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Reconstitution of cyc⁻ S49 membranes by in vitro translated $G_{s\alpha}$

Membrane anchorage and functional implications

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After ADP-ribosylation by cholera toxin which promotes dissociation of the subunits, the α-subunit of G_s ($G_{s\alpha}$) remained strongly associated with plasma membranes of wild-type S49 cells, since its interaction with the membrane was insensitive to 1 M KCl. Its association with the membrane was partially disrupted by 6 M urea and totally abolished by treatment with alkali at pH ≥11.5. In vitro translated $G_{s\alpha}$ could interact with plasma membranes from the cyc⁻ mutant of S49 cells as revealed by its cosedimentation with the membrane fraction and incubation of reconstituted membranes with GTPγS did not alter anchorage of $G_{s\alpha}$. The characteristics of the association of in vitro translated $G_{s\alpha}$ with cyc⁻ membranes after GTPγS treatment, i.e. sensitivity to 1 M KCl, 6 M urea and alkali treatment, were very similar to those described for the ADP-ribosylated form in wild-type membranes. Restoration of the coupling between the adrenergic receptor and adenylate cyclase further confirmed the vectorial reconstitution of cyc⁻ membranes by in vitro translated α-subunit of G_s.

Guanyl nucleotide-binding protein; Membrane anchorage; Translation; Adenylate cyclase

1. INTRODUCTION

GTP-binding proteins are involved in the transduction of an extracellular signal to intracellular effectors like cGMP phosphodiesterase, adenylate cyclase, phospholipase C and ionic channels [1-3]. Signal-transducing GTP-binding proteins display the same oligomeric structure characterized by the association of three distinct subunits (α,β,γ). Stimulation of a receptor coupled to those transducers induces exchange of GDP by GTP on the α-subunit which in turn promotes dissociation of the GTP-liganded α-subunit from the $\beta\gamma$ subunits. In most systems, interaction of the activated α-subunit with a specific effector generates a diffusible second messenger which leads to the cascade of intracellular events corresponding to the biological response [1-3].

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As far as the α-subunits are concerned, there is a strong interdependency between their functional state (GDP and GTP forms) and their structural organization (heterotrimer vs monomer) which might reflect distinct modes of association with the membrane. In the resting state, the requirement of detergent for solubilization of the oligomeric form has been attributed to the properties of the $\beta\gamma$ subunits which may anchor the α-subunit in the membrane [4-6]. Such a statement is corroborated by the primary structure of the α-subunits from various GTP-binding proteins which contain neither a signal peptide nor a hydrophobic domain [7-10]. In the activated state, dissociation of the heterotrimeric complex is not accompanied by release of the monomeric α-subunit in the cytoplasm suggesting that the α-subunit itself can interact with the membrane directly or through some unknown components [1-3].

We decided therefore to study the association of the α-subunit of G_s, the GTP-binding protein involved in the activation of adenylate cyclase [11],

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with plasma membranes of wild-type S49 mouse lymphoma cells [12]. Interestingly, a phenotypic variant of S49 cells termed cyc$^-$ has been isolated [13] which lacks the α-subunit of G_s ($G_{s\alpha}$) as revealed by the absence of substrates for ADP-ribosylation with cholera toxin [14] and by the failure to detect a messenger RNA hybridizing with the $G_{s\alpha}$ cDNA [15]. In a first approach, we analyzed the association of $G_{s\alpha}$ with wild-type membranes after ADP-ribosylation by cholera toxin. As a second procedure we synthesized $G_{s\alpha}$ in the reticulocyte lysate and used it for reconstitution experiments with membranes from the cyc$^-$ mutant.

We report here that the properties of in vitro associated $G_{s\alpha}$ with cyc$^-$ membranes are very similar to those of in vivo associated $G_{s\alpha}$ in wild-type membranes and that the effectiveness of the in vitro reconstitution has been confirmed at the functional level by restoration of adenylate cyclase activation by GTPγS or isoproterenol.

2. MATERIALS AND METHODS

2.1. *Membrane preparation*

Plasma membranes were prepared from wild-type and cyc$^-$ S49 cells (both cell lines kindly donated by M. Siat and Dr B. Harris) as described [16].

2.2. *ADP-ribosylation*

Activation of cholera toxin (0.5 mg/ml) was carried out for 30 min at 37°C in 25 mM dithiothreitol. S49 membranes (5 mg protein/ml) were incubated for 30 min at 37°C with 50 μg/ml of the activated toxin and 1 μCi [^{32}P]NAD (800–1000 Ci/mmol; Du Pont-New England Nuclear) in 170 mM potassium phosphate buffer (pH 6.8), 10 mM $MgCl_2$, 1 mM ATP, 0.1 mM GTP and 10 mM thymidine.

2.3. *Immunoprecipitation*

The translation products were immunoprecipitated for 1 h at room temperature with an antiserum raised against the 10 carboxy-terminal residues of the $G_{s\alpha}$ subunit (a generous gift from Dr Milligan) or directed against the 29th–39th amino acids of the $G_{s\alpha}$ subunit (kindly provided by P. Brabet) according to [17].

2.4. *Construction of transcription plasmid*

The cDNA coding for the α-2 (long) subunit of human liver G_s (a generous gift from Dr J. Codina and Dr L. Birnbaumer) was excised from the replicative form of M13mp 18-αs-2 by digestion with *Kpn*I and *Xba*I. The αs-2 *Kpn*I-*Xba*I fragment was then inserted into *Kpn*I/*Xba*I-digested pIBI 31.

2.5. *In vitro transcription*

The plasmid pIBI-$G_{s\alpha\text{-}2}$ was linearized with *Kpn*I, transcribed into its messenger RNA with T_7 polymerase and purified as in [18].

2.6. *In vitro translation*

Cell-free translation of 100 ng $G_{s\alpha}$ mRNA was performed at 30°C for 45 min in a reticulocyte lysate. A typical translation medium contained 14 μl Promega nuclease-treated reticulocyte lysate, 1 μl of each of 19 amino acids at 1 mM except methionine, 3 μl of 10 μM [^{35}S]methionine (1100 Ci/mmol; Du Pont-New England Nuclear) and 2 μl mRNA (50 ng/μl).

2.7. *Reconstitution assay*

1 vol. translation medium was mixed with 3 vols cyc$^-$ membranes (1.3 mg protein/ml) and incubated for 30 min at 37°C. The reconstitution medium was treated with 0.1 mM GTPγS and 10 mM $MgCl_2$ for 10 min at 37°C and then centrifuged in a Beckman airfuge at 122 000 × g_{av} for 10 min. The pellet was resuspended in the same volume of 20 mM Na^+-Hepes, buffer (pH 8) containing 2 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT and homogenized in a Dounce homogenizer (10 strokes). Aliquots were incubated in the presence of the various effectors and then centrifuged in a Beckman airfuge as described above. The supernatant (S) was diluted with an equal volume of 2× sample buffer whereas the pellet (P) was respended in sample buffer by heating at 50°C for 30 min.

2.8 *Adenylate cyclase assay*

In order to decrease the inactivation of adenylate cyclase, the reconstitution assay was carried out for 30 min at 30°C and aliquots of the reconstitution medium were directly assayed for cAMP formation according to [19]. [^{32}P]cAMP was quantified as in [20].

2.9 *SDS-polyacrylamide gel electrophoresis*

Samples were analyzed on 9% acrylamide gels according to [21]. Dried gels were exposed to Kodak XAR-5 film at −70°C.

3. RESULTS

3.1. *Mode of association of ADP-ribosylated $G_{s\alpha}$ with plasma membranes from wild-type S49 cells*

To study the nature of the interaction between activated $G_{s\alpha}$ and the membrane, we labeled $G_{s\alpha}$ with [^{32}P]NAD by ADP-ribosylation of membranes with cholera toxin, a treatment known to act on the activated form of G_s [22]. As previously described in this cell line [14], two main substrates were specifically ADP-ribosylated by the cholera toxin.

Although promoting dissociation of the α-subunit from the $\beta\gamma$ subunits [23], ADP-ribosylation did not induce release of the two forms of $G_{s\alpha}$ (fig.1A). High ionic strength, e.g. 1 M KCl present, did not affect the association of ADP-

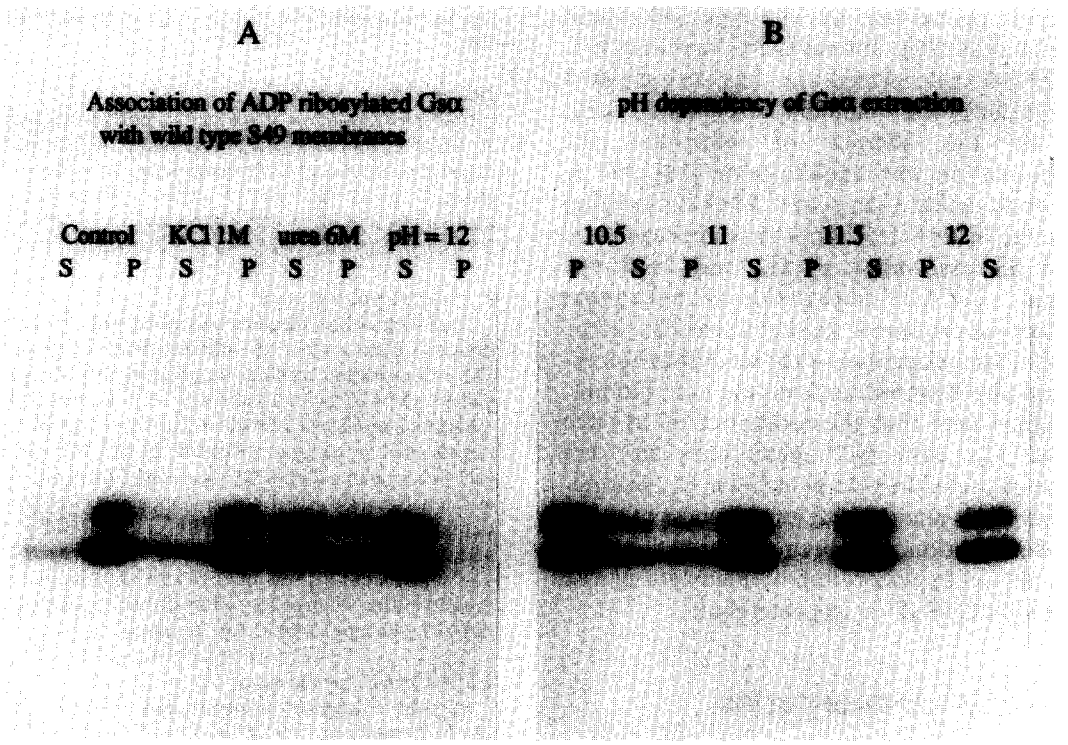

Fig.1. Following ADP-ribosylation by cholera toxin, the different effectors were added to the membranes for 10 min at 4°C except for urea treatment which was carried out at room temperature. Membranes were then centrifuged and the supernatant or pellet fraction analyzed as described in section 2.

ribosylated $G_{s\alpha}$ with the membrane (fig.1A) whereas 6 M urea was able to release more than half of $G_{s\alpha}$ in the supernatant fraction (fig.1A). Finally, alkali treatment at pH 12 of wild-type membranes extracted 100% of $G_{s\alpha}$ (fig.1A).

As shown in fig.1B, the pH dependency of $G_{s\alpha}$ extraction was indeed characterized by a narrow range of pH values: 0% of $G_{s\alpha}$ was extracted at pH $\leq$ 10.5 and 100% of $G_{s\alpha}$ was recovered in the supernatant fraction at pH $\geq$ 11.5.

Interestingly, the various conditions that had been studied after ADP-ribosylation produced the same effect on the interaction of the two forms of G_s with the membrane.

3.2. *In vitro translation of $G_{s\alpha}$ and its association with cyc$^-$ membranes*

Translation of $G_{s\alpha\text{-}2}$ messenger RNA in a reticulocyte lysate generated a major band which migrated slightly above that of the 43 kDa marker ovalbumin; other bands of lower molecular mass were also present (fig.2A). The comparative migration of ^{35}S-labeled $G_{s\alpha}$ from in vitro translation and of ^{32}P-labeled $G_{s\alpha}$ forms after ADP-ribosylation clearly showed that the former corresponded to the high molecular mass form present in wild-type S49 cells despite a slight difference in migration which can be attributed to the presence of the ADP-ribose moiety.

An antiserum raised against the 10 carboxy-terminal residues of $G_{s\alpha}$ immunoprecipitated all the translation products (fig.2B) whereas an antiserum raised against the 29th–39th amino-terminal residues of $G_{s\alpha}$ recognized only the upper band (fig.2B), suggesting that the lower bands might correspond to initiation of translation at internal AUG codons.

To investigate whether association of $G_{s\alpha}$ (long form) with cyc$^-$ membranes can occur posttranslationally, we reconstituted plasma membranes from the cyc$^-$ mutant of S49 cells with in vitro translated $G_{s\alpha}$. As shown in fig.2C, most translation products

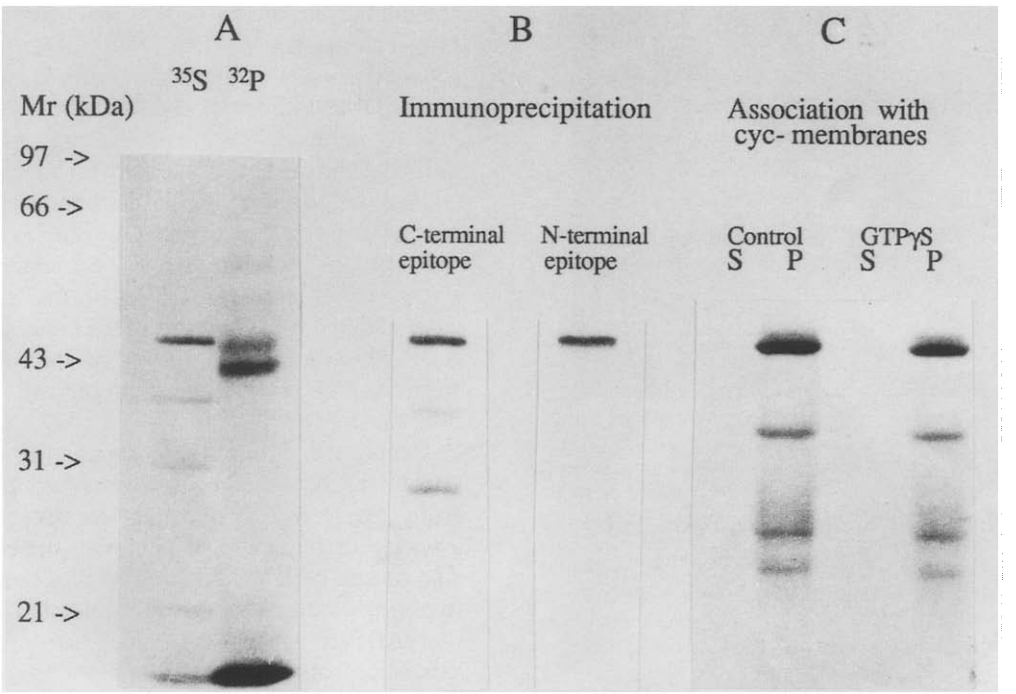

Fig.2. (A) Comparative migration of ^{35}S-labeled $G_{s\alpha}$ and [^{32}P]ADP-ribosylated $G_{s\alpha}$. After translation of $G_{s\alpha}$ messenger RNA in reticulocyte lysate supplemented with [^{35}S]methionine, the translation products were run in parallel with trichloroacetic acid precipitates of wild-type membranes of S49 cells which had been ADP-ribosylated by cholera toxin in the presence of [^{32}P]NAD. (B) Immunoprecipitation of the translation products by antibodies raised against an N-terminal sequence (amino acids 29–39) or a C-terminal sequence (amino acids 385–394). (C) Subsequent to reconstitution, cyc$^-$ membranes were incubated at 37°C for 10 min in the absence (control) or presence of 0.1 mM GTPγS and 10 mM $MgCl_2$.

cosedimented with the membrane fraction and this interaction remained unimpaired by treatment with GTPγS.

3.3. *Mode of association of in vitro translated $G_{s\alpha}$ with plasma membranes from cyc$^-$ S49 cells*

In order to verify that the in vitro reconstitution model could reproduce the in vivo association of activated $G_{s\alpha}$ with the membrane, we investigated the influence of the various conditions tested using GTPγS-treated reconstituted membranes on the association of ADP-ribosylated $G_{s\alpha}$ (fig.3).

High ionic strength did not affect the interaction of in vitro translated $G_{s\alpha}$ with cyc$^-$ membranes whereas 6 M urea as well as alkali extraction did release most of the polypeptide in the supernatant fraction (fig.3).

The labeled band which migrated slightly above $G_{s\alpha}$ in the supernatant fractions from urea and alkali treatments (fig.3) correspond to a 45 kDa

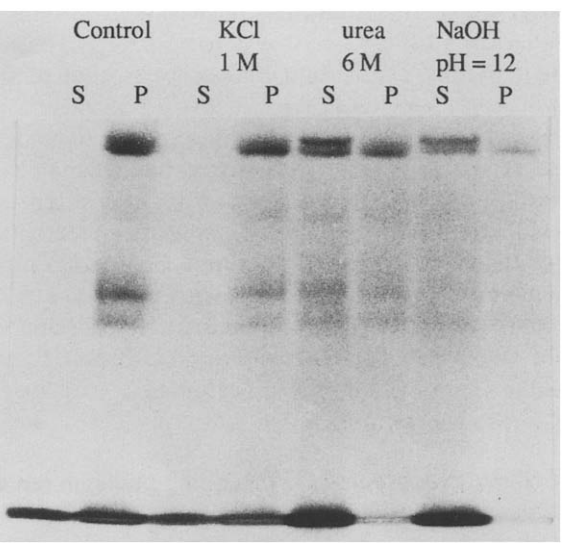

Fig.3. After reconstitution, cyc$^-$ membranes were incubated at 37°C for 10 min in the presence of 0.1 mM GTPγS and 10 mM $MgCl_2$. Addition of the various effectors was carried out as described in fig.1.

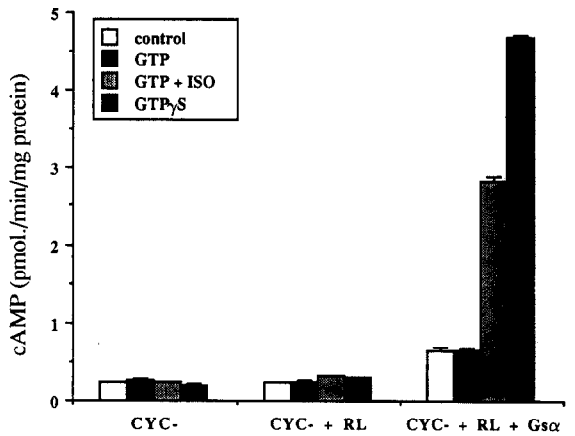

Fig.4. Adenylate cyclase activity was assayed by measuring cAMP formation at 30°C for 30 min in the presence of 10 mM $MgCl_2$ according to [19]. The effect of various effectors was checked on cyc^- membranes (CYC-), cyc^- membranes supplemented with reticulocyte lysate (CYC- + RL) and cyc^- membranes reconstituted by reticulocyte lysate programmed with $G_{s\alpha}$ messenger RNA (CYC- + RL + $G_{s\alpha}$). Final concentrations: GTP, 0.1 mM; isoproterenol (ISO), 10 μM; GTPγS, 10 μM.

protein species usually reported in reticulocyte lysates after denaturation [24].

3.4. *Reconstitution of adenylate cyclase activation*

A corollary to the effective reconstitution of endogenous association with the plasma membrane is reversion of the functional defect corresponding to switching of the variant phenotype to that of the wild type.

As shown in fig.4, the addition of reticulocyte lysate to cyc^- membranes altered neither the basal activity of adenylate cyclase nor its insensitivity to GTPγS or isoproterenol. On the other hand, the reticulocyte lysate programmed with $G_{s\alpha}$ messenger RNA not only increased basal activity of adenylate cyclase but also restored stimulation of the enzyme by isoproterenol and GTPγS.

4. DISCUSSION

Signal-transducing GTP-binding proteins represent a group of oligomeric proteins whose assembly and anchorage in the membrane remain to be elucidated. Furthermore, the anchorage of resting and activated forms of the α-subunits may correspond to distinct modes of association. Even though the anchorage of the GDP form of the α-subunit may be indirect via the other subunits, the dissociated α-subunit after activation is not released from the membrane [1-3].

It is therefore not surprising that ADP-ribosylation of the α-subunit of G_s, which induces dissociation [23], does not promote the release of ADP-ribosylated $G_{s\alpha}$ from the membrane as previously described in human erythrocyte membranes [25]. Even in the case of rat liver plasma membranes where 30% of $G_{s\alpha}$ is found in the supernatant after ADP-ribosylation [26], most of the activated α-subunit of G_s is still able to interact with the membrane.

The nature of this interaction and the possible involvement of other components can be deduced from the effect of various treatments on the association of activated $G_{s\alpha}$ with the membrane. The failure of 1 M KCl (or 1 M NaCl; not shown) to abolish binding of $G_{s\alpha}$ to the membrane reveals that ADP-ribosylated $G_{s\alpha}$ does not behave as an extrinsic protein and that its association with the membrane is strong. In contrast to a previous report [25], the significant release induced by a protein perturbant such as 6 M urea would strengthen the participation of protein-protein interactions and may suggest the involvement of a proteinaceous component through which the activated α-subunit of G_s would be anchored in the membrane. Finally, ADP-ribosylated $G_{s\alpha}$ can be alkali-extracted, demonstrating that it is not an integral membrane protein. This result is at variance with [25] but in agreement with the primary structure deduced from cDNA sequencing [7-10] which reveals neither a signal sequence nor any long stretch of hydrophobic amino acids.

In order to address the problem of the assembly and anchorage of GTP-binding proteins, we have developed an in vitro model based on reconstitution of cyc^- membranes (devoid of any $G_{s\alpha}$) by in vitro translated $G_{s\alpha}$.

Qualitatively, this model reproduces the mode of interaction of activated $G_{s\alpha}$ in wild-type membranes: after GTPγS treatment of reconstituted membranes, the activated α-subunit of G_s is not released by 1 M KCl and its interaction with the membrane is sensitive to urea and alkali treatment. Although posttranslational association of $G_{s\alpha}$ with cyc^- membranes can occur in vitro, it does not preclude that the in vivo assembly of the three

subunits takes place at the same subcellular location. Indeed, the requirement of detergents for solubilizing GTP-binding protein tends to support the idea that at least one of the subunits may be an integral membrane protein; since the amino acids sequences of α- and β-subunits do not contain hydrophobic domains, the γ-subunit remains the last candidate as an integral membrane protein and therefore the assembly may also occur at the level of the rough endoplasmic reticulum.

Functionally, the effectiveness of association is demonstrated by restoration of adenylate cyclase activation by GTPγS and isoproterenol, in agreement with a recent report [27]. Reconstitution of cyc$^-$ membranes by in vitro translated $G_{s\alpha}$ is confirmed by the following findings: reconstitution does not occur in the absence of cyc$^-$ membranes and there is a strict relationship between the amount of messenger RNA and the activity of reconstitution (not shown). Quantitative analysis of wild-type and reconstituted membranes reveals that basal as well as stimulated activity of adenylate cyclase in reconstituted membranes was lower than those determined in wild-type membranes, although the stimulation factor for GTPγS or isoproterenol observed in reconstituted membranes was similar to that found in wild-type membranes (not shown). For the case of 100% of synthesized $G_{s\alpha}$ being associated with the membrane, we calculated that 150 fmol of in vitro translated $G_{s\alpha}$ were incorporated per mg protein in cyc$^-$ membranes. Previous quantitation of $G_{s\alpha}$ in wild-type membranes after ADP-ribosylation by cholera toxin gave an estimate of 100–200 fmol/mg protein [14] whereas a recent antibody-based technique [28] suggests a considerably higher value of 18.9 pmol/mg protein. If the latter is the correct value, effective reconstitution can be achieved with less than 5% of the total amount of $G_{s\alpha}$ reported in wild-type membranes, suggesting that adenylate cyclase rather than the GTP-binding protein may be limiting in the coupling reaction.

Finally, we have shown that the long form of $G_{s\alpha}$ can reconstitute hormone-stimulated adenylate cyclase as recently reported for the short and long forms [27]. Since the long form is also able, like the short one, to activate Ca^{2+} channels [29], the functional significance of at least two forms of $G_{s\alpha}$ remains to be clarified.

Our in vitro reconstitution model will provide a useful tool for discussing the different elements which are involved in the assembly and anchorage of GTP-binding proteins, especially the interaction of the activated α-subunits with the membrane. Indeed, the influence of genetic or proteolytic alterations of the protein as well as the effects of various treatments of the membranes can shed some light on the peptide domains responsible for this interaction. As far as the α-subunit of G_s is concerned, it will be interesting to verify in the case of the activated state whether a discrete amino acid sequence is alone sufficient for anchorage in the membrane or if a posttranslational modification, different from or equivalent to the myristoylation described for the α-subunits of G_i and G_o [30], is necessary for conferring binding to the membrane.

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