

# A Surface-Exposed Region of $G_{s\alpha}$ in Which Substitutions Decrease Receptor-Mediated Activation and Increase Receptor Affinity

GALINA GRISHINA and CATHERINE H. BERLOT

Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, Connecticut

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## ABSTRACT

The mechanism by which receptors activate G proteins is unclear because a connection between the receptor and the nucleotide binding site has not been established. To investigate this mechanism, we evaluated the roles in receptor interaction of three potential receptor contact sites in  $\alpha_s$ : the  $\alpha 2/\beta 4$ ,  $\alpha 3/\beta 5$ , and  $\alpha 4/\beta 6$  loops. Substitutions of  $\alpha_{i2}$  homologs for  $\alpha_s$  residues in the  $\alpha 2/\beta 4$  loop and alanine substitutions of residues in the  $\alpha 4/\beta 6$  loop do not affect activation by the  $\beta_2$ -adrenergic receptor. However, replacement of five  $\alpha_s$  residues in the  $\alpha 3/\beta 5$  loop region with the homologous  $\alpha_{i2}$  residues decreases receptor-mediated activation of  $\alpha_s$  and increases the affinity of  $G_s$  for this receptor. The substitutions do not alter guanine nucleotide binding or hydrolysis, or activation by aluminum fluoride, indi-

cating that the effects on receptor interaction are not due to a destabilization of the guanine-nucleotide bound state. In a model of the receptor-G protein complex, the  $\alpha 3/\beta 5$  loop maps near the second and third intracellular loops of the receptor. The effects of the  $\alpha 3/\beta 5$  substitutions suggest that the wild-type residues may be receptor contact sites that are optimized to ensure the reversibility of receptor-G protein interactions. Furthermore, the  $\alpha 3/\beta 5$  region corresponds to an exchange factor contact site in both EF-Tu and Ras, suggesting that the mechanisms by which seven-transmembrane receptors and exchange factors catalyze nucleotide exchange may share common elements.

Heterotrimeric G proteins transmit signals from cell surface receptors to intracellular effector proteins that modulate a wide variety of physiological processes (Neer, 1995). The  $\alpha$ - and  $\beta\gamma$ -subunits of G proteins are associated in the inactive GDP-bound form. Receptors activate G proteins by catalyzing replacement of GDP by GTP on the  $\alpha$ -subunit. Binding of GTP leads to dissociation of the receptor from  $\alpha \cdot \text{GTP}$  and  $\beta\gamma$ , each of which can transmit signals to effectors.

Our understanding of the mechanism of receptor-mediated activation of G proteins is incomplete. The predicted membrane-facing side of the heterotrimer includes the amino and carboxyl termini of the  $\alpha$ -subunit and places the nucleotide binding site too far away to contact the receptor (Fig. 1). Therefore, receptors are thought to stimulate nucleotide exchange through currently undefined conformational changes transmitted from the sites of receptor binding to the nucleotide binding pocket (Bohm et al., 1997; Bourne, 1997).

The  $\alpha$ -subunits consist of two domains: a GTPase domain that resembles that of EF-Tu and Ras, and a helical domain consisting of  $\alpha$ -helices and connecting loops (Fig. 1). The bound nucleotide is buried between these domains. Three regions in the GTPase domain (switches I–III; Fig. 1) assume different conformations in the structures of guanosine 5'-O-(3-thiotriphosphate) (GTP $\gamma$ S)-bound versus GDP-bound  $\alpha$ -subunits (Noel et al., 1993; Coleman et al., 1994; Lambright et al., 1994; Mixon et al., 1995). These regions are likely to play a role in receptor-mediated activation, because switches I and II contact  $\beta\gamma$  (Lambright et al., 1996), with which  $\alpha$  must associate to be activated by receptors (Fung, 1983), and mutations that disrupt interactions between switch III and the helical domain impair receptor-mediated activation (Grishina and Berlot, 1998; Marsh et al., 1998; Warner et al., 1998).

Previous studies have identified regions of  $\alpha_s$  that play a role in activation by the  $\beta_2$ -adrenergic receptor (Hildebrandt et al., 1991; Codina and Birnbaumer, 1994; Iiri et al., 1997; Grishina and Berlot, 1998; Marsh et al., 1998; Warner et al., 1998). Of these regions, many are near the nucleotide, and only the extreme carboxyl terminus is likely to be a receptor contact site. However, the carboxyl terminus is not always necessary or sufficient to confer receptor specificity (Lee et

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<sup>1</sup> Residue numbering throughout is according to the long splice variant of  $\alpha_s$ .

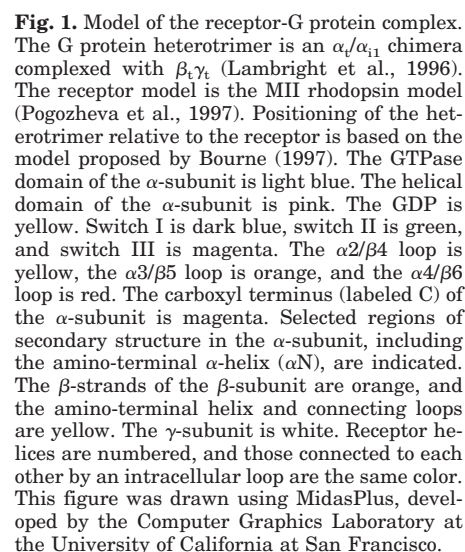
<sup>2</sup> The  $\alpha_s$  residue 354 is not shown because it represents an insertion of sequence relative to that of the  $\alpha_i/\alpha_{i1}$  chimera in the model.

**ABBREVIATIONS:** GTP $\gamma$ S, guanosine 5'-O-(3-thiotriphosphate); ICYP, iodocyanopindolol; DTT, dithiothreitol.

In the crystal structure of  $\alpha_s$  complexed with the catalytic domains of adenylyl cyclase (Tesmer et al., 1997), the  $\alpha 2/\beta 2$  and  $\alpha 3/\beta 5$  regions contact adenylyl cyclase, but the  $\alpha 4/\beta 6$  loop does not. Therefore, using alanine-scanning mutagenesis, we reevaluate the role of the  $\alpha 4/\beta 6$  loop in adenylyl cyclase activation as well as test its role in receptor-mediated activation. None of the alanine substitutions cause specific defects in adenylyl cyclase activation or in receptor-mediated activation, suggesting that the loop plays an indirect role in

Replacement of  $\alpha_8$  residues with  $\alpha_{12}$  homologs in the  $\alpha 3/\beta 5$  loop, but not the  $\alpha 2/\beta 4$  loop, decreases receptor-mediated activation and increases affinity for the  $\beta_2$ -adrenergic receptor. The effects of the  $\alpha 3/\beta 5$  substitutions are not due to a destabilization of the nucleotide-bound state, suggesting that the wild-type residues may be receptor contact sites that are optimized to ensure the reversibility of receptor-G protein association. The correspondence of the  $\alpha 3/\beta 5$  region to an exchange factor contact site in both EF-Tu and Ras suggests that the mechanisms by which seven-transmembrane receptors and exchange factors catalyze nucleotide exchange may share common elements.

**Materials.** The mammalian expression vector pcDNA I/Amp was obtained from InVitrogen (Carlsbad, CA). The bacterial expression vector pQE60, plasmid maxi kits, and Ni<sup>2+</sup> NTA resin were obtained from Qiagen (Santa Clarita, CA). Q Sepharose Fast Flow resin and ECL Western blotting detection reagents were obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Isoproterenol, 1-methyl-3-isobutylxanthine, cAMP, ATP, tosylphenylalanyl chloromethyl ketone-treated trypsin (T-8642), and Lubrol-PX were obtained from Sigma Chemical Co. (St. Louis, MO). Complete, EDTA-free protease inhibitor cocktail tablets were obtained from Boehringer Mannheim (Indianapolis, IN). Nitrocellulose filters for the GTP-γS binding assay were obtained from Millipore (Bedford, MA). Norit-SA3 was obtained from Aldrich (Milwaukee, WI). NuPAGE Bis-Tris 4–12% gels were



obtained from NOVEX (San Diego, CA). [2-<sup>3</sup>H]Adenine was obtained from Amersham Pharmacia Biotech (Little Chalfont, UK). [<sup>35</sup>S]GTP $\gamma$ S, [ $\gamma$ -<sup>32</sup>P]GTP, and [<sup>125</sup>I]iodocyanopindolol (ICYP) were obtained from New England Nuclear (Boston, MA).

**Construction of  $\alpha_s$  Mutant Constructs.** For expression in mammalian cells,  $\alpha_s$  mutant constructs were generated from the rat  $\alpha_s$  cDNA and contain an epitope, referred to as the EE epitope, that was generated by mutating  $\alpha_s$  residues DYVPSD (189–194)<sup>1</sup> to EYMPTE (single-letter amino acid code, mutated residues are underlined). For expression in *Escherichia coli*,  $\alpha_s$  mutant constructs were generated from the long splice variant of bovine  $\alpha_s$  containing a carboxyl-terminal hexahistidine tag, which was a generous gift from Alfred Gilman. All mutations were generated by oligonucleotide-directed in vitro mutagenesis using the Bio-Rad Muta-Gene kit except for those in  $\alpha_s(\alpha 3/\beta 5)$ , which were produced by subcloning a mutagenic oligodeoxynucleotide cassette. Subcloning and mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing.

**cAMP Accumulation Assay.** To determine the abilities of  $\alpha_s$  mutant constructs to activate adenylyl cyclase, the constructs were transiently expressed in COS-7 cells using DEAE-dextran under the control of the cytomegalovirus promoter in the expression vector pcDNA 1/Amp, and intracellular cAMP levels in cells labeled with [<sup>3</sup>H]adenine were determined as described previously (Medina et al., 1996).

To determine the abilities of  $\alpha_s$  mutant constructs to become activated by endogenous  $\beta_2$ -adrenergic receptors, the constructs were introduced by electroporation into a subclone of *cyc*<sup>−</sup> S49 lymphoma cells, which lack endogenous  $\alpha_s$  (Harris et al., 1985), that stably expresses Simian virus 40 large T antigen. cAMP accumulation in the presence of 1 mM 1-methyl-3-isobutylxanthine (a phosphodiesterase inhibitor) and in the presence or absence of the agonist isoproterenol (0.1 mM) was measured after labeling with [<sup>3</sup>H]adenine as described previously (Marsh et al., 1998).

**Membrane Preparations from COS-7 Cells and Trypsin Assay.** COS-7 cells were transiently transfected with  $\alpha_s$  mutant constructs using DEAE dextran as described above. Membranes were prepared 48 h after transfection as described previously (Medina et al., 1996). The trypsin resistance assay was performed as described previously (Berlot and Bourne, 1992). Samples were resolved by SDS-polyacrylamide gel electrophoresis (10%), transferred to nitrocellulose, and probed with the anti-EE monoclonal antibody as described previously (Medina et al., 1996). The antigen-antibody complexes were detected using an anti-mouse horseradish peroxidase-linked antibody according to the ECL Western blotting protocol.

**Preparation of Stable Cell Lines.** The  $\alpha_s$  constructs were expressed in *cyc*<sup>−</sup> *kin*<sup>−</sup> S49 lymphoma cells, which lack endogenous  $\alpha_s$  (*cyc*<sup>−</sup>) and in which cAMP-dependent protein kinase is inactivated (*kin*<sup>−</sup>), and cell membranes were prepared as described previously (Grishina and Berlot, 1998).

**Adenylyl Cyclase Assay.** Adenylyl cyclase activity in membranes of *cyc*<sup>−</sup> *kin*<sup>−</sup> S49 lymphoma cell lines expressing  $\alpha_s$  constructs was measured and used to determine EC<sub>50</sub> values for stimulation of adenylyl cyclase by GTP $\gamma$ S in the presence and absence of isoproterenol as described previously (Grishina and Berlot, 1998).

**Receptor Binding Assay.** Competition between isoproterenol and [<sup>125</sup>I]ICYP for binding to  $\beta_2$ -adrenergic receptors in membranes of *cyc*<sup>−</sup> *kin*<sup>−</sup> S49 lymphoma cell lines expressing  $\alpha_s$  constructs was measured as described by Grishina and Berlot (1998). The experimental data were analyzed for competition at two sites by nonlinear least-squares curve fitting as described by Grishina and Berlot (1998).  $K_L$  and  $K_H$ , the low- and high-affinity dissociation constants, were allowed to vary under the two conditions.

**Expression and Purification of  $\alpha_s$  from *E. coli*.** Both  $\alpha_s$  and  $\alpha_s(\alpha 3/\beta 5)$  in the plasmid pQE60 were expressed in *E. coli* strain JM109. Cultures were grown,  $\alpha_s$  expression was induced, and lysates were produced as described previously (Lee et al., 1994), except that Complete, EDTA-free protease inhibitor cocktail tablets were in-

cluded in the lysis buffer. The supernatant from a 30-min, 25,000g centrifugation was applied to a Ni<sup>2+</sup> NTA column that had been equilibrated with buffer A (50 mM Tris, pH 8.0, 20 mM 2-mercaptoethanol, 50  $\mu$ M GDP, 1 mM phenylmethylsulfonyl fluoride, and 1 Complete, EDTA-free protease inhibitor cocktail tablet/50 ml). The column was washed sequentially with buffer A containing 500 mM NaCl and buffer A containing 50 mM NaCl and 10 mM imidazole before elution with buffer A containing 50 mM NaCl, 150 mM imidazole, and 10% glycerol. The protein was concentrated and exchanged into buffer B (50 mM Tris, pH 8.0, 1 mM EDTA, 2 mM dithiothreitol (DTT), and 10% glycerol) containing 50  $\mu$ M GDP. The protein was then applied to a Q Sepharose Fast Flow column. The column was washed with buffer C (50 mM Tris, pH 8.0, 1 mM EDTA, 5 mM MgCl<sub>2</sub>, 14.5 mM 2-mercaptoethanol, 25  $\mu$ M GDP, and 10% glycerol), and  $\alpha_s$  was eluted with buffer C containing a linearly increasing gradient of NaCl (0–300 mM). Peak fractions were concentrated, exchanged into buffer B containing 10  $\mu$ M GDP, snap-frozen in liquid nitrogen, and stored at −80°C at a concentration greater than 2 mg/ml.

**GTP $\gamma$ S Binding Assays.** To measure association of GTP $\gamma$ S, 100 nM  $\alpha_s$  or  $\alpha_s(\alpha 3/\beta 5)$  was incubated at 20°C with 1  $\mu$ M [<sup>35</sup>S]GTP $\gamma$ S (5 × 10<sup>4</sup> cpm/pmol) in a buffer containing 25 mM HEPES (pH 8.0), 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 100 mM NaCl, and 1 mM DTT. At various times, aliquots (50  $\mu$ l; 5 pmol) were withdrawn and immediately filtered under vacuum on nitrocellulose filters. The filters were rinsed twice with 10 ml of ice-cold Stop Buffer (25 mM Tris, pH 8.0, 100 mM NaCl, and 25 mM MgCl<sub>2</sub>). Apparent on rates of GTP $\gamma$ S binding ( $k_{app}$ ) were calculated by a nonlinear least-squares fit to the equation:

$$B = B_{max} (1 - e^{-kt}).$$

To measure dissociation of GTP $\gamma$ S, 100 nM  $\alpha_s$  or  $\alpha_s(\alpha 3/\beta 5)$  was incubated with 1  $\mu$ M [<sup>35</sup>S]GTP $\gamma$ S as described above for 50 min. Dissociation of [<sup>35</sup>S]GTP $\gamma$ S was initiated by the addition of unlabeled GTP $\gamma$ S to a final concentration of 100  $\mu$ M. At various times, aliquots (50  $\mu$ l; 5 pmol) were withdrawn and added to 2 ml of ice-cold Stop Buffer and maintained on ice until all samples were collected. Samples were then filtered on nitrocellulose filters as described above.

**Single Turnover GTPase Assay.** One-hundred nanomolar  $\alpha_s$  or  $\alpha_s(\alpha 3/\beta 5)$  was incubated at 20°C with 1  $\mu$ M [<sup>32</sup>P]GTP (2 × 10<sup>4</sup> cpm/pmol) in a buffer containing 50 mM HEPES (pH 8.0), 1 mM EDTA, and 1 mM DTT. After 30 min, the first aliquot (50  $\mu$ l; 5 pmol) was withdrawn, and MgCl<sub>2</sub> and GTP were added to final concentrations of 10 mM and 100  $\mu$ M, respectively. Aliquots were withdrawn at various times and added to 750  $\mu$ l of ice-cold 5% (w/v) Norit-SA3 in 50 mM NaH<sub>2</sub>PO<sub>4</sub>. Samples were microcentrifuged, and [<sup>32</sup>P]Pi released in the supernatant was determined by liquid scintillation counting. Catalytic rates of GTP hydrolysis ( $k_{cat}$ ) were calculated by a nonlinear least-squares fit to the equation:

$$P = P_{max} - (P_{max} - P_0)(e^{-kt})$$

where  $P$  is the amount of phosphate released at time  $t$ ,  $P_{max}$  is the maximum amount of phosphate released, and  $P_0$  is the amount of phosphate released during the 30-min preincubation in the absence of MgCl<sub>2</sub>.  $P_{max}$  for  $\alpha_s$  and  $\alpha_s(\alpha 3/\beta 5)$  was 1.8 and 2.7 pmol, respectively.

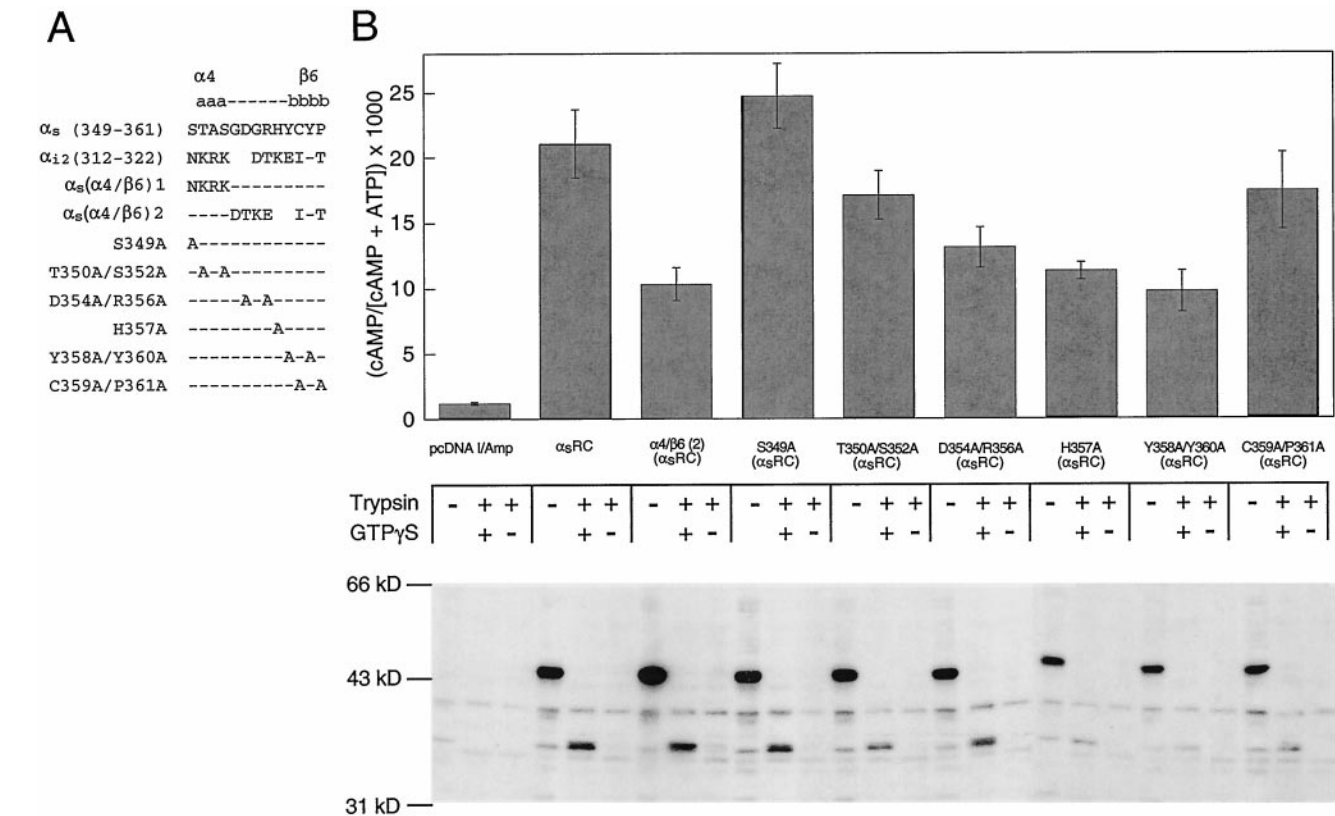
**Trypsin Assay Using Purified  $\alpha_s$ ,  $\alpha_s$  or  $\alpha_s(\alpha 3/\beta 5)$  (2.7  $\mu$ M)** was incubated at 30°C for 30 min in a buffer containing 20 mM HEPES (pH 8.0), 2 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM DTT, 0.025% Lubrol-PX, and activators as indicated. Tosylphenylalanyl chloromethyl ketone-treated trypsin was then added to a final concentration of 0.4 mg/ml, and the mixture was incubated for 5 min at 30°C. The digestion was terminated by adding soybean trypsin inhibitor to a final concentration of 1 mg/ml. The samples were resolved by polyacrylamide gel electrophoresis on a NuPAGE Bis-Tris 4–12% gel. Proteins were visualized by staining with Coomassie Blue.



Results

**Role of  $\alpha 4/\beta 6$  Loop in Adenylyl Cyclase Activation.** Substitutions of  $\alpha_{12}$  residues for  $\alpha_s$  residues in the  $\alpha 4/\beta 6$  loop were shown previously to decrease activation of adenylyl cyclase by  $\alpha_s$  (Berlot and Bourne, 1992). However, in the crystal structure of  $\alpha_s$  complexed with the catalytic domains of adenylyl cyclase (Tesmer et al., 1997), the  $\alpha 4/\beta 6$  loop does not contact adenylyl cyclase. In the structure of  $\alpha_s$  (Sunahara et al., 1997), the  $\alpha 3/\beta 5$  and  $\alpha 4/\beta 6$  loops are closer together than in the corresponding structures of  $\alpha_t$  (Noel et al., 1993) and  $\alpha_{i1}$  (Coleman et al., 1994), in part because of an interaction between Trp277 in  $\alpha 3/\beta 5$  and His357 in  $\alpha 4/\beta 6$ . The proximity of these loops suggested that the effects of mutations in the  $\alpha 4/\beta 6$  loop might be due to changes in the conformation of the  $\alpha 3/\beta 5$  loop, which contacts adenylyl cyclase (Tesmer et al., 1997) and is important for its activation (Itoh and Gilman, 1991; Berlot and Bourne, 1992). To test this hypothesis, we determined the effects of substituting alanine residues in the  $\alpha 4/\beta 6$  loop (Fig. 2A). The ability of mutant  $\alpha_s$  constructs to stimulate cAMP accumulation was measured after transient transfection in COS-7 cells. All constructs contained a GTPase-inhibiting mutation, referred to as the RC mutation, that replaces Arg201 with cysteine and causes

constitutive activation, facilitating detection of adenylyl cyclase-activating ability above that of  $\alpha_s$  endogenous to COS-7 cells, as described previously (Berlot and Bourne, 1992). Figure 2B (top) shows the effects of the alanine substitutions on adenylyl cyclase activation. S349A $\alpha_s$  and T350A/S352A $\alpha_s$  did not exhibit defects in effector activation. These constructs span the region mutated in  $\alpha_s(\alpha 4/\beta 6)1$  (Fig. 2A), which exhibited reduced activity due to decreased expression levels (Berlot and Bourne, 1992). The remainder of the mutant constructs span the region mutated in  $\alpha_s(\alpha 4/\beta 6)2$  (Fig. 2A), which exhibited a specific defect in adenylyl cyclase activation (Berlot and Bourne, 1992) (see below). Significant decreases in the ability to activate adenylyl cyclase ( $P < .05$ ) were caused by alanine substitutions of His357 and of Tyr358 and Tyr360 together (Fig. 2B). We tested the specificity of the mutations that reduced stimulation of cAMP synthesis by determining the expression levels of the mutants in COS-7 cell membranes and their abilities to undergo an activating conformational change that is measured as the acquisition of resistance to trypsin digestion (Berlot and Bourne, 1992). In the presence of GTP $\gamma$ S, trypsin removes a short segment from the amino terminus of  $\alpha_s$  but leaves the remainder of the protein intact (Fig. 2B,



**Fig. 2.** Adenylyl cyclase activation by  $\alpha_s$  constructs with substitutions in the  $\alpha 4/\beta 6$  loop. A, panel of  $\alpha_s$  mutant constructs with substitutions in the  $\alpha 4/\beta 6$  region. The top sequence is that of  $\alpha_s$ . Below that is the sequence of  $\alpha_{i2}$ . Residues that are identical with  $\alpha_s$  residues are represented by dashes.  $\alpha_s(\alpha 4/\beta 6)1$  and  $\alpha_s(\alpha 4/\beta 6)2$  are mutant  $\alpha_s$  constructs with decreased abilities to activate adenylyl cyclase (Berlot and Bourne, 1992). The  $\alpha_{i2}$  substitutions in  $\alpha_s(\alpha 4/\beta 6)2$  were based on an alignment of the  $\alpha_s$  and  $\alpha_{i2}$  sequences that differs from the current alignment, which is based on the crystal structures of  $\alpha_s$  (Sunahara et al., 1997) and  $\alpha_{i1}$  (Coleman et al., 1994). The remaining constructs contain alanine substitutions as indicated. B, top, cAMP accumulation in  $10^6$  COS-7 cells transfected with 1.5  $\mu$ g of vector alone or vector containing  $\alpha_s$ RC or the indicated mutant constructs. cAMP levels in [ $^3$ H]adenine-labeled cells were determined as described in *Experimental Procedures*. Each value represents the mean  $\pm$  S.E. of three independent experiments. Bottom, expression and trypsin sensitivity of these constructs. COS-7 cells ( $6.25 \times 10^6$ ) were transfected with 3  $\mu$ g plasmid/ $10^6$  cells of vector alone or vector containing  $\alpha_s$ RC or the indicated constructs, and membranes were prepared, treated with trypsin, and immunoblotted as described in *Experimental Procedures*. The first lane in each set is the control (no trypsin). The second and third lanes show the result of trypsin digestion in the presence or absence, respectively, of GTP $\gamma$ S. Similar results were obtained in two additional experiments.

bottom). In the absence of GTP $\gamma$ S, trypsin cleaves  $\alpha_s$  into small fragments not seen on the gel. Although all of the constructs assumed the activated conformation, those with decreased activities exhibited decreases in expression level (Fig. 2B, bottom). This qualitative link between activities and expression levels suggests that the alanine substitutions do not cause specific defects in adenylyl cyclase activation.

The effects of alanine substitutions in the  $\alpha 4/\beta 6$  loop suggest that residues in this loop are not directly involved in activation of adenylyl cyclase. Instead, the effector-activating defect of  $\alpha_s(\alpha 4/\beta 6)2$  is most likely due to altered interactions between the  $\alpha 3/\beta 5$  and  $\alpha 4/\beta 6$  loops. The importance of interactions between  $\alpha 3/\beta 5$  and  $\alpha 4/\beta 6$  for  $\alpha_s$  function is underscored by the observation that substitution of cysteine or alanine for Trp277 in the  $\alpha 3/\beta 5$  loop (which contacts His357) eliminates adenylyl cyclase activation. Substitution with cysteine does not affect expression level or the ability to assume the activated conformation (Itoh and Gilman, 1991), whereas substitution with alanine results in undetectable levels of expression (data not shown). It is also noteworthy that the  $\alpha 4/\beta 6$  loop contains two glycines that were replaced by  $\alpha_{i2}$  residues in  $\alpha_s(\alpha 4/\beta 6)2$ . These glycines may contribute a conformational flexibility to this  $\alpha_s$  region that is important for activation of adenylyl cyclase.

#### Role of $\alpha 4/\beta 6$ Loop in Receptor-Mediated Activation.

Because  $\alpha_s$  mutants with alanine substitutions in the  $\alpha 4/\beta 6$  loop did not exhibit defects in ability to activate adenylyl cyclase when expression level was controlled for, we tested their responses to  $\beta_2$ -adrenergic receptors by measuring receptor-dependent cAMP accumulation in transiently transfected *cyc*<sup>-</sup> S49 lymphoma cells, which lack endogenous  $\alpha_s$  (Harris et al., 1985). We used receptor-independent cAMP accumulation due to versions of the  $\alpha_s$  mutants containing the RC mutation (the RC versions) to normalize for expression level, as described previously (Grishina and Berlot, 1998; Marsh et al., 1998). For all of the constructs shown, the plasmid doses required to produce similar receptor-independent activities of the RC versions in *cyc*<sup>-</sup> cells (Fig. 3B) were consistent with their activities and expression levels in COS-7 cells (Fig. 2B). Y358A/Y360A $\alpha_s$  could not be evaluated in this assay due to the unexpectedly low activity of its RC version in *cyc*<sup>-</sup> cells.

None of the alanine substitution mutants tested exhibited defects in receptor-stimulated cAMP accumulation (Fig. 3A). T350A/S352A $\alpha_s$  was previously tested in this assay and exhibited normal receptor-mediated activation (Marsh et al., 1998). Therefore, we conclude that the  $\alpha 4/\beta 6$  loop of  $\alpha_s$  is not involved in receptor-mediated activation.

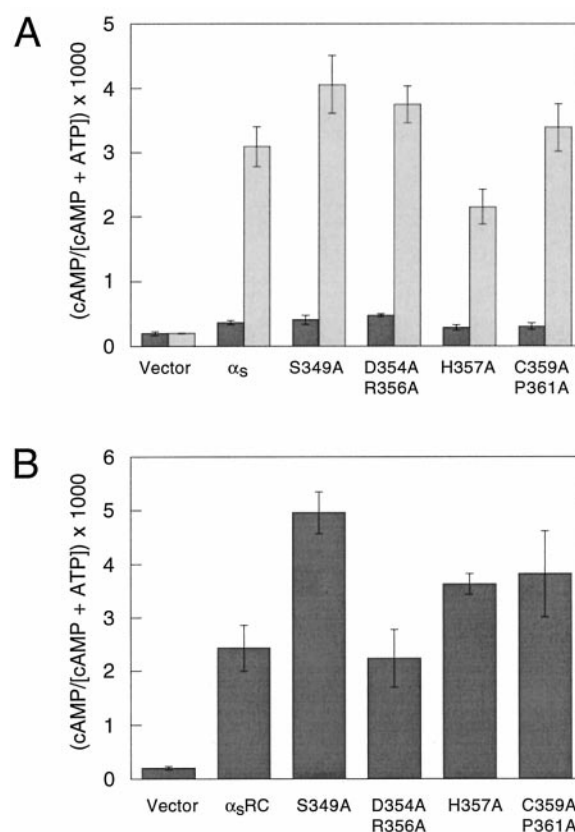
**Mutations in  $\alpha 3/\beta 5$  Region but Not  $\alpha 2/\beta 4$  Region of  $\alpha_s$  Decrease Receptor-Mediated Activation.** To test the roles of the  $\alpha 2/\beta 4$  and  $\alpha 3/\beta 5$  loop regions in receptor-mediated activation, we examined an  $\alpha_s$  construct referred to as  $\alpha_s(\alpha 2/\beta 4)$ , containing three substitutions of  $\alpha_{i2}$  homologs for  $\alpha_s$  residues (Q236H/N239E/D240G) in the  $\alpha 2$  helix and  $\alpha 2/\beta 4$  loop, and an  $\alpha_s$  construct referred to as  $\alpha_s(\alpha 3/\beta 5)$ , containing five  $\alpha_{i2}$  homolog substitutions (N271K/K274D/R280K/T284D/I285T) in the  $\alpha 3$  helix and  $\alpha 3/\beta 5$  loop, after stable expression in *cyc*<sup>-</sup> S49 lymphoma cells. (Figure 9 shows the locations of these substitutions in the secondary structure of  $\alpha_s$ .)

Stimulation of  $G_s$  by the  $\beta_2$ -adrenergic receptor increases the apparent affinity of  $\alpha_s$  for GTP $\gamma$ S. This can be measured as an isoproterenol-dependent decrease in the half-maximal

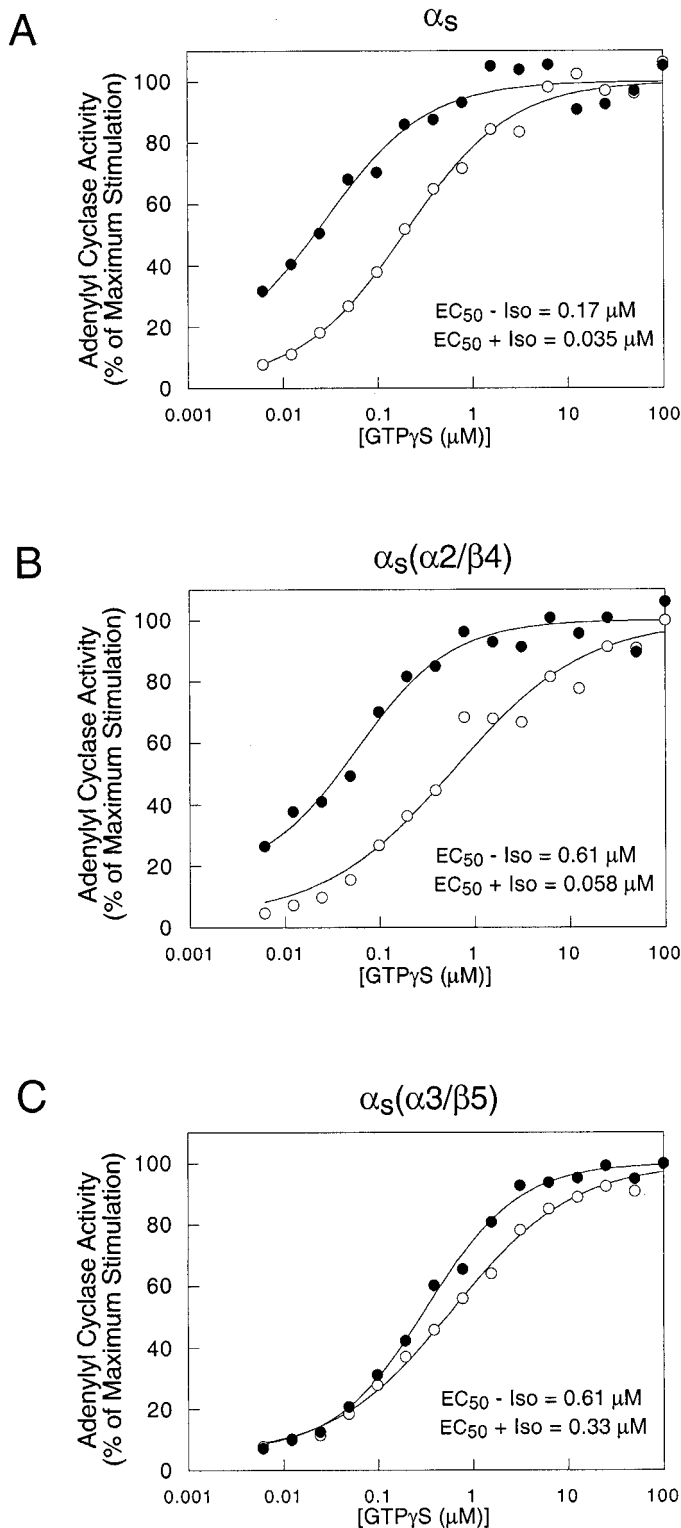
effective concentration (EC<sub>50</sub>) for GTP $\gamma$ S stimulation of adenylyl cyclase (Fig. 4A). We used this response to receptor stimulation as the read-out for receptor-mediated activation of  $\alpha_s(\alpha 2/\beta 4)$  and  $\alpha_s(\alpha 3/\beta 5)$ .

Although mutations in the  $\alpha 2/\beta 4$  and  $\alpha 3/\beta 5$  regions decrease adenylyl cyclase activation by  $\alpha_s$  (Itoh and Gilman, 1991; Berlot and Bourne, 1992), sufficient activity remained to allow determination of the EC<sub>50</sub> value for GTP $\gamma$ S activation of  $\alpha_s(\alpha 2/\beta 4)$  and  $\alpha_s(\alpha 3/\beta 5)$ .  $\alpha_s(\alpha 3/\beta 5)$  exhibited a substantially smaller isoproterenol-dependent decrease in the EC<sub>50</sub> for GTP $\gamma$ S stimulation of adenylyl cyclase (approximately 2-fold) than did  $\alpha_s$  and  $\alpha_s(\alpha 2/\beta 4)$ , which exhibited approximately 5- and 10-fold decreases, respectively (Fig. 4).

Taken together with the results of a previous study, the results with  $\alpha_s(\alpha 3/\beta 5)$  suggest that mutations in the  $\alpha 3/\beta 5$  loop rather than the  $\alpha 3$  helix are responsible for the defect in receptor-mediated activation. Substitutions of  $\alpha_{i2}$  homologs for the  $\alpha 3$  residues Asn271 and Lys274 in combination with  $\alpha_{i2}$  homolog substitutions of Leu266, Gln267, Ala269, and



**Fig. 3.** Receptor-mediated activation of  $\alpha_s$  proteins with mutations in the  $\alpha 4/\beta 6$  loop. A, cAMP accumulation in *cyc*<sup>-</sup> cells transiently transfected with the indicated mutants in the  $\alpha_s$  context. Cells were electroporated with 30  $\mu$ g of vector alone and of vector containing  $\alpha_s$  and S349A $\alpha_s$ , 45  $\mu$ g of vector containing D354A/R356A $\alpha_s$  and C359A/P361A $\alpha_s$ , and 60  $\mu$ g of vector containing H357A $\alpha_s$ . At these plasmid doses, similar amounts of receptor-independent cAMP accumulation were produced by versions of the constructs containing the RC mutation (B). cAMP values from unstimulated cells and from cells stimulated with 0.1 mM isoproterenol are dark gray and light gray, respectively. B, receptor-independent cAMP accumulation in *cyc*<sup>-</sup> cells containing the indicated mutants in the  $\alpha_s$ RC context. For each mutant, the same amount of plasmid was used as is indicated in A for the corresponding  $\alpha_s$  mutant. cAMP levels in [<sup>3</sup>H]adenine-labeled cells were determined as described in *Experimental Procedures*. All values represent the mean  $\pm$  S.E. of three independent experiments.



**Fig. 4.**  $\alpha_s(\alpha3/\beta5)$  exhibits a defect in receptor-mediated activation. Adenylyl cyclase activities in membranes of *cyc<sup>-</sup>* cells stably expressing  $\alpha_s$ , clone 3 (A),  $\alpha_s(\alpha2/\beta4)$ , clone 1 (B), or  $\alpha_s(\alpha3/\beta5)$ , clone 1 (C) were determined in the presence of the indicated concentrations of GTP $\gamma$ S in the presence (●) or absence (○) of 100  $\mu$ M isoproterenol (Iso). Expression levels of these clones are shown in Fig. 5. Data points represent the mean values from three independent experiments and are expressed as the percentage of the maximum observed adenylyl cyclase activity. EC<sub>50</sub> values were calculated as described previously (Grishina and Berlot, 1998).

Leu270 (see Fig. 9B) did not impair receptor-mediated activation (Marsh et al., 1998). The remaining residues mutated in  $\alpha_s(\alpha3/\beta5)$ , Arg280, Thr284, and Ile285, are located in the  $\alpha3/\beta5$  loop.

**Mutations in  $\alpha3/\beta5$  Region but Not  $\alpha2/\beta4$  Region of  $\alpha_s$  Increase Apparent Affinity of  $\alpha_s$  for  $\beta_2$ -Adrenergic Receptor.** To determine whether the decreased receptor-mediated activation of  $\alpha_s(\alpha3/\beta5)$  was due to altered receptor binding, we used a competitive binding assay that measures an  $\alpha_s$ -dependent increase in the affinity of the  $\beta_2$ -adrenergic receptor for the agonist isoproterenol (Grishina and Berlot, 1998). The high-affinity isoproterenol-binding state of the receptor, which requires the presence of  $G_s$  in the nucleotide-free state, reflects receptor- $G_s$  interaction. Isoproterenol binding is measured in competition with the antagonist ICYP, which binds to the receptor with the same affinity in the presence and absence of  $G_s$ .

We measured receptor affinities in membranes from three  $\alpha_s$ -expressing clones and two clones each expressing  $\alpha_s(\alpha2/\beta4)$  and  $\alpha_s(\alpha3/\beta5)$  (Fig. 5A). The binding results from  $\alpha_s$  clone 3,  $\alpha_s(\alpha2/\beta4)$  clone 1, and  $\alpha_s(\alpha3/\beta5)$  clone 1 are shown (Fig. 5, B–D). Similar results were obtained with the other clones, demonstrating that within the range of expression levels examined, binding profiles were independent of expression level (data not shown).

In the presence of 300  $\mu$ M GTP, receptors from  $\alpha_s$ -expressing cells were predominantly in the low-affinity state (Fig. 5B). In the absence of GTP,  $\alpha_s$  caused the appearance of high-affinity binding sites for isoproterenol on the receptor (Fig. 5B). In cells expressing  $\alpha_s(\alpha2/\beta4)$ , the affinity of the receptor for isoproterenol was similar to that in  $\alpha_s$ -expressing cells (Fig. 5C). However, in  $\alpha_s(\alpha3/\beta5)$ -expressing cells, the affinity of the receptor for isoproterenol in both the presence and absence of GTP was greater than that in  $\alpha_s$ -expressing cells (Fig. 5D). A similar pattern of increased high-affinity binding in cells expressing  $\alpha_s(\alpha3/\beta5)$  compared with  $\alpha_s$  was obtained when the assay was performed using 30  $\mu$ M GTP $\gamma$ S (data not shown). The simplest explanation for the increased affinity of the receptor for isoproterenol in the presence of  $\alpha_s(\alpha3/\beta5)$  compared with  $\alpha_s$  is that the affinity of  $\alpha_s(\alpha3/\beta5)$  for the receptor is greater than that of  $\alpha_s$ .

**$\alpha_s(\alpha3/\beta5)$  Exhibits Normal Guanine Nucleotide Handling Properties.** There is precedent for mutations that both decrease receptor-mediated activation of  $\alpha_s$  and impair guanine nucleotide binding (Iiri et al., 1997; Warner et al., 1998) and/or hydrolysis (Warner and Weinstein, 1999). In addition, because the affinity of G proteins for receptors is greatest in the nucleotide-free state, changes in nucleotide binding could affect apparent receptor affinity in the presence of nucleotide. Therefore, using purified  $\alpha_s$  and  $\alpha_s(\alpha3/\beta5)$ , we investigated whether the mutations in  $\alpha_s(\alpha3/\beta5)$  are associated with intrinsic defects in guanine nucleotide handling.

The rates of association of GTP $\gamma$ S to  $\alpha_s$  and  $\alpha_s(\alpha3/\beta5)$  were similar (Fig. 6A). Because the rate of GTP $\gamma$ S association is limited by the rate of GDP dissociation, the mutations in  $\alpha_s(\alpha3/\beta5)$  do not appear to increase GDP dissociation. The stability of GTP $\gamma$ S binding is also unaffected by the mutations in  $\alpha_s(\alpha3/\beta5)$ , because dissociation of GTP $\gamma$ S from both  $\alpha_s$  and  $\alpha_s(\alpha3/\beta5)$  was undetectable (Fig. 6B). The intrinsic rates of GTP hydrolysis of  $\alpha_s$  and  $\alpha_s(\alpha3/\beta5)$  were also the same (Fig. 6C). These results indicate that the decreased



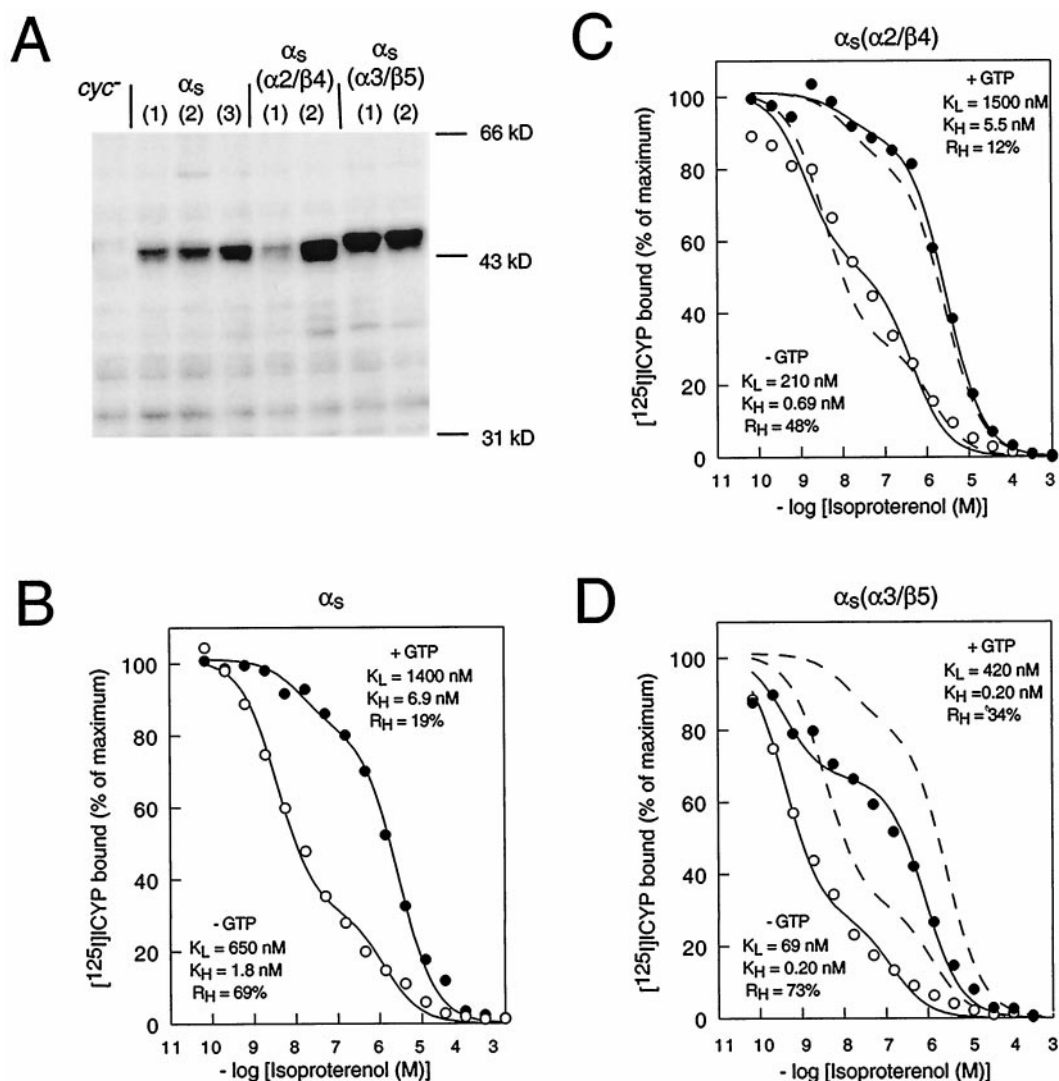
receptor-mediated activation and increased receptor affinity of  $\alpha_s(\alpha 3/\beta 5)$  are not consequences of a destabilization of the guanine nucleotide-bound state.

**$\alpha_s(\alpha 3/\beta 5)$  Exhibits Normal Activation by  $\text{AlF}_4^-$ .** Some  $\alpha_s$  mutations (Hildebrandt et al., 1991; Codina and Birnbaumer, 1994; Iiri et al., 1997; Grishina and Berlot, 1998; Warner et al., 1998) that decrease receptor-mediated activation also decrease activation by  $\text{AlF}_4^-$ , which activates  $\alpha$ -subunits by mimicking the  $\gamma$ -phosphate of GTP in the postulated transition state intermediate of the GTPase reaction (Coleman et al., 1994; Sondek et al., 1994). In contrast,  $\text{AlF}_4^-$  induced the trypsin-resistant activated conformation in  $\alpha_s(\alpha 3/\beta 5)$  to the same extent as  $\text{GTP}\gamma\text{S}$  did and to the same extent as in  $\alpha_s$  (Fig. 7). Because activation by  $\text{AlF}_4^-$  requires that the nucleotide binding site contain GDP and be in an appropriate conformation, this result further supports the conclusion that the mutations in  $\alpha_s(\alpha 3/\beta 5)$  specifically alter

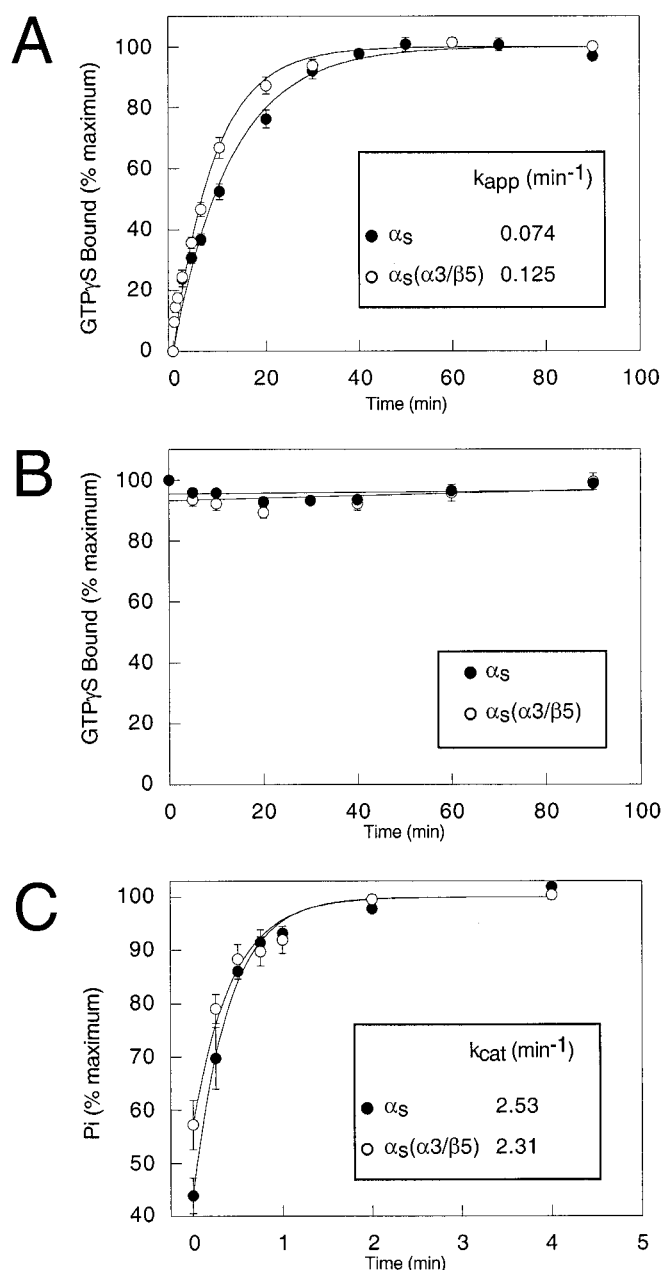
receptor-mediated activation and receptor binding without causing global structural distortions in  $\alpha_s$ .

**Mapping of Residues Important for the  $\text{G}_s$ - $\beta_2$ -Adrenergic Receptor Interaction onto a Receptor-G Protein Model.** We visualized the results of this and previous studies of the  $\alpha_s$  residues important for interaction with the  $\beta_2$ -adrenergic receptor using a model of the receptor-G protein complex with essentially the same constraints as the model proposed by Bourne (1997). The amino terminus of the  $\alpha$ -subunit and the carboxyl terminus of the  $\gamma$ -subunit, which contain lipid modifications important for membrane attachment, face the membrane. The third intracellular loop of the receptor is located at the interface between  $\alpha$  and  $\beta\gamma$ , near regions shown by mutagenesis and peptide studies to be important for receptor interaction (Bourne, 1997): the amino and carboxyl termini of  $\alpha$  and the carboxyl terminus of  $\beta$ .

In this model, the residues substituted in the  $\alpha 3/\beta 5$  loop



**Fig. 5.** Competition between isoproterenol and  $[^{125}\text{I}]\text{ICYP}$  for binding to the  $\beta_2$ -adrenergic receptor. A, immunoblot showing expression levels of  $\alpha_s$ ,  $\alpha_s(\alpha 2/\beta 4)$ , and  $\alpha_s(\alpha 3/\beta 5)$  in stable  $\text{cyc}^-$  cell lines. Membranes of  $\alpha_s$ , clone 3, (B),  $\alpha_s(\alpha 2/\beta 4)$ , clone 1 (C), or  $\alpha_s(\alpha 3/\beta 5)$ , clone 1 (D), were incubated with  $[^{125}\text{I}]\text{ICYP}$  (75 pM) and the indicated concentrations of isoproterenol in the presence (●) or absence (○) of 300  $\mu\text{M}$  GTP. Values represent the mean values of two independent experiments. The solid lines represent a nonlinear least-squares fit to the data, as described previously (Grishina and Berlot, 1998).  $K_L$  and  $K_H$  are the low- and high-affinity dissociation constants, respectively, and %  $R_H$  is the percentage of receptors in the high-affinity form. In C and D, the binding curves for membranes from  $\alpha_s$ -expressing cells, from B, are redrawn as dotted lines. Similar results for each construct were obtained in two additional experiments using the other cell lines in A.

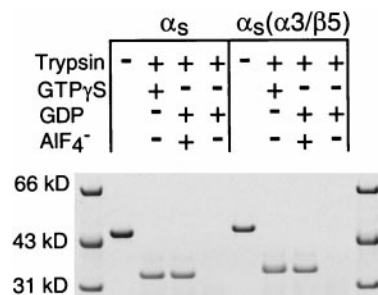


**Fig. 6.** Biochemical properties of  $\alpha_s$  and  $\alpha_s(\alpha3/\beta5)$ . A, rates of GTP $\gamma$ S binding.  $\alpha_s$  (●) or  $\alpha_s(\alpha3/\beta5)$  (○) (100 nM each) was incubated at 20°C with 1  $\mu$ M [ $^{35}$ S]GTP $\gamma$ S ( $5 \times 10^4$  cpm/pmol). At the times indicated, aliquots (50  $\mu$ l; 5 pmol) were withdrawn and filtered on nitrocellulose filters as described in *Experimental Procedures*. Apparent on rates of GTP $\gamma$ S binding ( $k_{app}$ ) were calculated as described in *Experimental Procedures*. Data points represent the mean  $\pm$  S.E. of six experiments. B, dissociation of GTP $\gamma$ S.  $\alpha_s$  (●) or  $\alpha_s(\alpha3/\beta5)$  (○) (100 nM each) was incubated at 20°C with 1  $\mu$ M [ $^{35}$ S]GTP $\gamma$ S as described in A for 50 min. Dissociation of [ $^{35}$ S]GTP $\gamma$ S was initiated by the addition of unlabeled GTP $\gamma$ S to a final concentration of 100  $\mu$ M. At the times indicated, aliquots (50  $\mu$ l; 5 pmol) were withdrawn and filtered on nitrocellulose filters as described in *Experimental Procedures*. Data points represent the mean  $\pm$  S.E. of four experiments. The values of  $k_{off}$  for both  $\alpha_s$  and  $\alpha_s(\alpha3/\beta5)$  were indistinguishable from 0. C,  $k_{cat}$  for the hydrolysis of GTP.  $\alpha_s$  (●) or  $\alpha_s(\alpha3/\beta5)$  (○) (100 nM each) was incubated at 20°C with 1  $\mu$ M [ $^{32}$ P]GTP ( $2 \times 10^4$  cpm/pmol) for 30 min in the presence of 1 mM EDTA. After withdrawal of the first aliquot, MgCl<sub>2</sub> and GTP were added to final concentrations of 10 mM and 100  $\mu$ M, respectively. At the times indicated, aliquots (50  $\mu$ l; 5 pmol) were withdrawn, and [ $^{32}$ P]Pi released was determined as described in *Experimental Procedures*. Catalytic rates of GTP hydrolysis ( $k_{cat}$ ) were calculated as described in *Experimental Procedures*. Data points represent the mean  $\pm$  S.E. of six experiments.

(280, 284, and 285, red in Fig. 8) are close to both the second and third intracellular loops of the receptor, indicating that they could be receptor contact sites. Substitutions of the residues in the  $\alpha3$  helix (271 and 274, pink in Fig. 8), in combination with additional substitutions, do not impair receptor-mediated activation (Marsh et al., 1998). The  $\alpha3/\beta5$  loop is in close proximity to both the  $\alpha2/\beta4$  and  $\alpha4/\beta6$  loops, suggesting that receptor-initiated signaling between the  $\alpha3/\beta5$  loop and the nucleotide binding pocket may involve one or both of these adjacent loops. The  $\alpha3/\beta5$  loop residues exhibit the same degree of solvent exposure in the presence or absence of  $\beta\gamma$ . However, the residue equivalent to Arg280 is  $\sim 4$  Å away from a  $\beta_t$  residue (Arg314) with which it forms a water-mediated hydrogen bond. Thus, this region may influence interaction with  $\beta\gamma$ .

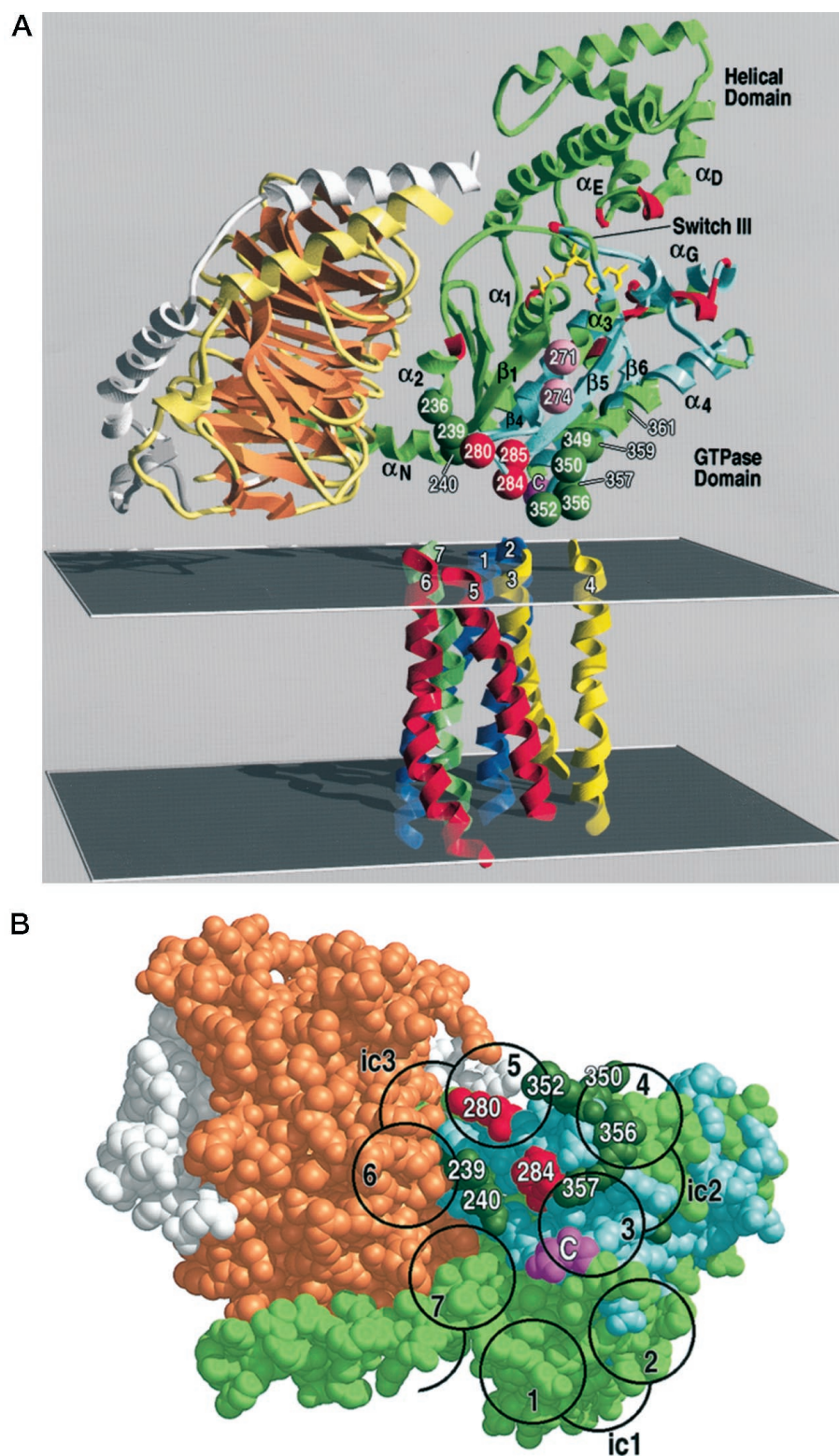
Other than the  $\alpha3/\beta5$  loop region, the only  $\alpha_s$  region close to the receptor in which substitutions alter receptor interactions is the carboxyl terminus (Marsh et al., 1998) (C, magenta in Fig. 8). The other  $\alpha_s$  residues in which substitutions disrupt receptor interactions (red in Fig. 8A), located in  $\alpha1$  (Hildebrandt et al., 1991), the  $\alpha D/\alpha E$  loop (Codina and Birnbaumer, 1994; Grishina and Berlot, 1998),  $\alpha2$  (Iiri et al., 1997), the  $\beta4/\alpha3$  loop including switch III (Grishina and Berlot, 1998; Marsh et al., 1998; Warner et al., 1998), the  $\beta5/\alpha G$  loop (Codina and Birnbaumer, 1994), the  $\alpha G/\alpha4$  loop (Marsh et al., 1998), and the  $\beta6/\alpha5$  loop (Marsh et al., 1998), are farther from the receptor and in many cases near the nucleotide and/or buried, indicating that they probably are not receptor contact sites.

Shown in green are  $\alpha_s$  regions that do not specify interaction with the  $\beta_2$ -adrenergic receptor. Among these regions is the  $\alpha4/\beta6$  loop, which does not appear to mediate receptor binding, because alanine substitutions throughout (residues 349, 350, 352, 354, 356, 357, 359, 361) do not affect receptor-mediated activation (Fig. 3; Marsh et al., 1998). The other regions shown in green were tested by replacing  $\alpha_s$  residues with  $\alpha_{i2}$  homologs (Masters et al., 1988; Marsh et al., 1998; present study). Homolog substitutions in these regions, which include the amino terminus and the  $\alpha2/\beta4$  loop (residues 236, 239, 240), do not rule out these regions as being receptor contact sites, because residues that are identical in  $\alpha_s$  and  $\alpha_{i2}$  were not changed.



**Fig. 7.** Effects of GTP $\gamma$ S and GDP/AlF<sub>4</sub><sup>-</sup> on tryptic cleavage; 2.7  $\mu$ M  $\alpha_s$  or  $\alpha_s(\alpha3/\beta5)$  was incubated with 12.5  $\mu$ M GTP $\gamma$ S, 12.5  $\mu$ M GDP plus 25  $\mu$ M AlCl<sub>3</sub> and 12.5 mM NaF, or 12.5  $\mu$ M GDP and then treated with trypsin as described in *Experimental Procedures*. The  $\alpha_s$  and trypsin-resistant fragments of  $\alpha_s$  were resolved by polyacrylamide gel electrophoresis and visualized by staining with Coomassie Blue. Similar results were obtained in two additional experiments.





**Fig. 8.** Mapping of residues important for  $G_s\beta_2$ -adrenergic receptor interaction onto a receptor-G protein model.  $\alpha_s$  residues are mapped onto the model of the receptor-G protein complex shown in Fig. 1A, ribbon diagram. The  $\beta$ -strands of the  $\beta$ -subunit are orange, and the amino-terminal helix and connecting loops are yellow. The  $\gamma$ -subunit is white. The GDP is yellow.  $\alpha_s$  residues in which substitutions with the homologous  $\alpha_{i2}$  residues were shown previously to leave receptor-mediated activation intact (Masters et al., 1988; Grishina and Berlot, 1998; Marsh et al., 1998) are green.  $\alpha_s$  residues that have not been tested for their roles in receptor interaction are light blue. The residues mutated in  $\alpha_s(\alpha 3/\beta 5)$  are red spheres ( $\alpha 3/\beta 5$  loop) or pink spheres ( $\alpha 3$  helix). Additional residues in which mutations alter receptor interaction (Hildebrandt et al., 1991; Codina and Birnbaumer, 1994; Iiri et al., 1997; Grishina and Berlot, 1998; Marsh et al., 1998) are red, except for the carboxyl terminus (labeled C), which is magenta. Residues in the  $\alpha 2/\beta 4$  and  $\alpha 4/\beta 6$  loops, in which substitutions with  $\alpha_{i2}$  homologs and alanine residues, respectively, do not alter receptor interaction, are green spheres. The numbers on the spheres represent  $\alpha_s$  residue numbers. Receptor helices are numbered, and those connected to each other by an intracellular loop are the same color. B, space-filling model viewing the heterotrimer surface that faces the receptor. The receptor helices are outlined in black, and the intracellular loops (i.c.1–3) and carboxyl-terminal tail are indicated. Colors are as in A except that  $\beta$  is entirely orange. These figures were drawn using MidasPlus, developed by the Computer Graphics Laboratory at the University of California at San Francisco.

## Discussion

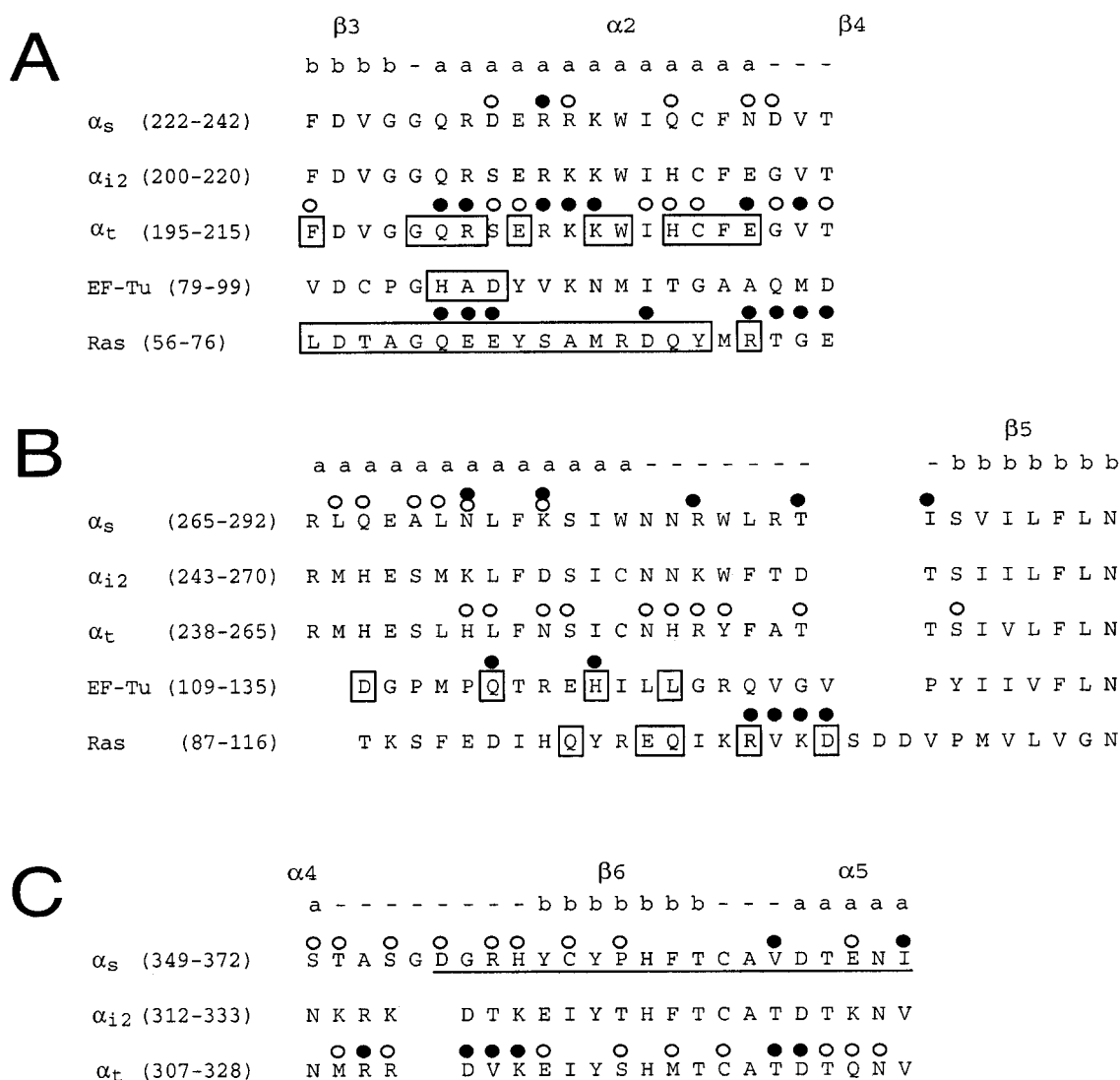
We have identified a region of the G protein  $\alpha$ -subunit, the  $\alpha 3/\beta 5$  loop, in which mutations decrease receptor-mediated activation of  $\alpha_s$  and increase the apparent affinity of  $G_s$  for the  $\beta_2$ -adrenergic receptor. Based on its location, this region

is likely to be a receptor contact site (Fig. 8). This region and the extreme carboxyl terminus are the only regions of  $\alpha_s$  that map near the receptor in which substitutions have been shown to alter receptor interaction. Of the other regions that map nearby, the  $\alpha 4/\beta 6$  loop does not appear to mediate receptor binding, because extensive substitutions with ala-

nines do not affect receptor-mediated activation (Fig. 3). Substitutions of  $\alpha_s$  residues in the amino terminus (Masters et al., 1988) and the  $\alpha 2/\beta 4$  loop (Figs. 4 and 5), which are also near, with the homologous  $\alpha_{i2}$  residues indicate that these regions do not specify interaction with the  $\beta_2$ -adrenergic receptor, but comprehensive alanine-scanning mutagenesis will be needed to determine whether they are required for receptor binding.

The fact that replacement of  $\alpha_s$  residues in the  $\alpha 3/\beta 5$  loop region with  $\alpha_{i2}$  homologs increases receptor affinity is surprising but suggests that the wild-type residues may be optimized to ensure reversibility of receptor-G protein inter-

action during the GTPase cycle. This interpretation is consistent with previous observations that substitutions in the sequences of peptides based on the carboxyl terminus of  $\alpha_t$  (Martin et al., 1996) or a  $\beta$ -subunit region that interacts with phospholipase C (Buck et al., 1999) can cause increased affinities for rhodopsin or phospholipase C, respectively. If the  $\alpha 3/\beta 5$  loop region is a receptor contact site, it should be possible to find mutations that decrease receptor affinity. Further studies will be directed at testing the effects of substitutions that change the critical residues in different or opposite ways from the original mutations.



**Fig. 9.** Alignment of GTPase sequences in switch II, the  $\alpha 3/\beta 5$  loop, and the  $\alpha 4/\beta 6$  loop. A, sequences of  $\alpha_s$ ,  $\alpha_{i2}$ ,  $\alpha_t$ , EF-Tu, and Ras in switch II. B, sequences of  $\alpha_s$ ,  $\alpha_{i2}$ ,  $\alpha_t$ , EF-Tu, and Ras from the beginning of  $\alpha 3$  to the end of  $\beta 5$ . C, sequences of  $\alpha_s$ ,  $\alpha_{i2}$ , and  $\alpha_t$  from the  $\alpha 4/\beta 6$  loop to the beginning of  $\alpha 5$ . Residue numbers of  $\alpha_s$ ,  $\alpha_{i2}$ ,  $\alpha_t$ , EF-Tu, and Ras are indicated in parentheses. Elements of secondary structure in  $\alpha_s$ , determined from the structure of  $\alpha_s$ -GTP $\gamma$ S (Sunahara et al., 1997), are indicated by a ( $\alpha$ -helices), b ( $\beta$ -strands), and dashes (turns and loops). In the sequence of  $\alpha_s$ , mutations of residues with filled circles over them impaired receptor-mediated activation (present report; Iiri et al., 1997; Marsh et al., 1998), mutations of residues with open circles above them did not impair receptor-mediated activation (present report; Masters et al., 1988; Marsh et al., 1998), the effects of mutations in residues with both open and filled circles over them were context-dependent, as described in the text, and the sequence of a peptide that can mimic the effects of  $\alpha_s$  on the  $\beta$ -adrenergic receptor (Rasenick et al., 1994) is underlined. In the sequence of  $\alpha_t$ , boxed residues represent residues that contact  $\beta_3$ , based on the structure of a heterotrimeric G protein (Lambright et al., 1996), mutations of residues with filled circles over them impaired receptor-mediated activation (Onrust et al., 1997), and mutations of residues with open circles above them did not impair receptor-mediated activation (Onrust et al., 1997). In the sequence of EF-Tu, boxed residues contact EF-Ts in the structures of EF-Tu·EF-Ts complexes (Kawashima et al., 1996; Wang et al., 1997) and mutations of residues with filled circles over them impaired exchange factor-mediated activation (Jonák et al., 1998; Zhang et al., 1998). In the sequence of Ras, boxed residues contact Sos in the structure of Ras·Sos (Boriack-Sjodin et al., 1998) and mutations of residues with filled circles over them impaired exchange factor-mediated activation (Mistou et al., 1992; Créchet et al., 1996; Leonardsen et al., 1996).

The defects in  $\alpha_s(\alpha 3/\beta 5)$  are different from those of several previously described  $\alpha_s$  mutants with defects in receptor-mediated activation (shown in red in Fig. 8). Two mutant  $\alpha_s$  constructs with substitutions at the interface of the GTPase and helical domains, N254D/M255L/I257L/R258A $\alpha_s$  in switch III and N167R $\alpha_s$  in the  $\alpha$ D/ $\alpha$ E loop also exhibit an apparent increase in receptor affinity and decreased receptor-stimulated binding of GTP $\gamma$ S, but in contrast to  $\alpha_s(\alpha 3/\beta 5)$ , these constructs exhibit a decreased response to  $\text{AlF}_4^-$  (Grishina and Berlot, 1998). Defects in the response to  $\text{AlF}_4^-$  could be due to an altered nucleotide binding site, because the response requires bound GDP. Indeed, R258A $\alpha_s$  exhibits increased rates of both GDP dissociation (Warner et al., 1998) and GTP hydrolysis (Warner and Weinstein, 1999). Other mutations that decrease activation by both receptors and  $\text{AlF}_4^-$  include D173K in the  $\alpha$ D/ $\alpha$ E loop and K293D in the  $\beta 5/\alpha$ G loop (Codina and Birnbaumer, 1994), R231H in  $\alpha 2$  (Iiri et al., 1997), and S54N in  $\alpha 1$  (Hildebrandt et al., 1991). In contrast to the mutations in  $\alpha_s(\alpha 3/\beta 5)$ , each of these mutations appears to alter the nucleotide binding site.

Our data, combined with previous results, indicate that switch II does not determine the receptor specificity of  $\alpha_s$ . The  $\alpha 2/\beta 4$  loop is located in the carboxyl-terminal part of switch II. Involvement of the amino-terminal part of switch II in receptor specificity was ruled out previously by an  $\alpha_{12}/\alpha_s$  chimera that substituted  $\alpha_{12}$  residues for  $\alpha_s$  residues 1 to 235 and that exhibits normal receptor-mediated activation (Masters et al., 1988) (Fig. 9A). However, switch II undoubtedly plays an important role in the response to receptor stimulation, because residues in this region bind to  $\beta\gamma$ , with which  $\alpha$ -subunits must be associated to interact with receptors (Fung, 1983). In support of such a role, substitution of histidine for a buried residue in switch II of  $\alpha_s$ , R231 (Figs. 8 and 9A), reduces receptor-mediated activation, possibly by disrupting the interaction of this residue with  $\alpha 3$  and switch III (Iiri et al., 1997). Furthermore, alanine substitutions of  $\alpha_t$  residues in switch II (Fig. 9A) disrupt activation by rhodopsin (Onrust et al., 1997). Based on their locations, most of these substitutions probably disrupt  $\beta\gamma$  interaction.

Our data suggest that the  $\alpha 4/\beta 6$  loop of  $\alpha_s$  does not regulate binding to or activation by the  $\beta_2$ -adrenergic receptor. Others have suggested that this region is a receptor contact site, because a synthetic peptide corresponding to  $\alpha_s$  residues 354 to 372, which extends from the  $\alpha 4/\beta 6$  loop to the beginning of  $\alpha 5$  (Fig. 9C), can mimic the effects of  $\alpha_s$  on the  $\beta_2$ -adrenergic receptor (Rasenick et al., 1994). However, within the region spanned by the peptide, the only substitutions that we have found to impair receptor-mediated activation of  $\alpha_s$  are of buried residues in the  $\beta 6/\alpha 5$  loop and the beginning of  $\alpha 5$  (Fig. 9C; Marsh et al., 1998), which could not be receptor contact sites. In the case of  $\alpha_t$ , alanine substitutions of residues in the  $\alpha 4/\beta 6$  loop impaired activation by rhodopsin (Onrust et al., 1997; Fig. 9C). Interestingly, the  $\alpha 4/\beta 6$  loop is positioned differently in the structures of  $\alpha_s$  (Sunahara et al., 1997) and  $\alpha_t$  (Noel et al., 1993; Lambright et al., 1994, 1996). These differences in structure may explain why mutations in this region of  $\alpha_t$  but not  $\alpha_s$  impair receptor-mediated activation.

In EF-Tu and Ras, both the  $\alpha 2/\beta 4$  and  $\alpha 3/\beta 5$  loop regions are exchange factor contact sites (Fig. 9), suggesting that the mechanisms by which seven-transmembrane receptors and exchange factors catalyze nucleotide exchange may share common elements. In the structures of EF-Tu-EF-Ts (Ka-

washima et al., 1996; Wang et al., 1997), a phenylalanine residue from EF-Ts is inserted in between the helices that correspond to  $\alpha 2$  and  $\alpha 3$  in the  $\alpha$ -subunit. The resulting conformational changes, which involve the phosphate binding loop, disrupt hydrogen bonds to  $\text{Mg}^{2+}$  and the  $\beta$ -phosphate of GDP. In the structure of Ras-Sos (Boriack-Sjodin et al., 1998), Sos binds to the phosphate-binding loop, switches I and II, and  $\alpha 3$  and the  $\alpha 3/\beta 5$  loop of Ras. Binding of Sos to Ras causes conformational changes in switches I and II and introduces residues that block magnesium binding and overlap the site where the  $\alpha$ -phosphate of GDP would bind.

By analogy to the actions of EF-Ts and Sos, receptors may alter the relative orientations of  $\alpha 2$ /switch II (via interaction with  $\beta\gamma$ ) and  $\alpha 3$  (via interaction with the  $\alpha 3/\beta 5$  loop) to disrupt interactions with the bound GDP and  $\text{Mg}^{2+}$ . In support of such receptor-stimulated conformational changes, comparison of the structures of the heterotrimer and the activated  $\alpha$ -subunit indicates activation-dependent alterations in the positions of  $\alpha 2$  and  $\alpha 3$ . In the heterotrimer (Lambright et al., 1996), binding of  $\beta\gamma$  to switch II opens a gap between  $\alpha 2$  and  $\alpha 3$ . Upon GTP binding, this gap closes and switch II comes into contact with  $\alpha 3$  and switch III (Noel et al., 1993; Coleman et al., 1994). Receptor-dependent changes in the positions of  $\alpha 2$  and  $\alpha 3$  could then be transmitted across the domain interface via switch III to enable release of the bound nucleotide, which is buried between the GTPase and helical domains. In support of this postulated mechanism, we have shown previously that interactions between switch III and the  $\alpha$ D/ $\alpha$ E loop at the domain interface are involved in receptor-mediated activation (Grishina and Berlot, 1998). Additional conformational changes induced by binding of the receptor to the carboxyl terminus of the  $\alpha$ -subunit could be transmitted to the  $\beta 6/\alpha 5$  loop region to loosen contacts with the guanine ring of GDP. Mutations in the  $\beta 6/\alpha 5$  loop of  $\alpha_t$  (Onrust et al., 1997) and  $\alpha_s$  (Marsh et al., 1998) disrupt receptor-mediated activation, indicating the importance of this region in nucleotide binding.

A more complete understanding of receptor-catalyzed nucleotide exchange by heterotrimeric G proteins awaits the solving of a structure of a seven-transmembrane receptor-heterotrimeric G protein complex. In the meantime, postulated connections between receptor binding sites and the nucleotide binding site can be tested by additional functional studies of  $\alpha$ -subunit mutants with substitutions in positions likely to transmit receptor-initiated conformational changes.

#### Acknowledgments

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**Send reprint requests to:** Dr. Catherine H. Berlot, Department of Cellular and Molecular Physiology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520-8026. E-mail: catherine.berlot@yale.edu