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

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Abstract. To elucidate the structural basis for membrane attachment of the α subunit of the stimulatory G protein (Gsα), mutant Gsa 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 (A3-28) or with 59 amino terminal residues deleted (A1-59) resulted in immunoreactive proteins which localized primarily to the particulate fraction. Similarly, mutants with 10 (A385-394), 32 (A353-384), or 42 (A353-394) 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 Gsa 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 heterotrimeric 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),

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

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Materials and Methods

Construction of Mutant Gsα

The cDNAs coding for rat Gsα (17) were kindly provided by Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD), and both the 47-kDa Gsα-1 containing 394 amino acid residues, and the 42-kDa 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 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.

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 the 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.
A further deletion in the carboxy terminal sequence of Gsa-3 was made by removing the sequence coding for 32 amino acid residues from Gly-353 to Gin-384 to construct (A353–384)Gsa-3. The 3' primer used to make the deletion was a 60-mer, 5'-TCA CTT AAG TTA GAG CAG CTC GTA TTG GCCG AAG ATG CAT GCG ACT AGC-3', which contained the sequences for six amino acid residues upstream to the deletion, 10 carboxy terminal residues, a termination codon, and the restriction site for AfblI, sequentially. The 5' primer was the same as that used in constructing (A385–394)Gsa-3. The PCR product and wild type Gsa-3-containing plasmid were cut with XmalI and AfblI, and then ligated with T4 DNA ligase.

Expression of Deleted Forms of Gsa 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 Gsa 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 recenterfuged 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.

Protein samples were solubilized and separated on 12.5% or 15% SDS-polyacrylamide gels, and then transferred onto nitrocellulose paper (3). The wild type and mutant Gsa proteins were detected with the peptide specific antibodies, RM and GCL. The RM antibody was generated against the amino terminal 16 residues from Gly-2 to Lys-17. The Gsa was visualized by treating the blot either with peroxidase labeled secondary antibody, or with [125I]protein A followed by autoradiography (13).

Results

Amino Terminal Deletion Mutants of Gsa

To evaluate the expression and membrane targeting of various deletion mutants of Gsa, 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 Gsa, principally localized to the particulate fraction, were visualized at 47 and 42 kD, respectively. These correspond to the known splice variants of Gsa (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 Gsa-1 and Gsa-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 Gsa-1 resulted in an increase in the density of the lower portion of the 47-kD band, demonstrating that the Gsa-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 Gsa-1 to construct (A3-28)Gsa-1, and the cDNA was cloned into the pCD-PS vector for transfection into COS-7 cells. We chose to delete this portion of Gsa since the amino terminus has been implicated in α subunit membrane binding and interaction with the βγ complex (20, 28, 29). The cells transfected with pCD-(A3-28)Gsa-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 (A3–28)Gsa-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 Gsa cDNA also contained a small amount of wild type Gsa, suggesting that the mutant Gsa 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 Gsa-1, the vast majority of (A3–28)Gsa-1 protein localized to the particulate fraction. In addition to the major ~41.5-kD (A3–28)Gsa-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 Gsa-I (WT), (Δ3-28)Gsot-1, (Δ3-28)Gsot-3, and (Δ1-59)Gsot-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 Gsa 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 Gsot-I, indicating that these proteins were not specific to the mutant Gsot. The bands at 38 and 34 kD most likely correspond to Gsot-1 proteins in which synthesis has started at the second, Met-60, or the third, Met-110, initiation codon, respectively.

Since (Δ3-28)Gsot-I 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 Gsot-3, and the deletion mutant was expressed in COS cells. Cells transfected with wild type Gsot-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 Gsot-3 seen in this figure was not typical of most experiments). Cells transfected with the Gsot-1 amino-terminal deletion mutant (Δ3-28)Gsot-1, 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)Gsot-3 deletion mutant. The 37- and 34-kD bands presumably represent Gsot-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)Gsot-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)Gsot-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)Gsot-I protein and the wild type Gsot subunits. These results showed that deletion of the amino terminal 26 residues did not lead to cytosolic localization of either Gsot-I or Gsot-3.

A novel cDNA (16) of canine Gsot, (Δ1-59)Gsot-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 band found in COS cells transfected with the wild type or (Δ3-28)Gsot-1 and probably reflects translation initiation at Met-60. The (Δ1-59)Gsot-I 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)Gsot-1, (Δ385-394)Gsot-3, and (Δ3-28/Δ353-384)Gsot-3 with RM and GCL antibody. The particulate fraction of the COS cells transfected with (1) (Δ1-59)Gsot-1, (2) (Δ385-394)Gsot-3, and (3) (Δ3-28/Δ353-384)Gsot-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 Gsot was visualized by incubating the blot with [125I]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 decapptide 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 Gin-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.

**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 Gin-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)Gsc-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 Gsc-3 protein was released by this treatment. When the particulate fractions were incubated with urea at 37°C, about half of the endogenous Gsc 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 Gsc, but only a small proportion of the mutant protein.

To analyze the solubility of the various mutant forms of Gsc 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 Gsc were extracted into the detergent phase under the conditions we used. The transfected wild type Gsc-3 showed slightly less extractability (Fig. 7, lane 2). Compared with endogenous, wild type Gsc-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 Gsc 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 Gsc-deficient CYC membranes, Audigier and co-workers (1, 20) showed that deletion of amino-terminal residues 2–29 from Gsc 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 Gsc. The authors interpreted these results as defining a critical role for the carboxy terminus of Gsc 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 Gsc from the particulate fraction of COS cells with sodium cholate. The particulate fraction of COS cells transfected with (1) vector alone; (2) wild type Gsc-3; (3) (Δ3-28)Gsc-3; (4) (Δ1-59)Gsc-1; (5) (Δ353-384)Gsc-3; (6) (Δ353-394)Gsc-3; or (7) (Δ3-28/Δ353-384)Gsc-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 [125I]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 carboxy-terminal 14 residues of Gilα promoted membrane binding of an otherwise soluble amino-terminal deletion mutant of Gilα.

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, transfected 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. 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 Go subunits. Mutation of Gilα (18) or Gsα (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 Go 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 Gα which when expressed in this system were localized to the cytosol or readily solubilized from the particulate fraction (15). Since transient expression of Go 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/effecter 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.

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