Mutations at the Domain Interface of Gsα Impair Receptor-mediated Activation by Altering Receptor and Guanine Nucleotide Binding

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G protein α subunits consist of two domains, a GTPase domain and a helical domain. Receptors activate G proteins by catalyzing replacement of GDP, which is buried between these two domains, with GTP. Substitution of the homologous α2 residues for four α1 residues in switch III, a region that changes conformation upon GTP binding, or of one nearby helical domain residue decreases the ability of α1 to be activated by the β-adrenergic receptor and by aluminum fluoride. Both sets of mutations increase the affinity of α1 for the β-adrenergic receptor, based on an increased amount of high affinity binding of the β-adrenergic agonist, isoproterenol. The mutations also decrease the rate of receptor-mediated activation and disrupt the ability of the β-adrenergic receptor to increase the apparent affinity of GTP for the GTP analog, guanosine 5’-O-(3-thiotriphosphate). Simultaneous replacement of the helical domain residue and one of the four switch III residues with the homologous α2 residues restores normal receptor-mediated activation, suggesting that the defects caused by mutations at the domain interface are due to altered interdomain interactions. These results suggest that interactions between residues across the domain interface are involved in two key steps of receptor-mediated activation, promotion of GTP binding and subsequent receptor-G protein dissociation.

Heterotrimeric G proteins transmit signals from cell surface receptors to effector proteins that modulate a wide variety of cellular processes (1, 2). The α and βγ 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. Receptor-catalyzed nucleotide exchange is thought to involve an “opening” of the guanine nucleotide binding pocket that facilitates GDP release and increases the relative affinity for GTP compared with GDP (3, 4). The transient empty state of the G protein has a high affinity for the hormone-receptor complex. However, this state is short-lived due to the high intracellular concentration of GTP. Binding of GTP leads to dissociation of the receptor from α-GTP and βγ, 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 βγ.

The α subunit structures consist of two domains, a GTPase domain that resembles the oncogene protein p21ras and a helical domain consisting of α helices and connecting loops. The bound GDP is buried between the two α subunit domains, suggesting that the helical domain may present a barrier to GDP release. Three regions in the GTPase domain (switches I–III) assume different conformations in the structures of GTPγS-bound versus GDP-bound α subunits (5–8). Switches I and II correspond to conformational switch regions in the structures of both p21ras and EF-Tu. Like the helical domain, switch III, which is located at the interface of the two domains, is unique to the structures of heterotrimeric G protein α subunits. The conformational switch regions are important for the interaction of α subunits with effectors (9), βγ (10, 11), and RGS (regulators of G protein signaling) regulators of G proteins (12). Most likely, they play a role in receptor-mediated activation as well.

We previously identified a cluster of four switch III residues2 in α1 at the interface between the GTPase and helical domains in which substitutions with α2 homologs in the mutant construct N254D/M255L/I257L/R258A decreased receptor-mediated activation of adenylyl cyclase in transiently transfected cells (13). The activation defect caused by substituting α2 residues for these α1 residues was corrected by replacing the helical domain of α1 with that of α2 in a chimera, α1α2, in which α2 homologs were substituted for α1 residues 62–235, extending from the end of the α1 helix to the end of the α2 helix (13). Thus, matching α2 residues on both sides of the domain interface of α1 restored receptor-initiated activation.

We now report a detailed analysis of the activation defects caused by these switch III substitutions and of a mutation that replaces a nearby helical domain residue, Asn167, with the homologous α2 residue, arginine. Measurements in stably transfected cells of isoproterenol binding to the β-adrenergic receptor, and the time course and dose-dependence of adenylyl cyclase stimulation by the hydrolysis-resistant GTP analog, GTPγS, in the presence and absence of isoproterenol indicate that the mutations increase the affinity of α1 for the β-adrenergic receptor, decrease the rate of receptor-mediated activation, and block receptor-stimulated increases in GTPγS affinity. Additional mutational analysis refines the nature of the interdomain interactions that play a role in receptor-mediated activation by demonstrating that of the switch III substitutions, R255A alone causes a defect in receptor-mediated activation, that this defect is corrected when the helical domain of α1 is replaced with that of α2, and that the defect caused by the N167R substitution is corrected when combined with the N254D substitution but not the R258A substitution. These results suggest that interdomain interactions are involved in the transmission of signals between the receptor and the guanine nucleotide binding pocket.

1 The abbreviation used is: GTPγS, guanosine 5’-O-(3-thiotriphosphate).

2 Residue numbering throughout is according to the long splice variant of αs.
**EXPERIMENTAL PROCEDURES**

**Construction of a Subunit Mutants—**α subunit constructs were generated from rat α1 cDNA (14). Chimeric α subunits were constructed from rat α1 cDNA and mouse α2 cDNA (15). 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 (16), which was generated by mutating α1 residues DYYPDS (189–194) to EYMPTE and α2 residues SDYIPTQ (166–172) to EYMPTE (single letter amino acid code; mutated residues are underlined). This epitope does not affect the ability of α to activate adenylyl cyclase in response to stimulation by the β-adrenergic receptor (17).

The α1 cDNA was subcloned into the expression vector, pcDNA I/Amp (Invitrogen), as a HindIII fragment. N167Rα, N167Aα, N254Dα, M255Lα, I257Lα, R258Aα, and N254D/M255L/I257L/R258Aα were produced by oligonucleotide-directed in vitro mutagenesis (18) using the Bio-Rad Muta-Genie kit. N167R/N254Dα and N167R/R258Aα were produced by ligating AlwI fragments containing either the N254D or the R258A mutations into N167Rα in place of the analogous fragment to produce an α1 cDNA containing both mutations. Construction of the αα1 chimera, in which α1 residues 62–235 are replaced by the homologous α2 residues, has been described elsewhere (13). R258Aα was produced by ligating a BamHI fragment containing the R258A mutation into αα1 in place of the analogous fragment.

Receptor-independent cAMP accumulation in transiently transfected cells was measured after introducing a second mutation (RC) that substitutes cysteine for the arginine at position 201 and causes constitutive activation by decreasing GTPase activity (19). αRC versions of N254Dα, M255Lα, I257Lα, and R258Aα were produced by ligating BamHI fragments containing the mutations into αRC in place of the analogous fragment. αRC versions of N167Rα and N167Aα were produced by ligating AlwI fragments containing the RC mutation into N167Rα and N167Aα, respectively, in place of the analogous fragment. αRC versions of N167R/N254Dα, N167R/R258Aα, and N167R/R258Aα were produced by ligating AlwI fragments containing the RC fragment and either the N254D or the R258A mutation into N167Re in place of the analogous fragment. The αα1RC version of R258Aα was produced by ligating a BamHI fragment containing the R258A mutation into αα1 in place of the analogous fragment.

Preparation of Stable Cell Lines—αα1 constructs were subcloned as HindIII fragments into the retroviral vector pMV7 (20) and then stably expressed as described (21), in a subclone of cysc S49 lymphoma cells, cysc-kin (22), in which cAMP-dependent protein kinase is inactivated. Single colonies containing the pMV7 vector were obtained using limiting dilution in microtiter wells and selection in G418 (1 mg/ml). Clones expressing αα1 constructs were identified by immunoblotting with the anti-EE monoclonal antibody. Cell membranes were prepared after nitrogen cavitation as described (23).

Immunoblots—25 μg of membrane proteins were resolved by SDS-polyacrylamide electrophoresis (10%), transferred to nitrocellulose, and probed with a monoclonal antibody to the EE epitope (16). The antigen-antibody complexes were detected using an anti-mouse horseradish peroxidase-linked antibody according to the ECL Western blotting protocol (Amersham Pharmacia Biotech).

**Adenylyl Cyclase Assay—**Conversion of [γ-32P]ATP to [γ-32P]cAMP in the presence of various activators was measured as described (23). Membranes were incubated at 30 °C. Reactions shown in Figs. 1 and 4 were preincubated for 5 min in the absence of [γ-32P]ATP and then incubated for 30 min. For the time courses shown in Fig. 3, membranes were preincubated in the absence of [γ-32P]ATP and activators for 6 min. At time = 0, [γ-32P]ATP and either GTP•S or GTP•S and isoproterenol were added and aliquots were removed at the indicated times for cAMP determination. To determine EC50 values for stimulation of adenylyl cyclase by GTP•S shown in Fig. 4, the observed adenylyl cyclase activity was fitted to the equation,

\[ Y = b + \frac{a - b}{1 + (X/d)^c} \]  

(1)

where X is the concentration of GTP•S, Y is the observed adenylyl cyclase activity, a is the adenylyl cyclase activity observed in the absence of GTP•S, b is the maximum observed adenylyl cyclase activity, c is the half-maximal effective concentration (EC50) of GTP•S, and d is the slope factor.

**Receptor Binding Assay—**Membranes were incubated with 75 pm [125I]ICYP in competition with a range of concentrations of isoproterenol (10−11 to 10−3 M) in the presence or absence of 300 μM GTP for 1 h at 30 °C as described (24). At the end of this time, the membranes were diluted and washed on Whatman GF/C filters, and bound [125I]ICYP was measured. The experimental data were analyzed for competition at two sites by nonlinear least-squares curve fitting as described (24). K1 and K2, the low and high affinity dissociation constants, respectively, were assumed to be the same in the presence and absence of GTP. When K1 and K2 were allowed to vary in the two conditions, improved fits to the data were obtained. Therefore, the two-state model may be an oversimplification of receptor behavior, as has been suggested (25).

**cAMP Accumulation Assay in Transiently Transfected cysc S49 Lymphoma Cells—**α subunit constructs were introduced by electroporation into a subclone of cysc S49 lymphoma cells (26) that stably expresses Simian virus 40 large T antigen, and cAMP accumulation was measured after labeling with [3H]adenine as described (13). Nucleotides were separated on ion-exchange columns (27), and cAMP accumulation was measured as [3H]cAMP/[3H]ATP + [3H]cAMP × 1000. Receptor-independent cAMP accumulation was determined by measuring basal cAMP levels in cells transfected with the αRC versions of the mutant constructs.

**RESULTS**

**Mutations at the Domain Interface of α Decrease Activation by the β-Adrenergic Receptor and by Aluminum Fluoride—**The ability of N254D/M255L/I257L/R258Aα to be activated by the β-adrenergic receptor and by AlF4−, which mimics the γ-phosphate of GTP, was measured after expression in cysc S49 lymphoma cells (26), which lack endogenous α1 (28). At equal expression levels (Fig. 1A), adenylyl cyclase activity stimulated by both isoproterenol and by AlF4 was reduced by 80% in membranes of cells expressing N254D/M255L/I257L/R258Aα.
compared with membranes of \(\alpha_s\)-expressing cells (Fig. 1B). Stimulation by the hydrolysis-resistant GTP analog, GTP\(\gamma\)S, not only was intact, but increased by almost 2-fold.

Because the activation defect of N254D/M255L/I257L/R258A was assessed as decreased when the helical domain consisted of \(\alpha_s\) residues (13), we analyzed the x-ray crystal structures of \(\alpha_s\) subunits to identify nearby helical domain residues that might be responsible for the conditional defect of these switch III substitutions. Comparison of the GTP\(\gamma\)S- and GDP-bound \(\alpha_s\) subunit structures (5-8) reveals slight changes in the positions of helical domain residues in the \(\alpha\)/\(\gamma\)E loop, which is in contact with switch III in the GTP\(\gamma\)S-bound form. In \(\alpha_s\), the only residue in this loop that is close to any of the four residues and differs in the sequences of \(\alpha_s\) is Asn\(^{167}\). Mutation of Asn\(^{167}\) to the homologous \(\alpha_{1\beta}\) residue to produce N167R\(\alpha_s\) decreased both isoproterenol-stimulated and AIF\(\gamma\)S-stimulated adenylyl cyclase activity by 60% in membranes of cells expressing equal amounts of protein compared with membranes of \(\alpha_s\)-expressing cells (Fig. 1). As with N254D/M255L/I257L/R258A\(\alpha_s\), stimulation by GTP\(\gamma\)S was increased ~2-fold (Fig. 1).

**Mutations at the Domain Interface of \(\alpha_s\)**

**Increase the Apparent Affinity of \(\alpha_s\) for the \(\beta\)-Adrenergic Receptor**—Because N254D/M255L/I257L/R258A\(\alpha_s\) and N167R\(\alpha_s\) exhibited decreased receptor-mediated activation, we used a competitive binding assay to determine whether these mutant \(\alpha_s\) subunits exhibit alterations in binding to the \(\beta\)-adrenergic receptor. This assay measures an \(\alpha_s\)-dependent increase in the affinity of the \(\beta\)-adrenergic receptor for the agonist, isoproterenol (24, 29), which occurs in the absence of bound guanine nucleotide. The high affinity hormone binding state of the receptor is thought to reflect its interaction with \(G_s\) in the nucleotide-free state. In the presence of GTP, receptors in membranes of \(\alpha_s\)-expressing cells were predominantly in the low affinity state (Fig. 2A). In the absence of GTP, \(\alpha_s\) caused the appearance of high affinity binding sites for isoproterenol on the receptor (Fig. 2A). Like \(\alpha_{1\beta}\), both N254D/M255L/I257L/R258A\(\alpha_s\) and N167R\(\alpha_s\) increased the affinity of the \(\beta\)-adrenergic receptor for isoproterenol in the absence of GTP compared with in its presence (Fig. 2, B and C). However, in membranes of cells expressing these constructs, the affinity of the receptor for isoproterenol in both the presence and absence of GTP was greater than in membranes from \(\alpha_s\)-expressing cells, due to decreases in \(K_a\) and \(K_H\), the low and high affinity dissociation constants, respectively, as well as increases in the percentage of receptors in the high affinity form, % \(R_H\). This increase in hormone-receptor binding was particularly striking in cells expressing N167R\(\alpha_s\), in which 50% of the receptors were in the high affinity form in the presence of GTP. The simplest explanation of the increased amount of hormone-receptor binding observed in the presence of N254D/M255L/I257L/R258A\(\alpha_s\) and N167R\(\alpha_s\) is that these mutant \(\alpha_s\) subunits increase the affinity of \(G_s\) for the receptor in both the nucleotide-bound and -free states.

**Mutations at the Domain Interface of \(\alpha_s\)**

**Decrease the Rate of Activation by the \(\beta\)-Adrenergic Receptor**—Because dissociation of \(G_s\) from the activated receptor must precede adenylyl cyclase activation, we investigated whether N254D/M255L/I257L/R258A\(\alpha_s\) and N167R\(\alpha_s\) exhibited altered rates of receptor-mediated activation. To estimate relative rates of receptor-mediated activation, we determined the effects of isoproterenol on the time courses of adenylyl cyclase activation by GTP\(\gamma\)S. In membranes of cells expressing \(\alpha_s\), GTP\(\gamma\)S activated adenylyl cyclase with a time lag that was greatly reduced by isoproterenol (Fig. 3A). This decreased time lag reflects receptor-stimulated increases in the rates of GDP dissociation and GTP\(\gamma\)S binding. In the absence of isoproterenol, GTP\(\gamma\)S activated ad-

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**FIG. 2. Competition between isoproterenol and \([\text{125}^I]\text{Iodocyanopindolol} for binding to the \(\beta\)-adrenergic receptor.** Membranes of cye- cells stably expressing \(\alpha_s\) (A), N254D/M255L/I257L/R258A\(\alpha_s\) (B), or N167R\(\alpha_s\) (C) were incubated with \([\text{125}^I]\text{ICYP} (75 \text{ pM}) and the indicated concentrations of isoproterenol, in the presence (filled symbols) or absence (open symbols) of 500 \(\mu\)M GTP. Values represent the means of duplicate determinations in a single experiment, which is representative of two such experiments. The solid lines represent a nonlinear least-squares fit to the data according to the two-state model for receptor activation (40). \(K_a\) 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. \(K_a\) and \(K_H\) were assumed to be the same in the presence and absence of GTP. In B and C, the binding curves for membranes from \(\alpha_s\)-expressing cells, from \(\alpha_s\), are redrawn as dotted lines. Similar results for each construct were obtained in two additional experiments using a second independent clone of stably transfected cye- cells. The binding assay and data analysis were performed as described (24).
enylyl cyclase in membranes containing N254D/M255L/I257L/R258A αs or N167R αs with somewhat shorter time lags than in αs membranes (Fig. 3, B and C). Isoproterenol increased the activation rates of these mutants, but not to the same extent as for αs. Thus, in the presence of isoproterenol, the time lags of the mutants were longer than that of αs (Fig. 3, B and C). N167R αs, which caused the appearance of the greatest amount of high affinity binding to the receptor (Fig. 2C), exhibited the longest time lag in the presence of isoproterenol. Thus, N254D/M255L/I257L/R258A αs and N167R αs exhibit decreased rates of receptor-mediated activation, which could reflect decreased rates of GTP-dependent dissociation from receptors. Alternatively, or in addition, decreased rates of receptor-mediated activation could be due to defects in receptor-stimulated GTP binding, which we investigated as described below.

Mutations at the Domain Interface of αs Disrupt the Ability of the β-Adrenergic Receptor to Promote Binding of GTPγS—Receptors stimulate guanine nucleotide exchange on G proteins by increasing the rate of GDP release and by causing a preference for GTP compared with GDP (3, 4). For αs, this results in an approximately 8-fold decrease in the half-maximal effective concentration (EC50) for GTPγS stimulation of adenylyl cyclase in the presence of isoproterenol compared with in its absence (Fig. 4A). In the absence of isoproterenol, N254D/M255L/I257L/R258A αs and N167R αs exhibited EC50 values for GTPγS stimulation of adenylyl cyclase that were slightly lower than that of αs (Fig. 4, B and C). However, these EC50 values were

Fig. 3. N254D/M255L/I257L/R258A αs and N167R αs exhibit increased time lags for activation by isoproterenol. Membranes of cyc cells stably expressing αs (A), N254D/M255L/I257L/R258A αs (B), or N167R αs (C) were preincubated in reaction mix at 30 °C for 6 min. At time 0, [γ32P]ATP and either 100 μM GTPγS (triangles) or 100 μM each of isoproterenol (Iso) and GTPγS (circles) were added. Aliquots were removed at the indicated times for determination of adenylyl cyclase activity. Data points represent the means of duplicate determinations in a single experiment. Similar results were obtained in four additional experiments.

Fig. 4. Isoproterenol does not decrease the EC50 for activation of N254D/M255L/I257L/R258A αs and N167R αs by GTPγS. Adenylyl cyclase activities in membranes of cyc cells stably expressing αs (A), N254D/M255L/I257L/R258A αs (B), or N167R αs (C) were determined in the presence of the indicated concentrations of GTPγS, in the presence (filled symbols) or absence (open symbols) of 100 μM isoproterenol (Iso). Data points represent the means from three independent experiments and are expressed as the percentage of the maximum observed adenylyl cyclase activity. EC50 values were calculated as described under “Experimental Procedures.”
unchanged by isoproterenol (Fig. 4, B and C) so that in the presence of isoproterenol, their apparent affinities for GTP•S were less than that of αs, Thus, although isoproterenol increases the rate at which these mutant α subunits exchange nucleotide (Fig. 3), it does not increase their apparent affinities for GTP.

Localization of a Single Switch III Residue on the GTPase Side of the Domain Interface That Is Important for Receptor-mediated Activation of αs—We individually tested each of the four switch III residues that were mutated in N254D/M255L/I257L/R258Aαs, to determine their roles in receptor-mediated activation. Receptor-dependent stimulation of cAMP synthesis was measured in transiently transfected cys- S49 lymphoma cells. The only substitution that decreased receptor-mediated activation was R258A (Fig. 5A). Receptor-independent cAMP accumulation was also measured after introducing a second mutation (the RC mutation) that substitutes cysteine for the arginine at position 201 (19). αsRC has decreased GTPase activity and is constitutively activated. R258AαsRC produces receptor-independent cAMP accumulation similar to that of αsRC, indicating that, as is the case for N254D/M255L/I257L/R258Aαs, R258Aαs can activate adenylyl cyclase when it is in the GTP-bound form.

Complementation of the Activation Defect of R258Aαs—Because the activation defect of N254D/M255L/I257L/R258Aαs was corrected by replacing the helical domain of αs with that of α12 in a chimera, αsα12, in which α12 homologs were substituted for αs residues 62–235, (Fig. 6) (13), we investigated whether introducing the single homolog substitution (R258A) responsible for the defect of N254D/M255L/I257L/R258Aαs into αsα12 would result in normal activation properties. R258Aαsα12 exhibited activation properties similar to those of αs rather than those of R258Aαs (Fig. 5A). Thus, the defect produced by the R258A substitution appears to be due to an alteration in interactions with αs residue(s) in the helical domain.

Combining the N167R and R258A Substitutions Results in an Additive Defect in Receptor-mediated Activation—We investigated the effect of combining the substitutions on each side of the domain interface, N167R and R258A, that caused significant decreases in receptor-mediated activation. Although Asn167 and Arg258 are both at the domain interface, they are not close enough to make contact in the x-ray crystal structures of α subunits (see Fig. 6). N167R/R258Aαs exhibits a more severe activation defect (Fig. 5B) than either N167Rαs (Fig. 5C) or R258Aαs (Fig. 5A) does, although receptor-independent activation by N167R/R258AαsRC is normal. Because the defects of the N167R and R258A substitutions are additive, the contributions of these substitutions to defects in receptor-mediated activation are independent. Furthermore, some other residue(s) in the helical domain other than the α12 homolog of Asn167 must be responsible for the suppression of the R258A defect in R258Aαsα12.

Combining the N167R and N254D Substitutions Corrects the Defect of the N167R Substitution—According to the x-ray crystal structures of α subunits, Asn167 is close to Asn254 (see Fig. 6). Therefore, we hypothesized that the N167R substitution might cause a conditional defect, depending on the identity of the residue at position 254. According to this hypothesis, replacing Asn254 with aspartate should correct the defect caused by the N167R mutation. Indeed, we found that an α subunit with both substitutions, N167R/N254Dαs, exhibits activation properties similar to those of αs (Fig. 5B). Thus, the N254D substitution, which on its own does not disrupt receptor-mediated activation, corrects the activation defect caused by the N167R substitution.

Substitution of Asn167 by Alanine Does Not Cause a Defect in Receptor-mediated Activation of αs—To further investigate the mechanism by which the N167R substitution causes a defect in receptor-mediated activation, we determined the effect of mutating Asn167 to alanine. Alanine substitutions eliminate the side chain beyond the β carbon but generally do not alter the main chain conformation or impose significant electrostatic or steric effects (30). Therefore, if the activation defect resulting from the N167R substitution is due to a steric or electrostatic incompatibility with Asn254, then alanine substitution might not cause an activation defect. However, if the N167R substitution removes a favorable interaction between Asn167 and Asn254, then alanine substitution should also cause a defect. Because N167Aαs exhibited normal receptor-stimulated cAMP production (Fig. 5C), the activation defect of N167Rαs appears to be due to a steric or electrostatic incompatibility that is reversed by the N254D substitution.
DISCUSSION

Our analysis of \( \alpha_i \) mutants with substitutions at the interface of the GTPase and helical domains suggests that interdomain interactions play a role in the bi-directional transmission of signals between receptors and the nucleotide binding site. Interaction between activated receptors and G proteins promotes GTP binding by accelerating GDP release and increasing the relative affinity for GTP compared with GDP (3, 4). Conversely, nucleotide binding decreases the affinity of G proteins for receptors (24, 29). Substitution of the homologous \( \alpha_i \) residues for four \( \alpha_i \) residues (Asn\( ^{254} \) Meq\( ^{255} \) Ile\( ^{257} \) and Arg\( ^{258} \)) in switch III of the GTPase domain or of one nearby helical domain (Asn\( ^{167} \)) in the \( \alpha\alpha\beta \) heterotrimer causes defects in both directions of this communication process. Signal transmission from the receptor to the nucleotide binding site is defective in that the affinities of these \( \alpha_i \) mutants for GTP\( _{\gamma} \) are unchanged by isoproterenol. Conversely, altered communication between the guanine nucleotide binding pocket and the receptor binding site is demonstrated by high affinity hormone-receptor binding in the presence of 300 \( \mu \)M GTP.

Contacts between the \( \alpha\alpha\beta \) heterotrimer-based \( \alpha \) subunit model shown in Fig. 6, the helical
domain side of the interface “above” the GDP consists of the αD/E loop. Moving up from the GDP toward the top of the α subunit, the corresponding GTPase side of the interface consists of the β5/αG, βα/α3, and αG/α4 loops. Closest to the GDP, a salt bridge interaction between Asp172 in the carboxy-terminal portion of the αD/E loop and Lys293 in the β5/αG loop (Fig. 6, dark blue) is required for activation by the β-adrenergic receptor and by AIF4, but not by GTP·S (31). These residues are highly conserved among α subunits, and Lys293 is located in the NKKXD motif, which is important for GDP binding by monomeric GTPases. Mutation of Asp273 increases GDP affinity, consistent with the idea that the mutation “frees” Lys293 from Asp172 to interact with GDP, whereas mutation of Lys293 decreases GDP affinity. Because Asp172 and Lys293 are adjacent to the bound guanine nucleotide, they are more directly involved in regulating guanine nucleotide binding than the residues mutated in our study are. The effects of mutating Asp273 and Lys293 on receptor affinity and receptor-dependent changes in GDP affinity have not been determined.

Arg258 (Fig. 6, red) is located further up, in the βα/α3 loop, which includes switch III. Interestingly, a mutation that substitutes tryptophan for Arg258 was found in a patient with Albright hereditary osteodystrophy, and R258Wαs exhibited more severe defects than did R258Aαs. The defect caused by the R258A substitution is suppressed (Fig. 5A) by substituting the entire helical domain of α2 for that of αs in the αs2/αs chimera (Fig. 6), but we have not identified the helical domain residue(s) responsible. In addition to Asn167, there are two other αs residues in the αD/E loop, Ile172 and Cys174, that differ in the sequences of αs and αs2. Cys174 is not close to Arg258 when modeled onto the heterotrimeric G protein structures (10, 11) or in the structure of αs-GTP·S (32). However, in the latter structure, the side chains of Ile172 and Arg258 are within ~4 Å of each other (see Fig. 7).

Asn167 (Fig. 6, red) is located further away from the GDP in the αD/E loop. The activation defect caused by replacing Asn167 with its αs homolog (arginine) is corrected (Fig. 5B) by simultaneously replacing Asn254 (Fig. 6, magenta) in the βα/α3 loop with its αs homolog (aspartate). In the α subunit structures, the corresponding residues are hydrogen bonded to each other via the side chain of the residue corresponding to Asn167 and either the side chain (in αs (5, 7) and an αs/αs2 chimera complexed with β2γ2 (10)) or the backbone carbonyl (in αs (9, 32) and αs2 (6)) of the residue corresponding to Asn254 (see Fig. 7). The N254D substitution in αs might correct the defect caused by the N167R substitution via a charge neutralization mechanism. This idea is supported by the fact that all α subunits with an arginine at the position corresponding to Asn167 (αs1, αs11, αs14, αs15, αs16, and αs3) have an aspartate at the position corresponding to Asn254 and by the observation (Fig. 5C) that the N167A substitution in αs leaves receptor-mediated activation intact. It is not surprising that the activation defect of N167R/ R258Aαs is worse than those of N167Rαs and R258Aαs (Fig. 5), because these residues are not within contact distance. Because the two mutations cause additive defects, the two residues also do not appear to influence each other through electrostatic or steric effects (33).

We previously found that substitution of αs residues 304, 305, and 307–311 in the αG/α4 loop (furthest from the nucleotide on the GTPase side of the interface in Fig. 6) disrupts receptor-mediated activation in the context of αs, but not αs2 (13). Of the mutated αs residues, only Lys305 and Tyr311 are close to the interface in the structure of αs-GTP·S (32). Although interactions between residues in switch II and the αD/E loop are important for receptor-mediated activation, the known receptor binding sites of αs, the carboxyl terminus of αs (13, 23, 34) and possibly the α4/β6 loop (34), are not near this interface. α Subunits bind to βγ, which is required for receptor-mediated activation, via switches I and II and the amino terminus (10, 11), which are also not near this interface. Thus, receptors initiate activating signals at a significant distance from the domain interface, possibly via an interaction between switches II and III.

N254D/M255L/I257L/R258Aαs and N167Rαs exhibit two characteristics in the absence of receptor stimulation that are not normal and that resemble those of wild-type αs upon activation by hormone-bound receptors. They exhibit slightly elevated basal rates of activation (Fig. 3) and somewhat increased basal affinities for GTP·S (Fig. 4). The basal activation rates of these αs mutants, which reflect basal nucleotide exchange rates, are not nearly as elevated as in the αs mutant, A366Sαs, which is both thermolabile and constitutively activated (35). However, increased rates of basal GDP dissociation in N254D/ M255L/I257L/R258Aαs and N167Rαs would account for their observed defects in activation by aluminum fluoride, which requires the presence of bound GDP.

The defects in guanine nucleotide and receptor binding of N254D/M255L/I257L/R258Aαs and N167Rαs may be interrelated. For instance, decreased receptor-stimulated GDP binding would stabilize the high affinity hormone-receptor-G protein complex, which forms when the G protein is in the nucleotide-free state. Conversely, higher affinity receptor-G protein interactions could decrease nucleotide binding, because activated receptors can cause dissociation of both GDP and GTP analogs (36, 37). However, although there are other reported αs mutants with defects in GTP binding and receptor-mediated activation, there is no precedent for an associated increase in receptor affinity. R231Hαs, containing a mutation in the α2 helix (38), and S54Nαs, containing a substitution in the α1 helix (39), exhibit impaired activation by receptors and AIF4 but can be activated by GTP·S. The affinities of these αs mutants for GTP are decreased upon receptor stimulation. The affinity of R231Hαs for the receptor is normal, and the affinities of S54Nαs and of A366Sαs, which exhibits accelerated GDP release (35), were not determined. The defect of S54Nαs appears to be due to altered interactions with the bound Mg2+, with which Ser243 interacts. Thus, the activation defects of these αs mutants are distinct from those of N254D/M255L/I257L/ R258Aαs and N167Rαs.

Further studies will be required to elucidate how the α subunit domain interface mediates communication between the residues responsible for receptor binding and the guanine nucleotide binding pocket. This type of regulation appears to be unique to heterotrimeric G proteins, which interact with seven-transmembrane-spanning receptors, compared with monomeric GTPases, which lack switch III and the helical domain and which utilize different exchange factors.

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