Amino- and Carboxy-Terminal Deletion Mutants of Gsα Are Localized to the Particulate Fraction of Transfected COS Cells

Yong-Sung Juhnn, Teresa L. Z. Jones, and Allen M. Spiegel

Molecular Pathophysiology Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

Abstract. To elucidate the structural basis for membrane attachment of the α subunit of the stimulatory G protein (Gsα), mutant Gsα cDNAs with deletions of amino acid residues in the amino and/or carboxy termini were transiently expressed in COS-7 cells. The particulate and soluble fractions prepared from these cells were analyzed by immunoblot using peptide specific antibodies to monitor distribution of the expressed proteins. Transfection of mutant forms of Gsα with either 26 amino terminal residues deleted (Δ3-28) or with 59 amino terminal residues deleted (Δ1-59) resulted in immunoreactive proteins which localized primarily to the particulate fraction. Similarly, mutants with 10 (Δ385-394), 32 (Δ353-384), or 42 (Δ353-394) amino acid residues deleted from the carboxy terminus also localized to the particulate fraction, as did a mutant form of Gsα lacking amino acid residues at both the amino and carboxy termini (Δ3-28)/(Δ353-384). Mutant and wild type forms of Gsα demonstrated a similar degree of tightness in their binding to membranes as demonstrated by treatment with 2.5 M NaCl or 6 M urea, but some mutant forms were relatively resistant compared with wild type Gsα to solubilization by 15 mM NaOH or 1% sodium cholate. We conclude that: (*a*) deletion of significant portions of the amino and/or carboxyl terminus of Gsα is still compatible with protein expression; (*b*) deletion of these regions is insufficient to cause cytosolic localization of the expressed protein. The basis of Gsα membrane targeting remains to be elucidated.

GUANINE nucleotide binding proteins (G proteins)[1] involved in signal transduction are heterotrimers composed of a GTP-binding α subunit and a βγ subunit complex. G proteins constitute one family in the GTP-binding protein superfamily (5, 6, 14, 22, 24). The G proteins transduce extracellular signals into intracellular effects by transferring the information received by various receptors for hormones, photons and odorants, to effectors such as adenylyl cyclase for the stimulatory G protein (Gs) and inhibitory G proteins (Gi), cGMP phosphodiesterase for retinal transducin, and phospholipases and ion channels for other G proteins. The specificity of receptor–effector coupling results primarily from the α subunit. G protein α subunits are known to be localized to the cytosolic face of the plasma membrane. The α subunits, with the exception of transducin, are tightly bound to the membrane, so that detergents are required to release them (37). However, the α subunit amino acid sequences contain no hydrophobic membrane spanning domains to account for their attachment.

Posttranslational modification of proteins with lipids is one way to increase the hydrophobicity of the molecules and thus promote membrane association (38). Fatty acylation with myristate was found in the α subunits of Gi and Go (9), and failure of membrane attachment was observed with mutant forms (substitution of alanine for glycine in position 2) of Gi (18) and Go (27) which were incapable of undergoing *N*-myristoylation. Isoprenylation is another type of lipid modification observed in G proteins. The γ subunit of transducin was found to be modified by farnesylation (12, 23) and the γ subunit of neural G proteins by geranylgeranylation (26, 39). A mutant γ subunit lacking this lipid modification lost its ability to target to the membrane (35). However, no such lipid modification has been observed in the α subunit of Gs (Gsα), nor was it found to contain either myristate or palmitate (9). Thus the molecular mechanism of its anchorage to the membranes remains unclear.

Sternweis (36) suggested that the α subunit might be anchored to the membrane by the βγ subunit complex, which is more hydrophobic and incorporates spontaneously into phospholipid vesicles. The α subunit of G proteins is believed to be separated from the βγ subunit complex when it is activated by receptors or GTP analogs such as GTPγS. Gsα was observed to remain membrane associated even upon activation. There is some evidence for translocation of Gsα from the membrane after agonist treatment of cells (32),

Dr. Yong-Sung Juhnn's present address is Department of Biochemistry, Seoul National University, Seoul 110-790, Korea.

1. *Abbreviations used in this paper*: G protein, guanine nucleotide binding proteins; Gi, inhibitory G proteins; Gs, stimulatory G protein; PCR, polymerase chain reaction.

but prolonged incubation of membranes with nonhydrolyzable GTP analogs was needed before significant release of Gsα from the membrane was observed (25). These observations argue against a simple model of Gsα anchorage by the βγ complex. Therefore, other mechanisms are needed to explain the tight binding of Gsα to the membrane.

In an effort to determine which Gsα domains are essential for membrane binding, we constructed mutant forms of Gsα with deletions in the amino terminal, carboxy terminal, or both terminal sequences using the polymerase chain reaction (PCR). The mutants were expressed in COS cells, and the intracellular localization of the mutated proteins was determined by separating the cells into particulate and soluble fractions, and immunoblotting with peptide-specific antibodies.

Materials and Methods

Construction of Mutant Gsαs

The cDNAs coding for rat Gsα (17) were kindly provided by Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD), and both the 47-kD Gsα-1 containing 394 amino acid residues, and the 42-kD Gsα-3 containing 379 residues (8), were cloned into the pCD-PS vector which contains simian virus 40-derived DNA sequences permitting expression of cloned genes in eukaryotes (4). The deletion mutants of Gsα were constructed using the PCR, and the schematic map of deletions in the Gsα mutants is shown in Fig. 1. The oligonucleotide primers for PCR were synthesized with a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA), and the tritylated oligomers were purified on NENsorb Prep columns (New England Nuclear, Du Pont, Boston, MA). The reaction mixture contained 10 mM Tris-Cl, pH 8.4, 50 mM KCl, 100 μg/ml gelatin, 2–4 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate, 25 pmol of each upstream and downstream oligonucleotide primer, 200 ng template DNA, and 1.25 U of *Thermus aquaticus* (*Taq*) polymerase (Perkin-Elmer Cetus, Norwalk, CT) in a total volume of 50 μl. 25 cycles consisting of denaturation at 96°C for 1 min, annealing at 45°C for 1 min, and primer extension at 72°C for 3 min were performed, followed by one final extension reaction. The PCR products were analyzed by agarose gel electrophoresis, and DNA of the proper size was recovered from the gel.

(Δ3–28)Gsα-1. The nucleotide sequences coding for the amino terminal 26 residues from Cys-3 to Lys-28 were deleted from the cDNA of Gsα-1 to construct (Δ3–28)Gsα-1. The sequence of the 5′ primer was 5′-TAT AAC GGC CGA TGG GCC AGC TGC AGA AGG-3′, and was designed to contain a restriction site for XmaIII, codons for the first two amino acid residues of Gsα, and then that for Gln-29 to delete the nucleotide sequences coding for the intervening 26 amino acid residues. The 3′ primer, 5′-ATG AAG TAC TTG GCC CGG-3′, complementary to the wild type Gsα-1 sequence, was positioned downstream to the restriction site for MluI. The PCR was performed using Gsα-1 cDNA in pCD plasmid, pCD-Gsα-1, as the template. The purified mutant PCR fragment was then ligated into pCD-Gsα-1 in place of the wild type sequence using the single recognition sites for XmaIII and MluI.

(Δ3–28)Gsα-3. The same nucleotide sequence deleted in (Δ3–28)Gsα-1 was also deleted in Gsα-3 by using the same oligonucleotide primers but with pCD-Gsα-3 as the template DNA.

(Δ1–59)Gsα-1. A novel cDNA (16) of canine Gsα-1 coding for the sequences from the second initiation site, Met-60, was a generous gift from Dr. Yoshihiro Ishikawa (Massachusetts General Hospital, Boston, MA). The cDNA insert was cloned into the pCD-PS plasmid to construct pCD-(Δ1–59)Gsα-1.

(Δ385–394)Gsα-3. The carboxy terminal 10 residues from Gsα-3 were deleted using PCR to construct (Δ385–394) Gsα-3. Note that throughout the paper we number Gsα residues based on the length (394 amino acids) of Gsα-1 (8). Thus, for example, we designate the mutant in which the carboxy-terminal decapeptide of Gsα-3 is deleted (Δ385–394)Gsα-3. The sequence of the 3′ primer was 5′-TCA ACT CTT AAG TTA CTG GAT GAT GTC ACG GCA-3′, which contains the sequence coding from Cys-379 to Gln-384 followed by a termination codon and the recognition site for AflII. The 5′ primer, 5′-CCG CAG CCC GGC CGC GCC CCG CCG CCG ATG-3′, was a wild type sequence upstream to the restriction site of XmaIII. The mutant PCR fragment was ligated into the pCD-Gsα-3 at the unique restriction sites for XmaIII and AflII.

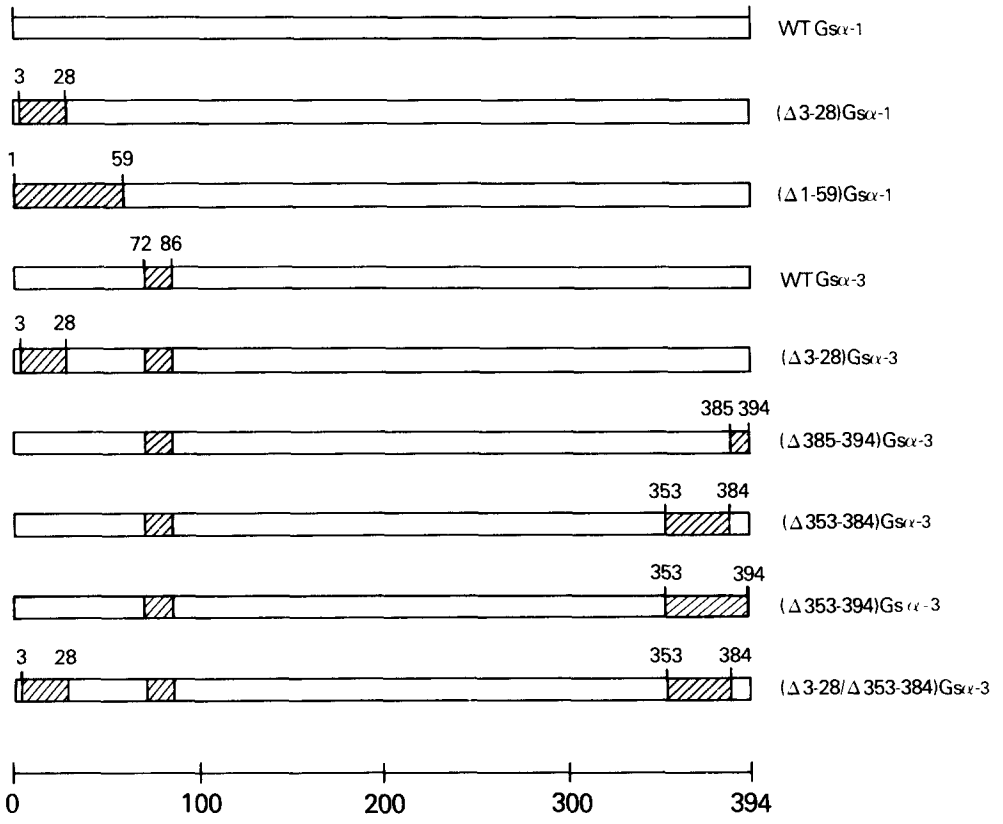

Figure 1. Schematic map of the wild type and mutant forms of Gsα. The hatched bars represent the deleted amino acid residues, and the numbers indicate the first or last residue of the deletion. Residues 72–86 correspond to the alternatively spliced portion of Gsα (8). Gsα-1 and Gsα-3 refer to 2 of the 4 forms derived by alternative splicing (8), and have 394 and 379 residues, respectively. The scale at bottom, and the numbering for all mutants throughout the paper, use the number of residues found in Gsα-1.

(Δ353–384)Gsα-3. A further deletion in the carboxy terminal sequence of Gsα-3 was made by removing the sequence coding for 32 amino acid residues from Gly-353 to Gln-384 to construct (Δ353–384)Gsα-3. The 3′ primer used to make the deletion was a 60-mer, 5′-TCA ACT CTT AAG TTA GAG CAG CTC GTA TTG GCG AAG ATG CAT GCG ACT AGC AGT GCT GAT-3′. It contained the sequences for six amino acid residues upstream to the deletion, 10 carboxy terminal residues, a termination codon, and the restriction site for AflII, sequentially. The 5′ primer was the same as that used in constructing (Δ385–394)Gsα-3. The PCR product and wild type Gsα-3-containing plasmid were cut with XmaIII and AflII, and then ligated with T4 DNA ligase.

(Δ353–394)Gsα-3. The carboxy terminal 42 residues, from Gly-353 to Leu-394, were deleted using PCR. The 3′ primer was 5′-TCA ACT CTT AAG TTA ACT AGC AGT GCT GAT-3′, which contained the sequences for six amino acids upstream to the deletion, a termination codon, and the restriction site for AflII. The 5′ primer was the same as that employed in construction of (Δ385–394)Gsα-3. The PCR product was digested with XmaIII and AflII, and then ligated to the wild type cDNA prepared by digestion with the same enzymes.

(Δ3–28/Δ353–384)Gsα-3. A double deletion mutant was constructed by ligating the XmaIII–MluI fragment of (Δ3–28)Gsα-3 into the plasmid containing (Δ353–384)Gsα-3.

Preparation of Mutant Gsα DNA

A competent *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg, MD) was transformed with the ligation mixture of various mutants and the transformed cell colonies were selected on an LB agar plate containing 100 μg/ml ampicillin. Plasmid DNA purified from each colony was analyzed with the proper restriction enzyme to screen for the specific mutant Gsα DNA. DNA from positive clones was then sequenced using the dideoxynucleotide chain termination method to insure the correct mutation (33). Plasmids were purified using a cesium chloride gradient method (33).

Expression of Deleted Forms of Gsα in COS Cells and Preparation of the Cell Fractions

Transformed monkey kidney cells, COS-7, were transfected with the pCD-PS plasmid with and without the cDNA of wild type and mutated Gsα by the DEAE-dextran method (10). COS-7 cells were maintained in DME with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biofluids, Rockville, MD).

After 48 hours the transfected COS cells were harvested, pelleted and resuspended in four volumes of homogenization buffer composed of 20 mM Tris-Cl, pH 8.0, 1 mM PMSF, 1 mM EDTA, 10 μg/ml leupeptin, 1 μg/ml aprotinin, and 1 mM DTT. The cells were homogenized by passing through a 25-guage needle 15 times. The cell lysate was centrifuged at 600 *g* for 5 min, and the supernatant was centrifuged at 100,000 *g* for 60 min at 4°C. The resulting supernatant was transferred to a fresh tube, the pellet was suspended in the original volume of buffer, and both fractions were recentrifuged and separated. The washed pellet was resuspended in three volumes of the homogenization buffer and designated as the particulate (P) fraction, and the supernatant was designated as the soluble (S) fraction.

The protein concentration was determined by the dye binding method (7) with BSA (Bio-Rad Laboratories, Cambridge, MA) as the reference standard.

Treatment of the Particulate Fraction with Salts and Sodium Hydroxide

The particulate fraction of the COS cells was centrifuged at 430,000 *g* for 15 min at 4°C in a TLA100-2 rotor (Beckman Instruments Inc., Palo Alto, CA), and the pellet was resuspended in homogenization buffer. Approximately 75-μg aliquots of the suspension were treated for 20 min with distilled water at 4°C (control), or 2.5 M NaCl at 4°C, or 6 M urea at 37°C, each to a final volume of ~50 μl. The samples were centrifuged at 430,000 *g* for 15 min to prepare the pellet and the supernatant. The same quantity of the particulate fraction was treated with 15 mM sodium hydroxide solution containing 2 mM EDTA and 0.2 mM DTT, and the sample was centrifuged immediately, followed by neutralization of each fraction as described (2).

Solubilization of Various Forms of Gsα with Sodium Cholate

100-μg aliquots of the particulate fraction of COS-7 cells expressing various mutant forms of Gsα were resuspended in homogenization buffer containing 1% (wt/vol) sodium cholate and incubated at 25°C for 1 h. The mixture was centrifuged at 100,000 *g* for 1 h to prepare pellet and soluble fractions.

SDS-PAGE and Immunoblot

Protein samples were solubilized and separated on 12.5% or 15% SDS–polyacrylamide gels, and then transferred onto nitrocellulose paper (31). The wild type and mutant Gsα proteins were detected with the peptide specific antibodies, RM and GCL. The RM antibody was generated against the carboxy terminal decapeptide of Gsα (34), and GCL was generated against the amino terminal 16 residues from Gly-2 to Lys-17. The Gsα was visualized by treating the blot either with peroxidase labeled secondary antibody, or with [^{125}I]protein A followed by autoradiography (13).

Results

Amino Terminal Deletion Mutants of Gsα

To evaluate the expression and membrane targeting of various deletion mutants of Gsα, we transfected COS cells with cDNAs and measured specific immunoreactivity in particulate and soluble fractions. The COS cell fractions were prepared by ultracentrifugation of the cell lysate, followed by washing each fraction to minimize cross contamination. When the untransfected COS cells were fractionated and analyzed by SDS-PAGE and western blot, two forms of endogenous Gsα, principally localized to the particulate fraction, were visualized at 47 and 42 kD, respectively. These correspond to the known splice variants of Gsα (8). The 47-kD band was less dense than the 42-kD band, and it sometimes resolved into two bands indicating that this fraction was likely composed of Gsα-1 and Gsα-2 (8). Endogenous immunoreactivity is unchanged (Fig. 2) after mock transfection or transfection with vector alone (pCD-PS). Transfection of the cells with wild type Gsα-1 resulted in an increase in the density of the lower portion of the 47-kD band, demonstrating that the Gsα-1 had been overexpressed after transfection (Fig. 2, *left*).

The nucleotide sequence coding for 26 amino acid residues from Cys-3 to Lys-28 was deleted from the cDNA of rat Gsα-1 to construct (Δ3–28)Gsα-1, and the cDNA was cloned into the pCD-PS vector for transfection into COS-7 cells. We chose to delete this portion of Gsα since the amino terminus has been implicated in α subunit membrane binding and interaction with the β/γ complex (20, 28, 29). The cells transfected with pCD-(Δ3–28)Gsα-1 exhibited a new band, which was resolved narrowly from the 42-kD protein and detected only with the carboxy terminal antibody, RM, but not with amino terminal antibody, GCL. This new band of (Δ3–28)Gsα-1 protein was visualized in the particulate fraction of the transfected COS cells, and a faint band was detected in the soluble fraction (Fig. 2, *left*). The soluble fraction of the cells transfected with wild type Gsα cDNA also contained a small amount of wild type Gsα, suggesting that the mutant Gsα was membrane associated to a degree similar to the wild type. Since the total mass of protein was generally more than twice as great in the particulate fraction as in the cytosol, and since a proportionately greater amount of the total membrane protein was comprised of Gsα, the vast majority of (Δ3–28)Gsα-1 protein localized to the particulate fraction. In addition to the major ~41.5-kD (Δ3–28)Gsα-1 protein, two bands were detected with RM antibody (but not GCL) at 38 and 34 kD. These lower molecular

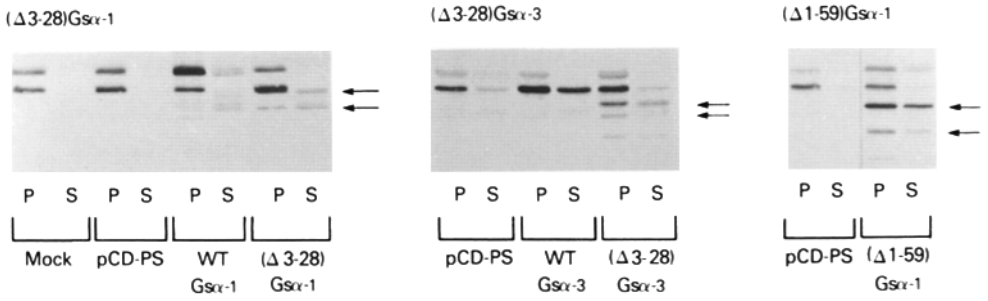

Figure 2. Immunoblot analysis of the amino terminal deleted forms of Gsα in transfected COS cells. The COS cells were transfected without DNA (Mock), vector only (*pCD-PS*), wild type Gsα-1 (*WT*), (*Δ3-28*)Gsα-1, (*Δ3-28*)-Gsα-3, and (*Δ1-59*)Gsα-1 by the DEAE-dextran method. The particulate fraction (*P*) and the soluble fraction (*S*) were prepared from the COS cells by centrifuging the 600 *g* supernatant at 100,000 *g* for 60 min separation and repeat centrifugation. 50 μg of protein was separated by 12.5% SDS-PAGE and transferred onto nitrocellulose paper. The blot was incubated with RM antibody directed against the carboxy terminal decapeptide, and then with peroxidase labeled goat anti-rabbit IgG antibody. The Gsα was then visualized by incubating the blot with the substrate mixture containing hydrogen peroxide and 4-chloro-1-naphthol. The upper arrows indicate the deletion mutant Gsα proteins, and the lower arrows indicate proteins presumptively originating by translation from downstream initiation sites.

weight bands were also observed on the blot of COS cells transfected with wild type Gsα-1, indicating that these proteins were not specific to the mutant Gsα. The bands at 38 and 34 kD most likely correspond to Gsα-1 proteins in which synthesis has started at the second, Met-60, or the third, Met-110, initiation codon, respectively.

Since (Δ3–28)Gsα-1 was poorly resolved from the endogenous 42-kD form of Gsα, we deleted the same 26 amino terminal residues (from Cys-3 to Lys-28) from the 42 kD Gsα-3, and the deletion mutant was expressed in COS cells. Cells transfected with wild type Gsα-3 showed increased expression of the 42-kD band predominantly but not exclusively localized to the membrane (Fig. 2, *center*; the relatively large proportion of wild type Gsα-3 seen in this figure was not typical of most experiments). Cells transfected with the Gsα-3 amino-terminal deletion mutant ([Δ3–28]Gsα-3) exhibited, in addition to the two endogenous forms of Gsα, three new proteins with molecular masses of 39, 37, and 34 kD on SDS-PAGE (Fig. 2, *center*). These three proteins were detected with RM antibody but could not be detected with GCL antibody directed against amino terminal residues. Of these, the predominant one at 39 kD most likely represents the (Δ3–28)Gsα-3 deletion mutant. The 37- and 34-kD bands presumably represent Gsα-3 proteins which use the second Met-60 or the third Met-110 as the initiation codon in their translation. The lower of these two bands comigrated with the 34-kD protein expressed in the (Δ3–28)Gsα-1 transfected cells, as would be predicted by a common initiation site downstream of the alternatively spliced residues 72–86 (8).

Most of the (Δ3–28)Gsα-3 expressed in the COS cells localized to the particulate fraction, though a small fraction was also visualized in the soluble fraction, as with the (Δ3–28)Gsα-1 protein and the wild type Gsα subunits. These results showed that deletion of the amino terminal 26 residues did not lead to cytosolic localization of either Gsα-1 or Gsα-3.

A novel cDNA (16) of canine Gsα, (Δ1–59)Gsα-1, which codes for the amino acid sequence beginning with the second initiation codon, Met-60, was transfected into COS cells. The immunoblot of these cell fractions exhibited two new bands with molecular masses of 38 and 34 kD in addition to the two endogenous forms of Gsα (Fig. 3, *right*). The 38-kD protein was visualized only with RM antibody, and was not detected with GCL antibody (Fig. 3, lane *1*). This 38-kD protein comigrates with the 38-kD band found in COS cells transfected with the wild type or (Δ3–28)Gsα-1 and probably reflects translation initiation at Met-60. The (Δ1–59)Gsα-1 protein expressed in the COS cells localized to the particulate fraction to approximately the same extent as the other mutants. Thus, the large deletion which includes part of the

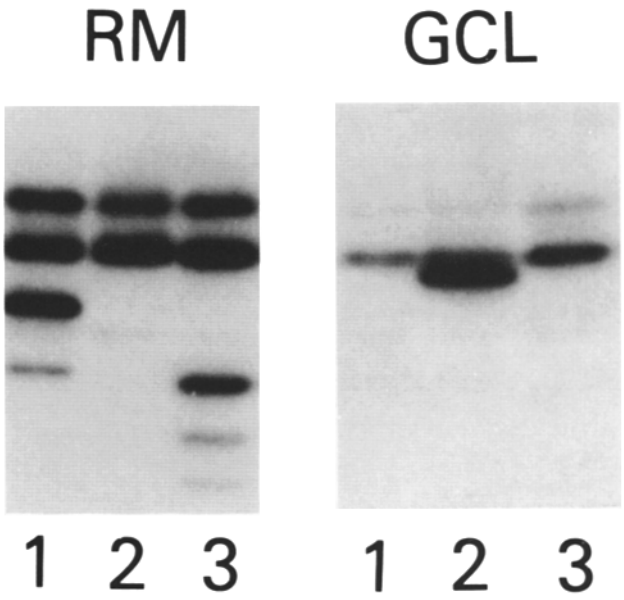

Figure 3. Reactivity of (Δ1-59)Gsα-1, (Δ385-394)Gsα-3, and (Δ3-28/Δ353-384)Gsα-3 with RM and GCL antibody. The particulate fraction of the COS cells transfected with (*1*) (Δ1-59)Gsα-1, (*2*) (Δ385-394)Gsα-3, and (*3*) (Δ3-28/Δ353-384)Gsα-3, were separated on 12.5% SDS-PAGE and immunoblotted with RM antibody directed against the carboxy terminal decapeptides or GCL antibody directed against the amino terminal 16 residues. The Gsα was visualized by incubating the blot with [^{125}I]protein A, followed by autoradiography. Under the conditions we employed, RM is significantly more sensitive than GCL (note that the latter, for example, barely detects the endogenous upper form of Gsα).

putative GTP-binding domain does not lead to cytosolic localization.

Carboxy-terminal Mutants

V8 protease digestion of S49 cell membranes has been reported to release Gsα from the membrane to the soluble fraction, and this has been ascribed to a critical protease-sensitive region near the carboxy terminus (1). We therefore chose to delete portions of the carboxy terminus to evaluate their contribution to Gsα membrane localization. The carboxy terminal 10 residues from Arg-384 to Leu-394 were deleted from 42-kD Gsα-3 to construct (Δ385–394)Gsα-3, and COS cell expression was again used to assess membrane localization. The transfected cells expressed the mutant Gsα as a 40-kD protein (Fig. 4), which was visualized with GCL antibody directed against the amino terminal residues, but not with RM antibody generated against the carboxy decapeptide deleted in this mutant (Fig. 3, lane *2*). The major portion of the (Δ385–394)Gsα-3 protein was localized to the particulate fraction, demonstrating that deletion of the 10 carboxy terminal residues of Gsα does not cause cytosolic localization of the protein.

A deletion near, but not including, the carboxy terminus was made by removing 32 residues from Arg-353 to Gln-384 to prepare (Δ353–384)Gsα-3. COS cells transfected with this construct expressed a 38-kD protein (Fig. 4). This band was visualized by both RM and GCL antibodies. The (Δ353–384)Gsα protein was localized primarily to the particulate fraction. The carboxy terminal 42 residues from Arg-353 to Leu-394 were deleted to construct (Δ353–394)Gsα-3. COS cells transfected with this mutant were observed to express a 36-kD protein on immunoblots with GCL antibody (Fig. 4). The expressed (Δ353–394)Gsα-3 protein was still localized to the particulate fraction.

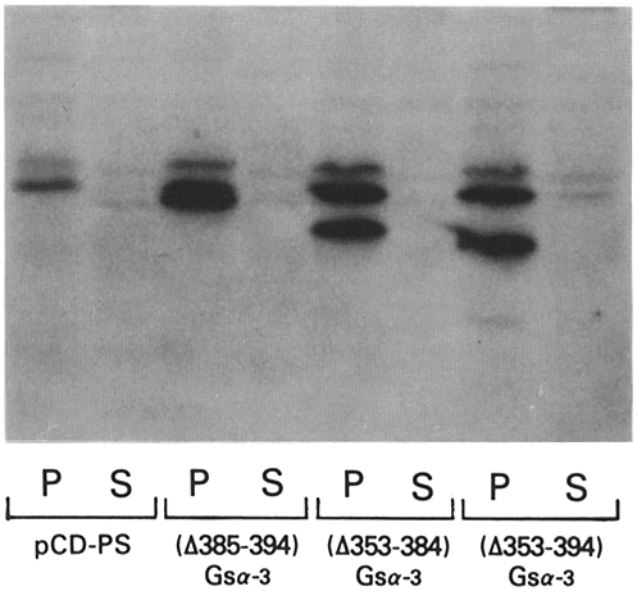

Figure 4. Immunoblot analysis of carboxy terminal deletion mutants of Gsα transfected in COS cells. 100 μg of protein from the particulate (*P*) and the soluble (*S*) fraction were separated by SDS-PAGE and immunoblotted with GCL antibody directed against the amino terminal 16 residues. The Gsα was visualized by incubating the blot with [^{125}I]protein A followed by autoradiography.

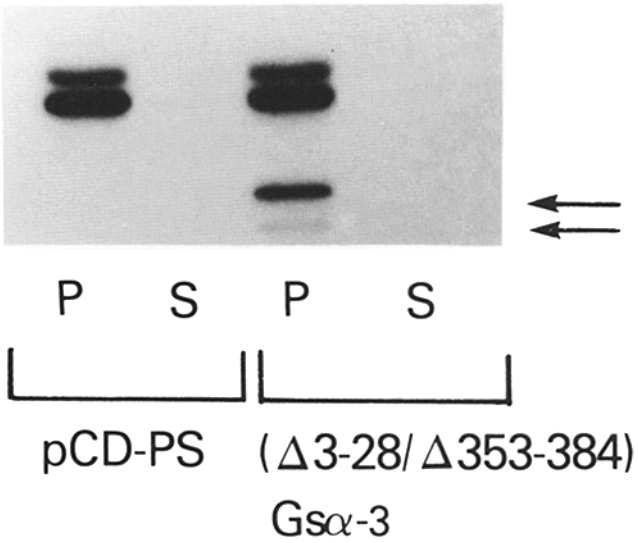

Figure 5. Immunoblot analysis of (Δ3-28/Δ353-384)Gsα-3 expressed in COS cells by transfection. 50 μg of protein from the particulate (*P*) and the soluble (*S*) fraction were separated on 15% SDS-PAGE and transferred onto nitrocellulose paper. The blot was incubated with RM antibody directed against the carboxy terminal decapeptide, and the Gsα was visualized by incubating the blot with [^{125}I]protein A followed by autoradiography. The upper arrow indicates the double deletion mutant form of Gsα, and the lower arrow indicates protein presumptively originating from a downstream initiation site.

A Double Deletion Mutant

Since some models of the 3-D structure of an α subunit suggest that the amino- and carboxy-termini are in close proximity and are oriented toward the plasma membrane (3), we made a double deletion mutant of Gsα-3 that combined the deletion of the 26 amino terminal residues, from Cys-3 to Lys-28, and the deletion of the 32 residues near the carboxy-terminus, from Gly-353 to Gln-384. We reasoned that lack of both the amino terminal and the carboxy terminal residues may cause Gsα to localize to the cytosol. Preservation of the last 10 amino acids permitted detection of the mutant protein with RM antibodies. When the double deletion mutant, (Δ3–28/Δ353–384)Gsα-3, was expressed in the COS cells by transfection, a major new band migrating at 34 kD was visualized with RM antibody (Fig. 5, *right*). This protein could not be detected with GCL antibody (Fig. 3, lane *3*). The double deletion mutant Gsα was mainly localized to the particulate fraction, indicating that even the loss of residues at the amino and near the carboxy terminus does not cause cytosolic localization.

Characterization of Gsα Membrane Binding

To assess further the membrane binding of the deletion mutant Gsα proteins, the particulate fractions of the COS cells transfected with the (Δ3–28/Δ353–384)Gsα-3 cDNA were treated with 2.5 M NaCl, 6 M urea, or 15 mM NaOH (Fig. 6). This concentration of NaCl is enough to release loosely

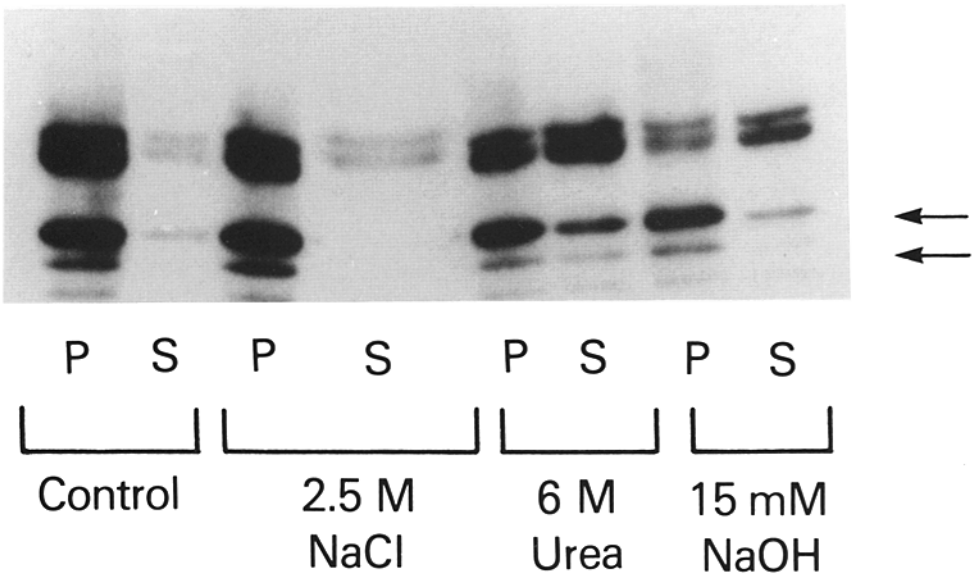

Figure 6. Treatment of the particulate fraction of COS cells transfected with (Δ3-28/Δ353-384)Gsα-3 with salts and sodium hydroxide. The particulate fraction, 75 μg total protein, was treated with 2.5 M sodium chloride, 6 M urea, or 15 mM sodium hydroxide, and the preparations were centrifuged at 430,000 *g* for 15 min to separate the pellet (*P*) and the supernatant (*S*). The samples were analyzed as indicated in the legend of Fig. 5. Arrows indicate proteins described in Fig. 5.

bound proteins, including peripheral membrane proteins, but little of the endogenous wild type and none of the mutated Gsα protein was released by this treatment. When the particulate fractions were incubated with urea at 37°C, about half of the endogenous Gsα immunoreactivity localized to the soluble fractions, whereas much less of the mutant protein was released. Treatment of the particulate fraction with sodium hydroxide released most of the endogenous forms of Gsα, but only a small proportion of the mutant protein.

To analyze the solubility of the various mutant forms of Gsα expressed in COS cells, the particulate fractions were subjected to extraction with the detergent cholate. Sodium cholate has been used to solubilize G protein α subunits from membranes in the initial step of purification (37). At least half of the endogenous forms of Gsα were extracted into the detergent phase under the conditions we used. The transfected wild type Gsα-3 showed slightly less extractability (Fig. 7, lane *2*). Compared with endogenous, wild type Gsα-3, all the deletion mutants showed a reduction in susceptibility to cholate solubilization. The carboxy terminal deletion mutants were substantially more resistant to solubilization by cholate than amino terminal deletion mutants (Fig. 7).

Discussion

G protein α subunits including Gs are tightly bound to the cell membrane despite lacking hydrophobic, membrane-spanning domains. For pertussis toxin-sensitive α subunits including the various forms of Gi and Go, the amino terminus, site of co-translational myristoylation, appears to be critical for membrane targeting (11, 18, 27). Since Gsα does not undergo myristoylation, the mechanism of its membrane attachment is unclear. Using a reconstitution assay involving binding of in vitro translated α subunits to Gsα-deficient CYC^- membranes, Audigier and co-workers (1, 20) showed that deletion of amino-terminal residues 2–29 from Gsα did not prevent binding of the in vitro translated protein to CYC^- membranes. Treatment of reconstituted membranes with V8 protease released a soluble 43-kD fragment of Gsα. The authors interpreted these results as defining a critical role for the carboxy terminus of Gsα in membrane binding, but direct evidence, including sequence identification of the proteolytic fragment, was not provided (1). In work published (21) after the present paper was first submitted, the same authors provided evidence for a critical role for

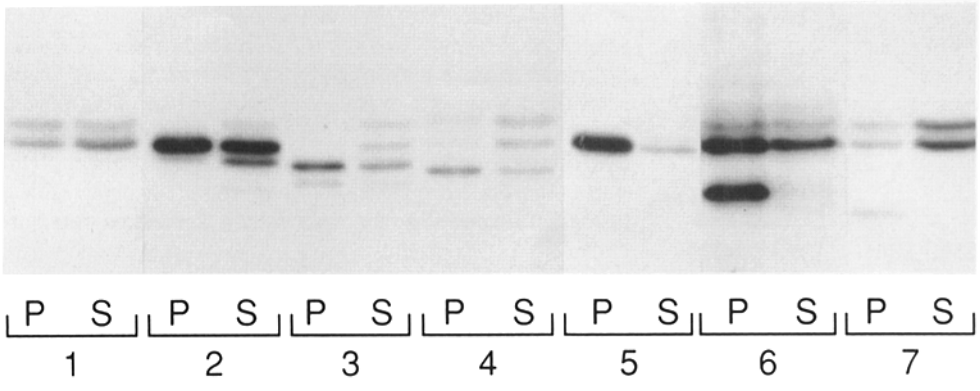

Figure 7. Solubilization of various forms of Gsα from the particulate fraction of COS cells with sodium cholate. The particulate fraction of COS cells transfected with (*1*) vector alone; (*2*) wild type Gsα-3; (*3*) (Δ3-28)Gsα-3; (*4*) (Δ1-59)-Gsα-1; (*5*) (Δ353-384)Gsα-3; (*6*) (Δ353-394)Gsα-3; or (*7*) (Δ3-28/Δ353-384)Gsα-3 was incubated for 1 h at 25°C in homogenization buffer containing 1% cholate. The mixture was centrifuged for 1 h at 100,000 *g* to prepare pellet (*P*) and soluble (*S*) fractions which were analyzed by SDS-PAGE and immunoblot using RM antibody for all samples except those in panel 6 for which GCL antibody was used. Antibody binding was detected by incubating the blot with [^{125}I]-protein A followed by autoradiography.

residues 367–376 near the carboxy terminus of Gsα in membrane binding. Substitution of this segment of Gsα for the carboxyl-terminal 14 residues of Gilα promoted membrane binding of an otherwise soluble amino-terminal deletion mutant of Gil.

In the present work, we sought to define the relative importance of amino and carboxy-terminal regions of Gsα in membrane binding. Our approach was to construct deletion mutant cDNAs, transfect these acutely in COS cells, and monitor expression in particulate vs. soluble fractions. Deletion of substantial portions of the amino terminus, carboxy terminus, or both (Fig. 1) was still compatible with expression of stable protein in COS cells. In every case tested, the protein product was localized primarily to the particulate fraction, and in similar proportion to the endogenous forms of Gsα or to transfected wild type Gsα cDNAs. These results are consistent with those of Audigier and coworkers (1, 20) for the amino terminal deletion mutants and for the carboxyl-terminal 385–394 deletion mutant (1, 21), but not for the 353–384 and 353–394 deletion mutants. The latter encompass the putative site of V8 protease cleavage that led to release of Gsα from membranes (1) as well as the 367–376 residues claimed to be critical for membrane association (21).

There are several possible reasons for the latter discrepancy. The methods used to study membrane association are quite different. In the assay employed by Audigier and co-workers in vitro translated protein is incubated with membranes which are then sedimented, and the proportion of membrane bound, labeled protein determined (19). Even for wild type Gsα, the proportion of protein that becomes membrane bound is substantially less than 100% (21). The relationship between this assay and membrane targeting of intracellularly synthesized protein is not clear, although in vitro synthesized Gsα has been shown to be biologically active (30). It is also important to point out that the ability of residues 367–376 of Gsα to promote membrane association of soluble Gilα does not prove that this region alone is critical for Gsα membrane binding. Additional regions could also be important. The same authors did not directly assess the ability of forms of Gsα deleted at the carboxy terminus to bind to membranes.

Acute transfection of COS cells to monitor expression of mutant proteins has the advantage of more closely approximating intracellular synthesis and targeting, but this method too is far from physiologic in that proteins are often substantially overexpressed, and could potentially be aberrantly localized. Nevertheless, this method has been very useful in elucidating the critical role of myristoylation in membrane binding of certain Gα subunits. Mutation of Gilα (18) or Goα (27) that precludes myristoylation leads to completely cytosolic localization of the product even when overexpressed in COS cells. Thus, the fact that all the deletion mutants of Gsα localized to the particulate fraction could indicate that Gsα membrane attachment is different from that of myristoylated α subunits in that several regions are critical for membrane binding.

It is also possible, however, that particulate localization of the expressed proteins does not reflect specific plasma membrane association. Resistance of the deletion mutants of Gsα to solubilization by alkali or sodium cholate may reflect localization of these particular mutant forms of the protein to a relatively inaccessible compartment, or protein aggregation. Overexpression of Gsα in Sf-9 cells using a recombinant baculovirus produced a protein found in the particulate fraction that was resistant to solubilization by 1% sodium cholate. This contrasts with various forms of Giα which when expressed in this system were localized to the cytosol or readily solubilized from the particulate fraction (15). Since transient expression of α subunits in COS cells has been used by many investigators to assess G protein function, the present results suggest that one must be cautious in correlating the appearance of immunoreactivity in the particulate fraction with correct localization of functional protein to receptor/effector interaction sites.

In summary, our results show that amino and carboxy terminal deletion mutants of Gsα can be expressed in COS cells, and they are localized to the particulate fraction. These results confirm that the amino terminus of Gsα is not critical for membrane binding, but leave open the question of whether the carboxy terminus, including residues 367–376, is critical in this respect. Additional studies are required to define the molecular basis for Gsα membrane targeting.

We are grateful to Dr. Regina M. Collins for providing the COS-7 cells for transfection, to Dr. Cecille Unson and Dr. Paul Goldsmith for peptides and antibodies, and to Dr. John J. Merendino Jr. for helpful discussions and review of the paper.

Received for publication 24 October 1991 and in revised form 20 July 1992.

References

1. Audigier, Y., L. Journot, C. Pantaloni, and J. Bockaert. 1990. The carboxy terminal domain of Gsα is necessary for anchorage of the activated form in the plasma membrane. *J. Cell. Biol.* 111:1427–1435.
2. Baldwin, S. A., and G. E. Lienhard. 1989. Purification and reconstitution of glucose transporter from human erythrocytes. *Methods Enzymol.* 174: 39–50.
3. Berlot, C. H., and H. R. Bourne. 1992. Identification of effector-activating residues of $G_{s\alpha}$. *Cell.* 68:911–922.
4. Bonner, T. I., A. C. Young, M. R. Brann, and N. J. Buckley. 1988. Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron.* 1:403–410.
5. Bourne, H. R., D. A. Sanders, and F. McCormick. 1990. The GTPase superfamily: a conserved switch for diverse cell functions. *Nature (Lond.).* 348:125–132.
6. Bourne, H. R., D. A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.).* 349:117–127.
7. Bradford, M. M. 1976. A rapid and sensitive methods for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.
8. Bray, P., A. Carter, C. Simons, V. Guo, C. Pukett, J. Kamholz, A. Spiegel, and M. Nirenberg. 1986. Human cDNA clones for four species of $G_{s\alpha}$ signal transduction protein. *Proc. Natl. Acad. Sci. USA.* 83:8893–8897.
9. Buss, J. E., S. M. Mumby, P. J. Casey, A. G. Gilman, and B. M. Sefton. 1987. Myristoylated α subunit of guanine nucleotide-binding regulatory proteins. *Proc. Natl. Acad. Sci. USA.* 84:7493–7497.
10. Cullen, B. R. 1987. Use of eukaryotic expression technology in the functional analysis of cloned genes. *Methods Enzymol.* 152:684–704.
11. Eide, B., P. Gierschik, G. Milligan, I. Mullaney, C. Unson, P. Goldsmith, and A. Spiegel. 1987. GTP-binding proteins in brain and neutrophil are tethered to the plasma membrane via their amino termini. *Biochem. Biophys. Res. Commun.* 148:1398–1405.
12. Fukada, Y., T. Takao, H. Ohguro, T. Yoshizawa, T. Akino, and Y. Shimonishi. 1990. Farnesylated γ subunit of photoreceptor G protein indispensable for GTP-binding. *Nature (Lond.).* 346:658–660.
13. Gierschik, P., G. Milligan, M. Pines, P. Goldsmith, J. Codina, W. Klee, and A. Spiegel. 1986. Use of specific antibodies to quantitate the guanine nucleotide binding protein Go in brain. *Proc. Natl. Acad. Sci. USA.* 83:2258–2262.
14. Gilman, A. G. 1987. G-proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615–649.
15. Graber, S. G., R. A. Figler, and J. C. Garrison. 1992. Expression and purification of functional G protein α subunits using a baculovirus expression system. *J. Biol. Chem.* 267:1271–1278.

16. Ishikawa, Y., C. Bianchi, B. Nadal-Ginard, and C. J. Homcy. 1990. Alternative promoter and 5'-exon generate a novel Gsα mRNA. *J. Biol. Chem.* 265:8458–8462.
17. Jones, D. T., and R. R. Reed. 1988. Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. *J. Biol. Chem.* 262:14241–14249.
18. Jones, T. L. Z., W. F. Simonds, J. J. Merendino Jr., M. M. Brann, and A. M. Spiegel. 1990. Myristoylation of an inhibitory GTP-binding protein α subunit is essential for its membrane attachment. *Proc. Natl. Acad. Sci. USA.* 87:568–572.
19. Journot, L., J. Bockaert, and Y. Audigier. 1989. Reconstitution of *cyc*-S49 membranes by *in vitro* translated Gsα. Membrane anchorage and functional implications. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 251:230–236.
20. Journot, L., C. Pantaloni, J. Bockaert, and Y. Audigier. 1991. Deletion within the amino-terminal region of Gsα impairs its ability to interact with βγ subunits and to activate adenylate cyclase. *J. Biol. Chem.* 266:9009–9015.
21. Journot, L., C. Pantaloni, M. -A. Poul, H. Mazarguil, J. Bockaert, and Y. Audigier. 1991. Amino acids 367–376 of the Gsα subunit induce membrane association when fused to soluble amino-terminal deleted Gilα subunit. *Proc. Natl. Acad. Sci. USA.* 88:10054–10058.
22. Kaziro, Y., H. Itoh, T. Kozasa, M. Nakafuku, and T. Satoh. 1991. Structure and function of signal transducing GTP-binding proteins. *Annu. Rev. Biochem.* 60:349–400.
23. Lai, R. K., D. Perex-Sala, F. J. Canada, and R. R. Rando. 1990. The γ subunit of transducin is farnesylated. *Proc. Natl. Acad. Sci. USA.* 87: 7673–7677.
24. Lochrie, M. A., and M. I. Simon. 1988. G protein multiplicity in eukaryotic signal transducin system. *Biochemistry.* 27:4957–4965.
25. Milligan, G., and C. G. Unson. 1989. Persistent activation of the α subunit of Gs promotes its removal from the plasma membrane. *Biochem. J.* 260:837–841.
26. Mumby, S. M., P. J. Casey, A. G. Gilman, S. Gutowski, and P. C. Sternweis. 1990. G protein γ subunits contain a 20-carbon isoprenoid. *Proc. Natl. Acad. Sci. USA.* 87:5873–5877.
27. Mumby, S. M., R. O. Heukeroth, J. I. Gordon, and A. G. Gilman. 1990. G-protein α subunit expression, myristoylation, and membrane association in COS cells. *Proc. Natl. Acad. Sci. USA.* 87:728–732.
28. Navon, S. E., and B. K. K. Fung. 1987. Characterization of transducin from bovine retinal rod outer segments. Participation of the amino terminal region of Tα in subunit interaction. *J. Biol. Chem.* 262:15746–15751.
29. Neer, E. J., L. Pulsifer, and L. G. Wolf. 1988. The amino terminus of G protein α subunit is required for interaction with βγ. *J. Biol. Chem.* 263:8996–9000.
30. Olate, J., R. Mattera, J. Codina, and L. Birnbaumer. 1988. Reticulocyte lysates synthesize an active α subunit of the stimulatory G protein Gs. *J. Biol. Chem.* 263:10394–10400.
31. Pines, M., P. Gierschik, G. Milligan, W. Klee, and A. Spiegel. 1985. Antibodies against the carboxy-terminal 5-kDa peptide of the α subunit of transducin crossreact with the 40-kDa but not the 39-kDa guanine nucleotide binding protein from brain. *Proc. Natl. Acad. Sci. USA.* 82:4095–4099.
32. Ransnas, L. A., P. Svoboda, J. R. Jasper, and P. A. Insel. 1989. Stimulation of β-adrenergic receptors of S49 lymphoma cells redistributes the α subunit of the stimulatory G protein between cytosol and membranes. *Proc. Natl. Acad. Sci. USA.* 86:7900–7903.
33. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
34. Simonds, W. F., P. K. Goldsmith, C. J. Woodward, C. G. Unson, and A. M. Spiegel. 1989. Receptor and effector interactions of Gs. Functional studies with antibodies to the αs carboxy terminal decapeptide. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 249:189–194.
35. Simonds, W. F., J. E. Butrynski, N. Gautman, C. G. Unson, and A. M. Spiegel. 1991. G-protein βγ dimer. Membrane targeting requires subunit coexpression and intact γ C-A-A-X domain. *J. Biol. Chem.* 266:5363–5366.
36. Sternweis, P. C. 1986. The purified α subunits of Go and Gi from bovine brain require βγ for association with phospholipid vesicles. *J. Biol. Chem.* 261:631–637.
37. Sternweis, P. C., and J. D. Robishaw. 1984. Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. Biol. Chem.* 259:13806–13813.
38. Towler, D. A., J. I. Gordon, S. P. Adams, and L. Glaser. 1988. The biology and enzymology of eukaryotic protein acylation. *Annu. Rev. Biochem.* 57:69–99.
39. Yamane, H. K., C. C. Farnsworth, H. Xie, W. Howald, B. K. -K. Fung, S. Clarke, M. H. Gelb, and J. A. Glomset. 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.