Restricting the Mobility of $G_s\alpha$: Impact on Receptor and Effector Coupling

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ABSTRACT: The α-subunit of the stimulatory G protein, G_s, has been shown to dissociate from the plasma membrane into the cytosol following activation by G protein-coupled receptors (GPCR) in some experimental systems. This dissociation may involve depalmitoylation of an amino-terminal cysteine residue. However, the functional significance of this dissociation is not known. To investigate the functional consequence of $G_s\alpha$ dissociation, we constructed a membrane-tethered $G_s\alpha$ (tet$G_s\alpha$), expressed it in Sf9 insect cells, and examined its ability to couple with the β_2 adrenoceptor and to activate adenylyl cyclase. Compared to wild-type $G_s\alpha$, tet$G_s\alpha$ coupled much more efficiently to the β_2 adrenoceptor and the D1 dopamine receptor as determined by agonist-stimulated GTPγS binding and GTPase activity. The high coupling efficiency was abolished when $G_s\alpha$ was proteolytically cleaved from the membrane tether. The membrane tether did not prevent the coupling of tet$G_S\alpha$ to adenylyl cyclase. These results demonstrate that regulating the mobility of $G_s\alpha$ relative to the plasma membrane, through fatty acylation or perhaps interactions with cytoskeletal proteins, could have a significant impact on receptor−G protein coupling. Furthermore, by enabling the use of more direct measures of receptor−G protein coupling (GTPase activity, GTPγS binding), tet$G_S\alpha$ can facilitate the study for receptor−G protein interactions.

Heterotrimeric guanine nucleotide-binding proteins (G proteins) are the signal transducers which relay information from membrane-bound receptors to effector systems. Receptor activation stimulates exchange of GDP for GTP on the α-subunit, which then dissociates from the receptor and the $\beta\gamma$ dimer and interacts with effectors. The α-subunit remains active until the bound GTP is hydrolyzed to GDP by the intrinsic GTPase activity of the α-subunit (*1*−*3*).

G protein α-subunits have a variety of posttranslational lipid modifications (*4*). $G_i\alpha$, $G_o\alpha$, and $G_z\alpha$ are myristoylated by amide linkage at their N-terminal glycine residues (*5, 6*), and they are also palmitoylated at distinct sites (*7*). $G_s\alpha$[1] and $G_q\alpha$ are palmitoylated by a thioester bond at N-terminal cysteine residues (*8, 9*). These α-subunit modificaitons may be important for membrane attachment or interactions with $\beta\gamma$ subunits or effector molecules (*10, 11*). Prenylation of the γ subunit is also known to play a role in membrane anchorage of the $\beta\gamma$ dimer (*12*). Activation of the adenylyl cyclase activating G protein α subunit, $G_s\alpha$, by the nonhydrolyzable GTP analogues Gpp[NH]p and GTPγS, or stimulation via receptors induces palmitate turnover and the release of $G_s\alpha$-subunit from membrane to cytosol (*11, 13*−*17*). It has been suggested that a cycle of acylation and deacylation could be a mechanism for regulating the activity and membrane association of G protein α-subunits (*18, 19*); however, the functional consequence of α-subunit translocation from the plasma membrane has not been determined. Other investigators have found that activation of G protein α subunits causes them to concentrate in subdomains of the plasma membrane but not to be released from the membrane (*20*).

To examine the consequence of restricting the mobility of $G_S\alpha$ on receptor−G protein coupling, we coexpressed the β_2 adrenoceptor and the D1 dopamine receptor with a membrane-tethered $G_s\alpha$ (tet$G_s\alpha$), which cannot be released from the plasma membrane following receptor activation. We compared the functional properties of tet$G_S\alpha$ with native $G_S\alpha$ expressed in Sf9 insect cells. Our results suggest that regulating the mobility of $G_s\alpha$ in the plasma membrane may be important for controlling receptor−G protein coupling.

EXPERIMENTAL PROCEDURES

Materials. Rat $G_s\alpha$ long isoform cDNA was kindly provided by Dr. R. R. Reed (Johns Hopkins University, Baltimore, MD) (*21*). The baculovirus transfer vector pVL1392 and BaculoGold transfection kit were from Pharmingen (San Diego, CA). D1 dopamine receptor encoding virus was provided by Dr. M. Bouvier (University of Montreal). [γ-^{32}P]GTP (6000 Ci/mmol), [α-^{32}P]ATP (3000 Ci/mmol), and [^{35}S]GTPγS (1250 Ci/mmol) were from NEN-DuPont (Boston, MA). [^{3}H]Dihydroalprenolol ([^{3}H]DHA)

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[1] Abbreviations: GPCR, G protein-coupled receptor; β_2AR, β_2 adrenoceptor; D1R, D1 dopamine receptor; $G_s\alpha$, α-subunit of the stimulatory G protein; tet$G_s\alpha$, membrane-tethered $G_s\alpha$; [^{3}H]DHA, [^{3}H]-dihydroalprenolol; GTPγS, guanosine 5′-O-(3-thiotriphosphate); ISO, (−)isoproterenol; SAL, salbutamol; DOB, dobutamine; EPH, (−)ephedrine; DCI, dichloroisoproterenol; ALP, (−) alprenolol; ICI, ICI-118551; APB, (±)-chloro-APB hydrobromide.

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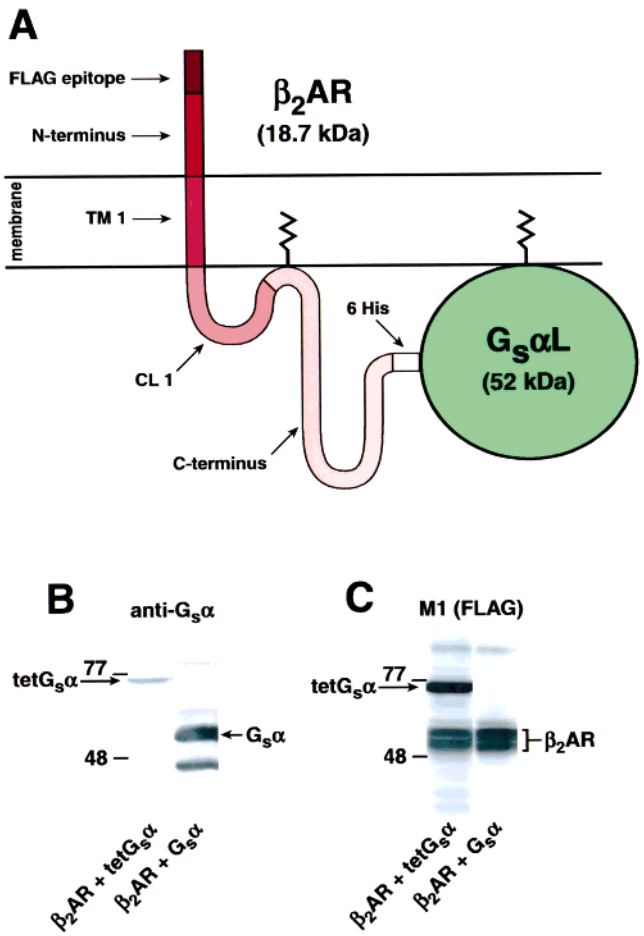


FIGURE 1: Schematic structure of membrane-tethered $G_s\alpha$ and its expression in Sf9 membranes. (A) Schematic structure of membrane-tethered $G_s\alpha$ ($tetG_s\alpha$). The FLAG epitope-tagged N-terminus, transmembrane domain 1 (TM1), cytoplasmic loop 1 (CL1), and C-terminus of the human β_2AR cDNA were linked to the N-terminus of $G_s\alpha$ by hexahistidine. (B) Detection of $G_s\alpha$ and $tetG_s\alpha$ coexpressed with β_2AR in Sf9 membranes by immunoblotting with anti-$G_s\alpha$ antibody. Minor band in the right lane shows degradation products of $G_s\alpha$. (C) Detection of the flag epitope-tagged $tetG_s\alpha$ and β_2AR in Sf9 membranes coexpressing β_2AR and $G_s\alpha$ or $tetG_s\alpha$ by immunoblotting with M1 monoclonal antibody. For both panels B and C, 50 μg of membrane protein was loaded/lane and resolved by 10% SDS−polyacrylamide gel electrophoresis then transferred to nitrocellulose. Numbers on the left are the molecular masses of marker proteins (kDa).

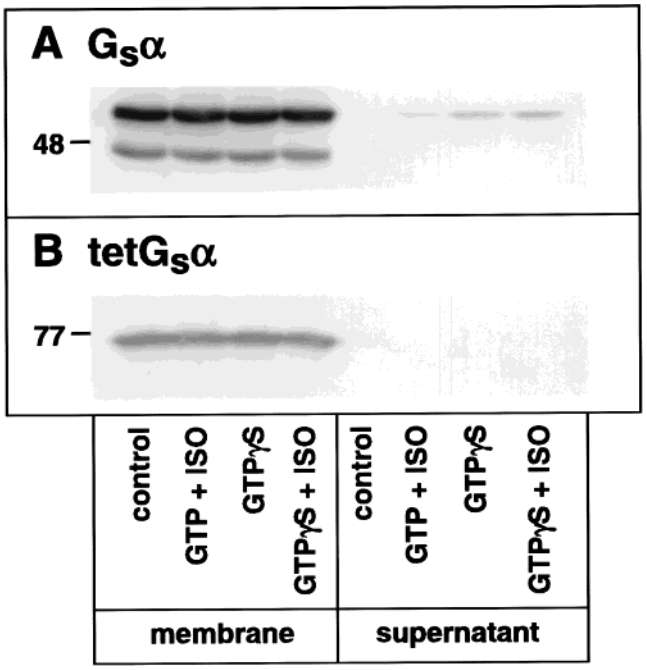


FIGURE 2: $G_s\alpha$ release from membrane to supernatant in membranes coexpressing β_2 adrenoceptor and $G_s\alpha$ or $tetG_s\alpha$. Immunoblots of supernatant and pellet fractions from membranes coexpressing β_2 adrenoceptor and $G_s\alpha$ (A) or β_2 adrenoceptor and $tetG_s\alpha$ (B) probed with anti-$G_s\alpha$ antibody. A total of 100 μg of each membranes were incubated at 37 °C with isoproterenol (100 μM) plus GTP (10 μM), with GTPγS (10 μM), or with isoproterenol (100 μM) plus GTPγS (10 μM) for 1 h. Following the incubation, the membranes were centrifuged and proteins from supernatants and pellets were resolved by 10% SDS−polyacrylamide gel electrophoresis followed by immunoblotting as described in the Experimental Procedures. Similar results were obtained in three different experiments.

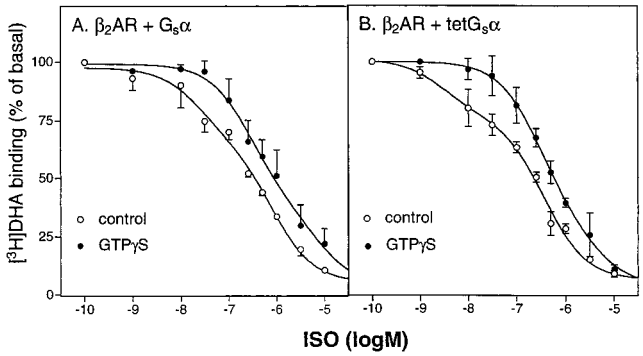


FIGURE 3: [^{3}H]DHA competition binding in Sf9 membranes coexpressing β_2 adrenoceptor and $G_s\alpha$ or $tetG_s\alpha$. Competition binding of [^{3}H]dihydroalprenolol (1 nM) with varying concentrations of isoproterenol in membranes coexpressing β_2 adrenoceptor with $G_s\alpha$ (A), and with $tetG_s\alpha$ (B) in the absence (○) or presence (●) of 10 μM GTPγS was performed as described in the Experimental Procedures. Expression levels of β_2 adrenoceptor in A and B are 7.4 and 4.8 pmol/mg, respectively. Data are expressed as percent of basal bound [^{3}H]DHA. Data shown are the mean ± SD of three independent experiments performed in triplicate.

(85−90 Ci/mmol) was from Amersham (Arlington Heigths, IL). Anti-$G_s\alpha$ antibody was from Calbiochem (San Diego, CA). Guanosine 5′-*O*-thiotriphosphate (GTPγS) was from Boehringer Mannheim (Mannheim, Germany). Sources of other materials have been described elsewhere (*22*, *23*).

Construction and Expression of tet$G_s\alpha$ DNA. To construct a membrane anchor, DNA sequences encoding amino acids 1−64 of the β_2 adrenoceptor (*24*−*26*) were fused with amino acids 343−412 of the β_2 adrenoceptor using a linker-adaptor. The linker-adaptor (which encoded the amino acid sequence TVTNYFR) connected the *Pst*I site in the DNA sequence encoding the first cytoplasmic loop with the *Ban*II site in the DNA sequence encoding the carboxyl-terminus. A cDNA encoding the $\beta_2G_s\alpha$ fusion protein (*22*, *23*) in pVL1392 (pVL1392 $\beta_2G_s\alpha$) was digested with *Nco*I and *Eco*RV to remove the sequence encoding amino acids 1−370 of the β_2 adrenoceptor. The *Nco*I−*Eco*RV fragment from the membrane anchor was ligated into the large *Nco*I−*Eco*RV fragment from pVL1392 $\beta_2G_s\alpha$ to produce $tetG_s\alpha$ in pVL1392 (Figure 1). To construct TEV-tetGsα (Figure 7), a sequence of 27 base pairs (ACT AGT GAA AAT CTT TAT TTC CAG GGA) encoding a Tobacco Etch Virus (TEV) protease cleavage site was inserted between the sequence encoding the membrane tether and the sequence encoding the amino-terminus of $G_S\alpha$, by overlap-extension PCR.

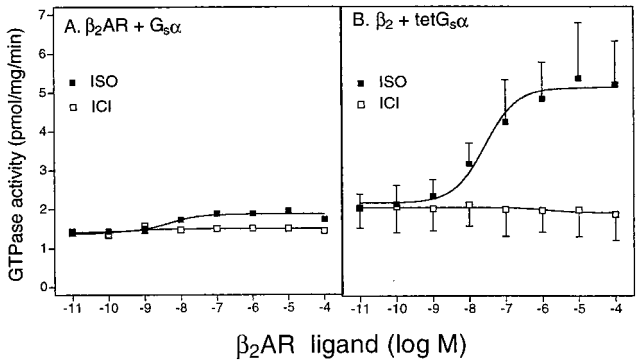


FIGURE 4: Comparison of the ligand regulation of GTPase activity in membranes coexpressing β_2 adrenoceptor and $G_s\alpha$, or tet$G_s\alpha$. GTP hydrolysis was measured with 100 nM [γ-^{32}P]GTP as substrate in the presence of isoproterenol (●) or ICI-118551 (○) in membranes (10 μg of protein) coexpressing β_2 adrenoceptor (7.4 pmol/mg) with $G_s\alpha$ (A) and β_2 adrenoceptor (4.8 pmol/mg) with tet$G_s\alpha$ (B). Data shown are the means ± SD of three independent experiments performed in triplicate.

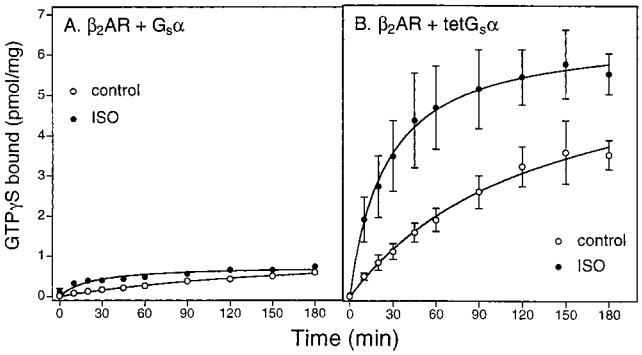
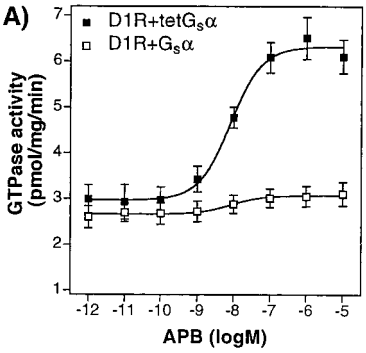


FIGURE 5: Time course of [^{35}S]GTPγS binding in membranes coexpressing β_2 adrenoceptor and $G_s\alpha$ or tet$G_s\alpha$. Membranes (15 μg of protein) coexpressing β_2 adrenoceptor and $G_s\alpha$ (A) or tet$G_s\alpha$ (B) were incubated with 1 nM [^{35}S]GTPγS and 1 μM GDP in the absence (○) or presence (●) of 10 μM isoproterenol as described in the Experimental Procedures. Expression levels of β_2 adrenoceptor in panels A and B are 7.4 and 4.8 pmol/mg, respectively. Data shown are the mean ± SD of three independent experiments performed in triplicate.



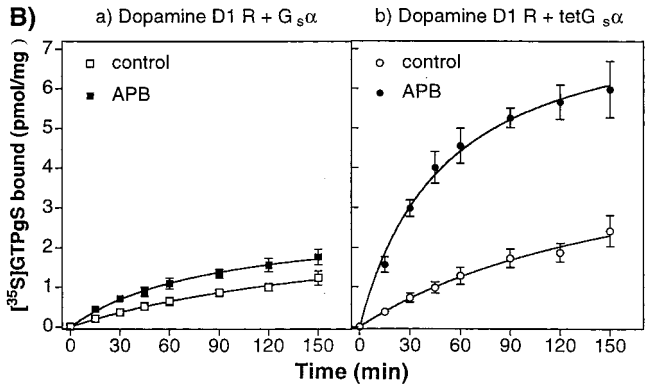


FIGURE 6: Regulation of G protein activity by the D1 dopamine receptor. (A) Comparison of the ligand regulation of GTPase activity in membranes coexpressing D1 dopamine receptor and $G_s\alpha$ or tet$G_s\alpha$. GTP hydrolysis was measured with 100 nM [γ-^{32}P]GTP as substrate in the presence of chloro-APB hydrobromide (APB) in membranes (10 μg of protein) coexpressing D1 dopamine receptor (4.9 pmol/mg) with $G_s\alpha$, and D1 dopamine receptor (5.0 pmol/mg) with tet$G_s\alpha$ as described in the Experimental Procedures. (B) Time course of [^{35}S]GTPγS binding in membranes coexpressing D1 dopamine receptor and $G_s\alpha$ or tet$G_s\alpha$. Membranes (15 μg of protein) coexpressing D1 dopamine receptor and $G_s\alpha$ (a), or tet$G_s\alpha$ (b) were incubated with 1 nM [^{35}S]GTPγS and 1 μM GDP in the absence (open symbol) or presence (closed symbol) of 1 μM of APB as described in the Experimental Procedures. Expression levels of D1 dopamine receptor in A and B are 4.9 and 5.0 pmol/mg, respectively. The expression levels of tet$G_s\alpha$ and $G_s\alpha$ in cells expressing the D1 receptor are similar to the level of tet$G_s\alpha$ in cells expressing the β_2AR (Figure 1) (data not shown). Data shown are the mean ± SD of three independent experiments performed in triplicate.

Cell Culture. Recombinant baculoviruses were generated and amplified as described (*22*, *23*). Virus stocks were characterized by infecting Sf9 cells seeded at 3.0×10^6 cells/mL with dilutions ranging from 1:50 to 1:500 to determine the dilution that produced the highest expression of recombinant protein. Cells were incubated in a 27 °C shaker (125 rpm) for 48 h. For coexpression studies, a series of Sf9 cell cultures (100−200 mL) was infected with a 1:500 dilution of a high titer β_2 adrenoceptor baculovirus stock and a 1:200−1:500 dilution of a high titer $G_s\alpha$ or tet$G_s\alpha$ baculovirus stock in order to achieve a desired receptor to G protein stoichiometry. Cultures having the desired expression levels of receptor and G protein were selected for study. Membranes were prepared as described previously (*22*, *23*). All of the β_2AR coexpression studies comparing WT-Gsα and tetGsα were done with the same batch of membranes.

[^{3}H]DHA Binding. Membrane (15−20 μg/tube) of protein from infected Sf9 insect cells were suspended in 500 μL of binding buffer (75 mM Tris/HCl, pH 7.4, 12.5 mM $MgCl_2$, and 1 mM EDTA), supplemented with 10 nM [^{3}H]DHA and 0.2% (w/v) BSA to determine β_2 adrenoceptor expression level. Nonspecific binding was assessed in the presence of 10 μM (−)-alprenolol. Incubations were performed for 60 min at 25 °C with shaking at 200 rpm. Competition binding experiments were carried out with 15−20 μg of membrane protein/tube with 1 nM [^{3}H]DHA in the presence of isoproterenol at various concentrations without or with GTPγS (10 μM) as described elsewhere (*22*, *23*).

GTPase Activity. For construction of dose response curves to ligands, assay tubes (100 μL) contained 10 μg of membrane protein, 0.1 μM [γ-^{32}P]GTP (0.1 μCi/tube), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP, 1 mM adenylyl imidodiphosphate, 5 mM creatine phosphate, 40 μg of creatine kinase, and 0.2% (w/v) BSA in 50 mM Tris/HCl, pH 7.4. Reactions were performed for 20 min at 25 °C and were terminated by the addition of 900 μL of a slurry consisting of 5% (w/v) activated charcoal and 50 mM NaH_2PO_4, pH 2.0, as described elsewhere (*22*). Reaction mixtures were centrifuged for 15 min at room temperature at 15000*g*. 700 μL of the supernatant of reaction mixtures were transferred, and [^{32}P]P_i was determined by liquid scintillation counting.

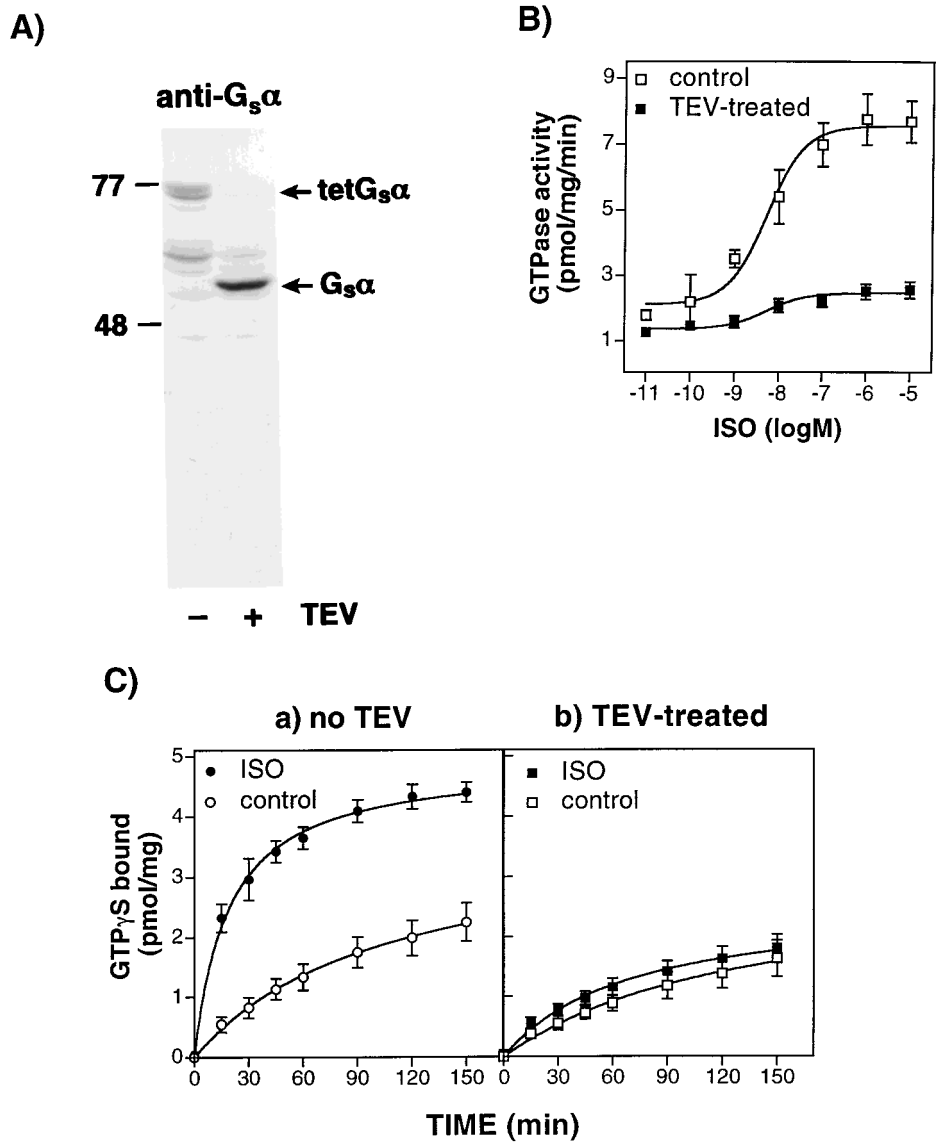


FIGURE 7: Reversal of membrane tethering by site-specific TEV cleavage. β_2AR (11.1 pmol/mg) was coexpressed with TEV-tetG$_s\alpha$, which encodes TEV protease cleavage site between receptor portion and the N-terminus of G$_s\alpha$ in Sf9 membranes. (A) Western detection of TEV protease treated-TEV-tetG$_s\alpha$ in Sf9 membranes (50 μg) with anti-G$_s\alpha$ antibody (1:1000). (B) Isoproterenol stimulated GTP hydrolysis was measured as described in the Experimental Procedures in membranes with (closed symbol) or without (open symbol) TEV protease treatment (mean ± SD, $n = 3$). (C) [^{35}S]GTPγS binding was performed as described in the Experimental Procedures in the absence (open symbol) or presence (closed symbol) of 1 μM isoproterenol (ISO) without (a) or with (b) TEV protease treatment for 30 min at room temperature.

[^{35}S]GTPγS Binding. Membranes were pelleted by a 15 min centrifugation at 4 °C and 15000*g* and resuspended in binding buffer. Sf9 membranes (10 μg of protein/tube) were suspended in 500 μL of binding buffer supplemented with 0.05% (w/v) BSA, 1 nM [^{35}S]GTPγS (0.25 μCi/tube), GDP (1 μM) with or without isoproterenol (10 μM). Incubations were performed at 25 °C and shaking at 200 rpm. Nonspecific binding was determined in the presence of 10 μM GTPγS and was less than 0.2% of total binding. Bound [^{35}S]-GTPγS was separated from free [^{35}S]GTPγS by filtration through GF/C filters, followed by three washes with 3 mL of cold binding buffer. Filter-bound radioactivity was determined by liquid scintillation counting.

Adenylyl Cyclase Activity. Assay tubes (50 μL) contained 15 μg of membrane protein, guanine nucleotides, and ligands as indicated in Figure 7 legend, 40 μM [α-^{32}P]ATP (2.5 μCi/tube), 2.7 mM mono(cyclohexyl)ammonium phosphoenolpyruvate, 0.125 IU of pyruvate kinase, 1 IU of myokinase, 0.1 mM cAMP and 5 mM $MgCl_2$, 0.4 mM EDTA, and 30 mM Tris/HCl, pH 7.4. Reactions were conducted for 20 min at 37 °C. Separation of [^{32}P]cAMP from [α-^{32}P]ATP was performed as described (*27*).

Western Blot Analysis. For translocation studies, membranes (100−200 μg of protein/reaction) were resuspended in binding buffer containing various combinations of compounds (100 μM GTP, 100 μM isoproterenol, and 100 μM GTPγS) as indicated in Figure 2. The membrane suspensions were incubated for 1 h at 37 °C. After centrifugation at 150000*g* for 30 min in Beckman TL100, the supernatant fractions were precipitated by 2% (w/v) deoxycholate/24% (w/v) trichloroacetic acid, and neutralized with 20 μL of 1 M Tris base solution. Precipitated protein was then dissolved in 50 μL of Laemmli sample buffer and proteins were separated by 10% SDS−polyacrylamide gel electrophoresis. Proteins were transferred onto nitrocellulose membranes and blotted with anti-G$_s\alpha$ antibody (1:1000). For general immu-

noblotting, membrane proteins (50 μg/lane) were loaded and analyzed with either M1 monoclonal antibody (1:1000) or anti-$G_s\alpha$ antibody (1:1000). Expression of $G_s\alpha$ and tet$G_s\alpha$ in Sf9 membranes was quantitated by densitometer.

Sucrose Density Gradient. For subcellular fractionation, pellets of Sf9 cells infected with baculovirus encoding β_2-AR and either tetGsα or WT-$G_s\alpha$ were homogenized in ice-cold buffer (20 mM Tris/HCl, pH 7.4, 3 mM $MgCl_2$, 1 mM EDTA), including protease inhibitor cocktail (Boehringer Mannheim) with 30−35 strokes. Two millilliters of homogenates (20−40 mg of total protein) was applied on top of the discontinuous sucrose density gradients consisting of 19, 23, 27, 31, 35, and 43% (g/100 ml) sucrose in 20 mM Tris/HCl, pH 8.0, 3 mM $MgCl_2$, 1 mM EDTA, and centrifuged for 30 min at 27 000 rpm in a Beckman SW28 rotor at 4 °C as described elsewhere (*28*). Fractions of 5 mL were collected, and proteins from 750 μL of each fractions were precipitated by 2% (w/v) deoxycholate/24% (w/v) trichloroacetic acid, and neutralized with 30 μL of 1 M Tris base solution. Precipitated protein was then dissolved in 70 μL of Laemmli sample buffer and separated by 10% SDS−polyacrylamide gel electrophoresis.

Miscellaneous. Protein concentration was determined using the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Data were analyzed by nonlinear regression, using the Prism program (GraphPad, San Diego, CA). Data are given as means ± SD of 3−4 independent experiments performed in triplicate.

RESULTS

Expression of β_2 Adrenoceptor, $G_s\alpha$, and tet$G_s\alpha$. To investigate the functional significance of $G_s\alpha$ dissociation from the plasma membrane following receptor activation, we designed a membrane tethered form of $G_s\alpha$ (tet$G_s\alpha$). The tet$G_s\alpha$ was constructed by fusing the amino-terminus of $G_s\alpha$ to the carboxyl-terminus of a single membrane-spanning protein. The single membrane-spanning protein was made by fusing the amino-terminal 64 amino amino acids of the β_2 adrenoceptor to its carboxyl-terminal 70 amino acids (Figure 1A). To evaluate the function of tet$G_s\alpha$, the β_2 AR was expressed in Sf9 insect cells together with either tet$G_s\alpha$ (tet$G_s\alpha$ membranes) or $G_s\alpha$ ($G_s\alpha$ membranes). Western blot analysis of membrane preparations is shown in Figure 1, panels B and C. Expression of $G_s\alpha$ and tet$G_s\alpha$ was detected using anti-$G_s\alpha$ antibody. The apparent molecular masses of $G_s\alpha$ and tet$G_s\alpha$ are 52 and 70 kDa, respectively (Figure 1B). The mobility of tet$G_s\alpha$ is consistent with the known molecular mass of $G_s\alpha$ (52 kDa) and the predicted molecular mass of the membrane-tether (18 kDa). The expression level of $G_s\alpha$ was approximately 10-fold higher than that of tet$G_s\alpha$ by densitometric quantitation. The tet$G_s\alpha$ and the β_2 adrenoceptor are both tagged at the amino-terminus with the FLAG epitope, and their expression was also examined using M1 monoclonal antibody (Figure 1C). The expression level of the β_2 adrenoceptor is similar in the two membrane preparations as shown by Western blot analysis (Figure 1C) and [^{3}H]DHA binding (7.4 pmol/mg in $G_s\alpha$ membranes; 4.8 pmol/mg in tet$G_s\alpha$ membranes). Moreover, based on immunoreactivity with the M1 antibody, tet$G_s\alpha$ expression level is comparable to that of β_2 adrenoceptor. Thus, in $G_s\alpha$ membranes, $G_s\alpha$ is approximately 10 times more abundant than the β_2 adrenoceptor. Immunoreactive bands corresponding to the β_2 adrenoceptor, $G_s\alpha$, or tet$G_s\alpha$ were not detected in uninfected cells with either anti-$G_s\alpha$ or M1 monoclonal antibody (data not shown).

Table 1: [^{3}H]DHA Competition Binding Properties of Isoproterenol in Membranes Coexpressing β_2AR and $G_s\alpha$ or Tet$G_s\alpha$

	β_2AR[a] + $G_s\alpha$	β_2AR[a] + tet$G_s\alpha$
K_h[b] (nM)	15.2 ± 8.9	4.2 ± 1.3
K_l[b] (μM)	0.8 ± 0.1	0.5 ± 0.1
$\%R_h$[c]	32.3 ± 7.5	28.6 ± 5.8
$K_{hGTP\gamma S}$[d] (μM)	27.9 ± 0.5	30.1 ± 1.0
$K_{lGTP\gamma S}$[d] (μM)	6.7 ± 1.5	3.6 ± 1.6

[a] Expression levels of β_2 adrenoceptor in coexpression with WT-$G_s\alpha$ or with tet$G_s\alpha$ were 7.4 and 4.8 pmol/mg protein, respectively. Competition binding isotherms were analyzed by nonlinear regression fitting to two-site binding. [b] K_h and K_l designate the dissociation constants for the high- and low-affinity state of the β_2 adrenoceptor, respectively. [c] $\%R_h$ indicates the percentage of high-affinity binding sites. [d] $K_{hGTP\gamma S}$ and $K_{lGTP\gamma S}$ designate the corresponding values obtained in the presence of GTPγS (10 μM). Data shown are the means ± SD ($n = 3$).

Agonist-Stimulated $G_s\alpha$ Release from Sf 9 Membranes. $G_s\alpha$ has been observed to translocate from the plasma membrane to the cytoplasm following receptor activation in several mammalian cell lines (*13*−*17*)] (*11*). As shown in Figure 2, this phenomenon is also observed in Sf9 membranes expressing wild-type $G_s\alpha$, but not in membranes expressing tet$G_s\alpha$. Only a small fraction of $G_s\alpha$ is released following incubation with isoproterenol plus GTP, with GTPγS alone, or with GTPγS plus isoproterenol. This may be a consequence of the large excess of $G_s\alpha$ relative to β_2 adrenoceptor in these membranes.

Receptor-G Protein Coupling. The efficiency of receptor−G protein coupling was assessed in several ways. GTP-sensitive, high-affinity agonist binding reflects the formation of the ternary complex between agonist, receptor, and guanine nucleotide-free G protein (*22, 23, 29*−*33*). We have previously observed that the β_2 adrenoceptor couples poorly to endogenous insect cell G proteins, i.e., no GTPγS sensitive, high-affinity binding is observed in the absence of coexpressed mammalian $G_s\alpha$ (*22, 23*). As shown in Figure 3, GTPγS-sensitive high-affinity binding of the agonist isoproterenol was observed in both $G_s\alpha$ and tet$G_s\alpha$ membranes. Approximately 30% GTPγS-sensitive high-affinity agonist binding sites were observed for the β_2 adrenoceptor in both $G_s\alpha$ and tet$G_s\alpha$ expressing membranes (Table 1). This was surprising since the ratio of G protein to receptor (moles of $G_s\alpha$ per moles of β_2 adrenoceptor) was ∼10 in $G_s\alpha$ membranes and ∼1 in tet$G_s\alpha$ membranes.

We next examined basal and agonist-stimulated GTPase activity in Sf9 membranes expressing β_2 adrenoceptor and either $G_s\alpha$ or tet$G_s\alpha$. GTPase activity measures the velocity of the complete G protein cycle (*1*) (*23*). There was a modest (∼1.6 − fold) elevation of basal GTPase activity in tet$G_s\alpha$ membranes compared to $G_s\alpha$ membranes; however, isoproterenol-stimulated GTPase activity in tet$G_s\alpha$ membranes was ∼2.8 − fold higher than the activity in $G_s\alpha$ membranes. There was no significant response to the inverse agonist ICI-118551 for either membrane preparation (Figure 4).

GTPγS binding measures the uptake of the nonhydrolyzable GTP analogue GTPγS to the α-subunit and is therefore not a steady-state assay (*1, 23*). However, this assay provides

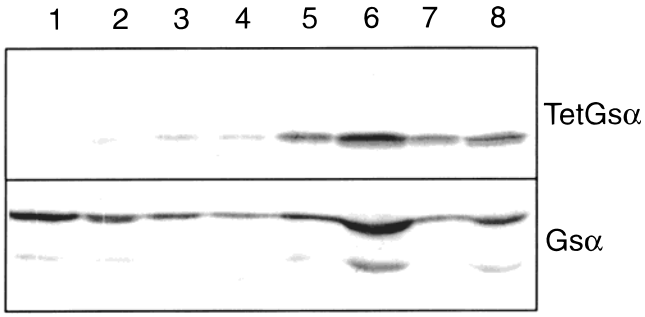


FIGURE 8: Sucrose density gradient analysis of the distribution of $G_s\alpha$ and tet$G_s\alpha$ in whole cell lysates. Whole-cell lysates were separated on a discontinuous sucrose gradient as described in the Experimental Procedures. Fractions were subjected to Western Blot analysis using antibody to $G_s\alpha$. Previous studies (*28*) have shown that fraction 1 contains predominantly cytosolic proteins, fractions 2−3 contain light vesicles including endoplasmic reticulum, fractions 4−6 contain plasma membranes, fraction 7 contains mitochondria, and fraction 8 is the pellet containing unbroken cells and nuclei.

information about the number of G proteins accessible to receptors during a given period of time (*1, 23*). Surprisingly, both basal and isoproterenol-stimulated GTPγS binding were much higher in tet$G_s\alpha$ membranes than in $G_s\alpha$ membranes, even though the tet$G_s\alpha$ membranes had a lower G protein expression level than $G_s\alpha$ membranes (Figure 5).

Efficient coupling with tet$G_S\alpha$ is not limited to the β_2 adrenoceptor. Basal and agonist-stimulated GTPγS binding and GTPase activity were both significantly greater in membranes expressing the D1 dopamine receptor and tet$G_S\alpha$ than in membranes expressing the D1 dopamine receptor and wild-type $G_S\alpha$ (Figure 6). In these experiments, tet$G_S\alpha$ and $G_S\alpha$ were expressed at similar levels as determined by Western Blot analysis.

The efficient coupling observed with tet$G_S\alpha$ may not be due to the membrane tether, but may reflect differences in the processing of a tethered $G_S\alpha$ during biosynthesis in Sf9 cells. To test this hypothesis, we generated a cleavable form of tet$G_S\alpha$ (TEVtet$G_S\alpha$) in which the carboxyl-terminus of the membrane tether is separated from the amino-terminus of $G_S\alpha$ by a cleavage site for Tobacco Etch Viurs (TEV) protease. TEV protease efficiently cleaves $G_S\alpha$ from the membrane tether (Figure 7A), and dramatically reduces the efficiency of coupling to the β_2 adrenoceptor as determined by GTPγS binding (Figure 7C) and GTPase activity (Figure 7B). Thus, it appears that $G_S\alpha$ released from TEVtet$G_S\alpha$ following TEV cleavage is not functionally superior to wild-type $G_S\alpha$ expressed in insect cells.

Another possible explanation for the observed difference in the efficiency of coupling of $G_s\alpha$ and tet$G_s\alpha$ could be a difference in the distribution of these two proteins in cellular membrane compartments. Experiments shown in Figures 3−7 were performed on a crude membrane fraction that contains plasma membrane as well as intracellular membrane organelles. To examine the distribution of $G_s\alpha$ and tet$G_s\alpha$ in insect cell membranes, we fractionated whole-cell lysates (including cytosolic proteins) from Sf9 cells expressing either $G_s\alpha$ or tet$G_s\alpha$ in a discontinuous sucrose density gradient and subjected the fractions to Western blot analysis using anti-$G_s\alpha$ antibody (See Figure 8). A significant amount of $G_s\alpha$ was observed in the lowest density fraction representing cytosolic proteins (fraction 1 and partly 2). No cytosolic pool

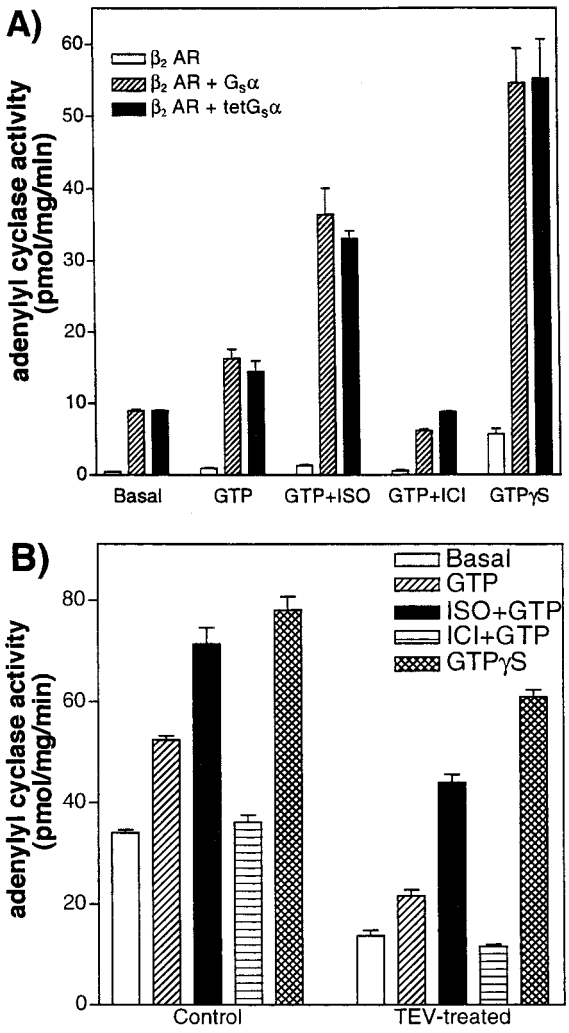


FIGURE 9: Regulation of adenylyl cyclase activity in membranes expressing β_2 adrenoceptor alone, or together with $G_s\alpha$ or tet$G_s\alpha$. (A) Adenylyl cyclase activity was determined in membranes (20 μg) expressing β_2 adrenoceptor alone or together with $G_s\alpha$ or tet$G_s\alpha$: (a) basal, (b) 1 μM GTP, (c) 1 μM GTP plus 1 μM isoproterenol, (d) 1 μM GTP plus 1 μM ICI-118551, and (e) 1 μM GTPγS. The data shown are the representative of four independent experiments (mean ± SD). Similar results were produced with different membrane preparations. (B) Adenylyl cyclase activity was determined (as described above) in membranes expressing tet$G_s\alpha$ having a TEV cleavage site between the tethered portion and $G_s\alpha$. Membranes were incubated with TEV or buffer control for 30 min at room temperature before performing adenylyl cyclase experiments.

of tet$G_s\alpha$ was observed. However, the relative distribution of $G_s\alpha$ immunoreactivity in the denser gradient fractions (fractions 3−8) representing cellular membrane components was nearly identical for $G_s\alpha$ and tet$G_s\alpha$ with the majority of both proteins sedimenting in fraction 6. Thus, it appears that differences in the distribution of $G_s\alpha$ and tet$G_s\alpha$ in cellular membrane compartments cannot explain the functional differences in receptor−G protein coupling.

G Protein−Effector Coupling. Figure 9A shows the comparison of receptor-mediated activation of adenylyl cyclase through $G_s\alpha$ and through tet$G_s\alpha$. The coupling observed in these experiments is predominantly through the coexpressed mammalian $G_s\alpha$ or tet$G_s\alpha$ rather than through the insect cell G protein. We have previously shown that coupling of the β_2 receptor to insect cell adenylyl cyclase through insect $G_s\alpha$-like G protein is inefficient and that the

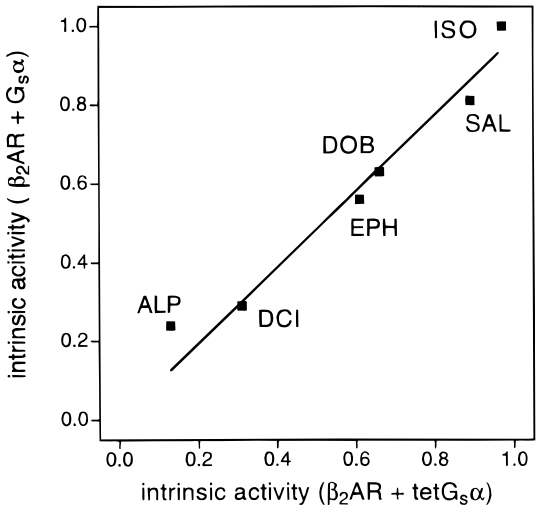


FIGURE 10: Intrinsic adenylyl cyclase activities of full and partial β_2 adrenoceptor agonists in membranes coexpressing β_2 adrenoceptor and $G_s\alpha$ or $tetG_s\alpha$. Adenylyl cyclase activity was measured in the membrane proteins (20 μg) coexpressing β_2 adrenoceptor and $G_s\alpha$ or $tetG_s\alpha$ as described in the Experimental Procedures. Reaction mixtures contained ligands at 1 μM to 1 mM as determined from saturated dose response curves: isoproterenol (10 μM), salbutamol (100 μM), dobutamine (100 μM), ephedrine (1 mM), dichloroisoproterenol (100 μM), and alprenolol (1 μM) were used. The intrinsic activities of ligands at β_2 adrenoceptor in $G_s\alpha$ membranes were plotted against their intrinsic activities at β_2 adrenoceptor in $tetG_s\alpha$ membranes.

coupling efficiency can be dramatically enhanced by coexpressing mammalian $G_s\alpha$ with the β_2 receptor (*22*, *23*). As shown in Figure 9A, basal, isoproterenol-stimulated, and ICI-118551-inhibited adenylyl cyclase activities are similar for $tetG_S\alpha$ and $G_S\alpha$ membrane preparations. Moreover, the intrinsic activities of various agonists and partial agonists are the same in the two membrane preparations (Figure 10). This is further confirmation that the $G_s\alpha$ component of $tetG_s\alpha$ is not affected by the membrane tether. We have previously shown that subtle functional differences between the short and long splice isoforms of $G_s\alpha$ are readily detected by differences in intrinsic activities of β_2 receptor agonists (*22*).

To further examine the effect of the membrane tether on coupling of $G_s\alpha$ to adenylyl cyclase, we studied membranes expressing $tetG_s\alpha$ with a TEV cleavage site between the tethered portion and $G_s\alpha$. As shown in Figure 7, TEV cleavage dramatically reduces coupling of receptor to $G_s\alpha$ as determined by GTPase activity and GTPγS binding. In contrast to the experiments shown in Figure 9A, receptor, adenylyl cyclase and G protein levels are identical in the two membrane preparations, the only difference being cleavage of the membrane tether. TEV cleavage has significant effects on basal and agonist activated adenylyl cyclase (Figure 9B).

DISCUSSION

In our present study we examined the functional consequence of restricting the mobility of $G_s\alpha$ by membrane tethering. $G_s\alpha$ has been shown to dissociate from the plasma membrane in several cell lines (*11*, *13*−*17*). This may in part be due to dissociation of $G_s\alpha$ from the $\beta\gamma$ subunit, but may also require hydrolysis of the thioester link between the N-terminal cysteine and palmitic acid. It has previously been shown that the amino-terminus of $G_s\alpha$ can be fused to the carboxyl-terminus of the β_2 adrenoceptor (*22*, *23*, *34*). We therefore used this approach to tether $G_s\alpha$ to the plasma membrane by using the first membrane-spanning domain of the β_2 adrenoceptor as a membrane anchor. We were surprised to find that $tetG_s\alpha$ coupled much more efficiently to the β_2 adrenoceptor than did the wild-type $G_s\alpha$, even though the density of wild-type $G_s\alpha$ was at least 10-fold higher than the density of $tetG_s\alpha$. The most striking functional differences between $tetG_s\alpha$ and wild-type $G_s\alpha$ were observed with respect to receptor-stimulated GTPase activity and GTPγS binding. The steady-state GTPase activity measures the ability of receptor to stimulate multiple G protein activation/inactivation cycle. These GTPase results can be explained by postulating that $tetG_s\alpha$ was capable of repeated cycles of interaction with an agonist-liganded receptor while the wild-type $G_s\alpha$ was only capable of a single interaction because after activation $G_s\alpha$ dissociated from the receptor. GTPase activity is therefore limited by the rate of diffusion of new $G_s\alpha$ molecules to receptor molecules. It should be noted that the fraction of $G_s\alpha$ that dissociates from insect cell membranes following activation (Figure 2) is relatively small. This may reflect the marked overexpression of $G_s\alpha$ relative to the β_2 adrenoceptor. Nevertheless, the experiment shown in Figure 2 does not constitute strong support for the hypothesis that the highly efficient coupling of $tetG_s\alpha$ relative to $G_s\alpha$ is solely due to the inability of $tetG_s\alpha$ to dissociate from the plasma membrane.

The functional difference between $G_s\alpha$ and $tetG_s\alpha$ was even more striking when GTPγS binding was examined (Figure 5). GTPγS binding in Sf9 membranes must be performed in the presence of 1 μM GDP to reduce the background binding of GTPγS to endogenous insect cell G proteins (*22*, *23*, *35*, *36*). Under these conditions, we observed very little β_2 adrenoceptor-stimulated GTPγS binding even with the large excess of $G_s\alpha$ (Figure 5A). However, both basal and isoproterenol-stimulated GTPγS binding were dramatically increased in membranes expressing $tetG_s\alpha$ (Figure 5B). This may in part due to more efficient coupling of receptor to the tethered G protein. It may also reflect the fact that GTPγS binding may not be irreversible. It has been shown that GTP analogues can dissociate from $G_s\alpha$ after the G protein dissociates from the receptor (*23*, *37*−*41*). This may indicate that restricting the mobility of $G_s\alpha$ would facilitate receptor-mediated rebinding of GTPγS to $tetG_s\alpha$ molecules that have lost GTPγS. Thus, by restricting the mobility of $G_s\alpha$ relative to the membrane, it is possible to increase the apparent binding capacity of the G protein for GTPγS.

The high efficiency of coupling between $tetG_S\alpha$ and the β_2 adrenoceptor suggests that restricting the mobility of $G_S\alpha$ promotes physical interactions with the receptor. However, other mechanisms for enhanced coupling must be considered. Tethering of $G_S\alpha$ to the membrane may alter its biosynthesis so that a greater proportion of $tetG_S\alpha$ expressed in insect cells is functional. Alternatively, $tetG_S\alpha$ may be more efficient than $G_S\alpha$ in competing with insect cell Gα-subunits for insect cell $\beta\gamma$ subunits following biosynthesis. To test these hypotheses, we generated a cleavable form of $tetG_S\alpha$ ($TEVtetG_S\alpha$) in which the carboxyl-terminus of the membrane tether is separated from the amino-terminus of $G_S\alpha$ by a cleavage site for Tobacco Etch Viurs (TEV) protease. TEV protease efficiently cleaves $G_S\alpha$ from the membrane

tether (Figure 7A) and dramatically reduces the efficiency of coupling to the β_2 adrenoceptor as determined by GTPγS binding (Figure 7C) and GTPase activity (Figure 7B). Thus, there is no evidence that the enhanced coupling efficiency of tet$G_S\alpha$ is due to more efficient biosynthetic processing of $G_S\alpha$.

Another possible mechanism for enhanced receptor−G protein coupling in tet$G_S\alpha$ membranes is the formation of a complex between β_2 adrenoceptor sequences (amino-terminus, TM1, and the carboxyl-terminus) in tet$G_S\alpha$ and complementary domains of the coexpressed β_2 adrenoceptor. If this were the case, we would expect the highly efficient coupling between receptor and tet$G_S\alpha$ would be restricted to the β_2 adrenoceptor. However, we also find that the D1 dopamine receptor couples more efficiently to tet$G_S\alpha$ than to wild-type $G_S\alpha$ (Figure 6). Nevertheless, we cannot rule out a nonspecific hydrophobic interaction between the transmembrane domain of tet$G_s\alpha$ and transmembrane domain(s) of both the β_2AR and the D1 dopamine receptor.

The poor coupling of $G_s\alpha$ compared to tet$G_s\alpha$ may reflect a relative deficiency of $\beta\gamma$ in cells overexpressing $G_s\alpha$. Thus, the membrane tether in tet$G_s\alpha$ may substitute for $\beta\gamma$. We have previously addressed this question when comparing the properties of β_2AR-$G_s\alpha$ fusion proteins to β_2AR and $G_s\alpha$ expressed as separate proteins (*23*). In these studies, we compared β_2AR expressed with $G_s\alpha$ alone or together with $\beta_1\gamma_2$. Coexpression of $\beta_1\gamma_2$ had a modest effect on high-affinity agonist binding (35% with Gsα alone compared to 43% with $G_s\alpha + \beta_1\gamma_2$), but no significant effect on β_2AR stimulation of GTPase activity, GTPγS binding, or adenyly cyclase. Therefore, it does not appear that the properties of tet$G_s\alpha$ are solely due to the tether functioning as a surrogate for $\beta\gamma$.

In contrast to the results observed in GTPase and GTPγS binding assays, the β_2 adrenoceptor-stimulated adenylyl cyclase in $G_s\alpha$ membranes and in tet$G_s\alpha$ membranes were similar. We have previously observed that the β_2 adrenoceptor couples inefficiently to insect cell adenylyl cyclase via the insect cell G_s-like G protein. However, the coupling efficiency can be dramatically improved if mammalian $G_s\alpha$ is coexpressed in insect cells with the β_2 adrenoceptor (*23*). Therefore, the basal and agonist-stimulated adenylyl cyclase activity in membranes expressing $G_s\alpha$ and tet$G_s\alpha$ is largely due to the mammalian G protein (Figure 9).

Experiments shown in Figure 9A demonstrate that tet$G_s\alpha$ can effectively interact with adenylyl cyclase. However, when considering the efficiency of receptor−G protein interactions (Figures 3−6), one might expect that tet$G_s\alpha$ would be even more effective at activating adenylyl cyclase. Since the GTPase and GTPγS binding studies reflect the efficiency of G protein activation, we might expect much more efficient basal and agonist stimulated adenylyl cyclase activation in tet$G_s\alpha$ membranes compared to $G_s\alpha$ membranes. This apparent reduction in efficiency of adenylyl cyclase activation in tet$G_s\alpha$ membranes may be due to the fact that adenylyl cyclase is limited and the relationship between picomoles of adenylyl cyclase activated and picomoles of GTPγS bound is not linear. It is also possible that the more restricted mobility of tet$G_S\alpha$ may limit access to adenylyl cyclase. Following activation, tet$G_s\alpha$ may only be able to activate adenylyl cyclase molecules in the immediate neighborhood of the receptor. In contrast, $G_s\alpha$ may be more free to diffuse in the plane of the membrane or through the cytosol to reach distant adenylyl cyclase molecules. Nevertheless, site-specific cleavage of the membrane tether with TEV protease significantly reduces basal and agonist activated adenylyl cyclase (Figure 9B), suggesting that even if membrane tethering restricts access to adenylyl cyclase, this limitation is overcome by greater effect of membrane tethering on enhancing receptor−G protein coupling.

CONCLUSION

Several studies suggest that the plasma membrane distribution of $G_s\alpha$ is altered following activation. We have examined the functional significance of $G_S\alpha$ redistribution using a membrane tethered form of $G_s\alpha$ called tet$G_s\alpha$. Compared to $G_s\alpha$, tet$G_s\alpha$ coupled more efficiently to the β_2 adrenoceptor and the D1 dopamine receptor. The membrane tether did not prevent the coupling of tet$G_S\alpha$ to adenylyl cyclase. Our results suggest that redistribution of $G_s\alpha$ from the membrane may be important for regulating receptor−G protein coupling. Our studies also provide evidence that cytoskeletal proteins that promote the physical association of receptor and G proteins may greatly enhance coupling.

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