing the $\gamma_2\text{C68S}$ protein exhibit a cytoplasmic labeling pattern that extends completely and relatively homogeneously between the cell margins at any particular focal plane (except where the label is excluded from organelles) (Figure 7B). Labeling by the antibodies was achieved for the wild-type γ_2 -expressing cells when they were fixed with either 3% or 10% formalin. In contrast, cells expressing the $\gamma_2\text{C68S}$ mutant protein required fixation in 10% formalin for labeling. Fixation of these cells in 3% formalin was presumably not sufficient to retain the soluble $\gamma_2\text{C68S}$ protein.

The presence of expressed wild-type γ_2 protein on the inner surface of the plasma membrane was confirmed in the following experiments. Cells grown on cover slips can be sonicated and washed such that the plasma membrane remains adherent to the cover slip with its internal surface exposed (Moore *et al.*, 1987). Analysis of the indirect immunofluorescent labeling of the internal surface of such membranes indicated that γ_2 or β_1 subunits were associated with the plasma membrane when expressed singly or together (Figure 8 and Table 1). The mutant $\gamma_2\text{C68S}$ subunit was not visualized in these membrane preparations, even when β_1 was overexpressed concurrently. These results are consistent with those from the cell fractionation experiments and indicate that the nonprenylated γ subunit

Figure 6. Fluorescence photomicrographs of whole cells transfected to express γ_2 subunits and stained with γ subunit-specific antibodies. (A and B) Cells labeled with antibodies that were preincubated with an irrelevant peptide. (C and D) Cells labeled with antibodies preincubated with the immunogen peptide. (A) Cells transfected with wild-type γ_2 cDNA exhibit an intense perinuclear label having the apparent distribution of endoplasmic reticulum. (B) A cell expressing the nonprenylated γ_2 mutant clearly demonstrates a cytoplasmic distribution. The staining pattern is relatively homogeneous and extends completely between cell margins except where the staining is excluded from organelles. Specificity is demonstrated by the marked reduction in staining intensity when the antibody was preincubated with the immunogen peptide before staining cells for expression of γ_2 (C) or γ_2 C68S (D). All cells were transfected with 1 μ g of DNA and were permeabilized and fixed with 10% formalin. Bar = 20 μ m.

does not form a stable interaction with the plasma membrane. Fewer plasma membranes labeled by β_1 antibodies were observed for cells transfected with both β_1 and γ_2 C68S cDNAs than for cells transfected with β_1 alone or β_1 and wild-type γ_2 subunits (Table 1). Based on these results, it is possible that the plasma membrane is the source of the increased cytosolic β_1 observed in western immunoblots of fractions from cells that were transfected with both β_1 and γ_2 C68S cDNAs (Figure 3, lane 12).

The location of overexpressed β subunits was examined further by indirect immunofluorescence of whole cells. The majority of cells overexpressing β_1 subunits displayed intense staining in the region of the Golgi apparatus; intracellular punctate labeling was also evident (Figure 9A). As with γ_2 subunits, a large fraction of the overexpressed β_1 protein was not localized to the plasma membrane. Reducing the amount of DNA used in the transfections from 1 μ g to 30 ng reduced the staining intensity but did not alter the distribution of

Figure 7. Fluorescence confocal images of cells expressing γ_2 and the nonprenylated γ_2 mutant. (A) COS cells were transfected with wild-type γ_2 cDNA and processed with γ -specific antibodies. The intense punctate label for wild-type γ_2 subunits is an intracellular pattern that is distributed chiefly in the perinuclear region. (B) A cell expressing the γ_2 C68S mutant protein exhibits a cytoplasmic distribution of label that extends relatively homogeneously between the cell margins except where it is excluded from organelles. Cultures were transfected with 1 μ g of DNA and fixed with 10% formalin. Bar = 10 μ m.

the label. When cells were transfected to express the β_2 subunit (with the Okayama-Berg vector) (Gao *et al.*, 1987), the distribution of label was similar to that seen with β_1 . Labeling was dependent on transfection with a β -subunit cDNA and was blocked by incubation of the antibodies with either the peptide used as antigen to produce the antibodies or with $\beta\gamma$ protein purified from bovine brain. Labeling was not blocked by an irrelevant peptide or by α_o protein purified from bovine brain. The same labeling pattern of β cDNA-transfected cells was obtained with both U-49 antibodies and with an antiserum generated against purified $\beta\gamma$ from brain (designated S-217) (Mumby *et al.*, 1986). The distribution of overexpressed β may not accurately reflect the normal location of the endogenous subunits. The nature

of the punctate staining of expressed β subunits is not known, but it could result from protein aggregation. Electron microscopic techniques will be required to determine if the structures corresponding to the punctate staining are vesicular.

Although the subcellular fractionation experiments described above indicated that expression of γ_2 C68S influenced the distribution of β_1 , there was no evidence that overexpression of β_1 influenced the distribution of γ_2 (either wild-type or C68S). However, immunofluorescence studies suggest a different conclusion. The distribution of either expressed wild-type γ_2 or γ_2 C68S (Figure 9C) resembled that of β_1 when γ and β were coexpressed. Indeed, double-labeling experiments (γ_2 labeled indirectly with fluorescein-conjugated second

Figure 8. Fluorescence micrographs of isolated plasma membranes from transfected COS cells. Transfected COS cells on coverslips were sonicated to leave only the plasma membranes attached to the coverslip, and the plasma membranes were processed for immunofluorescence. (A) Membranes from cells expressing wild-type γ_2 subunits demonstrate a relatively homogeneous distribution of indirectly labeled γ antibodies. (B) Membranes from cells expressing the γ_2 C68S mutant do not label with the γ antibodies. (C) Membranes from cells expressing β_1 subunits exhibit a distinctly punctate distribution with β_1 antibodies. (D) Membranes from cells cotransfected to express wild-type γ_2 and β_1 subunits were indirectly labeled with the γ_2 antibody (D). All cultures were transfected with 1 μ g of DNA. After sonication they were fixed with 10% formalin. Quantitation of the results is shown in Table 1. Bar = 10 μ m.

antibody; β_1 labeled directly with Texas Red-conjugated primary antibody) demonstrated that the intracellular distributions of both wild-type γ_2 and the γ_2 C68S mutant proteins were very similar to that for β_1 when β and γ subunits were coexpressed. When G protein α subunits (α_s , α_{i2} , α_o) were coexpressed with β_1 , they also acquired a distribution similar to that for β_1 . The influence of expression of β on the distribution of α and γ argues against the notion that expressed β is present only as misfolded aggregates. The basis for the discrep-

ancy between the observed location of coexpressed γ_2 C68S and β_1 in our cellular fractionation and immunocytochemical studies is not known. One possibility, however, is that association of γ_2 C68S with the β_1 -containing structures observed in whole cells by immunofluorescence is not stable and is disrupted when cells are homogenized and fractionated before western immunoblotting. Alternatively, stable $\beta_1\gamma_2$ C68S complexes may form but be released from membranes on homogenization and fractionation. This latter possibility

Table 1. Labeled plasma membranes from COS cells transfected to express G protein subunits

Transfection	Antibody	No. slides	Stained membranes (per 30 fields)*
γ_2	γ	6	45 ± 2
γ_2 C68S	γ	6	1 ± 0.3
β_1	β_1	8	38 ± 2
$\gamma_2 + \beta_1$	γ	6	48 ± 7
$\gamma_2 + \beta_1$	β_1	5	57 ± 5
γ_2 C68S + β_1	γ	7	0
γ_2 C68S + β_1	β_1	4	11 ± 2

* Data are expressed as the means $\pm$ SE.

could account for the results of tryptic digestion experiments, which indicate that β_1 is bound to nonprenylated γ_2 C68S in the soluble fraction derived from cells coexpressing these two proteins (Figure 4, E and F).

DISCUSSION

Heterotrimeric G proteins are generally considered to be associated with the inner face of the plasma membrane. Using two different approaches, we find that prenylation of γ subunits influences the association of both β and γ subunits with membranes. Inhibition of protein prenylation in Neuro 2A cells by compactin results in the distribution of β and γ subunits (but not α subunits) to the cytoplasm. Replacement of Cys⁶⁸ of the γ_2 subunit with Ser prevents prenylation of the mutant protein and prevents association of the expressed pro-

tein with the membrane fraction of transfected COS cells. When γ_2 C68S is expressed with β_1 , both proteins can be found in the cytoplasmic fraction by western immunoblotting. Results obtained by site-directed mutagenesis and western immunoblotting are in general agreement with those reported by Spiegel and coworkers (Simonds *et al.*, 1991).

The results of our immunofluorescence experiments corroborate most of the results obtained by western immunoblotting, leading us to conclude that wild-type γ_2 is membrane-associated, whereas the nonprenylated γ_2 C68S mutant is cytoplasmic (at least when expressed alone). The following observations support this conclusion. 1) The wild-type γ_2 protein was visualized after fixation with 3% formalin, whereas the γ_2 C68S mutant protein was not; 10% formalin was required to retain and detect the mutant protein in whole cells by immunofluorescence. 2) The wild-type γ_2 protein appeared to be associated with intracellular membranes, possibly the endoplasmic reticulum, by both routine and confocal microscopy; the nonprenylated γ_2 mutant protein was found in the cytoplasm. 3) The wild-type γ_2 subunit was associated with the inner aspect of isolated plasma membranes, whereas the γ_2 mutant protein was not.

Carboxyl-terminal processing of γ subunits is presumed to involve three steps, similar to those elucidated for p21^{ras}, a monomeric GTP-binding protein. Prenylation of p21^{ras} (at a cysteine 4 residues from the initial carboxyl terminus of the nascent protein) is thought to precede cleavage of the three terminal residues and methylation of the resulting carboxyl-terminal prenylated cysteine (Clarke *et al.*, 1988; Casey *et al.*, 1989; Gutierrez *et al.*, 1989; Hancock *et al.*, 1989). Two dif-

Figure 9. Fluorescence photomicrographs of cells expressing increased levels of β_1 subunits and/or γ C68S mutant subunits. (A) COS cells on coverslips were transfected with the β_1 cDNA and processed for immunofluorescence with β_1 -specific antibodies. Note the intense perinuclear stain for β_1 subunits in the Golgi region (arrows) and structures that appear to be intracellular vesicles. Cells overexpressing β_1 subunits are generally more rounded in shape than are other transfected cells. (B) Cells expressing the nonprenylated γ_2 mutant demonstrate a cytoplasmic distribution. (C) Cells transfected to express β_1 and γ_2 C68S were labeled with antibody to γ_2 . The distribution of the γ_2 mutant protein is similar to that for overexpressed β_1 . Bar = 50 μ m.

ferent brain G protein γ subunits are modified by an all-*trans*-geranylgeranyl moiety on carboxymethylated terminal cysteine residues (Yamane *et al.*, 1990). When we changed Cys⁶⁸ (4 residues from the carboxyl terminus of the deduced amino acid sequence) of γ_2 to Ser, [³H]mevalonate was not incorporated into the expressed protein. It thus seems that Cys⁶⁸ is almost certainly the site of γ_2 prenylation. Localization of a variety of forms of p21^{ras} to the plasma membrane also requires palmitoylation or a polybasic domain in addition to the CAAX motif (Hancock *et al.*, 1990). It is not yet known if additional covalent modifications or other features of the γ -subunit polypeptides are important for their association with the plasma membrane.

The bulk of protein carboxyl methyltransferase activity is associated with the endoplasmic reticulum (Stephenson and Clarke, 1991). When we overexpressed wild-type γ_2 , much of the protein appeared to be at the endoplasmic reticulum (by immunofluorescence). Perhaps carboxyl methylation of γ_2 subunits at the cytoplasmic face of the endoplasmic reticulum constitutes a restriction point in the processing of the protein. Overproduction of γ_2 might cause a concentration of the protein at this point in the pathway.

Lipid modifications of α and γ subunits are necessary for distribution of these proteins to membranes. Lipid modifications of β subunits have not yet been identified. The strong affinity of β subunits for γ may account for the targeting of β subunits to membranes. However, when β_1 subunits were expressed above the concentration of endogenous γ , they were nevertheless associated with the particulate fraction (as demonstrated by immunoblotting) and with isolated plasma membranes (as shown by immunofluorescence). The location of the intracellular (nonplasma membrane) β_1 remains unclear, but the protein may be in the Golgi apparatus and lysosomes or it may be aggregated. Electron microscopic techniques will be required to resolve this issue. We have been unable to visualize endogenous β (or α or γ) subunits by immunocytochemical techniques with our antisera, although the same antisera are suitable for western immunoblotting and immunoprecipitation (Mumby *et al.*, 1986; Buss *et al.*, 1987). The distribution of transiently expressed G protein subunits to structures in addition to the plasma membrane may obviously be a function of overexpression and may not represent the normal distribution. Despite the fact that such localization may be artifactual, it remains intriguing that this β -subunit protein appears capable of interacting with both α and γ . Perhaps more emphasis should be placed on evaluation of the normal distribution of β and the mechanisms by which the subunit associates with membranes. Malbon and coworkers have detected endogenous β subunits in A431 cells by immunofluorescence (Wang *et al.*, 1989). They observed a punctate staining pattern that they interpreted to be associated with the plasma membrane and within the cytoplasm.

The nature of the cytoplasmic staining was not addressed further (Wang *et al.*, 1989) but does resemble the staining pattern we observed in cells overexpressing β subunits.

Two lines of evidence have led us to conclude that prenylation of γ subunits is not an absolute requirement for interaction with β subunits. When Neuro 2A cells are treated with compactin to inhibit prenylation of γ , both β and γ subunits are found in the cytoplasmic fraction. Coexpression of β_1 subunits with γ_2 C68S mutant protein in COS cells resulted in an increase of β_1 in the cytoplasmic fraction. Results of limited tryptic digestion indicate that the β_1 subunit in the soluble fraction was associated with nonprenylated γ subunits. However, it is certainly possible that the affinity of β for unmodified γ subunits may be altered relative to that for normally processed γ subunits. Synthesis and purification of $\beta\gamma$ -subunit complexes with prenylated and unmodified γ polypeptides should be a useful way to answer such questions.

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REFERENCES

- Adams, G., and Rose, J. (1985). Structural requirements of a membrane-spanning domain for protein anchoring and cell surface transport. *Cell* 41, 1007-1015.
- Andersson, S., Davis, D.N., Dahlback, H., Jornvall, H., and Russell, D.W. (1989). Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* 264, 8222-8229.
- Buss, J.E., Mumby, S.M., Casey, P.J., Gilman, A.G., and Sefton, B.M. (1987). Myristoylated α subunits of guanine nucleotide-binding regulatory proteins. *Proc. Natl. Acad. Sci. USA* 84, 7493-7497.
- Casey, P.J., Soliski, P.A., Der, C.J., and Buss, J.E. (1989). p21^{ras} is modified by a farnesyl isoprenoid. *Proc. Natl. Acad. Sci. USA* 86, 8323-8327.
- Clarke, S., Vogel, J.P., Deschenes, R.J., and Stock, J. (1988). Posttranslational modification of the Ha-ras oncogene protein: evidence for a third class of protein carboxyl methyltransferases. *Proc. Natl. Acad. Sci. USA* 85, 4643-4647.
- Fukada, Y., Takao, T., Ohguro, H., Yoshizawa, T., Akino, T., and Shimonishi, Y. (1990). Farnesylated γ -subunit of photoreceptor G protein indispensable for GTP-binding. *Nature* 346, 658-660.

- Fung, B.K.-K. (1983). Characterization of transducin from bovine retinal rod outer segments. I. Separation and reconstitution of subunits. *J. Biol. Chem.* 258, 10495–10502.
- Fung, B.K.-K., and Nash, C.R. (1983). Characterization of transducin from bovine retinal rod outer segments. II. Evidence for distinct binding sites and conformational changes revealed by limited proteolysis with trypsin. *J. Biol. Chem.* 258, 10503–10510.
- Gao, B., Mumby, S.M., and Gilman, A.G. (1987). The G protein β_2 cDNA encodes the β_{35} subunit. *J. Biol. Chem.* 262, 17254–17257.
- Gautam, N., Baetscher, M., Aebersold, R., and Simon, M.I. (1989). A G protein gamma subunit shares homology with ras proteins. *Science* 24, 971–974.
- Gautam, N., Northup, J., Tamir, H., and Simon, M.I. (1990). G protein diversity is increased by associations with a variety of γ subunits. *Proc. Natl. Acad. Sci. USA* 87, 7973–7977.
- Gilman, A.G. (1987). G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56, 615–649.
- Glomset, J.A., Gelb, M.H., and Farnsworth, C.C. (1990). Prenylproteins in eukaryotic cells: a new type of membrane anchor. *Trends Biochem. Sci.* 15, 139–142.
- Green, N., Alexander, H., Olson, A., Alexander, S., Shinnick, T.M., Sutcliffe, J.G., and Lerner, R.A. (1982). Immunogenic structure of the influenza virus hemagglutinin. *Cell* 28, 477–487.
- Gutierrez, L., Magee, A.I., Marshall, C.J., and Hancock, J.F. (1989). Post-translational processing of p21^{ras} is two-step and involves carboxyl-methylation and carboxy-terminal proteolysis. *EMBO J.* 8, 1093–1098.
- Hancock, J.F., Magee, A.I., Childs, J.E., and Marshall, C.J. (1989). All *ras* proteins are polyisoprenylated but only some are palmitoylated. *Cell* 57, 1167–1177.
- Hancock, J.F., Paterson, H., and Marshall, C.J. (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21^{ras} to the plasma membrane. *Cell* 63, 133–139.
- Hurley, J.B., Fong, H.K.W., Teplow, D.B., Dreyer, W.J., and Simon, M.I. (1984). Isolation and characterization of a cDNA clone for the γ subunit of bovine retinal transducin. *Proc. Natl. Acad. Sci. USA* 81, 6948–6952.
- Jones, T.L.Z., Simonds, W.F., Merendino, J.J., Jr., Brann, M.R., and Spiegel, A.M. (1990). Myristoylation of an inhibitory GTP-binding protein α subunit is essential for its membrane attachment. *Proc. Natl. Acad. Sci. USA* 87, 568–572.
- Kunkel, T.A., Roberts, J.D., and Zabor, R.A. (1987). Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.* 154, 367–382.
- Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 227, 680–685.
- Lai, R.K., Perez-Sala, D., Canada, F.J., and Rando, R.R. (1990). The γ subunit of transducin is farnesylated. *Proc. Natl. Acad. Sci. USA* 87, 7673–7677.
- Linder, M.E., Pang, I.-H., Duronio, R.J., Gordon, J.I., Sternweis, P.C., and Gilman, A.G. (1991). Lipid modifications of G protein subunits: myristoylation of G_{oo} increases its affinity for $\beta\gamma$. *J. Biol. Chem.* 266, 4654–4659.
- Maltese, W.A., and Robishaw, J.D. (1990). Isoprenylation of C-terminal cysteine in a G-protein γ subunit. *J. Biol. Chem.* 265, 18071–18074.
- Moore, M.S., Mahaffey, D.T., Brodsky, F.M., and Anderson, R.G.W. (1987). Assembly of clathrin-coated pits onto purified plasma membranes. *Science* 236, 558–563.
- Mumby, S.M., and Buss, J.E. (1990). Metabolic radiolabeling techniques for identification of prenylated and fatty acylated proteins. *Methods* 1, 216–220.
- Mumby, S.M., Casey, P.J., Gilman, A.G., Gutowski, S., and Sternweis, P.C. (1990b). G protein γ subunits contain a 20-carbon isoprenoid. *Proc. Natl. Acad. Sci. USA* 87, 5873–5877.
- Mumby, S.M., and Gilman, A.G. (1991). Synthetic peptide antisera with determined specificity for G protein α or β subunits. *Methods Enzymol.* 195, 215–233.
- Mumby, S.M., Heuckeroth, R.O., Gordon, J.I., and Gilman, A.G. (1990a). G protein α subunit expression, myristoylation, and membrane association in COS cells. *Proc. Natl. Acad. Sci. USA* 87, 728–732.
- Mumby, S.M., Kahn, R.A., Manning, D.R., and Gilman, A.G. (1986). Antisera of designed specificity for subunits of guanine nucleotide-binding regulatory proteins. *Proc. Natl. Acad. Sci. USA* 83, 265–269.
- Mumby, S.M., Pang, I.-H., Gilman, A.G., and Sternweis, P.C. (1988). Chromatographic resolution and immunologic identification of the α_{40} and α_{41} subunits of guanine nucleotide-binding regulatory proteins from bovine brain. *J. Biol. Chem.* 263, 2020–2026.
- Northup, J.K., Smigel, M.D., Sternweis, P.C., and Gilman, A.G. (1983). The subunits of the stimulatory regulatory component of adenylate cyclase. Resolution of the activated 45,000-dalton (α) subunit. *J. Biol. Chem.* 258, 11369–11376.
- Robishaw, J.D., Kalman, V.K., Moomaw, C.R., and Slaughter, C.A. (1989). Existence of two γ subunits of the G proteins in brain. *J. Biol. Chem.* 264, 15758–15761.
- Russel, M., Kidd, S., and Kelley, M.R. (1986). An improved filamentous helper phage for generating single-stranded plasmid DNA. *Gene* 45, 333–338.
- Schmidt, C.J., and Neer, E.J. (1991). *In vitro* synthesis of G protein $\beta\gamma$ dimers. *J. Biol. Chem.* 266, 4538–4544.
- Schultz, A.M., Tsai, S.-C., Kung, H.-F., Oroszlan, S., Moss, J., and Vaughan, M. (1987). Hydroxylamine-stable covalent linkage of myristic acid in G_{oo}, a guanine nucleotide-binding protein of bovine brain. *Biochem. Biophys. Res. Commun.* 146, 1234–1239.
- Seabra, M.C., Reiss, Y., Casey, P.J., Brown, M.S., and Goldstein, J.L. (1991). Protein farnesyltransferase and geranylgeranyltransferase share a common α subunit. *Cell* 65, 429–434.
- Simonds, W.F., Butrynski, J.E., Gautam, N., Unson, C.G., and Spiegel, A.M. (1991). G-protein $\beta\gamma$ dimers. Membrane targeting requires subunit coexpression and intact γ C-A-A-X domain. *J. Biol. Chem.* 266, 5363–5366.
- Stephenson, R.C., and Clarke, S. (1991). Peptide substrates for a C-terminal farnesyl cysteine protein carboxyl methyltransferase. *FASEB J.* 5, A1181.
- Sternweis, P.C. (1986). The purified α subunits of G_o and G_i from bovine brain require $\beta\gamma$ for association with phospholipid vesicles. *J. Biol. Chem.* 261, 631–637.
- Sternweis, P.C., and Robishaw, J.D. (1984). Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* 259, 13806–13813.
- Wang, H.-Y., Berrios, M., and Malbon, C.C. (1989). Indirect immunofluorescence localization of β -adrenergic receptors and G proteins in human A431 cells. *Biochem. J.* 263, 519–532.
- Winslow, J.W., Van Amsterdam, J.R., and Neer, E.J. (1986). Conformations of the α_{39} , α_{41} , and $\beta\gamma$ components of brain guanine nucleotide-binding proteins. *J. Biol. Chem.* 261, 7571–7579.
- Yamane, H.K., Farnsworth, C.C., Xie, H., Howald, A., Fung, B.K.-K., Clarke, S., Gelb, M.H., and Glomset, J.A. (1990). Brain G protein γ subunits contain an all-*trans*-geranylgeranyl-cysteine methyl ester at their carboxyl termini. *Proc. Natl. Acad. Sci. USA* 87, 5868–5872.