A Highly Effective Dominant Negative $\alpha_s$ Construct Containing Mutations That Affect Distinct Functions Inhibits Multiple $G_s$-coupled Receptor Signaling Pathways*

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To investigate the subcellular organization of receptor-G protein signaling pathways, a robust dominant negative $\alpha_s$ mutant containing substitutions that alter distinct functions was produced and tested for its effects on $G_s$-coupled receptor activity in HEK-293 cells. Mutations in the $\alpha_{3B5}$ loop region, which increase receptor affinity, decrease receptor-mediated activation, and impair activation of adenylyl cyclase, were combined with G226A, which increases affinity for $\beta\gamma$, and A366S, which decreases affinity for GDP. This triple $\alpha_s$ mutant can inhibit signaling to $G_s$ from the luteinizing hormone receptor by 97% and from the calcitonin receptor by 100%. In addition, this $\alpha_s$ mutant blocks all signaling from the calcitonin receptor to $G_s$. These results lead to two conclusions about receptor-G protein signaling. First, individual receptors have access to multiple types of G proteins in HEK-293 cell membranes. Second, different G protein $\alpha$ subunits can compete with each other for binding to the same receptor. This dominant negative $\alpha_s$ construct will be useful for determining interrelationships among distinct receptor-G protein interactions in a wide variety of cells and tissues.

Stimulation of heterotrimeric G proteins by cell surface receptors activates signaling pathways that mediate specific responses to hormones and neurotransmitters. Cells express a wide variety of G protein-coupled receptors as well as numerous G protein $\alpha$, $\beta$, and $\gamma$ subunits. Many receptors can activate more than one type of G protein, and the G protein subunits can interact with many different types of receptors. The manner in which signaling specificity is maintained in the midst of this vast range of potential interactions is not well understood. This report investigates the interdependence of distinct signaling pathways activated by receptors with broad G protein specificities using a receptor-sequestering dominant negative G protein $\alpha$ subunit.

Many potential mechanisms could establish that distinct receptor-G protein interactions will be independent of each other. Among these, one possibility is that specific receptor-G protein complexes localize to separate membrane compartments (1, 2). Differential associations with particular proteins or lipids (3, 4) or covalent modifications such as phosphorylation (5) may result in subpopulations of receptors and G proteins that have restricted access to each other. Although G proteins are often expressed at much higher levels than their receptors are (6), there is evidence that different receptors utilize separate pools of G proteins (7, 8). An alternative potential mechanism for isolating distinct receptor-G protein interactions is that receptors utilize separate regions for binding different G proteins. If this is the case, then multiple types of receptor-G protein interaction can occur simultaneously without affecting each other. Localization of G protein-binding sites have indicated that separate receptor regions may specify interactions with distinct G proteins (9, 10).

Dominant negative G protein $\alpha$ subunits can test potential mechanisms for separating the distinct G protein interactions of broad specificity receptors. For instance, if interactions with different G proteins are localized to separate subcellular compartments and/or receptor regions, signaling from a receptor to one type of G protein $\alpha$ subunit will be blocked by a dominant negative version of that $\alpha$ subunit, whereas the other signaling pathways will be unperturbed. Alternatively, if each receptor has access to multiple types of G protein and these different G proteins can compete with each other for receptor binding, then a dominant negative $\alpha$ subunit will block all of the G protein signaling pathways activated by the receptor.

Several dominant negative G protein $\alpha$ subunits have been developed previously, but they inhibit G protein signaling incompletely and therefore are not optimal for investigating receptor-G protein signaling pathways. One dominant negative $\alpha_s$ mutant contains three substitutions that disrupt different $\alpha$ subunit functions, but it is extremely unstable (11), which contributes to its inability to inhibit signaling completely (12, 13). Xanthine-binding mutants of $\alpha_{1D}$, $\alpha_{1F}$, and $\alpha_{1G}$ can inhibit signaling of specific G protein-coupled receptor families, but inhibition of receptor-mediated phospholipase C stimulation is incomplete (14, 15). $\alpha$ subunit carboxyl-terminal fragments exhibit dominant negative activity (16), but inhibition of $G_s$ signaling is only partial (13).

This report describes the development of a highly effective dominant negative $\alpha_s$ mutant that contains substitutions that alter distinct $\alpha$ subunit functions, each of which should stabilize the receptor-bound, nucleotide-free state of $G_s$. One set of mutations, located in the $\alpha_{3B5}$ loop region, specifically increases receptor affinity and decreases receptor-mediated activation without affecting nucleotide handling (17) and also disrupts activation of adenylyl cyclase (18). G226A increases affinity for $\beta\gamma$ (19, 20), and A366S decreases affinity for GDP (21). Although A366S alone causes $\alpha_s$ to be thermolabile, $\alpha_{s}$($\alpha_{3B5}$/G226A/A366S), containing all three sets of mutations, is expressed at close to wild-type levels and blocks signaling from the luteinizing hormone receptor to $G_s$ by up to 97%. The
effects of αs(α3β5/G226A/A366S) on signaling by the calcitonin receptor to Gs and Gq are tested in transiently transfected HEK-293 cells. The results demonstrate that this dominant negative αs mutant can block multiple G protein signaling pathways, which indicates that each receptor has access to multiple types of G protein and that these G proteins can compete with each other for receptor binding.

**EXPERIMENTAL PROCEDURES**

Construction of αs Mutant Constructs—αs mutant constructs in the expression vector pcdNA/Amp (Invitrogen) were generated from the rat αs CDNA (22) containing the EE epitope (23), which was generated by mutating residues 189–194 (residues 189–194) to EYMPTE. Mutations were generated by oligonucleotide-directed in vitro mutagenesis using the Bio-Rad Muta-Gen e kit except for those in the α3β5 region, which were produced by subcloning mutant oligodeoxynucleotide cassettes. Subcloning and mutagenesis procedures were verified by restriction enzyme analysis and DNA sequencing.

**Transient Expression and Assays for cAMP Accumulation and Inositol Phosphate Formation—HEK-293 cells (ATCC, CRL-1573) (106 per 60-mm dish) were transfected with plasmids as described in the figure legends using 10 μg of LipofectAMINE 2000 Reagent (Invitrogen) according to the manufacturer’s instructions. 24 h after transfaction, the cells were replated in 24-well plates and labeled with either [3H]adenosine-5′-triphosphate or [3H]inositol, and solubilized with 5 mM LiCl and in the presence or absence of agonist as indicated in the figure legends. Inositol phosphate formation was measured in the presence of 3-isobutyl-1-methylxanthine (IBMX) and 10 μM 125I-labeled sCT (26, 27). cAMP accumulation was measured in the absence of IBMX (28), and GDP-binding results were detected using an anti-mouse horseradish peroxidase-linked antibody according to the ECL Western blotting protocol (Amersham Biosciences).

**RESULTS**

**Combining Substitutions in the α3β5 Loop Region of αs with Mutations That Alter βγ and GDP Binding Results in Highly Effective Dominant Negative Activity—**Replacing five αs residues in the α3 helix and the α3β5 loop (29) with the homologous αs residues (N271K, R280K, T284D, and I285T) resulted in an αs construct, αs(α3β5), that exhibits increased affinity for and a decreased ability to be activated by the βγ-adrenergic receptor (17). Independently, the mutations also disrupt activation of adenyl cyclase by αs (18). These properties suggested that αs(α3β5) might be able to sequester Gs-coupled receptors and exhibit dominant negative activity. Indeed, when αs(α3β5) was transiently expressed in HEK-293 cells that were co-transfected with plasmid encoding the rat luteinizing hormone receptor, cAMP accumulation in response to 20 ng/ml hCG (CR-127, National Hormone and Peptide Program) was inhibited by 42% (S.E. = 37%, n = 3) (Fig. 1).

With the goal of producing more effective dominant negative activity, additional mutations predicted to stabilize the receptor-G protein complex were introduced into αs in combination with the α3β5 substitutions. The effects of adding each of three mutations, G226A, E268A, and A366S, to αs(α3β5) were tested. When combined, these three mutations produce partial dominant negative activity in αs (11). G226A impairs activating conformational changes in switch II required for dissociation of αs from βγ (19, 20). E268A disrupts a salt bridge with Arg-231 that maintains the activated conformation of αs (30). A366S elevates basal GDP release, causing αs to be constitutively activated and to spend more time in the empty state (21). αs(α3β5/A366S) inhibited cAMP accumulation in response to 20 ng/ml hCG by 69% (S.E. = 1%, n = 3) (Fig. 1). When introduced into αs(α3β5), both G226A and A366S produced increased dominant negative activity (Fig. 1). αs(α3β5/
increased the dominant negative activity of $\alpha_5(\alpha_3\beta_5)$, G226A and A366S, with the $\alpha_3\beta_5$ substitutions resulted in a highly effective dominant negative $\alpha_5$ construct that inhibited the cAMP accumulation response to 20 ng/ml hCG by 97% (S.E. = 0.2%, $n = 3$) (Fig. 1). No further increase in dominant negative activity resulted when the E268A substitution was added to $\alpha_5(\alpha_3\beta_5)$ (Fig. 1).

The dominant negative $\alpha_5$ mutants, $\alpha_5(\alpha_3\beta_5)$, $\alpha_5$(G226A/E268A/A366S), and $\alpha_5$(G226A/G226A/A366S), decreased the effectiveness of signaling from the luteinizing hormone receptor to $G_s$ both by increasing the EC$_{50}$ value of the cAMP response to hCG and by decreasing the magnitude of cAMP responses to hCG (Fig. 2 and Table I). Compared with $\alpha_5(\alpha_3\beta_5)$ and $\alpha_5$(G226A/E268A/A366S), the effects of $\alpha_5(\alpha_3\beta_5)$ on both of these parameters were greater. These incremental effects of combining the $\alpha_3\beta_5$, G226A, and A366S mutations are consistent with their independent sites and mechanisms of action.

Substitutions in the $\alpha_3\beta_5$ Loop Rather Than in the $\alpha_3$ Helix Produce Dominant Negative Activity—To investigate how altering the $\alpha_3\beta_5$ loop region of $\alpha_5$ produces dominant negative activity, the effects of smaller numbers of substitutions in this region were tested. Of the five $\alpha_5$ homolog substitutions, two are located in the $\alpha_3$ helix (N271K and K274D) and three (R280K, T284D, and I285T) are in the $\alpha_3$ loop. Based on their location, only the loop residues are likely to interact directly with receptors (17). Separately testing the effects of substitutions in these two regions showed that the dominant negative effect is due predominantly to the substitutions in the $\alpha_3\beta_5$ loop, rather than those in the $\alpha_3$ helix (Fig. 3). $\alpha_5$(R280K/T284D/I285T) was only slightly less effective as a dominant negative than $\alpha_5(\alpha_3\beta_5)$ was, whereas $\alpha_5$(N271K/K274D) exhibited the same basal and receptor-stimulated cAMP accumulation as $\alpha_5$ did. Therefore, the dominant negative phenotype of $\alpha_5(\alpha_3\beta_5)$ appears to result from the alteration of a receptor contact site on $\alpha_5$.

Individual substitutions of each of the $\alpha_3\beta_5$ loop residues decreased receptor-mediated activation of $\alpha_5$ but did not produce dominant negative activity (Fig. 3). $\alpha_5$(R280K) exhibited very little basal or receptor-stimulated activity, whereas the activities of $\alpha_5$(T284D) and $\alpha_5$(I285T) were decreased relative to that of $\alpha_5$. The dominant negative activity produced by simultaneously substituting the three residues may result from additive defects in receptor interaction or may involve conformational changes in the loop due to interactions among the mutated residues.

The $\alpha_3\beta_5$ Substitutions Compensate for the Instability Caused by the A366S Mutation—A previously reported limitation of $\alpha_5$(G226A/E268A/A366S) is its instability (11), due to the A366S mutation, which increases the amount of time $\alpha_5$ spends in the thermolabile nucleotide-free state (21). To determine whether the greater dominant negative activity of $\alpha_5(\alpha_3\beta_5)$ (G226A/A366S) compared with $\alpha_5$(G226A/E268A/A366S) is due...
in part to a stabilizing effect of the α3β5 substitutions, the expression levels of these αc constructs in membranes of transfected HEK-293 cells were compared (Fig. 4). The expression level of αc(α3β5) was similar to that of αc, whereas that of αc(G226A/E268A/A366S) was much lower. The expression level of αc(α3β5/G226A/A366S) was reduced somewhat relative to that of αc(α3β5) and of αc, but was much higher than that of αc(G226A/E268A/A366S). Thus, the α3β5 substitutions appear to counteract the destabilizing effect of A366S.

αc(α3β5/G226A/A366S) Inhibits Signaling of the Calcitonin Receptor to Gs and Gq—To investigate how dominant negative αc activity affects the signaling pathways of Gs-coupled receptors that also interact with other G protein heterotrimers, signaling of the calcitonin receptor to Gs and Gq was monitored in the presence and absence of αc(α3β5/G226A/A366S). αc(α3β5/G226A/A366S) completely blocked Gs-mediated cAMP accumulation in response to up to 0.48 nM sCT (Fig. 5A). Even at saturating amounts of sCT, this response was inhibited by 82.3% (S.E. = 0.3%, n = 3). αc(α3β5/G226A/A366S) increased the EC50 for stimulation of cAMP accumulation by sCT by a factor of 10 (Table II). As shown previously (9, 31), the calcitonin receptor coupled less efficiently to Gs than to Gq (Fig. 5 and Table II). In the presence of αc(α3β5/G226A/A366S), αq-dependent inositol phosphate formation in response to all doses of sCT was blocked entirely (Fig. 5B)

The ability of a dominant negative αc mutant to block signaling from the calcitonin receptor to both Gs and Gq leads to two conclusions regarding calcitonin receptor-G protein signaling. First, each calcitonin receptor has access to both Gs and Gq for binding. Second, αc can compete with αq for binding to the calcitonin receptor.

DISCUSSION

Combining substitutions in three different regions of αc results in dominant negative αc activity that can inhibit signaling from Gs-coupled receptors by close to 100%. The substitutions affect distinct αc interactions as follows: increasing receptor affinity, decreasing receptor-mediated activation, and decreasing activation of adenylly cyclase (α3β5 substitutions), increasing affinity for βγ (G226A), or decreasing affinity for GDP (A366S). Together, these mutations appear to stabilize the nucleotide-free αβγ-receptor complex. The incremental increases in dominant negative activity that result from combining these mutations are consistent with their independent sites and mechanisms of action. Although the nucleotide-free state, which is increased by the A366S mutation, is inherently unstable, αc(α3β5/G226A/A366S) is expressed at a level close to that of wild-type αc. This is most likely because the increased receptor affinity caused by the α3β5 substitutions stabilizes the empty state, which has the highest receptor affinity (32). The

![FIG. 4. Expression levels of dominant negative αc mutant constructs. HEK-293 cells (6.25 × 10^6) were transfected with 6 μg of vector (pcDNA/Amp) or plasmid encoding αc(α3β5), αc(α3β5/G226A/A366S), αc(G226A/E268A/A366S), or αc. αc(G226A/E268A/A366S) is expressed at much lower levels than αc, whereas the expression level of αc(α3β5/G226A/A366S) is only slightly reduced relative to that of αc. Similar results were obtained in two additional experiments.](image)

![FIG. 5. αc(α3β5/G226A/A366S) inhibits signaling from the calcitonin receptor to Gs and Gq. 10^6 HEK-293 cells were co-transfected with 0.04 μg of plasmid encoding the rabbit C1a calcitonin receptor in pRKCMV (9) and 2 μg of vector (pcDNA/Amp) (filled circles) or plasmid encoding αc(α3β5/G226A/A366S) (open circles). The average receptor number per cell was 252,000 (S.E. = 61,600, n = 3). A, inhibition of receptor-dependent cAMP accumulation by αc(α3β5/G226A/A366S). CAMP accumulation was measured in the absence or presence of the indicated amounts of sCT. B, inhibition of receptor-dependent inositol phosphate (IP) formation by αc(α3β5/G226A/A366S). Inositol phosphate formation was measured in the absence or presence of the indicated amounts of sCT. All values represent the mean ± S.D. from triplicate determinations in a single experiment. Two other experiments gave similar results.

![TABLE II

| αc(α3β5/G226A/A366S) | cAMP accumulation | IP formation |
|----------------------|-------------------|--------------|
| Vector               | 0.49 ± 0.15       | 8.76 ± 0.90  |
| αc(α3β5/G226A/A366S) | 4.86 ± 0.97       | NA*          |

* NA, not applicable.
α3β5 substitutions, on their own, do not affect the nucleotide handling properties of purified αs (17).

The ability of dominant negative αs activity to block signaling of the calcitonin receptor to multiple G protein pathways suggests that, at least in HEK-293 cells, distinct receptor-G protein complexes are not strictly compartmentalized into separate membrane domains. However, some mechanisms for compartmentalizing distinct receptor-G protein signaling pathways might not be detected using a dominant negative αs mutant. For instance, association of receptor subpopulations with distinct βγ combinations could restrict potential α subunit interactions. Inactivation of specific G protein subunits using antisense (33–37) and ribozyme (38, 39) strategies has demonstrated a remarkable specificity of interaction between receptors, αβγ combinations, and effectors. In particular, in HEK-293 cells, ribozyme-mediated suppression of γs specifically reduced expression of β1 and disrupted activation of Gs by β-agonist but not prostaglandin E, receptors (38). Such βγ specificity requirements might be overcome by a dominant negative αs mutant with increased affinity for both receptors and βγ. In addition, although the ratio of Gs to its receptors in cells is generally −100:1 (6), factors important for compartmentalization might become limiting when a dominant negative αs mutant is overexpressed. If this is so, then coexpressing potential compartmentalization factors with this αs mutant would be predicted to narrow the range of its inhibitory capacities.

The effect of dominant negative αs activity on other G protein pathways may depend on the cell and/or receptor type. For instance, cell-specific factors such as the caveolins, which have been reported to interact preferentially with particular G protein subunits (3), may restrict the accessibility of G proteins to receptors. HEK-293 cells do not have caveoli, although β-agonist receptors and adenylyl cyclase V/LIi2 localize to low buoyant density membrane domains in these cells (40). In addition, membrane fractionation studies have provided evidence for microdomains in the plasma membranes of neuroblastoma cells (41) and neutrophils (42) that have differences in their G protein content, and polarized epithelial cells differentially sort G protein α subunits (43) and G protein-coupled receptors (44). It will be of interest to use the dominant negative αs mutant described here to sort out the potential role of intracellular compartmentalization in regulating G protein signaling pathways in a wide range of cells and tissues.

The ability of a dominant negative αs mutant to inhibit signaling to Gs provides a molecular insight into receptor-G protein interactions in that it demonstrates that different types of G proteins can compete for binding to the calcitonin receptor. Previous studies of calcitonin receptor isoforms containing insertions or deletions identified distinct regions important for specific interactions but did not rule out mutually exclusive binding of different α subunits. The first intracellular loop (45–47) and an intact seventh transmembrane helix (9) appear to be important for coupling of this receptor to αs but not αo. The ability of different G proteins to compete for receptor binding despite these differences in specificity requirements indicates that either there is overlap in the receptor binding sites for different α subunits or that binding of one type of α subunit to a receptor sterically or allosterically blocks the association of a different one.

Competition between different G proteins for receptor binding raises the possibility that changes in the expression level of a particular G protein may affect other G protein signaling pathways as well. Alterations in G protein α subunit expression levels can take place on several time scales. Short term changes in the expression level of αs due to decreased stability of the activated state have been observed (48). More long term changes in the expression levels of αs (49) and αo (50) can occur during and play a role in development. In addition, pseudohypoparathyroidism is associated with decreased levels of functional αs (51).

The dominant negative αs mutant described here will have many applications to the investigation of how receptor-G protein signaling is regulated. In cell types for which there is strong evidence of subcellular compartmentalization, such as neurons and polarized epithelia, it may inhibit receptor subpopulations and be useful for determining which proteins coexist in the same membrane microdomains. In addition, it can be used to investigate the role of receptor-G protein interactions in the targeting of these proteins. For instance, if receptors are involved in the targeting of G protein subunits, receptor sequestration by a dominant negative α subunit may alter the localization patterns of wild-type subunits. The localization patterns of dominant negative α subunits as well as those of wild-type G protein subunits and receptors can be studied using fusions of these proteins to green fluorescent protein (52–55).

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