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Receptor-Mediated Activation of $G_{s\alpha}$: Evidence for Intramolecular Signal Transduction

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ABSTRACT

To investigate the mechanism by which cell surface receptors activate heterotrimeric G proteins, we applied a scanning mutagenesis approach to the carboxyl-terminal 40% of α_s (residues 236–394) to identify residues that play a role in receptor-mediated activation. We identified four regions of sequence in which mutations significantly impaired receptor-dependent stimulation of cAMP synthesis in transiently transfected *cyc*$^-$ S49 lymphoma cells, which lack endogenous α_s. Residues at the carboxyl terminus are likely to be receptor contact sites. Buried residues near the bound GDP are connected to the carboxyl terminus by an α helix and may regulate GDP affinity. Residues in two adjacent loops of the GTPase domain at the interface with the helical domain, one of which includes a region, switch III, that changes conformation on GTP binding, are positioned to relay the receptor-initiated signal across the domain interface to facilitate GDP release. Consistent with this hypothesis, replacing the helical domain of α_s with that of α_{i2} in an $\alpha_s/\alpha_{i2}/\alpha_s$ chimera corrects the defect in receptor-mediated activation caused by α_{i2} substitutions on the GTPase side of the interface. Thus, complementary interactions between residues across the domain interface seem to play a role in receptor-catalyzed activation.

Heterotrimeric G proteins transmit hormonal and sensory signals received by cell surface receptors to effector proteins that produce a wide variety of cellular responses (Neer, 1995). Theα, β, and γ 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 α subunit, resulting in dissociation of α·GTP from $\beta\gamma$, each of which can transmit signals to effectors. Hydrolysis of GTP by the α subunit regulates the timing of deactivation and reassociation of α with $\beta\gamma$. As intermediaries between receptors and effectors, G proteins play a crucial role in determining the specificity, nature, and degree of amplification of transmitted signals. For example, G_s mediates stimulation of adenylyl cyclase by β-adrenergic receptors. However, the molecular determinants that specify receptor/G protein interactions and the mechanism by which these interactions lead to G protein activation are not well understood.

Studies of G protein function can be interpreted in the context of the x-ray crystal structures of GTPγS-bound (active) (Noel *et al*., 1993; Coleman *et al*., 1994) and GDP-bound (inactive) (Lambright *et al*., 1994; Mixon *et al*., 1995) $\alpha_s$ubunits and of $\alpha\beta\gamma$ heterotrimers (Wall *et al*., 1995; Lambright *et al*., 1996). The α subunits consist of two domains, a GTPase domain that resembles the oncogene protein, $p21^{ras}$, and a helical domain consisting of α helices and connecting loops. Because the bound nucleotide is buried in the cleft between these domains, receptor-mediated nucleotide exchange presumably involves a conformational change that opens the cleft. Comparison of the structures of GTPγS-bound and GDP-bound α subunits reveals three regions in the GTPase domain (switches I–III) that change conformation, which could be involved in the activation process.

All α subunit residues involved in associating with receptors and with $\beta\gamma$, which is required for receptor-mediated activation (Fung, 1983), have not been identified. X-ray crystal structures of the $\alpha\beta\gamma$ complex (Wall *et al*., 1995; Lambright *et al*., 1996) showed that two α subunit regions contact the β subunit, the amino-terminal α helix and a region that includes switches I and II. The functional importance of these regions has been demonstrated using proteolysis (Navon and Fung, 1987), mutagenesis (Miller *et al*., 1988; Journot *et al*., 1991), and cross-linking (Garcia-Higuera *et al*., 1996). Numerous genetic and biochemical studies, reviewed by Neer (1995), have implicated the carboxyl-terminal region of the α subunit in interaction with receptors. However, the locations of these $\beta\gamma$ and receptor-interacting residues, which are dis-

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ABBREVIATIONS: GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEK, human embryonic kidney.

tant from the domain cleft, have not revealed the mechanism of receptor-mediated G protein activation.

We exploited the differences in receptor specificities of α_s and α_{i2}, which are relatively divergent members of the α subunit family, sharing ~40% amino acid identity, to identify additional α subunit residues that mediate a response to receptor stimulation. Measurements of receptor-stimulated guanine nucleotide exchange in reconstituted phospholipid vesicles have demonstrated that the efficiency with which the β-adrenergic receptor regulates α_{i2} is ~10% of that seen for α_s (Rubenstein *et al.*, 1991). The α_s residues that specify interaction with the β-adrenergic receptor have been localized to the carboxyl-terminal 40% of α_s by means of an α_{i2}/α_s chimera (Masters *et al.*, 1988).

By examining a panel of α_s mutants in which clusters of residues were replaced by α_{i2} homologs or alanines, we identified four regions of sequence that are specifically required for receptor-mediated activation. Residues at the extreme carboxyl terminus are the most likely receptor contact residues. Buried residues near the guanine ring of the bound GDP, connected to the carboxyl terminus by an α helix, may transmit the signal from the receptor to modulate GDP affinity. Residues in two adjacent loops of the GTPase domain at the interface with the helical domain, one of which includes switch III, are positioned to relay the receptor-initiated signal across the domain interface to facilitate GDP release. Consistent with this hypothesis, replacing the helical domain of α_s with that of α_{i2} in an $\alpha_s/\alpha_{i2}/\alpha_s$ chimera corrects the defect in receptor-mediated activation caused by α_{i2} substitutions on the GTPase side of the interface. Thus, complementary interactions between residues across the domain interface seem to play a role in receptor-catalyzed activation.

Experimental Procedures

Materials. The expression vector pcDNA I/Amp was obtained from InVitrogen (Carlsbad, CA). Plasmids used for electroporation were prepared using Qiagen Plasmid Maxi Kits (Santa Clarita, CA). Isoproterenol, 1-methyl-3-isobutylxanthine, cAMP, and ATP were obtained from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium, minimal essential medium with Earle's salts, and geneticin were obtained from GIBCO BRL (Grand Island, NY). Horse serum was obtained from Hyclone (Logan, UT). [^{3}H]Adenine was obtained from Amersham (Little Chalfont, UK).

Construction of α subunit mutants and chimeras. The α_s mutant constructs were generated from rat α_s cDNA (Jones and Reed, 1987). Chimeric α subunits were constructed from rat α_s cDNA and mouse α_{i2} cDNA (Sullivan *et al.*, 1986). Subcloning and mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing. All α subunit constructs produced in this study contain an epitope, referred to as the EE epitope (Grussenmeyer *et al.*, 1985), which was generated by mutating α_s residues DYVPSD (189–194) to EYMPTE and α_{i2} residues SDYIPTQ (166–172) to EEYMPTE (single-letter amino acid code; mutated residues are underlined). This epitope does not affect the ability of α_s to activate adenylyl cyclase in response to stimulation by the β-adrenergic receptor (Wilson and Bourne, 1995).

The amino acid substitutions in the α_s mutant constructs produced in this study are shown in Figs. 1 and 5. To generate these constructs, the α_s cDNA was subcloned into the expression vector pcDNA I/Amp as a *Hin*dIII fragment. The mutations in $\alpha_s(1)$, $\alpha_s(2)$, $\alpha_s(10)$, $\alpha_s(11)$, $\alpha_s(12)$, $\alpha_s(13)$, $\alpha_s(14)$, $\alpha_s(15)$, $\alpha_s(16)$, and $\alpha_s(17)$ were introduced into the α_s cDNA by oligonucleotide-directed *in vitro* mutagenesis (Kunkel *et al.*, 1987) using the BioRad Muta-Gene kit (Hercules, CA). The mutations in $\alpha_s(3)$, $\alpha_s(4)$, $\alpha_s(5)$, $\alpha_s(6)$, $\alpha_s(7)$, $\alpha_s(8)$, and $\alpha_s(9)$ were introduced into the α_s cDNA by ligating *Bam*HI fragments from previously generated constructs (Berlot and Bourne, 1992) that contained these mutations into α_s in place of the analogous fragment. To produce $\alpha_s(2 + 6)$, $\alpha_s(6)$ was digested with *Bgl*II and *Eco*RV to yield a fragment containing the $\alpha_s(6)$ mutations, which was ligated into $\alpha_s(2)$ in place of the analogous fragment to produce an α_s cDNA containing both the $\alpha_s(2)$ and $\alpha_s(6)$ mutations.

Because receptor-dependent stimulation of cAMP synthesis was used to measure receptor-mediated activation of the mutant α_s constructs, any effects the mutations might have on receptor-independent cAMP synthesis were controlled for by measuring basal cAMP accumulation in response to parallel constructs (the α_sRC versions), in which substitution of cysteine for the arginine at position 201 (Landis *et al.*, 1989) inhibits GTPase activity and causes constitutive activation. α_sRC versions of the constructs were produced by ligating *Bam*HI fragments containing the mutations into α_sRC in place of the analogous fragment.

An α_{sis} chimera, in which α_s residues 62–235 are replaced by the homologous α_{i2} residues, was produced from α_s and an α_{is} chimera, in which α_s residues 1–235 are replaced by the homologous α_{i2} residues. The α_{i2} cDNA was subcloned into pcDNA I/Amp as an *Eco*RI fragment. To generate α_{is}, the α_s cDNA was digested with *Bam*HI and the fragment encoding α_s residues 236–394 and the 3′ untranslated region of α_s was ligated into α_{i2} in place of the analogous fragment. Then, α_{sis} was generated using polymerase chain reactions that produced DNA fragments with overlapping ends that were combined subsequently in a fusion polymerase chain reaction (Horton *et al.*, 1989). An RC version of α_{sis}, α_{sis}RC, was produced by substituting cysteine for Arg179, which causes constitutive activation of α_{i2} by inhibiting GTPase activity (Wong *et al.*, 1991). α_{sis}RC was derived from α_{is}RC, which was generated by ligating the *Bam*HI α_s fragment encoding α_s residues 236–394 and the 3′ untranslated region of α_s into α_{i2}RC in place of the analogous fragment. To produce α_{sis}RC, α_{i2}RC was digested with *Dra*III to yield a fragment containing the RC mutation, which was ligated into α_{sis} in place of the analogous fragment.

To introduce the $\alpha_s(1)$, $\alpha_s(2)$, $\alpha_s(6)$, and $\alpha_s(2 + 6)$ mutations into α_{sis}RC and α_{sis}, these mutations were first subcloned as *Bam*HI fragments into α_{i2}RC and α_{i2} in place of the analogous fragments. Digestion of these α_{i2}RC and α_{i2} constructs with *Dra*III yielded fragments containing the $\alpha_s(1)$, $\alpha_s(2)$, $\alpha_s(6)$, and $\alpha_s(2 + 6)$ mutations with or without the RC mutation, respectively, which were ligated into α_{sis}pcDNA I/Amp in place of the analogous fragments to produce α_{sis}RC and α_{sis} constructs, respectively, containing the desired mutations.

cAMP accumulation assay. Transient transfections were performed using a subclone of *cyc*$^-$ S49 lymphoma cells (Bourne et al., 1975) that stably expresses Simian virus 40 large T antigen. These cells were maintained in Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum and 0.6 mg/ml geneticin. Transient transfection of cells expressing TAg with vectors containing a Simian virus 40 origin of replication has been shown to maximize expression levels (Clipstone and Crabtree, 1992). Therefore, we used the expression vector, pcDNA I/Amp, which contains a Simian virus 40 origin of replication, as well as the cytomegalovirus promoter, to electroporate the *cyc*$^-$ cells.

The α subunit constructs were introduced into *cyc*$^-$ cells (2×10^7 cells in 1.0 ml of 20 mM HEPES-buffered minimal essential medium with Earle's salts without bicarbonate) by electroporation at room temperature using a GIBCO BRL Cell-Porator (capacitance setting, 1600 μF; voltage setting, 250 V; Grand Island, NY). After electroporation, the cells were added to 4.0 ml of Dulbecco's modified Eagle's medium containing 10% heat-inactivated horse serum in 60-mm tissue culture dishes. At 24 hr after electroporation, the cells were labeled with 12 μCi/ml [^{3}H]adenine. Then, 24 hr later, cAMP accumulation was measured. The cells first were washed in assay me-

dium (20 mM HEPES-buffered Dulbecco's modified Eagle's medium without bicarbonate). The cells were transferred to 24-well plates and incubated at 37° for 30 min in the same medium containing 1 mM concentration of the phosphodiesterase inhibitor 1-methyl-3-isobutylxanthine, with or without the addition of 0.1 mM isoproterenol (a saturating stimulus). During this incubation, the cells attached to the wells. Reactions were terminated by aspiration and the immediate addition of 5% trichloroacetic acid plus 1 mM concentration each of ATP and cAMP. Nucleotides were separated on ion exchange columns (Salomon *et al.*, 1974). cAMP accumulation was expressed as [^{3}H]cAMP/([^{3}H]ATP + [^{3}H]cAMP) × 1000.

Results

Panel of α_s mutant constructs for studying receptor-mediated activation. The 159-residue carboxyl-terminal segment of α_s (residues 236–394), which specifies interaction with the β-adrenergic receptor (Masters *et al.*, 1988), contains 59 amino acids that are identical in the sequence of α_{i2} and therefore do not specify interaction with this receptor. We previously demonstrated that mutations of residues in three adjacent regions of the α subunit structure, the $\alpha2/\beta4$,

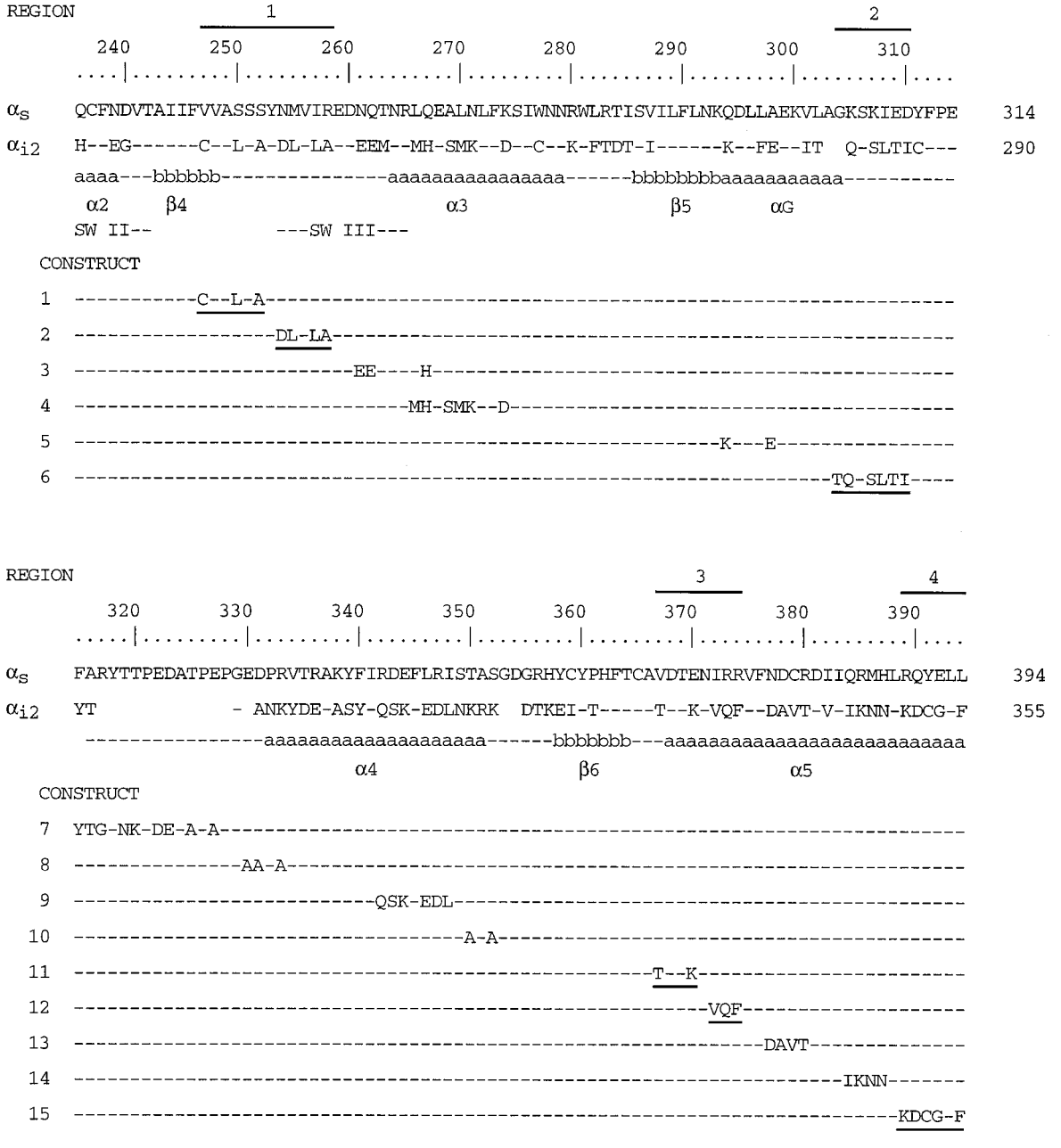

Fig. 1. Panel of α_s mutant constructs. All mutations are within the carboxyl-terminal 40% of the rat α_s sequence (Jones and Reed, 1987), which is depicted in two sections (residues 236–314 and 315–394). *Top lines*, sequence of α_s. *Second lines*, mouse α_{i2} (Sullivan *et al.*, 1986) in the corresponding region. *Dashes*, residues identical to those of α_s. *Numbered sequences*, individual mutant constructs. For each α_s mutant construct, mutated residues are shown by the single-letter amino acid code. *Dashes*, residues identical to those of α_s. *Underlined sequences (located in regions 1–4)*, mutations that disrupted receptor-mediated activation of α_s. Elements of secondary structure, determined from the structure of α_s·GTPγS (Sunahara *et al.*, 1997), are indicated: *a*, αhelices; *b*, βstrands; and *dashes*, turns and loops. Regions that switch conformation between the GDP-bound and GTPγS-bound forms of α_t (Lambright *et al.*, 1994) and α_{i1} (Mixon *et al.*, 1995) (switches II and III) are indicated. The alignment shown, which is based on the recently solved structure of α_s·GTPγS (Sunahara *et al.*, 1997), differs from our previous alignment (Berlot and Bourne, 1992) in the location of an insertion of α_s sequence relative to that of α_{i2}. In the previous alignment, α_s residues 324–336 were inserted between α_{i2} residues 299 and 300.

$\alpha 3/\beta 5$, and $\alpha 4/\beta 6$ loops (Noel *et al.*, 1993), disrupt the ability of α_s to activate adenylyl cyclase (Berlot and Bourne, 1992). Because the current study of receptor-mediated activation of α_s mutants used a cAMP accumulation assay, we did not test 16 residues in these loops. Of the remaining 84 nonidentical residues, 61 were changed in small clusters to α_{i2} homologs or to alanines using 15 α_s mutant constructs (Fig. 1).

Transient transfection assay for receptor-mediated activation of α_s. To test the abilities of mutant α_s proteins to be activated by the β-adrenergic receptor, we measured receptor-dependent stimulation of cAMP synthesis after transient transfection of *cyc*$^-$ S49 lymphoma cells (Bourne *et al.*, 1975), which lack endogenous α_s (Harris *et al.*, 1985). Basal cAMP levels in cells transfected with 10–90 μg of vector containing α_s varied linearly in proportion to the plasmid dose (Fig. 2A). Stimulation of these α_s-transfected cells with the β-adrenergic agonist isoproterenol produced increased cAMP levels that also exhibited a linear relationship to the amount of transfected plasmid (Fig. 2A). We also determined receptor-independent cAMP accumulation by measuring basal cAMP levels in cells transfected with versions of the α_s mutants in which Arg201 is mutated to cysteine. The α_s containing this mutation, α_sRC, exhibits constitutive activation due to inhibited GTPase activity (Landis *et al.*, 1989). As with α_s-transfected cells, basal cAMP levels in *cyc*$^-$ cells transfected with 10–90 μg of vector containing α_sRC varied linearly in proportion to the plasmid dose (Fig. 2B).

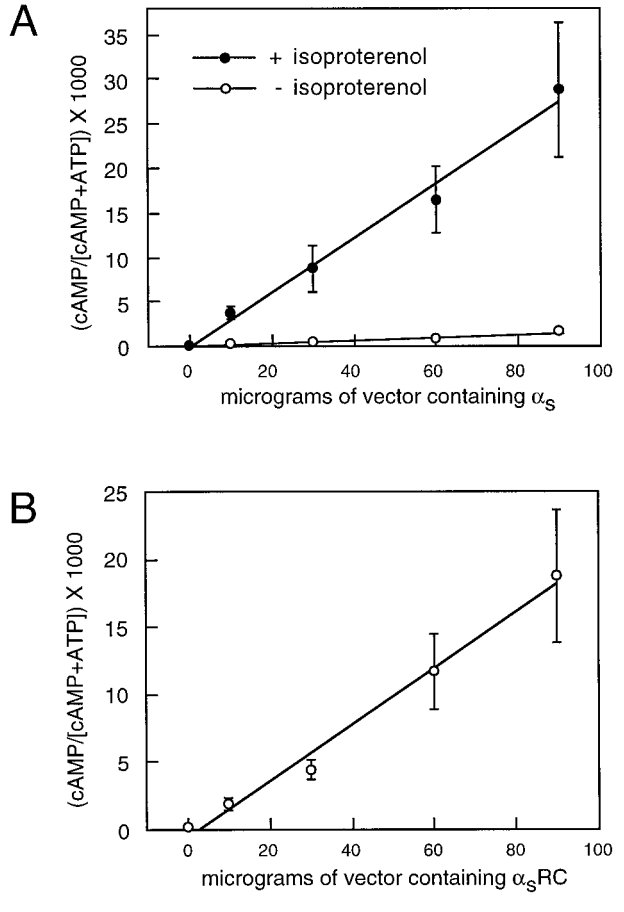

Fig. 2. Transient transfection assay for receptor-mediated activation of α_s. **A, cAMP accumulation in *cyc*$^-$ cells electroporated with the indicated doses of vector containing α_s. cAMP levels were measured in the presence and absence of 0.1 mM isoproterenol. B, Receptor-independent cAMP accumulation in *cyc*$^-$ cells electroporated with the indicated doses of vector containing α_sRC. For the 0-μg points, 30 μg of vector was used. cAMP levels in [^{3}H]adenine-labeled cells were determined as described in Experimental Procedures. All values represent the mean ± standard error of three independent experiments.**

We initially measured receptor-independent cAMP accumulation due to the α_sRC mutants using 30 μg of plasmid. At this plasmid dose, the activities of some of the α_sRC mutants were reduced compared with that of α_sRC. The expression levels of transiently expressed α_s proteins in *cyc*$^-$ cells were not high enough to be detected using an immunoblot but could be determined in transiently transfected HEK 293 cells. α_sRC mutants with reduced activities in *cyc*$^-$ cells had similarly reduced activities in HEK 293 cells. The activities of these α_sRC mutants directly correlated with their expression levels as determined by immunoblotting of HEK 293 cell membranes (data not shown). Because the activities of both α_s and α_sRC in *cyc*$^-$ cells were directly proportional to the amount of transfected plasmid (Fig. 2), it was possible to normalize the expression levels of these α_s and α_sRC mutant constructs to that of α_s and α_sRC by transfecting with increased amounts of plasmid.

To compare receptor-dependent activation of α_s mutants with that of α_s, we identified plasmid doses for which the activities of the α_sRC mutants were similar to those for 30 μg of the α_sRC-containing plasmid (Fig. 3B). At these plasmid doses, we compared receptor-dependent cAMP accumulation due to the corresponding α_s mutants with that for 30 μg of the α_s-containing plasmid (Fig. 3A). An assumption underlying this normalization procedure is that the substitutions in the mutant constructs have similar effects on the expression levels of α_s and α_sRC. This assumption is supported by the observation that the basal activity of α_sRC is ~10-fold greater than that of α_s (Figs. 2 and 3) and the basal activities of each of the α_sRC mutants also are ~10-fold greater than those of the corresponding α_s mutants (Fig. 3).

Of the four regions of sequence in which mutations disrupted receptor-mediated activation (see below), all except one of them (region 2) included at least one cluster of residues that did not decrease expression level. Studies using stably transfected cells confirmed that receptor-mediated activation of the region 2 mutant was decreased (see below). Thus, although a 9-fold range of plasmid doses was used for the transient transfection assay, our conclusions do not depend on the activities in this assay of the constructs with low expression levels.

Receptor-mediated activation of α_s mutant constructs. Receptor-stimulated cAMP accumulation due to 9 of the 15 α_s mutant constructs was similar to that of α_s (Fig. 3A). The other 6 constructs produced reduced receptor-dependent increases in cAMP levels and delineated four regions of sequence containing seven or fewer α_s mutations that disrupt the ability of α_s to be activated by the β-adrenergic receptor (Fig. 1). Region 1, defined by α_s(1) and α_s(2), contains V247, S250, S252, N254, M255, I257, and R258. Region 2, defined by α_s(6), contains G304, K305, and K307-Y311. Region 3, defined by α_s(11) and α_s(12), contains V367, E370, and I372-R374. Region 4, defined by α_s(15), contains R389-E392 and L394.

Because α_s(6) in region 2 was poorly expressed in transiently transfected cells, we established lines of *cyc*$^-$ cells

stably transfected with this α_s mutant construct to investigate further the role of region 2 in receptor-mediated activation. As expected from the results of the transient transfection assay, the expression levels obtained in α_s(6)-expressing lines, as determined by immunoblotting, were lower than those in α_s-expressing lines. In addition, a defect in receptor-mediated activation was seen in that isoproterenol-stimulated adenylyl cyclase activity was reduced relative to that stimulated by GTPγS (Grishina G and Berlot CH, unpublished observations). Thus, the mutations in region 2 impair receptor-dependent activation but also seem to decrease the stability of α_s.

Mapping of mutations that block receptor-mediated activation onto the structure of a heterotrimeric G protein. Because receptors interact with $\alpha\beta\gamma$ heterotrimers, we mapped the α_s residues in which mutations disrupted activation by the β-adrenergic receptor onto the x-ray crystal structure of an α_t/α_{i1} chimera complexed with $\beta_t\gamma_t$ (Lambright *et al.*, 1996) to visualize their positions in three dimensions (Fig. 4). The recently solved structure of α_s·GTPγS (Sunahara *et al.*, 1997) is very similar to the structures of α_t·GTPγS (Noel *et al.*, 1993) and α_{i1}·GTPγS (Coleman *et al.*, 1994), indicating that the structure of $\alpha_t/\alpha_{i1}\beta_t\gamma_t$ is a good model for the G_s heterotrimer. Structural features unique to

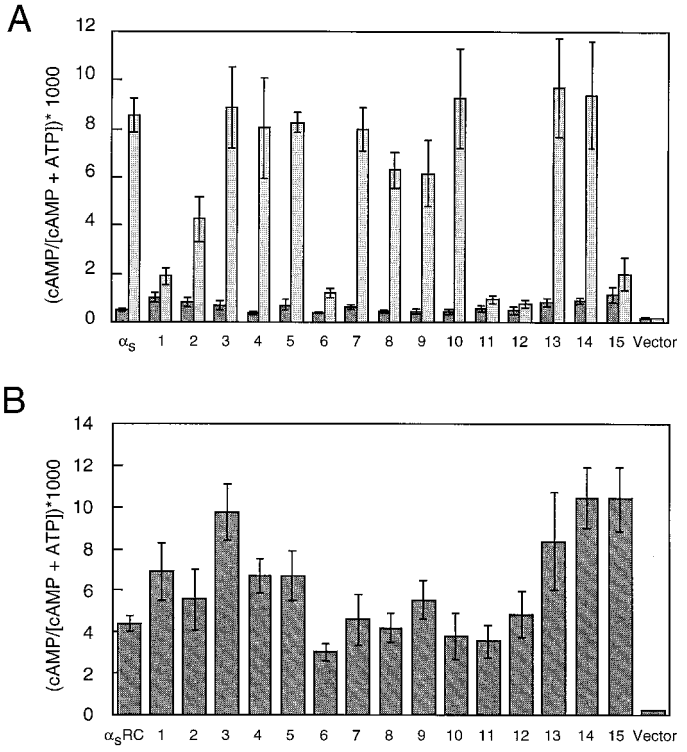

Fig. 3. Receptor-mediated activation of mutant α_s proteins. A, cAMP accumulation in *cyc*$^-$ cells containing the indicated mutants in the α_s context. Cells were electroporated with 20 μg of vector containing α_s (*2*), 30 μg of vector alone, 30 μg of vector containing α_s and α_s (*3* and *10–15*), 60 μg of vector containing α_s (*5*), 90 μg of vector containing α_s (*1, 4, 7, and 8*), 120 μg of vector containing α_s (*9*), and 180 μg of vector containing α_s (*6*). *Dark gray*, cAMP values from unstimulated cells. *Light gray*, cAMP values from cells stimulated with 0.1 mM isoproterenol. B, Receptor-independent cAMP accumulation in *cyc*$^-$ cells containing the indicated mutants in the α_sRC context. For each mutant, the same amount of plasmid was used as is indicated in A for the corresponding α_s mutant. cAMP levels in [^{3}H]adenine-labeled cells were determined as described in Experimental Procedures. All values represent the mean ± standard error of at least three independent experiments.

α_s that are relevant to the mutations that blocked receptor-mediated activation are discussed. Some of the mutations that blocked receptor-mediated activation map onto solvent-exposed residues that could potentially interact directly with the receptor, whereas others map onto residues that are buried and are more likely to mediate nucleotide exchange by propagating conformational changes within α_s.

The residues in regions 1 and 2 are located in the GTPase domain at the interface between the GTPase and helical domains. Region 1 extends from the middle of β4 to the middle of the β4/α3 loop and overlaps with switch III, which assumes different conformations in the structures of GTPγS-bound and GDP-bound α subunits (Noel *et al.*, 1993; Coleman *et al.*, 1994; Lambright *et al.*, 1994; Mixon *et al.*, 1995). Region 2 is located in the adjacent αG/α4 loop. Residues in the amino-terminal part of region 1, defined by α_s(1), are buried within the interior of the molecule, making contacts with other residues in the GTPase domain. In the carboxyl-terminal part of region 1, the side chains of N254, M255, and R258 in α_s(2) are in close proximity to residues in the helical domain. The residues in region 2 immediately precede a 12-residue insertion of sequence in α_s relative to α_t and α_i. However, comparison of the structures of α_s·GTPγS (Sunahara *et al.*, 1997), α_t·GTPγS (Noel *et al.*, 1993), and α_{i1}·GTPγS (Coleman *et al.*, 1994) reveals that the orientation of region 2 with respect to the helical domain is the same in all of the α subunits. In the structure of α_s·GTPγS (Sunahara *et al.*, 1997), the 12-residue insertion is located farther from the interface than the location of region 2 (Fig. 4A, *to the right of Region 2*). Of the region 2 residues, K305 and Y311 are closest to the interface, and all of the residues except for I308 are surface-exposed.

Region 3 is located near the guanine nucleotide binding pocket and includes residues in the β6/α5 loop and the beginning of α5. Three of the residues, E370, R373, and R374, are solvent-exposed, whereas two, V367 and I372 (shown in Fig. 4), are buried. V367 contacts the guanine ring of the bound nucleotide (Sunahara *et al.*, 1997). The residues in region 4, located at the extreme carboxyl terminus, were not visualized in the $\alpha_t/\alpha_{i1}\beta_t\gamma_t$ structure (Lambright *et al.*, 1996) and occupy different positions in the structures in which they were visualized. In the structure of α_t·GTPγS (Noel *et al.*, 1993), this region contacts the α2/β4 loop, whereas in the structures of α_s·GTPγS (Sunahara *et al.*, 1997), α_{i1}·GDP·AlF_4^-·RGS4 (Tesmer *et al.*, 1997), and $\alpha_{i1}\beta1\gamma2$ (Wall *et al.*, 1995), the extreme carboxyl terminus is distant from the rest of the α subunit. Region 4 is linked to region 3 by the α5 helix.

Mutations of buried, but not surface-exposed, residues in region 3 disrupt receptor-mediated activation. To determine the role of the buried and surface-exposed residues in region 3, we mutated separately each class of residues. We found that substitution of the buried residues with the homologous α_{i2} residues in α_s(16) specifically reduced receptor-mediated increases in cAMP production, whereas substitution of the surface-exposed residues with alanine residues in α_s(17) had no effect (Fig. 5). Therefore, this region does not seem to be a receptor contact site but instead probably is important for transmitting the receptor signal to the bound GDP. Of the two residues mutated in α_s(16), V367 is located in the β6/α5 loop, presumably in contact with the GDP, whereas I372 is near the beginning of

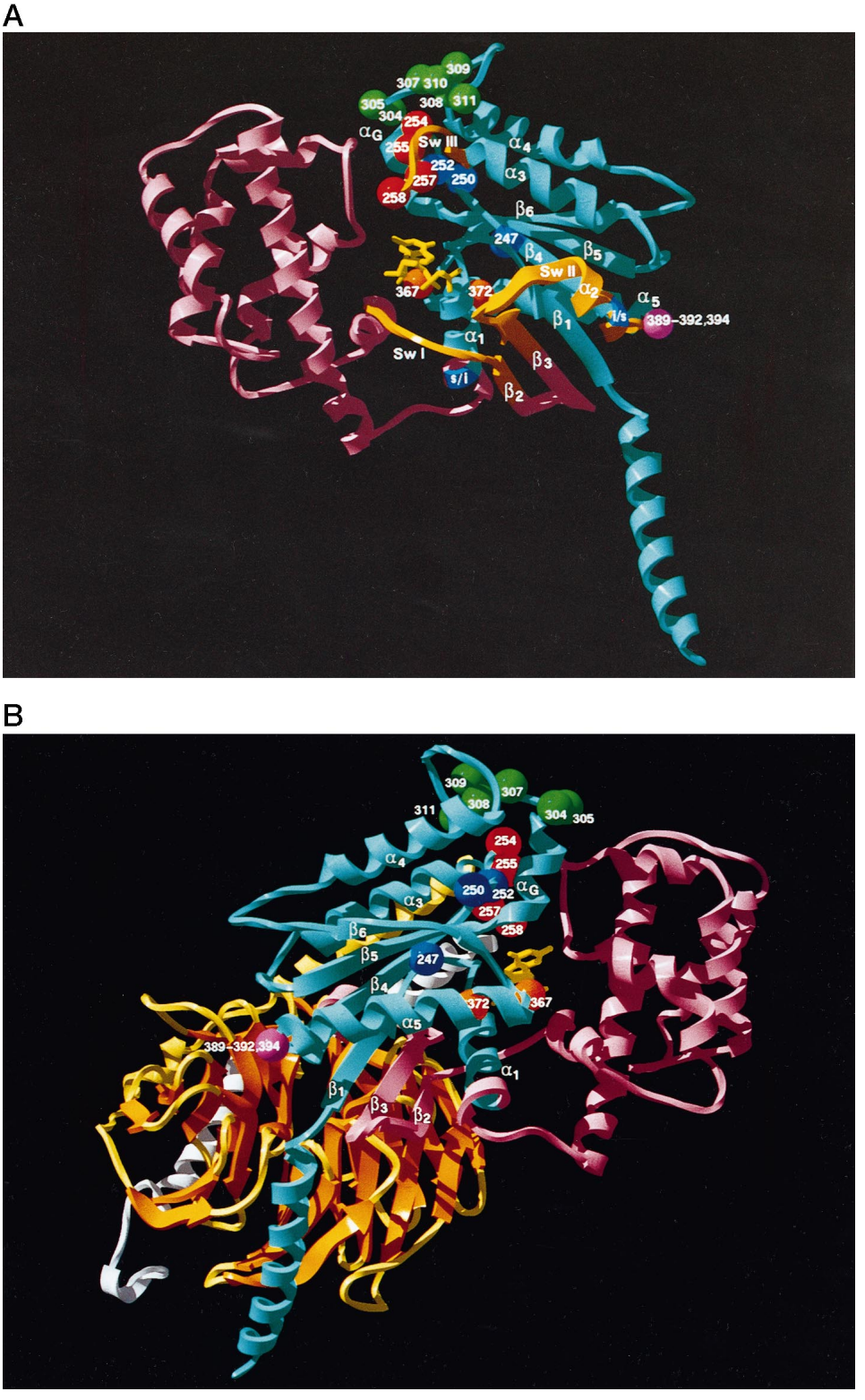

Fig. 4. Mapping of α_s mutations that disrupt receptor-mediated activation onto the structure of an $\alpha\beta\gamma$ heterotrimer. A, View of the α subunit. The $\beta\gamma$ subunits have been omitted for clarity. *Left of GDP (yellow)*, helical domain. *Right*, GTPase domain. *Blue spheres*, residues mutated in $\alpha_s(1)$ in region 1. *Red spheres*, residues mutated in $\alpha_s(2)$ in region 1. *Green spheres*, residues mutated in $\alpha_s(6)$ in region 2. *Orange spheres*, buried residues in region 3 mutated in $\alpha_s(16)$. Residues mutated in $\alpha_s(15)$ in region 4 are not seen in this structure, but the most carboxyl-terminal α_t/α_{i1} residue visualized (residue 343 at the end of $\alpha5$) is a *magenta sphere*. The amino-terminal portion of the α_{sis} chimera, consisting of α_s residues (*light blue*), extends from the amino terminus to the end of $\alpha1$. The middle portion of the chimera, consisting of α_{i2} residues (*pink*), extends from the α_s/α_{i2} junction (s/i) to the α_{i2}/α_s junction (i/s) at the end of $\alpha2$. The carboxyl-terminal portion of the chimera consists of α_s residues (*light blue*). *Gold*, switches I–III. *Numbers on the spheres*, α_s residue numbers. B, View of the heterotrimer. The model is rotated 180 degrees around the vertical axis relative to the view in A. *Gold*, βstrands in the β subunit. *Yellow*, amino-terminal α helix and the connecting loops. *White*, γsubunit. *Other colors*, as in A. X-ray coordinates of an α_t/α_{i1} chimera complexed with $\beta_t\gamma_t$ are from Lambright *et al.* (1996). These figures were drawn using MidasPlus, developed by the Computer Graphics Laboratory at UCSF.

α5. Because each of these residues was tested separately in α_s(11) and α_s(12), both regions seem to be involved in responding to receptor stimulation.

Defects caused by α_{i2} substitutions in regions 1 and 2 can be complemented by replacing the helical domain with α_{i2} residues. Based on the locations of the region 1 and 2 residues at the interface between the GTPase and helical domains, we hypothesized that they mediate receptor-dependent activation via interactions with helical domain residues. Mismatches across the domain interface between α_{i2} residues in the GTPase domain and α_s residues in the helical domain thus would be the cause of the reduced abilities of the region 1 and 2 α_s mutant constructs to be activated by receptor stimulation. According to this hypothesis, replacing the helical domain of α_s with that of α_{i2} would be expected to reverse the defect in receptor-mediated activation caused by the α_{i2} for α_s substitutions in regions 1 and 2. To test this prediction, we produced an α subunit chimera, α_{sis}, in which α_{i2} homologs are substituted for α_s residues 62–235, extending from the end of α1 to the end of α2 (see Fig. 4). The helical domain of this chimera is composed of α_{i2} residues, and the GTPase domain contains α_s residues.

The properties of α_{sis} were similar to those of α_s, with the exception of an elevation in basal activity. The expression levels of α_{sis}RC (the GTPase-inhibited version of α_{sis}) and α_sRC in membranes of transiently transfected HEK 293 cells were similar (data not shown). Receptor-independent cAMP accumulation due to α_{sis}RC also was similar to that of α_sRC (compare Fig. 6B with Fig. 2B). Stimulation of α_{sis}-transfected cells with isoproterenol resulted in cAMP levels simi-

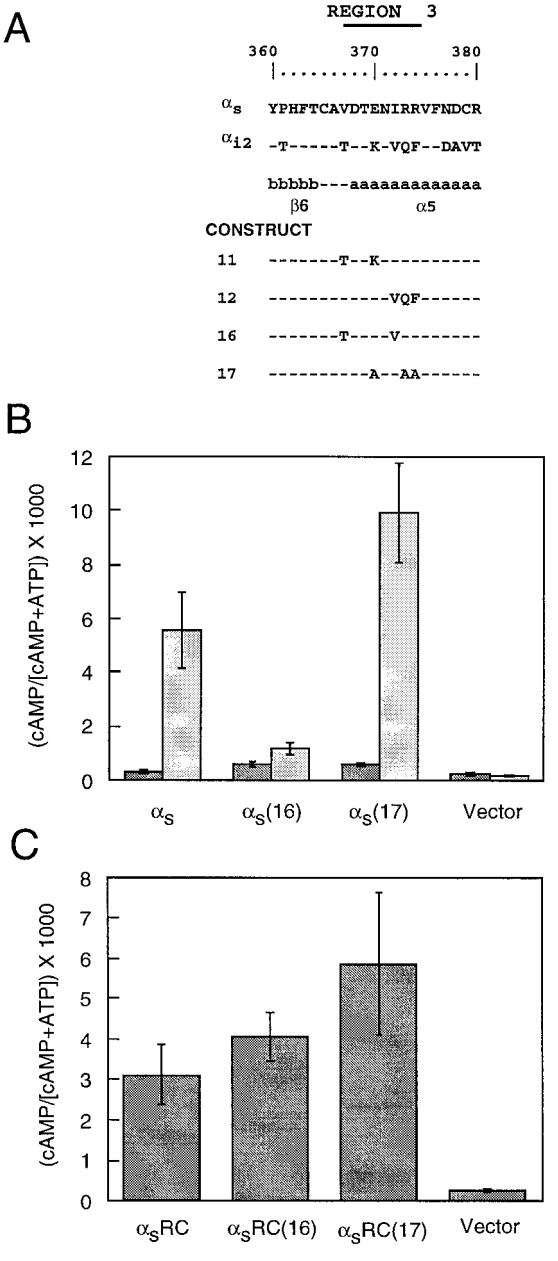

Fig. 5. Substitution of buried but not solvent-exposed residues in region 3 impairs receptor-mediated activation. A, Representation of α_s mutants as in Fig. 1. B, cAMP accumulation in *cyc*$^-$ cells containing the indicated mutants in the α_s context. Cells were electroporated with 30 μg of vector alone and of vector containing α_s and α_s(16) and with 45 μg of vector containing α_s(17). *Dark gray*, cAMP values from unstimulated cells. *Light gray*, cAMP values from cells stimulated with 0.1 mM isoproterenol. C, Receptor-independent cAMP accumulation in *cyc*$^-$ cells containing the indicated mutants in the α_sRC context. For each mutant, the same amount of plasmid was used as is indicated in B for the corresponding α_s mutant. cAMP levels in [^{3}H]adenine-labeled cells were determined as described in Experimental Procedures. All values represent the mean ± standard error of at least three independent experiments.

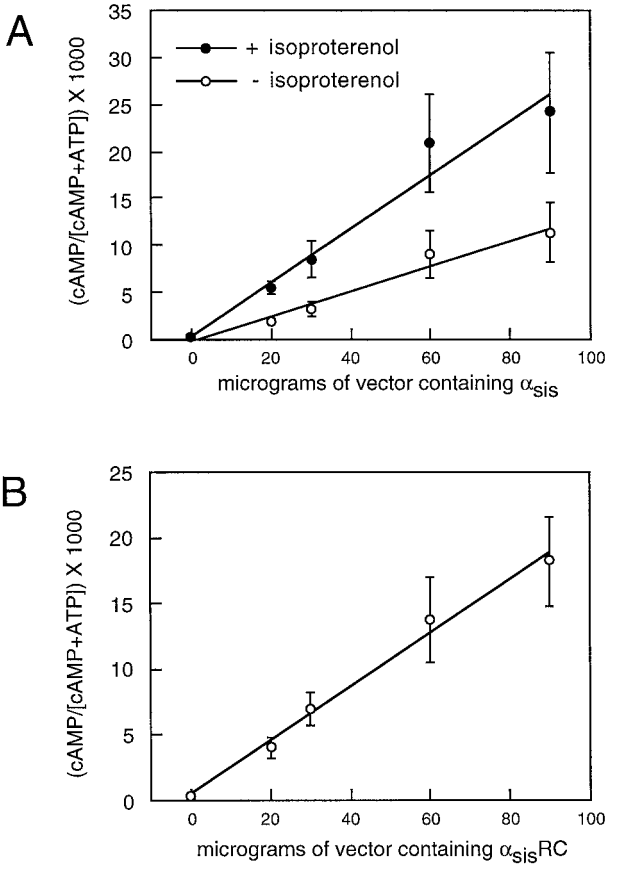

Fig. 6. Receptor-mediated activation of α_{sis}. A, cAMP accumulation in *cyc*$^-$ cells electroporated with the indicated doses of vector containing α_{sis}. cAMP levels were measured in the presence and absence of 0.1 mM isoproterenol. B, Receptor-independent cAMP accumulation in *cyc*$^-$ cells electroporated with the indicated doses of vector containing α_{sis}RC. For the 0-μg points, 30 μg of vector was used. cAMP levels in [^{3}H]adenine-labeled cells were determined as described in Experimental Procedures. Each value represents the mean ± standard error of three independent experiments.

lar to those in stimulated α_s-transfected cells (compare Fig. 6A with Fig. 2A). However, in the absence of β-adrenergic receptor stimulation, α_{sis} produced significantly elevated cAMP levels in transiently transfected cells relative to α_s (compare Fig. 6A with Fig. 2A). Adenylyl cyclase assays on membranes of *cyc*$^-$ cells stably transfected with α_s or α_{sis} confirmed that at equivalent expression levels, the basal activity of α_{sis} was elevated relative to that of α_s (Grishina G and Berlot CH, unpublished observations).

To determine whether the activation defects of the region 1 and 2 α_s mutant constructs, $\alpha_s(1)$, $\alpha_s(2)$, and $\alpha_s(6)$, could be due to mismatches between α_s and α_{i2} residues across the domain interface, we determined the effects of introducing the α_{i2} substitutions of these mutant constructs into α_{sis}. We also tested the effect of introducing the $\alpha_s(2)$ and $\alpha_s(6)$ mutations together, $\alpha_s(2 + 6)$, in both α_s and α_{sis}. We first identified plasmid doses of the GTPase-inhibited RC versions of these constructs that produced receptor-independent cAMP stimulation in transiently transfected *cyc*$^-$ cells comparable to that of 30 μg of α_sRC and of α_{sis}RC (Fig. 7B). Immunoblots of membranes from transiently transfected HEK 293 cells demonstrated that the expression levels of these constructs correlated with their activities (data not shown).

The impairment of receptor-mediated activation caused by two of the three clusters of α_{i2} substitutions at the domain interface was reversed in the context of α_{sis} (Fig. 7A). The isoproterenol-stimulated activities of $\alpha_{sis}(2)$ and $\alpha_{sis}(6)$ were similar to those of α_s and α_{sis}. Thus, matching α_{i2} residues across the domain interface seems to have corrected the decreased isoproterenol-stimulated activity of $\alpha_s(2)$ and $\alpha_s(6)$. Combining the $\alpha_s(2)$ and $\alpha_s(6)$ substitutions in $\alpha_s(2 + 6)$ resulted in a larger decrease in isoproterenol-stimulated activity than was observed for $\alpha_s(2)$ and $\alpha_s(6)$, suggesting that the defects in α_s caused by the two clusters of mutations are additive (compare Fig. 7 with Fig. 3). However, the isoproterenol-stimulated activity of $\alpha_{sis}(2 + 6)$ was similar to those of $\alpha_{sis}(2)$, $\alpha_{sis}(6)$, and α_s. In contrast, as was observed for $\alpha_s(1)$, the isoproterenol-stimulated activity of $\alpha_{sis}(1)$ was disrupted. The basal activities of $\alpha_{sis}(2)$ and $\alpha_{sis}(2+6)$ were not significantly different from that of α_{sis} ($p < 0.05$), but they were consistently intermediate between those of α_s and α_{sis}, suggesting that matching α_{i2} residues across the domain interface partially corrects the elevated basal activity of α_{sis}.

Discussion

In the analysis of α_s mutants presented here, we identified four regions of sequence that mediate activation by the β-adrenergic receptor. Two of these regions, the extreme carboxyl terminus (region 4) and the $\beta6/\alpha5$ loop (region 3), have been implicated previously in receptor/G protein interactions. However, current models of receptor-mediated activation based on these regions have not addressed the issue of how the bound GDP is released from its buried position between the GTPase and helical domains. The effects of mutations in the two other α_s regions identified in the current study (regions 1 and 2), located in the $\beta4/\alpha3$ and $\alpha G/\alpha4$ loops at the interface between these domains, suggest that interactions between residues across the domain interface may play a role in the response to receptor stimulation.

Region 4 (the extreme carboxyl terminus) is the most likely candidate for being a receptor contact site. This region was not visualized in the $\alpha_t/\alpha_{i1}\beta_t\gamma_t$ structure (Lambright *et al.*, 1996) and occupies different positions in the structures of α_t·GTPγS (Noel *et al.*, 1993), α_s·GTPγS (Sunahara *et al.*, 1997), α_{i1}·GDP·AlF_4^-·RGS4 (Tesmer *et al.*, 1997), and $\alpha_{i1}\beta1\gamma2$ (Wall *et al.*, 1995), which could indicate a high degree of mobility. In the structure of α_t·GTPγS (Noel *et al.*, 1993), this region contacts the $\alpha2/\beta4$ loop, which interacts with the β subunit in the heterotrimer structures (Wall *et al.*, 1995; Lambright *et al.*, 1996). Because receptors interact only with α subunits that are associated with $\beta\gamma$ (Fung, 1983), the carboxyl terminus of the α subunit may be located near the interface between $\alpha2/\beta4$ and the β subunit in the heterotrimer/receptor complex, so that receptors interact with both α and β in this region.

Residues at the extreme carboxyl termini of α subunits are

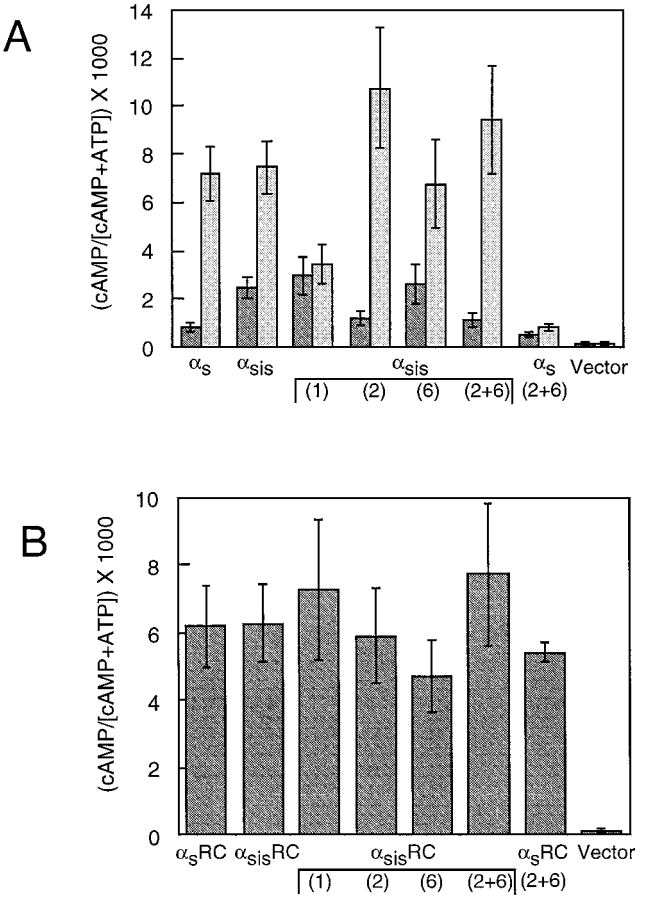

Fig. 7. Receptor-mediated activation of α_{sis} constructs containing α_{i2} substitutions at the domain interface. A, cAMP accumulation in *cyc*$^-$ cells containing the indicated clusters of mutations in the α_s or α_{sis} context. *Numbers in parentheses*, construct numbers in Fig. 1. Cells were electroporated with 20 μg of vector containing $\alpha_{sis}(2)$, 30 μg of vector alone and of vector containing α_s and α_{sis}, 40 μg of vector containing $\alpha_{sis}(2 + 6)$, 60 μg of vector containing $\alpha_{sis}(1)$ and $\alpha_{sis}(6)$, and 180 μg of vector containing $\alpha_s(2 + 6)$. *Dark gray*, cAMP values from unstimulated cells. *Light gray*, cAMP values from cells stimulated with 0.1 mM isoproterenol. B, Receptor-independent cAMP accumulation in *cyc*$^-$ cells containing the indicated clusters of mutations in the α_sRC or α_{sis}RC context. For each mutant, the same amount of plasmid was used as is indicated in A for the corresponding α_s or α_{sis} mutant. cAMP levels in [^{3}H]adenine-labeled cells were determined as described in Experimental Procedures. All values represent the mean ± standard error of at least three independent experiments.

sometimes, but not always, sufficient to confer receptor specificity (Conklin *et al.*, 1993, 1996; Lee *et al.*, 1995). For instance, although both the V_2 vasopressin and β_2-adrenergic receptors couple to α_s, only the V_2 vasopressin receptor can stimulate a chimeric α subunit in which the last five residues of α_q are replaced with the corresponding residues of α_s (Conklin *et al.*, 1996). Differences in the $\beta\gamma$ specificities of α subunits and receptors may dictate whether carboxyl-terminal swaps between α subunits will switch receptor specificities. Also, other regions of α_s, not identified in the current study, probably are receptor contact sites. For example, synthetic peptides corresponding to α_s residues 354–372 and 384–394, which extend from the α4/β6 loop to the beginning of α5 and from the end of α5 to the α subunit carboxyl terminus, respectively, can mimic the effects of α_s on the β_2-adrenergic receptor (Rasenick *et al.*, 1994). Our study of α_s, using homologous substitutions, does not rule out these regions as being receptor contact sites because residues that are identical in α_s and α_{i2} were not changed. Substitutions of surface-exposed α_s residues with alanine residues would be required to determine whether they are receptor contact sites. Furthermore, evaluation of receptor-mediated activation of α_s mutants with substitutions in regions important for adenylyl cyclase activation, which includes residues in the α4/β6 loop (Berlot and Bourne, 1992), would require an assay that is independent of adenylyl cyclase activation.

The results of our study suggest that the role of region 3 (the β6/α5 loop and the amino terminal portion of the α5 helix) in α_s is to regulate GDP affinity because substitutions of buried residues near the GDP, but not of adjacent solvent-exposed residues, disrupt receptor-mediated activation. GDP release by α_s constructs with these substitutions is not entirely blocked because receptor-independent activation of adenylyl cyclase by the GTPase-inhibited versions of the constructs is normal. Of the two region 3 residues in which substitutions decreased receptor-mediated activation, V367, which contacts the guanine ring of the GDP, also is near the helical domain. However, the importance of this proximity to the helical domain is unclear because the helical domain residues closest to V367 are conserved among α subunits and are superimposable on the structures of α_s, α_{i1}, and α_t. Substitutions of other buried residues in this region have been shown to affect guanine nucleotide exchange in several α subunits. The replacement of serine by alanine at position 366 of α_s causes constitutive activation by increasing GDP release (Iiri *et al.*, 1994). Also, the substitution of alanine for cysteine at position 325 of α_o decreases affinity for GDP (Thomas *et al.*, 1993). A mutagenesis study of α_t (Onrust *et al.*, 1997) identified two residues in the β6/α5 loop in which mutations reduced receptor-mediated activation, of which one, T323, corresponds to V367 in α_s.

Receptors activate G proteins by promoting GTP binding, which involves both accelerating GDP release and increasing the relative affinity for GTP compared with GDP (Florio and Sternweis, 1989). Our results suggest that the role of regions 1 and 2 in this process may be to relay conformational changes initiated by receptor/G protein binding across the domain interface rather than to specify interaction with the β-adrenergic receptor. Altered contacts between the two domains might facilitate nucleotide exchange by opening the cleft in which GDP is buried. This hypothesis is based on our observation that replacing α_s residues with α_{i2} homologs on the GTPase side of the domain interface impairs receptor-mediated activation of α_s, but not of a chimera, α_{sis}, in which the helical domain consists of α_{i2} residues. Thus, matching α_{i2} residues on both sides of the domain interface of α_s seems to restore receptor-initiated activation. As is sometimes the case with second-site suppressors of mutations, substitution of the helical domain of α_{i2} for that of α_s corrects the defects of the region 1 and 2 mutations but does not, on its own, cause a defect in receptor-mediated activation.

In support of the concept of communication between regions 1 and 2 and the helical domain, comparison of the structures of GTPγS-bound and GDP-bound α subunits (Noel *et al.*, 1993; Coleman *et al.*, 1994; Lambright *et al.*, 1994; Mixon *et al.*, 1995) shows that conformational changes in switch III (located in region 1) are associated with changes in the positions of helical domain residues in the αD/αE loop, which it contacts in the GTPγS-bound form. Although it is not clear how receptor/G protein interaction might lead to conformational changes in switch III, communication between switches II and III, which are closer together in the structures of GTPγS-bound versus GDP-bound α subunits, could be involved. In the structure of α_s·GTPγS (Sunahara *et al.*, 1997), the side chain of the switch III residue E259 is hydrogen-bonded to the backbone nitrogen of the switch II residue R228, and the side chain of the switch II residue R231 is hydrogen-bonded to the side chain of E268 in the α3 helix, adjacent to switch III. Substitution of a histidine for R231 in switch II of α_s leads to a decrease in receptor-mediated activation (Iiri *et al.*, 1997).

Our results also suggest that interdomain interactions may regulate the basal activity of α_s, because the basal activity of α_{sis} is elevated. From the experiments presented here, we cannot determine whether the increased basal activity of α_{sis} is due to increased nucleotide exchange or decreased GTPase activity. However, because the helical domain of α_s, when expressed on its own and reconstituted with the GTPase domain of α_s, stimulates GTPase activity and promotes binding of GTPγS (Markby *et al.*, 1993), interactions across the interface probably are important for both aspects of guanine nucleotide handling.

Other studies support the idea that interdomain communication plays a role in receptor-catalyzed G protein activation. A salt bridge interaction between D173 in the helical domain and K293 in the GTPase domain of α_s is required for receptor-mediated activation (Codina and Birnbaumer, 1994). A mutation within region 1 that substitutes tryptophan for arginine at position 258 in α_s, R258W, was found in a patient with pseudopseudohypoparathyroidism, and biochemical analysis revealed that although the mutant activated adenylyl cyclase normally in response to GTPγS, it exhibited attenuated responses to AlF_4^- and the β-adrenergic receptor (Warner DR and Weinstein LS, personal communication). Regions 1 and 2 may be important for receptor-mediated activation of other α subunits as well because a segment of α16 (residues 220–240), which overlaps with regions 1 and 2, contributes to the specificity of activation by the C5a receptor (Lee *et al.*, 1995).

Our study suggests that complementary interactions between α subunit residues across the interface between the GTPase and helical domains play a role in facilitating receptor-mediated activation. Biochemical studies using purified α_s mutant proteins with substitutions in these regions and

cell lines that stably express these α_s mutants will be required to more fully understand the nature of these intramolecular interactions and the mechanism by which receptor-catalyzed conformational changes lead to G protein activation.

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