A Dominant Negative Ga\(_s\) Mutant That Prevents Thyroid-stimulating Hormone Receptor Activation of cAMP Production and Inositol 1,4,5-Trisphosphate Turnover

COMPETITION BY DIFFERENT G PROTEINS FOR ACTIVATION BY A COMMON RECEPTOR*

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A Ser to Asn mutation at position 54 of the \(\alpha\) subunit of Ga\(_s\) (designated N54-\(\alpha\)) was characterized after transient expression of it with various components of the receptor-adenyl cyclase pathway in COS-1, COS-7, and HEK 293 cells. Previous studies of the N54-\(\alpha\) mutant revealed that it has a conditional dominant negative phenotype that prevents hormone-stimulated increases in cAMP without interfering with the regulation of basal cAMP levels (Cleator, J. H., Mehta, N. D., Kurtz, D. K., Hildebrandt, J. D. (1999) FEBS Lett. 243, 205-208). Experiments reported here were conducted to localize the mechanism of the dominant negative effect of the mutant. Competition studies conducted with activated \(\alpha^*_q\) (Q212L) showed that the N54 mutant did not work downstream by blocking the interaction of endogenous \(\alpha_q\) with adenyl cyclase. The co-expression of wild type or N54-\(\alpha\) along with the thyroid-stimulating hormone (TSH) receptor and adenyl cyclase isotypes Gs and G\(_q/11\) with respect to \(\beta^\gamma\) stimulation (AC II or AC III) revealed that the phenotype of the mutant is not dependent upon the presence of adenyl cyclase isoforms regulated by \(\beta^\gamma\). These studies ruled out a downstream site of action of the mutant. To investigate an upstream site of action, N54-\(\alpha\) was co-expressed with either the TSH receptor that activates both \(\alpha_q\) and \(\alpha_{q/11}\) or with the \(\alpha_{11}\)-adrenergic receptor that activates only \(\alpha_{q/11}\). N54-\(\alpha\) failed to inhibit \(\alpha_{11}\)-adrenergic receptor stimulation of inositol 1,4,5-trisphosphate production but did inhibit TSH stimulation of inositol 1,4,5-trisphosphate. These results show that Ga and G\(_q/11\) compete for activation by the TSH receptor. They also indicate that the N54 protein has a dominant negative phenotype by blocking upstream receptor interactions with normal G proteins. This phenotype is different from that seen in analogous mutants of other G protein \(\alpha\) subunits and suggests that either regulation or protein-protein interactions differ among G protein \(\alpha\) subunits.

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Heterotrimeric GTP-binding proteins or G proteins transmit signals from receptors on the surface of the cell to various intracellular effector enzymes. G proteins have traditionally been defined by their \(\alpha\) subunits, which consist of four families: Ga\(_s\), Ga\(_q/11\), and Ga\(_{15/16}\). One of the best-characterized \(\alpha\) subunits, Ga\(_s\), stimulates adenyl cyclase in response to receptor-mediated hormone stimulation (1-4). Inactive Ga\(_s\) consists of a GDP-bound \(\alpha\) subunit and a \(\beta^\gamma\) dimer. Upon receptor activation, the \(\alpha^*_q\)-GTP complex dissociates from the receptor and its \(\beta^\gamma\) dimer and then stimulates adenyl cyclase to increase cAMP levels in the cell. Certain isotypes of adenyl cyclase (e.g. AC II and AC IV) are also stimulated by \(\beta^\gamma\) in the presence of activated Ga\(_s\) (5, 6), whereas other isotypes of adenyl cyclase are inhibited by \(\beta^\gamma\) (e.g. AC I). Deactivation occurs when the \(\alpha\) subunit hydrolyzes its bound GTP to GDP, allowing it to reassociate with \(\beta^\gamma\).

G protein-coupled receptors (GPCRs)1 activate G proteins by catalyzing GTP exchange on the \(\alpha\) subunit. Although some GPCRs activate only one G protein isotype (e.g. \(\beta\)-adrenergic receptor stimulation of 

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The abbreviations used are: GPCR, G protein-coupled receptor; TSH, thyroid-stimulating hormone; PI, phosphatidylinositol; TSH-R, TSH receptor; \(\alpha^*_q\), activated \(\alpha^*_q\) Q212L mutant; VIP, vasoactive intestinal polypeptide; VIP-R, vasoactive intestinal polypeptide receptor; HEK, human embryonic kidney; GMP-P(NH)P, guanylyl-5'-yl imidodiphosphate; AC I and AC II, adenyl cyclase I and II; AR, adrenergic receptor; IP, inositol phosphate.
with a Ser to Asn mutation at this site (N54) also has an increased preference for GDP over GTP (23) and a conditional dominant negative phenotype (24). Its phenotype is conditional, because it prevents hormone-stimulated increases in cAMP, even though it itself activates adenyl cyclase downstream and increases basal cAMP levels (24).

The site analogous to Ser44 of αs in αs and αo is Ser47. Cys mutants of this site also have a dominant negative phenotype (25, 26). However, the mechanism in this case appears to be different from that of the N17-Ras mutant. In particular, others have suggested that C47-αs and C47-αo interfere with hormone activation of G proteins by tightly binding βy dimers (25, 26). These mutants are presumably not active themselves, because G protein subunit dissociation is required for activation (2). Further, their dominant negative phenotype is explained by their ability to sequester βy dimers away from other G protein α subunits and by the fact that intact G protein heterotrimers facilitate greatly receptor interactions. Because Ras mutants and αo mutants of this essential Ser residue involved in complexing Mg and nucleotide seem to have different mechanisms of action, we sought to determine the analogous mechanism for the N54-αs mutant. Surprisingly, the N54-αs mutant has a mechanism of action more similar to that of Ras than its more closely related αo counterparts. These results suggest differences either in the mechanism of regulation of αs and αo proteins by magnesium and nucleotides or in the protein-protein interactions of these different G proteins.

EXPERIMENTAL PROCEDURES

Materials—[^3H]Adenine, [^3H]inositol, donkey anti-rabbit horseradish peroxidase-linked antibody, and ECL reagents were purchased from Amersham Biosciences. Bovine TSH (Lot-AFP-5555B) was obtained from the National Hormone and Pituitary Program, NIDDK, National Institutes of Health. All of the other materials were of the highest quality grade available.

Construction of Vectors—cDNAs encoding the long form of Gαs, and the N54 mutation of Gαo in pMV7 (23) were subcloned into the HindIII site of the mammalian expression vector pcdNA3 (Invitrogen) driven by the cytomegalovirus promoter. The activated (Q212L) αs mutant (αs*) in the mammalian expression vector pCR-cytomegalovirus was kindly provided by R. Iyengar. Types II and III adenyl cyclase cDNAs (27, 28), kindly provided by Dr. R. Reed, were subcloned from Bluescript KS between the HindIII and BamHI sites of pcdNA1 and into the EcoRI site of pcdNA3, respectively. The rat TSH-R cDNA inserted in the simian virus 40 promoter-driven expression vector, pSG5 (29), was kindly provided by Dr. L. Kohn. The rat vasoactive intestinal polypeptide receptor (VIP-R) cDNA (30) in the expression vector CD58 under control of the cytomegalovirus promoter was a kind gift from Dr. S. Nagata. The αir-adrenergic receptor in the expression vector pMT2 under control of the SV40 major late promoter was kindly provided by Dr. D. Perez.

Transient Transfections and cAMP Determination—COS-1 and HEK 293 cells grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum at 37 °C were transfected with LipofectAMINE as previously described (24). Cyclic AMP was measured by use of the [^3H]Adenine uptake assay as described previously (24).

Immunoblotting—Samples were prepared by washing cells in phosphate-buffered saline followed by addition of Laemmli sample buffer containing 10% β-mercaptoethanol (31). Proteins were separated by SDS-polyacrylamide gel electrophoresis on 11% polyacrylamide gels by the procedure of Laemmli (31) and transferred to nitrocellulose using a Bio-Rad semi-dry transfer apparatus. Proteins were incubated with anti-αs antibody, ASC, produced according to the protocol of Spiegel and co-workers (32). Bands were visualized by ECL.

Inositol Phosphate Determination—Inositol phosphate production was measured by use of the [^3H]inositol uptake assay (33–36). Cells were labeled with 2 μCi/ml myo-[^3H]inositol 24 h prior to treatment with hormone. Cells were washed once with Dulbecco’s modified Eagle’s medium followed by incubation in Dulbecco’s modified Eagle’s medium/20 mM NaHepes (or Hanks’ balanced salt solution where indicated) containing 10 mM LiCl for 10 min prior to hormone treatment. Cells were incubated for 40 min at 35 °C in the presence or absence of the hormone. The reaction was terminated by the addition of 0.75 ml of ice-cold 10 mM formic acid to the cells. 0.75 ml of each reaction (1 well of a 24-well dish) was diluted in 3 ml of 20 mM ammonium hydroxide (pH of final solution, 8.0–9.0) and applied to regenerated Dowex anion-exchange columns (AG1-X8 resin, Bio-Rad 140-1444) followed by the addition of 1 ml of H2O. The combined sample and water wash was collected as the [^3H]inositol fraction. The column fractions were then washed with 4 ml of 40 mM ammonium formate in 0.1 M formic acid. The [^3H]inositol phosphate was collected by eluting the columns with 5 ml of 2 M ammonium formate in 0.1 M formic acid. Data are presented as the percent of total [^3H]inositol recovered as [^3H]inositol phosphates where total [^3H]inositol equals [^3H]inositol phosphates plus [^3H]inositol.

RESULTS

Previous characterization of the N54 mutant revealed that it possessed a conditional dominant negative phenotype with respect to the TSH-R and the β-adrenergic receptor (24). The nature of this phenotype is shown in Fig. 1A where the rat VIP-R (30) was co-expressed in COS-1 cells with either wild type or N54-αs. The expression of αs resulted in elevated basal cAMP levels with the mutant being slightly more effective than wild type. Co-expression with wild type αs was substantially more effective at increasing VIP-stimulated cAMP levels than co-expression with the N54-αs mutant. When basal cAMP levels were subtracted from those in the presence of VIP (Fig. 1A, right graph), N54-αs was seen to actually decrease VIP-stimulated cAMP levels. These results were similar to what is seen with the TSH receptor and the β-adrenergic receptors (Fig. 1B). Interestingly, whereas the expression of wild type αs increased
hormone-stimulated levels, N54-αs decreased both TSH and VIP-stimulated cAMP levels by ~50%. Based on dose-response curves for the expression of wild type and mutant αs (data not shown and Ref, 24), this appears to be a maximal effect of the mutant. The mutant also decreased stimulation of cAMP levels through the endogenous β-adrenergic receptor but to a lesser degree than for the transfected receptors. The lower inhibition seen in this case probably reflects the relative transfection efficiency, and stimulation through this receptor is probably blunted by ~50% as well.

Fig. 1 and our previous results (24) indicate that N54-αs interferes with receptor stimulation of adenylyl cyclase. The N17-Ras homolog of N54-αs interferes with Ras signaling by binding unproductively to its upstream regulator (16, 17, 37). Signaling by G proteins is more complex than for the monomeric G proteins, and hence, there are more possible sites of interaction for N54-αs. These studies did not support the idea that N54-αs blocks the ability of wild type αs to stimulate adenylyl cyclase.

Interestingly, it was observed that transfection of αs* in

Fig. 2. Co-expression of wild type or N54-αs with activated αs. A, effect of increasing amount of αs* expression on basal and TSH-stimulated cAMP levels in COS-1 cells. Cells were transfected with 0.2 μg of TSH-R and with the indicated amounts of αs* cDNA. Cells were stimulated with 3 nM TSH. Basal, wild type. B, co-expression of αs* with the N54 mutant in COS-1 cells transfected with TSH-R. Cells were transfected with 0.2 μg of TSH-R along with 450 ng of each αs cDNA as indicated. Cells were stimulated with 3 nM TSH. wt, wild type. C, percent of αs* + N54-αs compared with αs* alone in COS-1 and HEK 293 cells. This figure represents data from experiments from either COS-1 or HEK 293 cells transfected with αs* with activated N54-αs. Cells were transfected with a fixed amount of TSH-R cDNA into COS-1 cells and cAMP levels were measured in the absence or presence of TSH (Fig. 2A). As the amount of αs* cDNA increased, the difference between hormone-stimulated and basal cAMP levels decreased. At 450 ng of αs* cDNA transfected with the TSH-R, there was little hormone-stimulated activity, suggesting that the adenylyl cyclase in these cells was near maximally stimulated by the activated αs* mutant (Fig. 2A).

To examine whether the N54 mutant could inhibit activated αs* from stimulating adenylyl cyclase, αs* cDNA (450 ng) was co-transfected alone or with an equal amount of either wild type or N54-αs (Fig. 2B). As seen in Fig. 2B, a representative experiment conducted in COS-1 cells, the expression of αs* increased basal cAMP levels to a greater extent than did wild type or N54-αs. The co-expression of N54-αs with αs* failed to appreciably inhibit αs* from stimulating adenylyl cyclase under basal conditions or under hormone stimulation (Fig. 2B, compare αs* to αs* + N54-αs). A summary of experiments conducted in COS-1 cells is shown in Fig. 2C. Control immunoblots showed that mutant and wild type αs were actually expressed at ~10 times the level of the activated αs* protein in these experiments (Fig. 2D). Therefore, we sought to determine whether N54-αs had a mechanism analogous to its related αs proteins or one similar to the more distantly related Ras protein.

Co-expression of Wild Type or N54-αs with Activated αs, in COS-7 Cells—One possible mechanism of action of a dominant negative effect of N54-αs would be for it to bind to adenylyl cyclase, thus blocking stimulation by wild type αs. Although at first thought this mechanism might seem inconsistent with the fact that N54-αs has inherent basal activity (24), this is not necessarily true. For example, N54-αs could have a lower intrinsic efficacy for activating adenylyl cyclase than does wild type αs. Thus, its binding to adenylyl cyclase could activate the enzyme to a low level but preclude its interaction with an activated wild type αs with greater efficacy and generated by receptor activation of Gs. In fact, our previous data could be consistent with the idea that N54-αs does have less efficacy than does a persistently activated αs protein (24). To test whether this idea is probable, we determined whether N54-αs could effectively compete with an activated αs (Q212L-designated αs*) for stimulation of adenylyl cyclase. To establish conditions for this experiment, increasing amounts of αs* cDNA were transfected with a fixed amount of TSH-R cDNA into COS-1 and cAMP levels were measured in the absence or presence of TSH (Fig. 2A). As the amount of αs* cDNA increased, the difference between hormone-stimulated and basal cAMP levels decreased. At 450 ng of αs* cDNA transfected with the TSH-R, there was little hormone-stimulated activity, suggesting that the adenylyl cyclase in these cells was near maximally stimulated by the activated αs* mutant (Fig. 2A).
COS-1 cells resulted in an increase in the endogenous long form of αs (Fig. 2D). Although this is a potentially interesting result, we have not yet followed up. Because it was not clear how this induction affected the results of these experiments, we conducted the same experiment in HEK 293 cells, which do not show an increase in the expression of endogenous long form of αs upon expression of αs (Fig. 2D). The effect of transfection of αs alone compared with αs plus N54-αs on basal and TSH-stimulated cAMP levels in HEK 293 cells demonstrated that N54-αs did not prevent αs from stimulating adenylyl cyclase in these cells either (Fig. 2C).

Co-expression of Wild type or N54-αs with Adenylyl Cyclase II and III in COS-1 Cells—Another possible mechanism for the dominant negative effect of N54-αs might involve its interference with downstream effects of βγ. This could result from N54-αs irreversibly binding to βγ, as has been suggested to explain the phenotype of the analogous mutation in Goq (25). To determine whether the mutant interferes with the βγ regulation of downstream effectors, experiments were conducted with co-expressed adenylyl cyclase isotypes (AC II or AC III) that differ with respect to βγ regulation. AC II can be activated directly by βγ dimers in the presence of activated αs, whereas AC III is not regulated directly by βγ (5, 6).

AC II or AC III were co-expressed with the TSH-R and with αs (wild type or N54 mutant) in COS cells. Co-expression of AC II with the TSH-R increased TSH stimulation of cAMP accumulation 5-fold compared with transfection of the TSH-R alone (Fig. 3), whereas co-expression of AC III with the TSH-R only increased cAMP accumulation 3-fold in response to TSH. It is not clear whether these differences are due to differences in the level of expression of these two adenyl cyclase isofoms, differences in their inherent activities, or to differences in their regulation. Nevertheless, the differences in the inherent regulation of these two enzymes allowed us to test whether they differed also in their response to the presence of the αs mutants. When wild type αs was transfected with AC II, TSH-stimulated cAMP accumulation was decreased. This is consistent with the idea that TSH maximally stimulated AC II by a dual mechanism involving both αs and βγ and that expressed αs inhibited this maximal stimulation by binding βγ. In contrast, the expression of wild type αs with AC III increased cAMP accumulation in response to TSH, compatible with the idea that αs is rate-limiting for activation of AC III under these conditions. However, regardless of the response to wild type αs, transfection of the N54-αs mutant with either AC II or AC III decreased cAMP accumulation in response to TSH (Fig. 3, insets). This finding suggests that the effects of N54-αs on receptor regulation of cAMP are independent of the properties of the downstream adenylyl cyclase isofom producing the cAMP. This makes it unlikely that the mutant inhibits TSH stimulation of adenylyl cyclase by simply sequestering βγ and preventing the effects of βγ on downstream effectors. This idea is further supported by the fact that the mutant can inhibit TSH stimulation of adenylyl cyclase in HEK 293 cells, which reportedly lack an adenylyl cyclase that is stimulated by βγ (38).

Effect of Wild type αs or N54-αs on Signaling to Phospholipase-β through the α1B-Adrenergic Receptor in COS-7 Cells—In the case of the Ser45 mutant of αs, the analogous site to Ser45 of αs, it interferes not only with receptor regulation of αs-mediated signaling but also with signaling mediated through other G proteins, even for receptors that do not regulate Goq (25). This is one argument that the mutant works by sequestering βγ and preventing upstream receptor heterotrimer coupling. The β-adrenergic receptor, the TSH receptor, and the VIP receptor all couple to Goq. Presumably, a dominant negative effect of αs mediated by its binding to receptor would not directly interfere with signaling through receptors that do not couple to Goq. Therefore, we tested whether N54-αs blocked α1B-adrenergic receptor (α1B-AR) signaling to phospholipase Cβ through Goq. The α1B-adrenergic receptor is not known to couple to Goq. COS-7 cells were used for these experiments, because they reportedly lack a phospholipase Cβ isoform that is stimulated by βγ (39). The α1B-AR when expressed in COS-7 cells (Fig. 4A) had an EC50 for phenylephrine of ~1 μM (Fig. 4B). Increasing the expression of wild type αs slightly decreased the ability of the α1B-AR to increase inositol phosphate levels (Fig. 4C). In contrast, increasing the expression of N54-αs had no effect on the ability of the α1B-AR to stimulate phospholipase C (Fig. 4C). The expression of αs (Fig. 4) failed to alter phenylephrine stimulation of PI turnover in COS-7 cells (Fig. 4D), indicating that the effects of wild type αs cannot be accounted for by elevations in CAMP levels. Although these data are complicated by small effects of wild type αs protein on signaling through phospholipase (discussed below), they clearly demonstrate that N54-αs does not block α1B-AR stimulation of phospholipase C through Goq. This result supports the idea that N54-αs does not nonspecifically interfere with receptor regulation of downstream processes by sequestering βγ dimers necessary for receptor G protein coupling.

Co-expression of Wild Type or N54-αs with the TSH-R in COS-7 Cells: Effect on Inositol Phosphate Levels—Another possible mechanism for the dominant negative action of the N54 mutant could be by its upstream non-productive interaction (sequestration) with receptor. If this mechanism was responsible, we might hypothesize that for receptors that activate more than one kind of G protein, N54-αs would not only block recep-
Fig. 4. Inositol phosphate levels in COS-7 cells transfected with the $\alpha_{1P}$-adrenergic receptor. A, effect of transfection of COS-7 cells with different amounts of $\alpha_{1P}$-adrenergic receptor cDNA. Cells were stimulated with 100 $\mu$m phenylephrine. B, dose response of phenylephrine in COS-7 cells transfected with $\alpha_{1P}$-adrenergic receptor cDNA. Cells were transfected with 1 $\mu$m of $\alpha_{1P}$-AR cDNA and stimulated with the indicated amounts of phenylephrine. C, co-expression of $\alpha_{1P}$-adrenergic receptor with either wild type or N54-$\alpha_s$ in COS-7 cells. Cells were transfected with 0.2 $\mu$m of $\alpha_{1P}$-AR cDNA along with indicated amounts of either wild type or N54-$\alpha_s$ cDNA and stimulated with 100 $\mu$m phenylephrine (PE) for 40 min. This experiment was conducted three times with similar results. D, co-expression of $\alpha_{1P}$ with the $\alpha_{1B}$-AR in COS-7 Cells. Cells were transfected with 0.2 $\mu$m of $\alpha_{1B}$-AR along with 0.8 $\mu$m of $\alpha_{1P}$, wild type $\alpha_s$ (wt), or N54-$\alpha_s$. Cells were stimulated with 100 $\mu$m phenylephrine for 40 min at 35°C. This experiment was conducted twice with similar results.

DISCUSSION

The N54-$\alpha_s$ mutant was the first $\alpha_s$ mutation tested for its potential to have a dominant negative phenotype (23, 24). Its design was based upon the previous description of the phenotype of the N17 mutation in Ras (15, 40), a phenotype present in a large number of small G proteins with this substitution (18–22). Despite this strong conservation of phenotype among the small G proteins and the conservation of the site of mutation to that in the heterotrimeric G protein $\alpha$ subunits as well, it has been difficult to understand the phenotype of the $\alpha_s$ mutant. In part, this is because the N54-$\alpha_s$ mutant has inherent basal activity (23), which the N17-Ras mutant does not (15), and this has compromised acceptance of the mutant as a negative regulator of adenyl cyclase. Instead, we have more appropriately characterized the phenotype of N54-$\alpha_s$ as a conditional dominant negative, because it is negative with respect to hormone stimulation but not with respect to adenyl cyclase regulation (24). However, these studies did not disclose the mechanism of the dominant negative effect on hormone stimulation. This is a significant issue because a related analogous C47 or N47 mutation of $\alpha_s$ or $\alpha_i$ suggests a dominant negative phenotype of these proteins based upon their increased binding to $\beta y$ dimers as opposed to increased binding to the upstream receptor (25, 26, 41). Inability to resolve these issues has compromised the usefulness of the $\alpha_s$ mutant as an experimental reagent compared with that of these mutations in the small G proteins. Here we resolve the phenotype of the N54-$\alpha_s$ mutant and show that its dominant negative phenotype is different from that of the analogous $\alpha_i/o$ mutants. The differences in $\alpha_s$ and $\alpha_i/o$ described here suggest differences in the regulation of these proteins by guanine nucleotides and magnesium and/or the associated protein-protein interactions.

The N54-$\alpha_s$ mutant could inhibit receptor stimulation of adenyl cyclase by at least four different mechanisms. The mutant could 1) bind receptor non-productively, preventing receptor activation of wild type $\alpha_s$, 2) bind adenyl cyclase non-productively, blocking the activation of the enzyme by activated wild type $\alpha_s$, 3) sequester $\beta y$ released upon activation of wild type $G_o$, preventing stimulation of adenyl cyclase isomers activated by $\beta y$, or 4) sequester $\beta y$ released upon activation of endogenous G proteins, preventing their re-association with endogenous $\alpha$ subunits to form heterotrimers required for receptor recognition. The two downstream possibilities do not seem likely. The failure of the N54 mutant to
appreciably inhibited αs from activating adenylyl cyclase, even when expressed in vast excess (Fig. 2), makes unlikely the possibility that the mutant works by binding to adenylyl cyclase non-productively. In addition, the ability of the N54 mutant to inhibit TSH stimulation of adenylyl cyclase III, which is not activated by βγ, suggests that the N54 mutant does not function simply by sequestering βγ and preventing it from activating adenylyl cyclase. Finally, this mechanism could not account for the ability of the mutant to block TSH stimulation of inositol 1,4,5-trisphosphate production (Fig. 5).

N54-αs inhibition of TSH stimulation of cAMP accumulation and PI turnover in COS-7 cells suggests that the mutant binds non-productively to the TSH-R, preventing activation of Gi and Gq/11. The possibility that N54 non-specifically inhibits PI turnover in COS-7 cells suggests that the mutant binds non-productively to the TSH-R, preventing activation of Gs and PI turnover in COS-7 cells. The ability of N54 to inhibit TSH stimulation of cAMP accumulation and PI turnover in COS-7 cells suggests that the mutant binds non-productively to the TSH-R, preventing activation of Gi and Gq/11. The possibility that N54 non-specifically inhibits PI turnover in COS-7 cells suggests that the mutant binds non-productively to the TSH-R, preventing activation of Gs and PI turnover in COS-7 cells.

There are important similarities and important differences in the phenotypes of the N54-αs mutant and its αs and αi counterparts. Although not characterized as extensively as the N54-αs mutant, the C48-αs (26), C47-αs (25), and the N47-αs (41) mutants suggest a pattern of biochemical changes similar to those originally described for the N17-Ras mutant (15). These include decreased affinity for activating guanine nucleotides, altered preference for GDP over GTP (or its analogs), and decreased sensitivity to magnesium ion. These are all compatible with the role of the conserved Ser residue in complexing Mg2+ and the γ phosphate of GTP as disclosed in the x-ray structures of GTP-binding proteins (42–44). Outwardly, they all too have a similar phenotype as dominant negative inhibitors of signaling events. Importantly, however, there are real differences in the mechanism of the dominant negative phenotypes of the αs and αi mutants. The αs mutants are non-specific inhibitors of G protein signaling, acting both on receptors coupled to Gi and on receptors coupled to other G proteins such as Gq (25). This pattern was interpreted to be consistent with the αs mutants irreversibly complexing βγ dimers (25), although the effects on receptor interactions as well could probably not be ruled out. In contrast, the αs mutant is shown here to be a direct inhibitor of signaling specifically through Gi-coupled receptors, probably mediated primarily by its nonproductive binding to the receptor itself. Interestingly, the Giα subfamily may be different still, because the αs equivalent to N54-αs does not display a dominant negative phenotype at all (cited in Ref. 25). The different phenotypes of αs, αi, and αs with the mutations in this conserved Ser suggest differences secondary to their regulation by magnesium and/or guanine nucleotides and influences the ability of the proteins to interact with other proteins. Probably, these differences affect not only the interactions of the mutants, but they also translate into differences in the consequences of regulation of the wild type proteins as well.

Why mutation of the Ser of the first guanine nucleotide binding consensus sequence should have functionally different effects in different Gα subunit proteins is not clear. The crystal structures of αs (45) and αi in complex with a soluble adenylyl cyclase (46) disclose no grossly apparent differences in the interactions of this Ser with bound guanine nucleotide, Mg2+, or close elements of the protein as compared with the αs proteins. However, this is not completely diagnostic of differences in the proteins because it has also been suggested that known biochemical differences exist in effector interactions of the Gα proteins and αβγ interactions. For example, whereas αs requires only micromolar levels of free Mg2+ for binding of GMP/P(NH)P (a hydrolysis-resistant GTP analog), αi requires millimolar levels of free Mg2+ for the binding of GMP-P(NH)P (47, 48). Thus, differences in the interactions of Ser of the first guanine nucleotide binding consensus sequence may lead to subtle effects mediated by changes in side groups not immediately obvious in crystal structures. Alternatively, it is significant that there are not yet crystal structures for the Gs heterotrimer or for the GDP-ligated αs protein. It may be that the real differences in the Gα subunits are in their interactions with GDP rather than GTP. Perhaps the αs and αs proteins differ specifically in the role of Mg2+ in their interactions with GDP and that this results in differences in the regulation of their interactions with other proteins such as the G protein βγ dimer.

The ability of N54-αs to block both TSH-activation of cAMP accumulation and PI turnover has important implications regarding the TSH-R binding site for αs and αs. Point mutations of the TSH-R have been identified that cause constitutive activation of cAMP but not of PI turnover (49–51), which in the simplest sense suggests that the binding of αs and αs is separate or recognize different confirmations of the protein. An Ala to Ile mutation at position 623 (located in the third intracellular loop) of the TSH-R produces constitutive activation of cAMP (51); however, the substitution of Ala623 with either Lys...
Recently, a dominant negative mutant of Gαs containing three sets of separate mutations that affect distinct functions of Gαs has been characterized in HEK293 cells (52). The mutant contains five substitutions in the αβγδ loop of region, which increase receptor affinity, decrease receptor-mediated activation, and disrupt adenylyl cyclase activation (53), in addition to mutations G226A, which increases the affinity of a for βγ (54, 55), and A366S, which decreases affinity for GDP (56). The triple mutant inhibited the lutetinizing hormone and calcitonin receptors from activating adenylyl cyclase in addition to inhibiting the calcitonin receptor from activating PI turnover (52). Those data and ours presented here, using an entirely unrelated dominant negative mutation, suggest that individual receptors have access to multiple G proteins and that different G proteins can compete with each other for binding to the same receptor.

The ability of N54-αs to inhibit TSH stimulation of PI turnover by 50% (Fig. 5) is strikingly similar to the ability of N54-αs to inhibit TSH/VIP stimulation of cAMP accumulation by 50% (Fig. 1). This phenomenon is not just a coincidence of any specific set of conditions, because it is true of multiple pathways regulated by a single receptor (i.e. TSH-R regulation of cAMP or PI turnover), multiple receptors acting on the same pathway (TSH-R, VIP-R and, probably, β-adrenergic receptor), and receptors expressed in multiple cell types (HEK293 cells and COS-7 cells) and whether or not the mutant simultaneously affects basal signaling (for cAMP it does, but for PI turnover it does not). Thus, this phenomenon appears to represent a basic property of how N54-αs interacts with receptor systems. These results suggest that there is something about the mutant that precludes it from blocking 50% of the signaling through unmodified G proteins whether they are similar to or different from the mutant itself. We would suggest that this phenomenon relates to some fundamental property by which receptors interact with G proteins. It has been demonstrated that GPCRs (β-adrenergic receptor (57), 5–10 opioid (58), and metabotropic glutamate receptor (59)) receptors) can form dimers, suggesting that dimerization is involved in the activation of GPCRs. In addition, it has been demonstrated the GABA receptors are assembled as functional heterotrimeric complexes containing GABA1R1 and GABA2R2 (60–62). Although no evidence exists that either the TSH-R or VIP-R form dimers, perhaps the 50% increases the affinity of receptor interactions with receptor,
A Dominant Negative Goαs Mutant That Prevents Thyroid-stimulating Hormone Receptor Activation of cAMP Production and Inositol 1,4,5-Trisphosphate Turnover: COMPETITION BY DIFFERENT G PROTEINS FOR ACTIVATION BY A COMMON RECEPTOR

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