The Carboxy-Terminal Domain of Gsα Is Necessary for Anchorage of the Activated Form in the Plasma Membrane

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Abstract. GTP-binding proteins which participate in signal transduction share a common heterotrimeric structure of the αβγ-type. In the activated state, the α subunit dissociates from the βγ complex but remains anchored in the membrane. The α subunits of several GTP-binding proteins, such as Go and Gi, are myristoylated at the amino terminus (Buss, J. E., S. M. Mumby, P. J. Casey, A. G. Gilman, and B. M. Sefton. 1987. *Proc. Natl. Acad. Sci. USA.* 84:7493–7497). This hydrophobic modification is crucial for their membrane attachment. The absence of fatty acid on the α subunit of Gs (Gsα), the protein involved in adenylate cyclase activation, suggests a different mode of anchorage. To characterize the anchoring domain of Gsα, we used a reconstitution model in which posttranslational addition of in vitro–translated Gsα to cyc⁻ membranes (obtained from a mutant of S49 cell line which does not express Gsα) restores the coupling between the β-adrenergic receptor and adenylate cyclase. The consequence of deletions generated by proteolytic removal of amino acid sequences or introduced by genetic removal of coding sequences was determined by analyzing membrane association of the proteolyzed or mutated α chains. Proteolytic removal of a 9-kD amino-terminal domain or genetic deletion of 28 amino-terminal amino acids did not modify the anchorage of Gsα whereas proteolytic removal of a 1-kD carboxy-terminal domain abolished membrane interaction. Thus, in contrast to the myristoylated α subunits which are tethered through their amino terminus, the carboxy-terminal residues of Gsα are required for association of this protein with the membrane.

SIGNAL-TRANSDUCING GTP-binding proteins represent a family of heterotrimeric proteins (αβγ-type) which transduce an extracellular signal recognized by a membrane receptor to an intracellular effector. Stimulation of the receptor induces the replacement of GDP by GTP on the α subunit, promoting the dissociation of the GTP-liganded α subunit from the βγ subunits and the subsequent activation of the effector by the α subunit (2, 4, 8, 21).

The specificity of the interaction with the receptor and the effector is assigned to the α subunit which contains the nucleotide binding site. Consequently, the α subunits of various GTP-binding proteins share homologous domains related to the common features of the transduction process such as the nucleotide binding site and eventually the site for interaction with the βγ subunits. Alternatively, divergent domains are expected to specify the unique properties of each α subunit such as the receptor-binding domain or the effector-activating domain (2, 4, 8, 21).

Divergence may also affect some properties such as membrane anchorage of the activated α subunit when it is dissociated from the βγ complex. For example, visual phototransduction is carried out by a GTP-binding protein, transducin (T),[1] which is not strongly associated with the

membrane (15); moreover the GTP form of Tα subunit can be released from the disk membrane at 100 mM salt. Although it is very homologous to the Tα subunit, the α subunit of Go (22, 26) and the three Gi subtypes (3, 11) was shown to contain a myristic acid on the amino-terminal glycine (5). Trypsin digestion of GTPγS-treated membranes, from brain and neutrophil, removed a 2-kD amino-terminal domain on the α subunit of Go or Gi and the proteolytic deletion was accompanied by the release of the tryptic large fragment from the membrane (6). More recently, amino-terminal myristoylation of these proteins was shown to be essential for their membrane attachment (12, 19), suggesting that the amino-terminal myristic acid may be sufficient for the anchorage in the membrane of the activated form of these GTP-binding proteins.

Therefore, the domains involved in the interaction with the membrane, as well as the nature and the location of posttranslational modifications which contribute to or assign the membrane association, may differ from one GTP-binding protein to another.

Although the α subunit of Gs is not myristoylated (5), the interaction of Gsα with the membrane of wild-type S49 cells

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1. *Abbreviations used in this paper:* Gi, the guanine nucleotide-binding protein which mediates inhibition of adenylate cyclase; Go, a new GTP-binding protein from membranes of bovine brain; Gs, the guanine nucleotide-

binding protein which mediates activation of adenylate cyclase; GTPγS, guanosine 5′-(3-O-thio)-triphosphate; T, transducin from bovine rod outer segments.

is very strong. Indeed, even after activation by ADP ribosylation, membrane association of Gsα remains insensitive at high salt concentrations and requires alkali treatment for disruption (13).

We recently developed a model based upon the reconstitution of cyc⁻ membranes (obtained from a mutant of S49 cell line which does not express Gsα) by in vitro–translated Gsα where posttranslational addition of Gsα to cyc⁻ membranes was able to restore the coupling between the β-adrenergic receptor and adenylate cyclase (13, 23). In this model, we also found that Gsα, having bound to cyc⁻ membranes and then been activated by GTPγS, was not released from cyc⁻ membranes by high salt concentrations and became soluble only after alkali treatment (13). Consequently, regardless of the nature of the activation (ADP ribosylation or GTPγS) the activated α subunit of Gs is strongly associated with the membrane.

We decided to use this reconstitution model to characterize the anchoring domain of activated Gsα by developing a dual approach for generating deletions in the polypeptide sequence of the α chain: a proteolytic approach corresponding to the removal of peptide domains on reconstituted cyc⁻ membranes and a genetic approach based upon removal of coding sequences at the nucleotide level before reconstitution (see Fig. 1). The consequences of these deletions were then determined by analyzing the membrane association of the proteolytic fragments and the mutated α chains. The location of the domains removed by proteolysis was assigned by differential immunoprecipitation using antisera raised against an amino-terminal or a carboxy-terminal sequence (see Fig. 1).

In this paper, we report that proteolytic removal of a large amino-terminal domain or genetic deletion located in the amino-terminal domain does not impair the association of the Gsα activated form with the membrane. However, proteolytic removal of a small carboxy-terminal domain abolished the anchorage of Gsα in the membrane. This suggests that the carboxy-terminal residues of Gsα are necessary for the interaction with the membrane.

Materials and Methods

Construction of the Mutant (Δ 2-29)Gsα-2

The cDNA coding for the α-2 (long form) subunit of human liver Gs (a generous gift from Dr. J. Codina and Dr. L. Birnbaumer, Baylor College of Medicine, Houston, TX) was subcloned into the vector pIBI 31, as described previously (13). The plasmid pIBI-Gsα-2 (4,295 bp) was linearized by Nco I (which cuts in the sequence containing the ATG initiation codon) and then partially digested with Pvu II (four sites in the plasmid) in order to obtain a single cut by the enzyme. The digestion medium was phenol extracted, ethanol precipitated, and the recessed 3' termini from the Nco I digestion were filled in with the Klenow fragment of DNA polymerase. The fragments were then separated on a 0.5% agarose gel and the largest fragment (4,211 bp), corresponding to a deletion of 84 bp (coding for the amino acids 2 to 29) was purified by electroelution. The plasmid pIBI-(Δ 2-29)Gsα-2 was ligated with phage T4 DNA ligase and used for transformation of Escherichia coli strain TG1. Ampicillin-resistant clones were isolated and screened for the disappearance of one Pvu II site.

In Vitro Transcription of the Plasmids

After linearization by Kpn I, 10 μg of each plasmid was preincubated for 5 min at 37°C with 2 mM each of ATP, GTP, UTP, and CTP, 6 mM DTT, 6 mM MgCl₂, 40 mM Tris-HCl (pH 8), 2 mM spermidine, and 0.4 mM m7 G(5')ppp(5')C. Transcription was performed in the presence of 1,000 U

T7 RNA polymerase for 1 h at 37°C. The messenger RNAs were then purified as described previously (17).

In Vitro Translation of the Messenger RNAs

Cell-free translation of the messenger RNAs was carried out at 30°C for 45 min. A typical translation medium contained 7.5 μl of Promega nuclease-treated reticulocyte lysate, 0.5 μl of each of 19 amino acids at 1 mM except methionine, 1 μl of 10 μM [³⁵S]methionine (800–1,200 Ci/mmol; Du Pont Co.; New England Nuclear, Boston, MA), and 1 μl mRNA (2.5–5 ng/μl).

In the experiments where Gsα was activated by ADP ribosylation, in vitro translation was performed in the presence of unlabeled methionine.

Preparation of Membranes from S49 Cells

Plasma membranes were prepared by nitrogen cavitation of wild-type or cyc⁻ S49 cells (kindly donated by M. Siat and Dr. B. Harris, Merrel Dow Research Institute, Strasbourg, France) as described previously (25).

Reconstitution of Cyc⁻ Membranes by In Vitro–translated Gsα-2 and (Δ2-29)Gsα-2

1 vol of translation medium was incubated with 1 vol of cyc⁻ plasma membranes (2 mg protein/ml) for 30 min at 37°C. The reconstitution medium was then centrifuged for 5 min at 10,000 g and the membrane pellet resuspended in 1 vol of 20 mM Na⁺ Hepes (pH 8) buffer containing 2 mM MgCl₂, 1 mM EDTA, and 1 mM DTT.

ADP Ribosylation by Cholera Toxin

The cholera toxin-catalyzed ADP ribosylation was carried out on wild-type or reconstituted cyc⁻ membranes as described previously (13).

Proteolysis Experiments

Reconstituted membranes were digested with tosylphenylalanylchloromethyl ketone–treated trypsin (Sigma Chemical Co., St. Louis, MO) or V8 protease from Staphylococcus aureus (ICN K&K Laboratories, Inc., Danbury, CT) for 60 min at 30°C. The concentrations used for each enzyme are listed in the figure legends. Proteolysis with trypsin was terminated by the addition of 1 mM PMSF (Sigma Chemical Co.) and digestion with V8 protease was stopped by 2.5 mM diisopropylfluorophosphate (Sigma Chemical Co.).

Immunoprecipitation

Reconstituted membranes were solubilized by 1% SDS for 1 h at room temperature with agitation. Solubilized membranes were then heated to 100°C for 5 min and processed as described previously (1) except that the incubations with the antisera and protein A-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) were performed at 37°C for 1 h.

The antiserum raised against the 10 carboxy-terminal residues of the α subunit of Gs was a generous gift from Dr. Milligan, Glasgow University, Glasgow, Scotland, and the antiserum directed against the amino acids 29–39 of Gsα (referred to as NH₂-terminal antiserum or anti-N serum) was kindly provided by Dr. Brabet (Centre National de la Recherche Scientifique, and Institut de la Santé et la Recherche Medicale de Pharmacologie Endocrinologie).

SDS-PAGE

The various samples were resolved on 9 or 12% SDS-polyacrylamide gels by the method of Laemmli (16). After electrophoresis, the gels were fixed in 10% acetic acid and 35% methanol, treated with EN³HANCE (Du Pont Co.; New England Nuclear), dried under vacuum, and exposed to Kodak XAR-5 film at −70°C.

Results

Trypsin Proteolysis of ADP-ribosylated Wild-Type Membranes or Reconstituted cyc⁻ Membranes from S49 Cells

As previously reported (10), cholera toxin specifically radio-
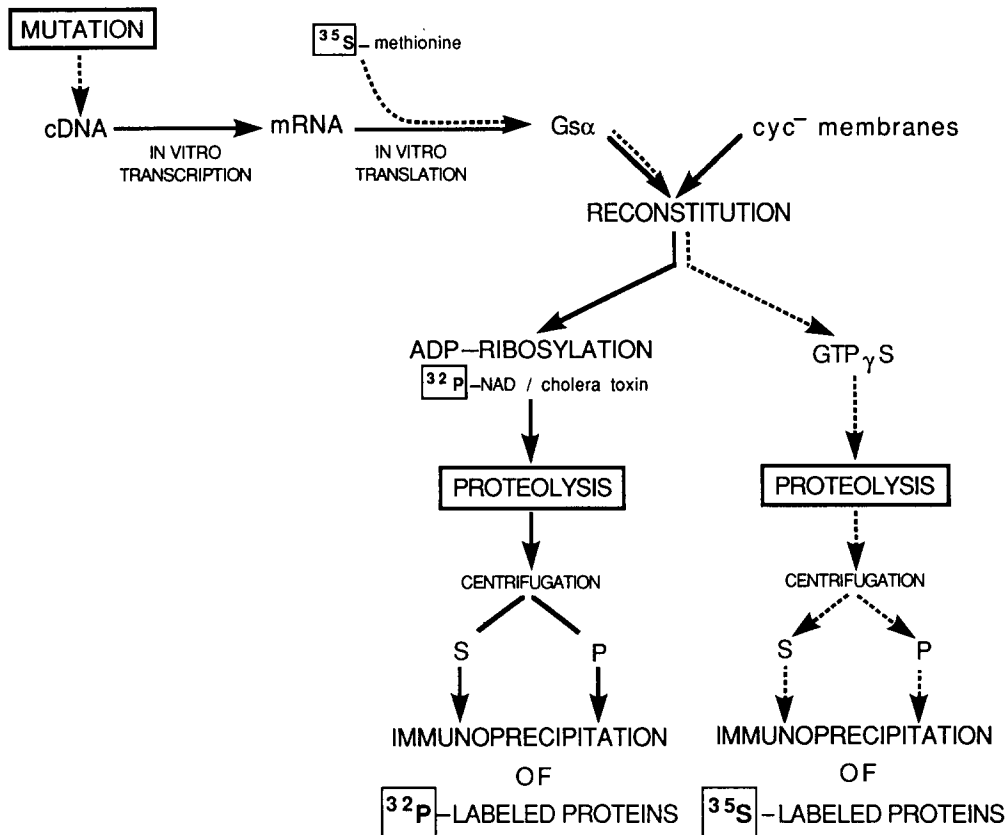
Figure 1. Schematic representation of the procedures used for the characterization of the anchoring domain in the α subunit of Gs (Gsα). The two main pathways following in vitro transcription are related to the mode of activation and the concomitant radiolabeling of Gsα. The straight line corresponds to the activation by ADP ribosylation where the translation is performed in the presence of unlabeled methionine and radiolabeling achieved during cholera toxin–catalyzed ADP ribosylation with ^{32}P[NAD]. The dotted line represents the activation by GTPγS where radiolabeling is performed during the in vitro translation with [^{35}S]methionine. Deletions, in boxes, of the Gsα polypeptide chain were produced either by genetic mutation at the cDNA level or by proteolytic digestion of reconstituted membranes.

labeled two main proteins in plasma membranes of wild-type S49 cells: a ^{32}P-labeled band migrated as a single band corresponding to a molecular mass of 42 kD (referred to as the short form) whereas the other ^{32}P-labeled band was resolved as a doublet of 45–46 kD (referred to as the long form) (Fig. 2 *A*).

Trypsin digestion of the ADP-ribosylated wild-type membranes revealed that the 42-kD ^{32}P-labeled band was insensitive to low concentrations of trypsin (below 1.25 μg/ml) (Fig. 2 *A*). On the other hand, the upper 45/46-kD ^{32}P-labeled doublet was degraded by low trypsin concentrations and there was a concomitant appearance of 37–38 kD ^{32}P-labeled tryptic fragments which were further degraded at higher trypsin concentrations (Fig. 2 *A*).

When cyc⁻ membranes were reconstituted by in vitro-translated Gsα long form and then ADP ribosylated with cholera toxin, only the upper 45/46-kD ^{32}P-labeled doublet was obtained. Trypsin proteolysis of the ADP-ribosylated reconstituted cyc⁻ membranes gave rise to the same 37–38-kD ^{32}P-labeled tryptic fragments whose appearance and disappearance were observed in the same range of trypsin concentrations that were active on wild-type membranes (Fig. 2 *B*).

### Membrane Association and Immunoprecipitation of the 37–38-kD Tryptic Fragments

After digestion by 0.6 μg/ml trypsin, ADP-ribosylated wild-type or reconstituted cyc-membranes were centrifuged and the distribution of undigested Gsα and its tryptic fragments was analyzed in the pellet and the supernatant fractions. As

previously reported (13), undigested ^{32}P-labeled isoforms of Gsα, the 42-kD and the 45/46-kD doublet, were recovered in the pellet fraction of wild-type membranes after ADP ribosylation (Fig. 3 *A*). We also show that in reconstituted cyc⁻ membranes, the ^{32}P-labeled 45/46-kD doublet corresponding to undigested Gsα is only found in the pellet fraction (Fig. 3 *B*).

Interestingly, the 37–38-kD ^{32}P-labeled tryptic fragments which were obtained from trypsin proteolysis of either wild-type or reconstituted cyc⁻ membranes were also recovered in the pellet fraction ((Fig. 3, *A* and *B*).

To define the location of the domains removed by trypsin, we solubilized and then immunoprecipitated the proteolyzed membranes with two different antisera, an antiserum raised against the ten COOH-terminal residues of Gsα and an antiserum directed against the amino acids 29–39 of Gsα referred to as NH₂-terminal antiserum. The 37–38-kD tryptic fragments were not immunoprecipitated by the NH₂-terminal antiserum (data not shown) but they were still recognized by antibodies raised against the 10 carboxy-terminal residues (Fig. 3, *A* and *B*). Consequently, these results suggested that the 37–38-kD tryptic fragments were generated by a deletion located within the amino-terminal domain, leaving the carboxy-terminal residues intact. It was noteworthy that the large amino-terminal deletion did not abolish the association of ADP-ribosylated tryptic fragments with the membrane.

Since activation of the α subunit can also be induced by GTPγS, we performed the same proteolysis experiments on reconstituted cyc⁻ membranes which had been treated by GTPγS.

(A) WILD-TYPE MEMBRANES   **(B)** RECONSTITUTED CYC⁻ MEMBRANES
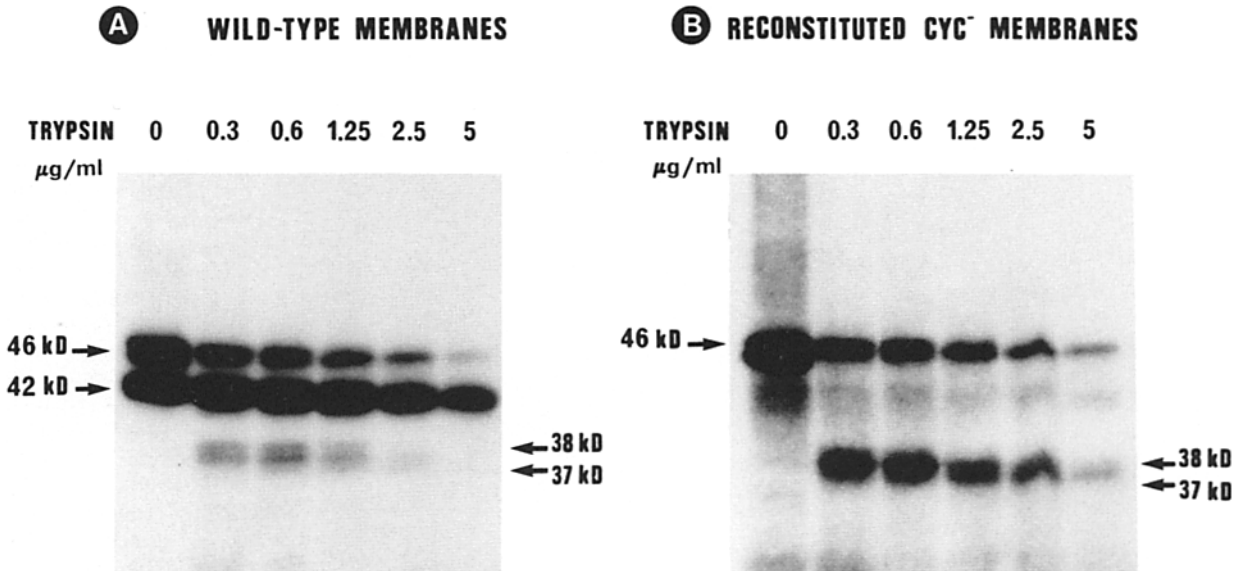
Figure 2. Trypsin proteolysis performed on wild-type membranes and reconstituted cyc⁻ membranes after ADP ribosylation. T7 polymerase transcription products were translated in reticulocyte lysate with unlabeled methionine and cyc⁻ membranes were reconstituted with the translation medium as described in Materials and Methods. Wild-type (*A*) or reconstituted cyc⁻ membranes (*B*) (1 mg protein/ml) were ADP ribosylated by cholera toxin in the presence of ^{32}P[NAD] as described previously (13). Trypsin digestion was then carried out for 60 min at 30°C at the indicated concentrations and proteolysis was stopped by addition of 1 mM PMSF. The ^{32}P-labeled proteins were separated by SDS-PAGE on a 9% acrylamide gel as shown previously (16) and visualized by autoradiography.

Trypsin Digestion of Reconstituted cyc⁻ Membranes after GTPγS Treatment

As previously described (13), translation of Gsα messenger RNA in a reticulocyte lysate gave rise to a major ^{35}S-labeled 46-kD band but also to bands of lower molecular mass which corresponded to initiation of translation at internal AUG codons. After reconstitution of cyc⁻ membranes, all ^{35}S-labeled translation products were associated with the membrane fraction (Fig. 4 *A*).

At low trypsin concentrations (<1.25 μg/ml), a major 37-kD ^{35}S-labeled tryptic fragment was rapidly generated. The 37-kD tryptic fragment was subsequently cleaved to yield a 36-kD ^{35}S-labeled fragment, at higher concentrations, which was further digested to a 35-kD ^{35}S-labeled fragment, at concentrations above 5 μg/ml trypsin (Fig. 4 *A*).

In contrast to the 37–38-kD tryptic fragments generated after trypsin digestion of reconstituted membranes which had been ADP ribosylated, the 36–37-kD tryptic fragments

(Fig. 4 *B*) as well as the 35-kD tryptic fragment (data not shown) obtained in similar conditions after GTPγS treatment were released in the supernatant fraction.

Interestingly, these soluble tryptic fragments were no longer immunoprecipitated by either the NH₂-terminal or the COOH-terminal antiserum (data not shown).

Many attempts to find proteolysis conditions of reconstituted membranes which would generate larger tryptic fragments and permit better characterization of the anchoring domain remained unsuccessful and thus we analyzed the effect of V8 protease on reconstituted cyc⁻ membranes which had been activated by GTPγS.

Staphylococcus aureus V8 Protease Digestion of Reconstituted cyc⁻ Membranes after GTPγS Treatment

Low concentrations of V8 protease (6.25 μg/ml) generated a major 43-kD ^{35}S-labeled proteolytic product which was

(A) WILD-TYPE MEMBRANES   **(B)** RECONSTITUTED CYC⁻ MEMBRANES
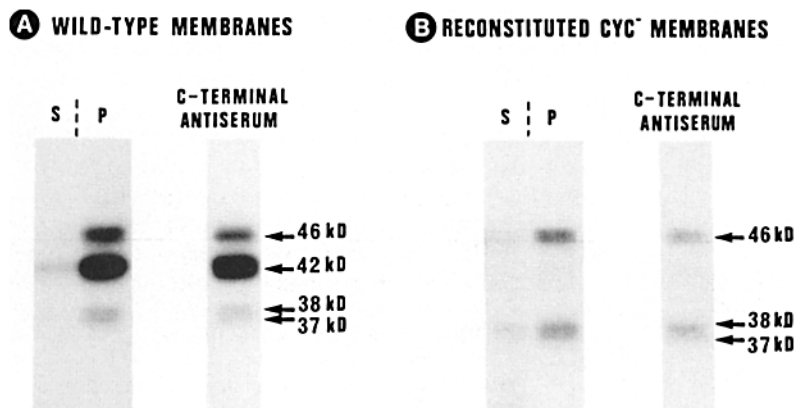
Figure 3. Membrane association and immunoprecipitation of the 37–38-kD ^{32}P-labeled tryptic fragments. After digestion, performed on wild-type (*A*) or reconstituted cyc⁻ membranes (*B*) with 0.6 μg/ml trypsin as described in Fig. 1, the medium was centrifuged at 10,000 g for 5 min and the pellet and the supernatant fractions were processed as described previously (13). The pellet fraction was also immunoprecipitated with an antiserum raised against the 10 carboxy-terminal residues of the Gsα subunit as described in Materials and Methods. The radioactive bands were resolved on a 9% acrylamide gel as shown previously (16) and visualized by autoradiography.
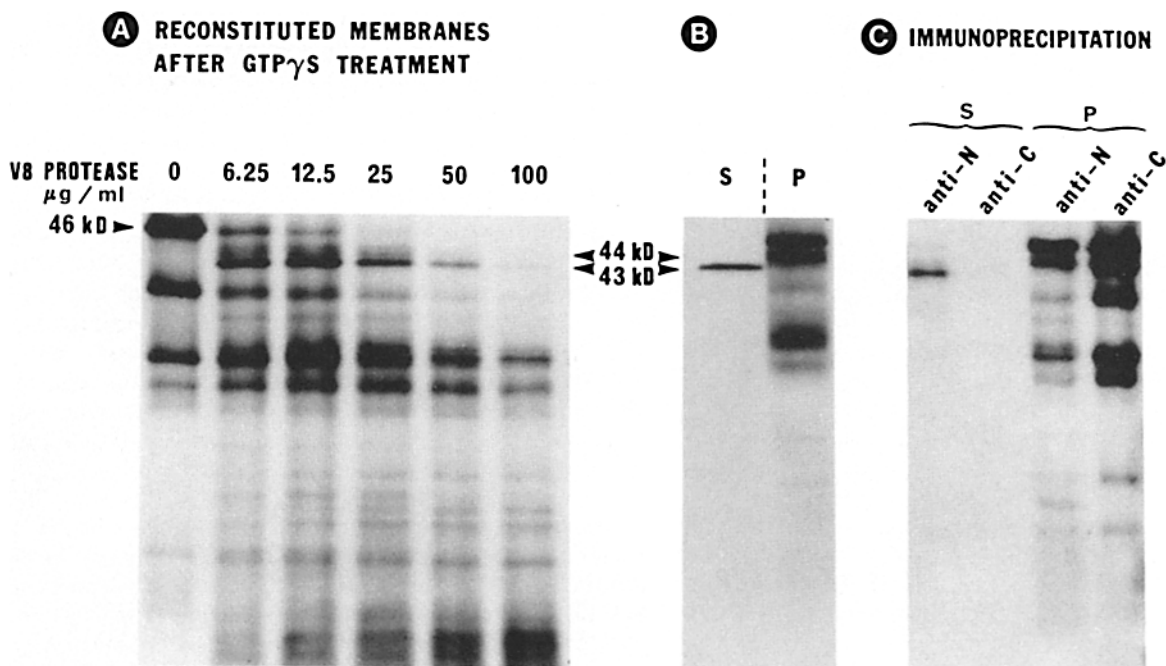
1430
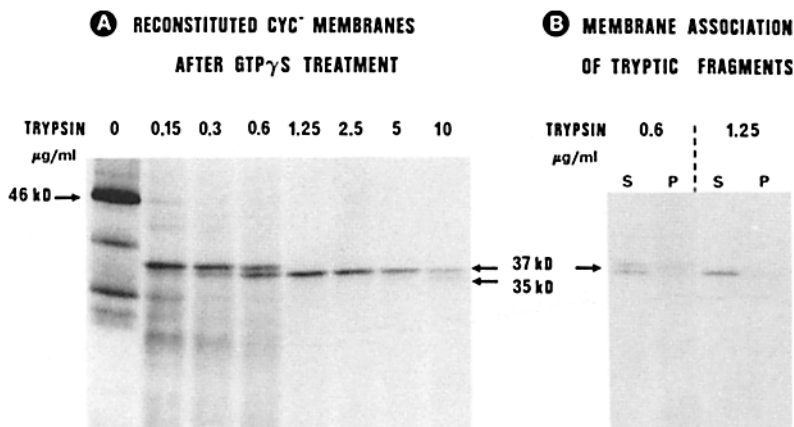
Figure 4. Trypsin digestion performed on reconstituted cyc⁻ membranes after GTPγS treatment. T7 polymerase transcription products were translated in reticulocyte lysate with ³⁵S-methionine and cyc⁻ membranes were reconstituted with the translation medium as described in Materials and Methods. Reconstituted membranes were incubated with 100 μM GTPγS + 10 mM MgCl₂ for 10 min at 37°C and then digested with the indicated concentrations of trypsin. Proteolysis was carried out for 60 min at 30°C and stopped by addition of 1 mM PMSF. The ³⁵S-labeled proteins were separated by SDS-PAGE on a 9% acrylamide gel as shown previously (16) and visualized by autoradiography. (A) Concentration dependency of trypsin digestion. (B) Membrane association of the 36–37-kD ³⁵S-labeled tryptic fragments. After digestion by 0.6 μg/ml or 1.25 μg/ml trypsin, the medium was centrifuged at 10,000 g for 5 min. The supernatant and the pellet fractions were processed as described before (13).

associated with a 44-kD ³⁵S-labeled fragment (Fig. 5 A). At higher concentrations (>12.5 μg/ml), the 44- and 43-kD bands progressively disappeared and were totally digested at concentrations >50 μg/ml (Fig. 5 A).

Since the domains removed by V8 protease were smaller than those removed by trypsin, it was interesting to study the association of the proteolytic fragments with the membrane and to locate the protease cleavage sites by immunoprecipitation.

Figure 5. *Staphylococcus aureus* V8 protease digestion performed on reconstituted cyc⁻ membranes after GTPγS treatment. T7 polymerase transcription products were translated in reticulocyte lysate with ³⁵S-methionine and cyc⁻ membranes were reconstituted with the translation medium as described in Materials and Methods. Reconstituted membranes were incubated with 100 μM GTPγS + 10 mM MgCl₂ for 10 min at 37°C and then digested with the indicated concentrations of S. aureus V8 protease. Digestion was carried out for 60 min at 30°C and terminated by addition of 2 mM diisopropylfluorophosphate. The ³⁵S-labeled proteins were separated by SDS-PAGE on a 12% acrylamide gel as shown previously (16) and visualized by autoradiography. (A) Concentration dependency of S. aureus V8 protease digestion. (B) Membrane association of the 44- and the 43-kD ³⁵S-labeled V8 protease fragments. After digestion by 12.5 μg/ml S. aureus V8 protease, the medium was centrifuged at 10,000 g for 5 min. The supernatant and the pellet fractions were processed as described before (13). (C) Immunoprecipitation of the supernatant and the pellet fractions. The ³⁵S-labeled proteins were immunoprecipitated by an antiserum directed against the amino acids 29–39 (referred to as anti-NH₂) or the 10 carboxy-terminal residues (referred to as anti-COOH) of the Gsα subunit as described in Materials and Methods.
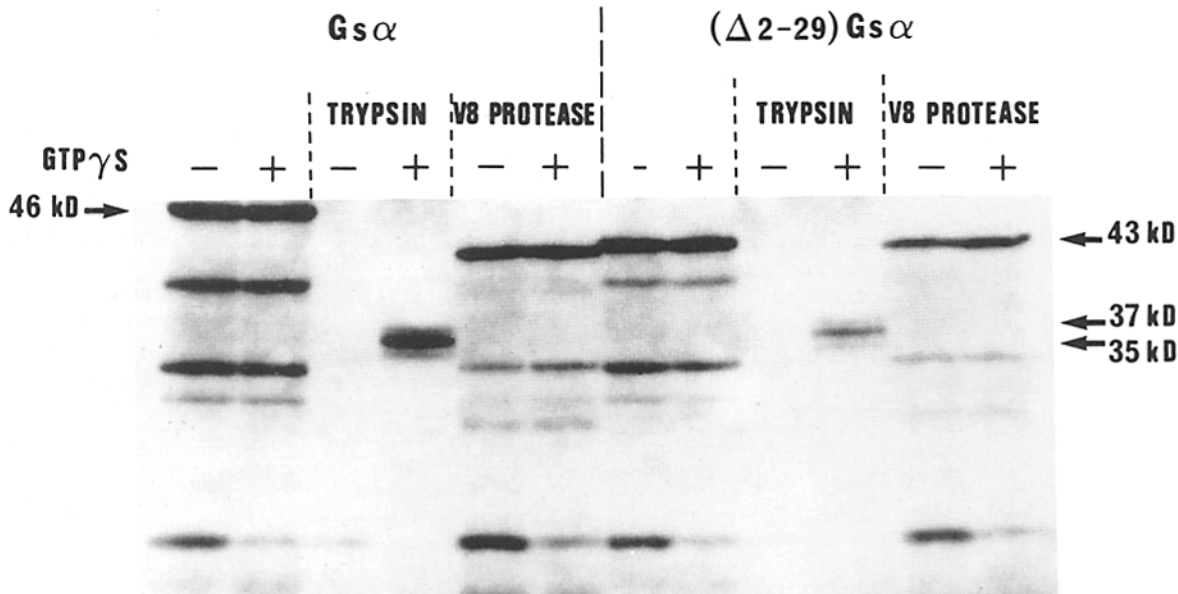
Figure 6. Proteolysis of in vitro–translated Gsα and (Δ2-29)Gsα with trypsin and *S. aureus* V8 protease. T7 polymerase transcription products were translated in reticulocyte lysate with ^{35}S-methionine as described in Materials and Methods. The translation medium was incubated for 10 min at 37°C in the absence or the presence of 100 μM GTPγS + 10 mM MgCl$_2$ and then digested with 100 μg/ml trypsin or with 12.5 μg/ml *S. aureus* V8 protease for 60 min at 30°C. Proteolysis was terminated as described in Materials and Methods. The ^{35}S-labeled proteins were separated by SDS-PAGE on a 9% acrylamide gel as shown previously (16) and visualized by autoradiography.

Membrane Association and Immunoprecipitation of the 44- and 43-kD V8 Protease Fragments

Centrifugation of the reconstituted cyc⁻ membranes, which had been treated with GTPγS and then digested with 12.5 μg/ml V8 protease, clearly showed that the 43-kD ^{35}S-labeled fragment was located in the supernatant fraction whereas the 44-kD ^{35}S-labeled fragment cosedimented with the membrane fraction (Fig. 5 *B*).

The same NH$_2$-terminal and COOH-terminal antisera, already mentioned for the analysis of the tryptic fragments, were used for immunoprecipitation of the solubilized membrane-bound 44-kD fragment and of the soluble 43-kD proteolytic product.

The soluble 43-kD fragment was no longer immunoprecipitated by the COOH-terminal antibodies but was still recognized by the NH$_2$-terminal antiserum raised against the amino acids 29–39 (Fig. 5 *C*). Interestingly, the membrane-bound 44-kD fragment was immunoprecipitated by the COOH-terminal antiserum suggesting that the domain removed by V8 protease was located at the NH$_2$-terminal end of the protein; it was also recognized by the antiserum raised against the amino acids 29–39 of Gsα, revealing that the 44-kD fragment derived from the α subunit of Gs after removal of NH$_2$-terminal amino acids (Fig. 5 *C*).

These results indicate that the 44-kD V8 protease fragment was generated by the removal of amino-terminal amino acids and that the 43-kD fragment resulted from a cleavage in the carboxy-terminal domain of Gsα.

As depicted in Fig. 1, our reconstitution model provided the unique opportunity to introduce a mutation at the cDNA level and to analyze its effect on membrane interaction of the mutated α subunit after in vitro transcription and in vitro translation. To determine whether the amino terminus was

involved in membrane anchorage, we synthesized a Gsα mutant which contained a deletion within the amino-terminal domain and we investigated whether the mutated protein was still able to be activated by GTPγS and interact with cyc⁻ membranes.

## Proteolysis Experiments on the Mutant (Δ2-29)Gsα

As expected from the size of the deletion (28 amino acids in the amino-terminal region), in vitro translation of (Δ2-29) Gsα gave rise to a major ^{35}S-labeled band which migrated with an apparent molecular mass between 43 and 44 kD (Fig. 6). Lower molecular weight ^{35}S-labeled bands corresponding to initiation of translation at internal AUG codons were also obtained and their electrophoretic migration was identical to that of the bands observed with Gsα (Fig. 6), in agreement with the location of the mutation.

Proteolysis performed on soluble (Δ2-29)Gsα with 100 μg/ml trypsin or 12.5 μg/ml V8 protease gave identical results to those obtained with soluble Gsα (Fig. 6). With trypsin, complete degradation was obtained in the absence of GTPγS whereas 35–37-kD tryptic fragments were generated in the presence of GTPγS. With V8 protease, the mutant was converted into the same 43-kD proteolytic product in the absence or in the presence of GTPγS (Fig. 6).

The resistance to trypsin digestion in the presence of GTPγS is a criterion indicating an effective GTPγS binding to the protein and visualizes the conformational change between the GDP form and the activated form of the α subunit (7, 9, 18, 27, 28). Consequently, we could assume that (Δ2-29)Gsα binds GTPγS and could be used to study the membrane interaction of the mutated protein activated by GTPγS.

Since both Gsα and (Δ2-29)Gsα generated the same 43-kD proteolytic fragment after V8 protease digestion, the

(Δ 2-29) Gs α

Control | GTPγS

S   P   S   P
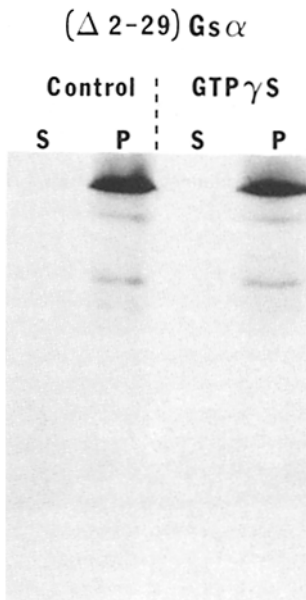
Figure 7. Membrane association of (Δ2-29)Gsα. T7 polymerase transcription products were translated in reticulocyte lysate with ^{35}S-methionine and cyc$^-$ membranes were reconstituted with the translation medium as described in Materials and Methods. Reconstitution of cyc$^-$ membranes by in vitro–translated (Δ2-29)Gsα was performed as described in Materials and Methods. Reconstituted membranes were incubated for 10 min at 37°C in the absence or the presence of 100 μM GTPγS and 10 mM MgCl$_2$ and then centrifuged at 10,000 g for 5 min. The supernatant and the pellet fractions were processed as described previously (13). The ^{35}S-labeled proteins were resolved on a 9% acrylamide gel as shown previously (16) and visualized by autoradiography.

overall conformation of the mutated protein was unlikely to be very different from that of the wild-type protein in the absence or the presence of GTPγS.

Membrane Association of the Mutant (Δ2-29)Gsα

After reconstitution of cyc$^-$ membranes by in vitro–translated (Δ2-29)Gsα, the major ^{35}S-labeled band, as well as the other translation products, was found associated with the pellet fraction and treatment of the reconstituted membranes with GTPγS did not modify the interaction with the membrane (Fig. 7). Consequently, removal of the 28 amino-terminal residues did not impair the anchorage of the mutated Gsα in the membrane either in GDP form or in GTPγS-liganded form.

Discussion

The validity of our in vitro reconstitution model has already been demonstrated by the functional restoration of isoproterenol-mediated activation of adenylate cyclase when cyc$^-$ membranes were reconstituted by in vitro–translated Gsα long form (13). We have extended these previous results by showing that the sensitivity of reconstituted cyc$^-$ membranes to trypsin proteolysis was undistinguishable from that of the wild-type membranes after ADP ribosylation, confirming that the correct association with the membrane of the Gsα long form could be reproduced posttranslationally.

Using this reconstitution model, we report the results of proteolysis experiments and genetic mutation which, in contrast to the amino-terminal domain of Goα and Giα (12, 19), show that the amino-terminal residues of Gsα do not play a significant role in the interaction of the activated form with the membrane. In addition, we provide strong evidence for the involvement of the carboxy-terminal domain in the membrane anchorage of activated Gsα.

The 37–38-kD tryptic fragments which are generated at low trypsin concentrations from ADP-ribosylated membranes were essentially recovered in the pellet fraction and were immunoprecipitated by antibodies raised against the 10 carboxy-terminal residues of Gsα but not by antibodies directed against the amino acids 29–39 of Gsα. These results show that an 8-kD deletion within the amino-terminal domain of Gsα long form does not affect the interaction of the ADP-ribosylated species with the membrane.

When similar proteolysis experiments were carried out on reconstituted cyc$^-$ membranes after GTPγS treatment, the resulting 35–37-kD tryptic fragments were released in the supernatant fraction and were no longer recognized by the carboxy-terminal antiserum. Therefore, in view of the membrane localization of the ADP-ribosylated 37–38-kD tryptic fragments which contain an intact carboxy-terminus, it was noteworthy that the release of 35–37-kD tryptic fragments from GTPγS-treated membranes was associated with a deletion located in the carboxy-terminal domain. The difference observed between the tryptic fragments, generated after ADP ribosylation or GTPγS treatment, revealed that a tryptic cleavage site in the COOH-terminal domain of ADP-ribosylated Gsα was not accessible to trypsin, suggesting that the active conformation resulting from each treatment was not identical in the membrane environment. Besides the structural change, corresponding to ADP ribose addition, a difference in the extent of dissociation between the ADP-ribosylated or the GTPγS-activated Gsα subunit and βγ subunits was already shown by their different rates of sedimentation in sucrose gradients (14). Consequently, although ADP ribosylation and GTP-γS treatment induce adenylate cyclase activation, ADP-ribosylated Gsα and GTPγS-liganded Gsα may differ in their mode of interaction with the effector.

Furthermore, V8 protease experiments performed on GTPγS-treated reconstituted membranes indicated that the 44-kD proteolytic fragment, which is immunoprecipitated by the COOH-terminal antiserum, still interacts with the membrane, suggesting that removal of a 2-kD amino-terminal domain does not modify the anchorage in the membrane.

Finally, the genetic deletion of 28 amino acids within the amino-terminal domain of Gsα did not alter the association of the mutated protein, (Δ2-29)Gsα, with the membrane. Consequently, proteolytic removal of a 2-kD amino-terminal domain (44-kD V8 protease fragment) and genetic deletion of 28 amino-terminal amino acids in (Δ2-29)Gsα were functionally equivalent to membrane interaction, although the
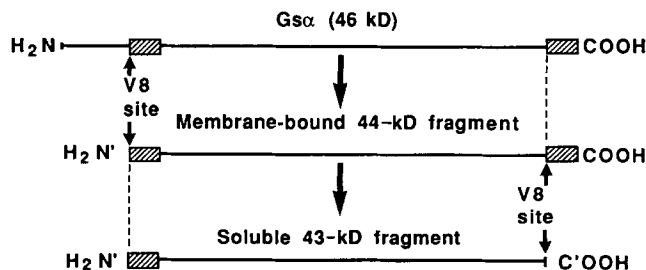
Figure 8. Schematic mapping of the V8 protease cleavage sites on Gsα long form. The hatched bars indicate the location of the amino acid sequences which are recognized by the NH$_2$-terminal antiserum and the COOH-terminal antiserum.

genetic deletion may not involve exactly the same amino acids that are removed by proteolysis.

All these points, which rule out the amino terminus as the anchoring domain, implied a different location for the domain involved in the membrane association of Gsα. Indeed, in V8 protease experiments, the 44-kD V8 protease fragment, which remained membrane bound, was recognized by the COOH-terminal antiserum whereas the 43-kD V8 protease fragment, which was released from the membrane, was not immunoprecipitated by the COOH-terminal antiserum. Taken together, these results suggested that removal of a carboxy-terminal domain promoted the release of Gsα from the membrane. Unfortunately, we were unable to demonstrate directly the importance of the carboxy terminus since a mutant with a deletion in the carboxy-terminal domain no longer bound GTPγS or at least did not achieve the activated conformation (data not shown). We are currently trying to construct mutants which are deleted in the carboxy-terminus but which still bind GTPγS.

With regard to the anchorage of the GTPγS-activated form, the location of the V8 protease-sensitive sites may be informative concerning the size of the carboxy-terminal domain and, eventually, the nature of the residues involved in the association with the membrane. We concluded that there are at least two distinct V8 cleavage sites; an amino-terminal site and a carboxy-terminal site. However, it was possible that the soluble 43-kD fragment was generated by either removal of a 3-kD carboxy-terminal domain or from cleavage at two distinct sites, eliminating simultaneously a 2-kD amino-terminal domain and a 1-kD carboxy-terminal domain. In this respect, the 44-kD fragment could represent an intermediate proteolytic product between 46-kD Gsα and the 43-kD fragment whose appearance would be related to the masking of the carboxy-terminal site by the membrane environment (see Fig. 8). As the digestion of soluble Gsα and (Δ2–29)Gsα by V8 protease generated the same 43-kD fragment, the removal of a 3-kD carboxy-terminal domain is unlikely since the size decrease would be additional to the deletion in the mutant and would generate a smaller fragment. Accordingly, the 43-kD fragment would correspond to the cleavage of an amino-terminal site and a carboxy-terminal site whereas the 44-kD fragment would not undergo the carboxy-terminal cleavage (see Fig. 8).

In this context, the amino-terminal deletion in (Δ2–29)Gsα would mimic the amino-terminal cleavage by V8 protease in Gsα. If we take into account the apparent size decrease between uncleaved Gsα (46 kD) and the membrane-bound 44-kD V8 protease fragment, the immunoprecipitation of the 44-kD fragment by the NH₂-terminal antiserum and the location of the V8 protease site in Tα (20), Glu²⁷ represents the most probable, albeit speculative, location for the amino-terminal site.

As far as the carboxy-terminal V8 protease site is concerned, the size of the cleaved domain is ∼1 kD and therefore restricts the location of the cleavage site to the last 10–15 amino acids: the most likely positions for cleavage would be Asp³⁷⁸ or Asp³⁸¹. Comparison of the aligned protein sequences of Gsα, Goα, the three Giα, and the two Tα proteins (11) indicates that Gsα diverges from this group of homologous proteins (Gsα retained only 40% identity with any of the other six). Gsα was notably divergent in the 25 carboxy-terminal residues (2, 4, 8, 11). For example, divergence be-

tween the carboxy-terminus of Gsα and that of Tα is exemplified by the absence of the carboxy-terminal V8 site in Tα (20). Therefore, it is less surprising that the domain required for anchorage of the activated α subunit may be located in the carboxy-terminal residues for Gsα instead of the amino-terminal residues as suggested for Goα or Giα (5, 12, 19). As a corollary, it is tempting to speculate that Gsα short form, which differs from the long form only by an internal stretch of 15 residues (24), is also tethered to the plasma membrane via its carboxy terminus.

In summary, we have shown that the carboxy terminus of Gsα was necessary for the association of the ADP-ribosylated or GTPγS-liganded α subunit with the membrane. In the case of GTPγS activation, the size of the carboxy-terminal domain required for the anchorage was restricted to ∼1 kD. We also demonstrated that a mutant containing a deletion within the amino-terminal domain was still able to bind GTPγS and to interact with the membrane after GTPγS treatment. It remains to be proven whether the carboxy-terminal domain is necessary and sufficient for the direct anchorage of GTPγS-liganded Gsα in the lipid bilayer or if it interacts with a protein component which is itself associated with the membrane. Furthermore, the question arises as to whether a posttranslational modification analogous to myristoylation may confer to the carboxy-terminal domain its ability to interact with the membrane.

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