

The N54- α_s Mutant Has Decreased Affinity for $\beta\gamma$ and Suggests a Mechanism for Coupling Heterotrimeric G Protein Nucleotide Exchange with Subunit Dissociation

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ABSTRACT

Ser54 of G_{α_s} binds guanine nucleotide and Mg^{2+} as part of a conserved sequence motif in GTP binding proteins. Mutating the homologous residue in small and heterotrimeric G proteins generates dominant-negative proteins, but by protein-specific mechanisms. For $\alpha_{i/o}$, this results from persistent binding of α to $\beta\gamma$, whereas for small GTP binding proteins and α_s this results from persistent binding to guanine nucleotide exchange factor or receptor. This work examined the role of $\beta\gamma$ interactions in mediating the properties of the Ser54-like mutants of G_{α} subunits. Unexpectedly, WT- α_s or N54- α_s coexpressed with α_{1B} -adrenergic receptor in human embryonic kidney 293 cells decreased receptor stimulation of IP3 production by a cAMP-independent mechanism, but WT- α_s was more effective than the mutant. One explanation for this result

would be that α_s , like Ser47 $\alpha_{i/o}$, blocks receptor activation by sequestering $\beta\gamma$; implying that N54- α_s has reduced affinity for $\beta\gamma$ since it was less effective at blocking IP3 production. This possibility was more directly supported by the observation that WT- α_s was more effective than the mutant in inhibiting $\beta\gamma$ activation of phospholipase C β 2. Further, in vitro synthesized N54- α_s bound biotinylated- $\beta\gamma$ with lower apparent affinity than did WT- α_s . The Cys54 mutation also decreased $\beta\gamma$ binding but less effectively than N54- α_s . Substitution of the conserved Ser in α_o with Cys or Asn increased $\beta\gamma$ binding, with the Cys mutant being more effective. This suggests that Ser54 of α_s is involved in coupling changes in nucleotide binding with altered subunit interactions, and has important implications for how receptors activate G proteins.

Introduction

Heterotrimeric G proteins mediate the effects of a vast array of extracellular signals on intracellular events. They consist of an α -subunit that reversibly binds a $\beta\gamma$ -dimer (Gilman, 1987; Birnbaumer, 1990; Spiegel, 1992; Neer, 1995; Hildebrandt, 1997; Hamm, 1998). Activated G protein-coupled receptor (GPCR) catalyzes the exchange of GDP for GTP on G_{α} , leading to the release of α -GTP and free $\beta\gamma$ from GPCR and on pathways to independently regulate effectors. Thus, G protein activation by GPCR involves a molecular mechanism coupling guanine nucleotide exchange to subunit dissociation (Gilman, 1987).

Ser54 of α_s is a key residue involved in Mg^{2+} and nucleotide binding, and is a highly conserved residue among GTP binding proteins (Sprang, 1997; Hamm, 1998). The S54N mutant of α_s (N54- α_s) has a conditional dominant-negative phenotype (Hildebrandt et al., 1991; Cleator et al., 1999). It has intrinsic

basal activity, increasing cAMP levels without agonist, but paradoxically blocks hormone stimulation of cAMP levels (Cleator et al., 1999). N54- α_s does this by binding receptor nonproductively, preventing GPCR signaling to other G proteins (Cleator et al., 2004). The analogous N17-Ras mutant also has dominant-negative properties based on nonproductive interaction with guanine nucleotide exchange factor, its equivalent of GPCR (Farnsworth and Feig, 1991), and this is true of many small G proteins (Feig, 1999). Homologous mutations of other G_{α} subunits also often have dominant-negative properties similar to those of N54- α_s . For example, studies of analogous S43C or S43N mutants of α_t suggest that they are also dominant-negative proteins by directly binding GPCR, in this case rhodopsin (Natochin et al., 2006).

Mutation of the Ser54 homolog in G_{α} proteins does not always, however, result in proteins with identical characteristics or mechanisms of action. The site analogous to Ser54 of α_s in α_{i2} is Ser48, and in α_o is Ser47. Cys mutants of these sites also have a dominant-negative phenotype (Slepak et al., 1993, 1995), but the mechanism in this case is different from those of N17-Ras (Farnsworth and Feig, 1991), N54- α_s (Cleator et al., 2004), and N43/C43- α_t (Natochin et al., 2006; Ramachandran and Cerione, 2011). C47- α_o and C48- α_{i2} prevent GPCR activation of G proteins by tightly binding $\beta\gamma$ (Slepak et al., 1993, 1995).

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ABBREVIATIONS: α_s^* , activated α_s ; α_{1B} -AR, α_{1B} -adrenergic receptor; ACS, auriculocondylar syndrome; b $\beta\gamma$, biotinylated- $\beta\gamma$; ET_AR, endothelin type A receptor; HEK293, human embryonic kidney 293; GPCR, G protein-coupled receptor; IP3, inositol triphosphate; N54- α_s , S54N mutant of α_s ; PE, phenylephrine; PLC β , phospholipase C β ; TSHR, thyroid-stimulating hormone receptor; VIP, vasoactive intestinal peptide.

Receptors most efficiently recognize the α -GDP $\beta\gamma$ complex (i.e., heterotrimer) (Fung, 1983; Yasuda et al., 1996). Thus, by diminishing the free $\beta\gamma$ pool in cells, these α -mutants can decrease the ability of all cellular GPCRs to signal to downstream G proteins. Alternatively, in cases where $\beta\gamma$ mediates the effects of a GPCR, such mutants can block downstream signaling by sequestering receptor-generated $\beta\gamma$.

Just recently, it was discovered that dominant-negative $G\alpha_{i3}$ subunits are involved in auriculocondylar syndrome (ACS), a rare condition that impairs craniofacial development. ACS is caused by interference of the endothelin type A receptor (ET_A R)/phospholipase $C\beta$ ($PLC\beta$) pathway that induces genes critical for craniofacial development (Marivin et al., 2016). This conclusion is supported by the finding that knockout of G_q or G_{11} in mice results in craniofacial features that resemble ACS (Offermanns et al., 1998; Dettlaff-Swiercz et al., 2005). In humans, mutations are found in genes encoding endothelin-1 (Gordon et al., 2013a), which is the ligand for the ET_A R and the downstream effector $PLC\beta_4$ (Rieder et al., 2012; Gordon et al., 2013b), but not G_q or G_{11} . Unexpectedly, mutations are found in $G\alpha_{i3}$ and are all clustered around the nucleotide binding pocket of $G\alpha_{i3}$. One of the mutations fully characterized was the $G\alpha_{i3}$ S47R mutant that, remarkably, is homologous to the N54- α_s , albeit an arginine (R) is substituted rather than an asparagine (N). The dominant-negative $G\alpha_{i3}$ S47R mutant preferentially binds GDP and sequesters its receptor ET_A R from activating G_q (Marivin et al., 2016), which is very similar to the way in which N54- α_s sequesters the thyroid-stimulating hormone receptor (TSHR) and prevents G_q activation (Cleator et al., 2004).

Here, we looked for an explanation for why the mechanism of action of the Ser54- α_s dominant negative is different from that of analogous $\alpha_{i2/o}$ mutants. These studies indicate that the optimal mutations in each protein have opposite effects on their $\beta\gamma$ -dimer interactions. These results have implications for the role of this site in translating Mg^{2+} and nucleotide binding into regulation of subunit interactions. N54- α_s binds GTP with lower affinity secondary to altered Mg^{2+} binding and binds nonproductively to a receptor imparting a dominant-negative phenotype. These results suggest that N54- α_s sequesters receptor in a $\beta\gamma$ -free state, which challenges the current view of the sequence of events in GPCR activation of heterotrimeric G proteins.

Materials and Methods

Materials. [3 H]inositol, [3 H]adenine, and [35 S]methionine were from Amersham BioSciences UK Ltd. (Little Chalfont, UK).

Construction of Vectors. cDNAs for α_s , N54- α_s , activated α_s (α_s^*), and α_{1B} -adrenergic receptor (α_{1B} -AR) were as described previously (Cleator et al., 1999, 2004). $G\beta$ and $G\gamma$ cDNAs were recently described (Dingus et al., 2005). A rat α_o cDNA in plasmid R/cytomegalovirus from Dr. Randall Reed (Johns Hopkins University School of Medicine) was transferred into pcDNA3.1(+) as an EcoRI-XbaI fragment. The β ARK-minigene in the pRK construct was obtained from Robert J. Lefkowitz, as described previously (Koch et al., 1994). The S47N and S47C mutants were generated from this construct using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The sense primer for S47C- α_o was "GGAGAATCAGGAAATGCAACCATTTGTGAAGCAG, changing codon 47 from AGC (Ser) to TGC (Cys). The S47N mutant was made with similar primers changing codon 47 to AAC (Asn). The S54C mutant of α_s was generated from the parent α_s -pcDNA vector using

the primer 5'-GCTTACAATGGTGCATTTGCCAGACTCTCCAG, changing codon 54 from AGC to TGC.

Cell Transfections and Inositol Phosphate Determination. Human embryonic kidney 293 (HEK293) cells were grown and transfected with LipofectAMINE (Thermo Fisher Scientific, Waltham, MA) as described previously (Cleator et al., 1999, 2004). Previous studies validated that WT- α_s and N54- α_s are expressed at comparable levels when cells are transfected with the same amount of cDNA (Cleator et al., 1999, 2004). IP $_3$ production was measured using a [3 H]inositol uptake assay (Cleator et al., 2004). Cells were labeled with 2 μ Ci/ml myo-[3 H] inositol 24 hours prior to experiments. Data are presented as the percentage of total [3 H]inositol recovered as [3 H]inositol phosphates.

In Vitro Transcription/Translation and Biotinylated- $\beta\gamma$ Binding Assay. WT or mutant α was synthesized in a 50- μ l solution containing 1 μ g cDNA, 10 μ Ci [35 S]Met, 1 mM cold Met, and 40 μ l of TNT Quick Coupled Transcription/Translation System (Promega, Madison, WI), as described previously (Dingus et al., 2005). The biotinylated- $\beta\gamma$ ($b\beta\gamma$) binding assay was as previously described (Dingus et al., 1994). Protein was translated at 30°C for 90 minutes (by which time there is little or no additional synthesis) and then frozen at -80°C until used in an experiment. Binding of α_s or α_o to $b\beta\gamma$ was in a 200- μ l solution containing 20 mM Hepes, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol, 100 mM NaCl, 100 μ M GDP, 0.5 μ Ci translation mixture, and 10 μ l of UltraLink Neutravidin Beads (Thermo Fisher Scientific, Waltham, MA) with 0.1 μ g of $b\beta\gamma$. Controls included [35 S]- α incubated with beads lacking $b\beta\gamma$. Samples were incubated at 4°C for 2 hours on a rotary shaker. Beads were collected in a Picofuge microfuge for 10 seconds and labeled proteins in the pellet separated on 11% SDS-PAGE gels. Fixed, dried gels were exposed for 1–2 days on a Molecular Dynamics (Sunnyvale, CA) phosphorimaging screen and analyzed with a Molecular Dynamics Storm Imager. Data were expressed as relative densities from a single autoradiogram with all samples from an experiment run on a single gel.

Results

Effect of Wild-Type- α_s or N54- α_s on Signaling to $PLC\beta_2$ through the α_{1B} -Adrenergic Receptor in HEK293 Cells. Previously, we showed that N54- α_s blocks G_s -coupled GPCRs from activating downstream G proteins (Cleator et al., 2004). Evidence for this included the observation that in COS-7 cells N54- α_s blocked signaling of receptors coupled to G_s , such as the TSHR, vasoactive intestinal peptide (VIP) receptor, and β -adrenergic receptor, but not those coupled primarily to G_q , such as the α_{1B} -AR. For the TSHR, which couples to both G_s and G_q (Allgeier et al., 1994), N54- α_s prevented TSH stimulation of both cAMP levels through G_s , and IP $_3$ levels through G_q (Cleator et al., 2004). Interestingly, in COS-7 cells, although N54- α_s had essentially no effect on phenylephrine (PE) stimulation of IP $_3$ levels through the α_{1B} -AR, wild-type- α_s actually suppressed agonist-stimulated IP $_3$ levels (Cleator et al., 2004). This effect was even more prominent in HEK293 cells, where the expression of wild-type- α_s suppressed PE-stimulated IP $_3$ levels by as much as 60% (Fig. 1). In this case, N54- α_s also decreased PE-stimulated IP $_3$ levels, but, as in COS-7 cells, N54- α_s was less effective than wild-type- α_s (Fig. 1A). Importantly, there was a fundamental difference in the effect of N54- α_s on signaling through the α_{1B} -AR (Fig. 1A) and its effects on TSHR, VIP receptor, and β -adrenergic receptor that signal through G_s . For G_s -coupled receptors, N54- α_s is more potent than wild-type- α_s at blocking receptor signaling (Cleator et al., 2004), although here, N54- α_s is less effective than wild-type- α_s . Because there was a difference in the efficacy of wild-type- α_s and N54- α_s

(Fig. 1B), and because this effect was opposite that for G_s-coupled receptors (Cleator et al., 2004), we wanted to understand the origin of this difference.

Activated Q213L- α_s (α_s^*) was expressed with the α_{1B} -AR in HEK293 cells to examine the possibility that increases in cAMP levels caused by wild-type- α_s or N54- α_s accounted for their ability to decrease α_{1B} -AR-mediated stimulation of IP₃ levels (Fig. 1B). Coexpression of α_s^* , which causes a greater cAMP increase than N54- α_s (Cleator et al., 1999), actually increased PE stimulation of IP₃ turnover (Fig. 1B), rather than inhibiting the response, as found with unactivated proteins. We therefore considered other possible mechanisms for an effect of α_s on PE-stimulated IP₃. One such mechanism would result from the expression of α_s suppressing free $\beta\gamma$ levels, as proposed for C47 mutants of the α_i -related proteins (Slepak et al., 1993, 1995). Such an effect would be consistent with, for example, studies showing that the expression of α_o (Yu et al., 1997) or α_t (Lustig et al., 1993) blocks $\beta\gamma$ -mediated

effects in cells. This hypothesis also implied, however, that the N54- α_s mutant has decreased affinity for $\beta\gamma$ compared with wild-type- α_s , since it was less effective in the suppression of PE stimulation of IP₃.

Wild-Type or N54- α_s Inhibition of $\beta_2\gamma_2$ Stimulation of PLC β_2 . Wild-type- α_s or N54- α_s inhibition of $\beta_2\gamma_2$ stimulation of PLC β_2 was used to study the $\beta\gamma$ binding affinity of the proteins in vivo. The ability of α_o to bind $\beta\gamma$, thus preventing $\beta\gamma$ activation of PLC β_2 , has been previously used to study in vivo binding of α_o (Yu et al., 1997). $\beta_2\gamma_2$ coexpressed with PLC β_2 in HEK293 cells caused a 4-fold to 5-fold increase in phosphatidylinositol turnover (Fig. 2A), similar to previously

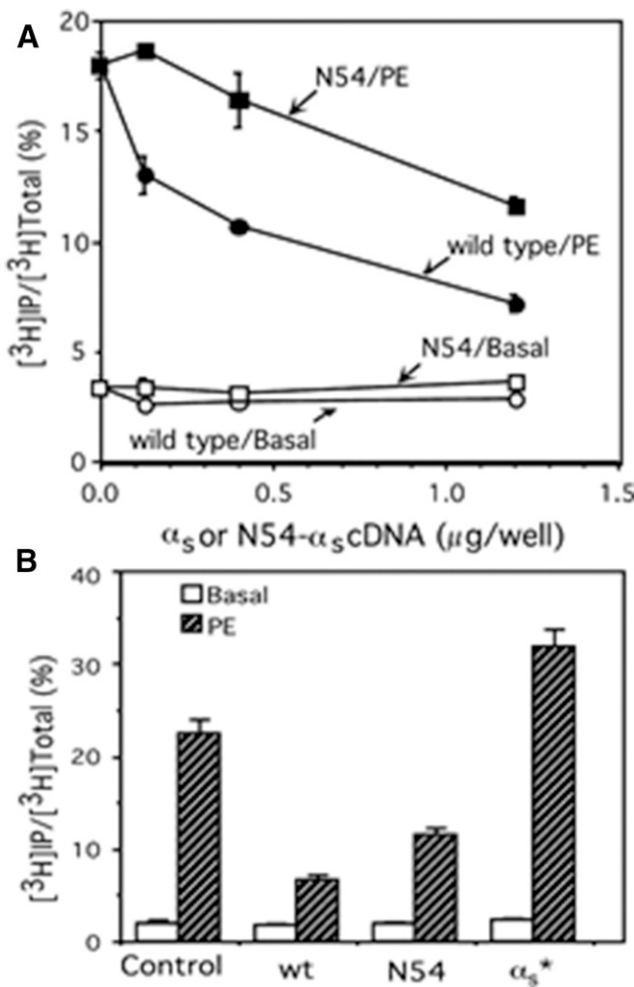


Fig. 1. (A) IP₃ levels in HEK293 cells transfected with α_{1B} -AR. Coexpression of α_{1B} -AR with α_s or N54- α_s . Cells were transfected with 0.2 μ g α_{1B} -AR cDNA and the indicated amounts of wild-type- α_s or N54- α_s cDNA, labeled with [³H]-inositol, as described in *Materials and Methods*, and stimulated 24 hours later with 100 μ M PE for 40 minutes. (B) Coexpression of α_s^* with the α_{1B} -AR. Cells were transfected with 0.2 μ g α_{1B} -AR and 0.8 μ g of α_s^* , wild-type- α_s , or N54- α_s . Cells were stimulated with 100 μ M PE for 40 minutes at 35°C. In each case, data are representative of three experiments. wt, wild type.

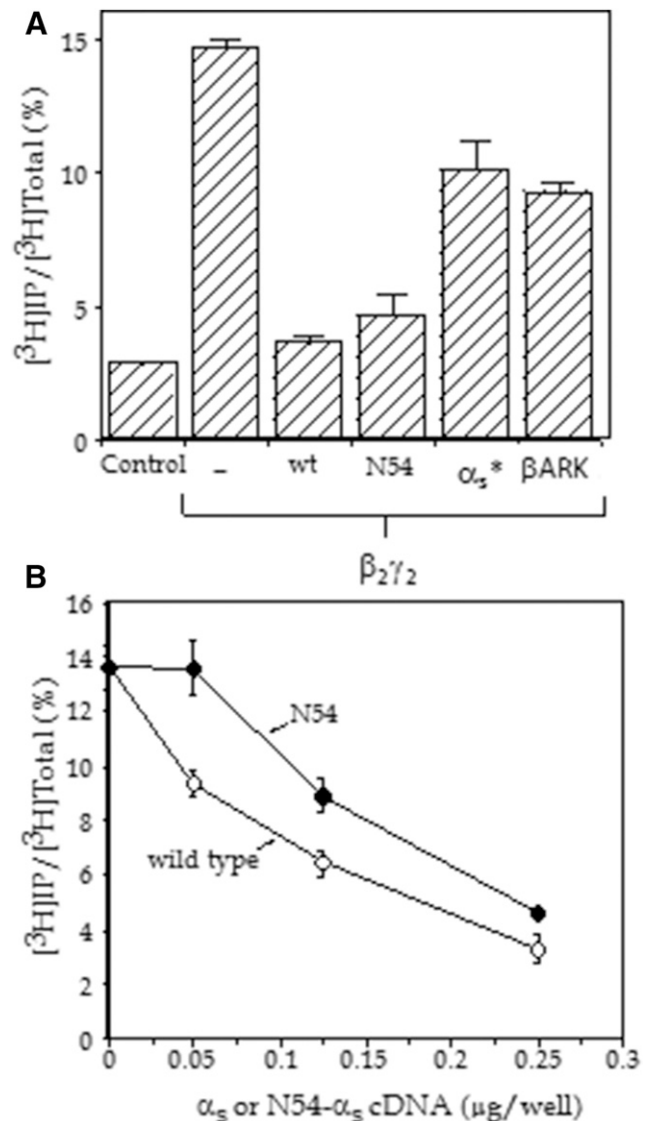


Fig. 2. Inhibition of $\beta\gamma$ stimulation of PLC β_2 in HEK293 cells by α_s and β ARK. (A) Cells were transfected with 0.4 μ g of PLC β_2 , 0.5 μ g of β_2 , 0.5 μ g of γ_2 , and 0.25 μ g of wild-type- α_s , N54- α_s , α_s^* , or β ARK cDNA, as indicated. Inositol phosphates were allowed to accumulate for 30 minutes at 35°C after the addition of LiCl. (B) Dose response of wild-type- α_s or N54- α_s inhibition of $\beta\gamma$ stimulation of PLC β_2 in HEK293 cells. Cells were transfected with 0.4 μ g of PLC β_2 , 0.5 μ g of β_2 , 0.5 μ g of γ_2 , and 0.25 μ g of α_s^* cDNA along with indicated amounts of either wild-type- α_s or N54- α_s cDNA. Inositol phosphates were allowed to accumulate for 30 minutes at 35°C after the addition of LiCl. Control cells transfected with PLC β_2 and α_s^* cDNA possessed a mean value of 2.137 ± 0.168 . wt, wild type.

reported results (Liu and Simon, 1996). At high levels of expression, wild-type- α_s and N54- α_s effectively inhibited $\beta_2\gamma_2$ stimulation of PLC β_2 (Fig. 2A).

Two possible mechanisms for inhibition effects of α_s subunits on $\beta\gamma$ stimulation of PLC β_2 , one mediated by α_s subunits complexing $\beta\gamma$ and the other resulting from downregulation of PLC β_2 after its phosphorylation by protein kinase A (Liu and Simon, 1996). cAMP has been shown to downregulate PLC β_2 through phosphorylation mediated by protein kinase A (Liu and Simon, 1996). α_s^* could be used to explore the contributions of cAMP-mediated inhibition of PLC β_2 , whereas the ability of the β ARK minigene to bind/sequester $\beta\gamma$ could be used to investigate the contributions of α_s complexing $\beta\gamma$ in inhibiting PLC β_2 . Exploring the relative contributions of these two mechanisms, we found that α_s^* decreased $\beta\gamma$ activation of PLC β_2 to a similar extent as the β ARK minigene, which scavenges $\beta\gamma$ (Koch et al., 1994). Neither α_s^* nor the β ARK minigene was as effective as wild-type- α_s or N54- α_s . One possible reason for the greater effect of wild-type- α_s and N54- α_s to inhibit $\beta\gamma$ -mediated PLC β_2 activation is that wild-type- α_s and N54- α_s have a dual effect of increasing cAMP and the ability to bind and sequester $\beta\gamma$. To nullify the inhibition of PLC β_2 by cAMP, α_s^* was coexpressed with wild-type- α_s and N54- α_s and $\beta_2\gamma_2$ -mediated PLC β_2 stimulation was measured (Fig. 2B). This strategy was used to measure the binding of wild-type- α_s or N54- α_s to $\beta\gamma$ while minimizing the confounding effects of cAMP. Wild-type- α_s was clearly more potent in inhibiting $\beta_2\gamma_2$ -mediated PLC β_2 stimulation compared with N54- α_s , suggesting that N54- α_s binds $\beta\gamma$ with less affinity than wild-type- α_s .

Binding of Wild-Type- α_s or N54- α_s to $\beta\gamma$.

To directly examine $\beta\gamma$ interactions, wild-type- α_s and N54- α_s were synthesized using an in vitro rabbit reticulocyte system and assayed for their ability to bind to $\beta\gamma$ immobilized on streptavidin beads (Dingus et al., 1994). Both proteins specifically bound $\beta\gamma$ (Fig. 3A). However, significantly less N54- α_s than wild-type- α_s bound to $\beta\gamma$ (Fig. 3B), closely paralleling the hypothesized reduced affinity of N54- α_s seen in the cellular data above. Attempts to conduct a full dose-response curve to determine the affinity of the proteins for $\beta\gamma$ were hindered by the relatively low amounts of protein synthesized (data not shown). At low concentrations, however, particularly at levels below the equilibrium dissociation constant (K_D) for binding, the amount of α bound is proportional to the affinity of the interaction. Thus, although saturation could not be reached with the low amounts of α_s synthesized, the difference in binding observed is compatible with the affinity of N54- α_s for $\beta\gamma$ being at least 3-fold lower than that of wild-type- α_s . The argument for a 3-fold decrease in apparent affinity of the mutant for $\beta\gamma$ is based upon the following: since we could not achieve saturation binding, our binding assays are likely at or below the K_D of the binding interaction. Significantly below the K_D for binding, the amount bound in a simple bimolecular interaction is directly proportional to the inverse of K_D [i.e., bound = free \times (B_{\max}/K_D)], or is directly proportional to (K_A) [i.e., bound = free \times $B_{\max} \times K_A$]. Thus, if binding of the mutant is only 33% of the binding of wild type, and the two have the same B_{\max} , as designed in the experiment, then the K_A of the mutant is 33% or less of that of mutant (i.e., the wild type has a 3-fold higher affinity). The difference is potentially greater than this because, to the degree the higher affinity interaction is saturated, the closer the weaker interaction approaches the same value as the higher affinity interaction, which then obscures the difference in affinities).

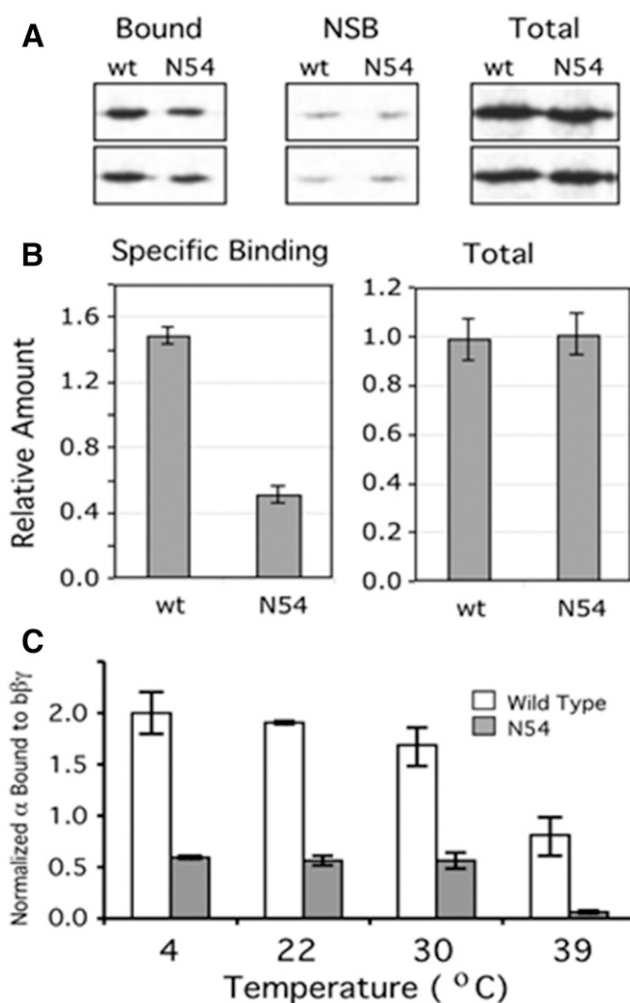


Fig. 3. Binding of α_s or N54- α_s to $\beta\gamma$. (A) Representative binding data. Results of a typical experiment in duplicate measuring binding of [35 S]- α_s to $\beta\gamma$. Total added [35 S]- α_s and protein bound to $\beta\gamma$ streptavidin beads (Bound), as described in *Materials and Methods*. Nonspecific binding (NSB) was [35 S]- α_s bound to beads lacking $\beta\gamma$. (B) Summary of binding data. Data are the mean \pm S.E.M. from three independent experiments. Bands were quantified after exposure on a PhosphorImager screen using ImageQuant (Molecular Dynamics). Graph on the left is bound α_s or N54- α_s minus the NSB. Graph on the right is the total input (Total) to each sample. Values were normalized in an experiment by dividing by the average values for wild-type- α_s and N54- α_s samples. (C) Stability of α_s and N54- α_s to heat denaturation. Wild-type- α_s and N54- α_s were synthesized by in vitro translation, as described in *Materials and Methods*; and then, in a subsequent treatment, incubated for 30 minutes at 4°C, 22°C, 30°C, or 39°C. After this incubation, binding to $\beta\gamma$ was determined at 4°C, as described above. Data are the mean \pm S.E.M. of four experiments. wt, wild type.

One potential complication of the results in Fig. 3B is the possibility that Ser54 mutants of α_s are more labile because of decreased affinity for guanine nucleotides (Hildebrandt et al., 1991; Cleator et al., 1999) and increased thermal denaturation of the nucleotide-free protein. This was evaluated by testing the susceptibility of wild-type and N54 mutant protein to temperature-dependent denaturation prior to assaying $\beta\gamma$ binding at 4°C (Fig. 3C). Thus, aliquots of previously synthesized wild-type- α_s and mutant N54- α_s were then incubated at different temperatures for 30 minutes and then evaluated in the $\beta\gamma$ binding assay. The expectation of this experiment was that, if N54- α_s is more temperature sensitive, less N54- α_s

would bind to $b\beta\gamma$ in a subsequent binding assay because of a reduced functional concentration. Although both proteins were sensitive to denaturation at 39°C (essentially a positive control for the denaturation protocol), and the mutant somewhat more so, both proteins were stable to temperatures up to 30°C, indicating that differences in stability did not explain the differences in the apparent affinity of wild-type- α_s and N54- α_s (Fig. 3B).

Binding of S54- α_s and S47- α_o Mutants to $b\beta\gamma$. The decreased binding of N54- α_s to $G\beta\gamma$, compared with wild-type- α_s , suggests differences in $\beta\gamma$ binding properties of α_s mutants compared with the analogous mutants of α_o and α_i (S47C), which have increased $\beta\gamma$ binding (Slepek et al., 1993, 1995). A possible explanation for this could be that substitution of the conserved Ser by Cys results in a different phenotype than for substitution with Asn. To test this idea, Ser54 in α_s was mutated to Cys (C54- α_s), whereas Ser47 of α_o was mutated to Asn or Cys (N47- α_o and C47- α_o , respectively) and $\beta\gamma$ binding evaluated. C54- α_s bound to $b\beta\gamma$ less well than wild type, although this effect was clearly not as great as for N54- α_s (Fig. 4A). The analogous mutations in α_o , in contrast to results with α_s , had increased affinity for $\beta\gamma$, with the C47- α_o mutant having a greater increase than the N47- α_o mutant (Fig. 4B). Thus, although the Asn substitution in α_s is more effective than is the Cys substitution, and the Cys substitution is more effective in α_o , in both cases the substitutions have phenotypic effects in the same direction.

Discussion

Ser54 of α_s and Ser17 of Ras are part of the Mg^{2+} binding site and first conserved guanine nucleotide binding domain found in both small and heterotrimeric G proteins

(Wittinghofer and Pai, 1991). N17-Ras is a dominant-negative protein with clear-cut properties that have made it a particularly important molecular tool for studying Ras signaling in cells (Feig, 1999). It binds nucleotides weakly, has increased preference for GDP, has high affinity for its upstream guanine nucleotide exchange factor, and does not activate downstream effectors (Feig, 1999). In contrast, the phenotype of N54- α_s is more complex, which limits the use of N54- α_s in cellular studies. N54- α_s has a conditional dominant-negative phenotype whereby it has increased intrinsic basal cAMP activity, but paradoxically decreases hormone stimulation of cAMP (Hildebrandt et al., 1991; Cleator et al., 1999). As a dominant-negative phenotype, N54- α_s also works upstream by binding the receptor nonproductively, preventing the activation of endogenous α_s and, in the case of the TSHR, endogenous $\alpha_{q/11}$ as well (Cleator et al., 2004). Surprisingly, as shown here, N54- α_s binds $\beta\gamma$ with lower apparent affinity than does wild-type- α_s . This is surprising because analogous α_o and α_i mutations (S47C) have increased apparent affinity for $\beta\gamma$ (Slepek et al., 1993, 1995).

Evidence for decreased association of N54- α_s with $\beta\gamma$ includes the data presented here in HEK-293 cells, as well as previous data in COS-7 cells (Cleator et al., 2004), showing that wild-type- α_s more effectively suppresses the stimulation of IP₃ levels through the G_q -coupled α_{1B} -AR than does N54- α_s (Fig. 1). In contrast, N54- α_s more effectively suppresses receptor increases in cAMP or IP₃ mediated by receptors coupled to G_s in addition to or instead of G_q (Cleator et al., 2004). Second, wild-type- α_s suppresses the stimulation of PLC β 2 more effectively than N54- α_s by $\beta_2\gamma_2$ coexpressed in HEK293 cells (Fig. 2). Third, under similar conditions, less N54- α_s binds to $b\beta\gamma$ than wild-type- α_s , which is consistent with a 3-fold or greater decrease in the affinity of N54- α_s for $\beta\gamma$

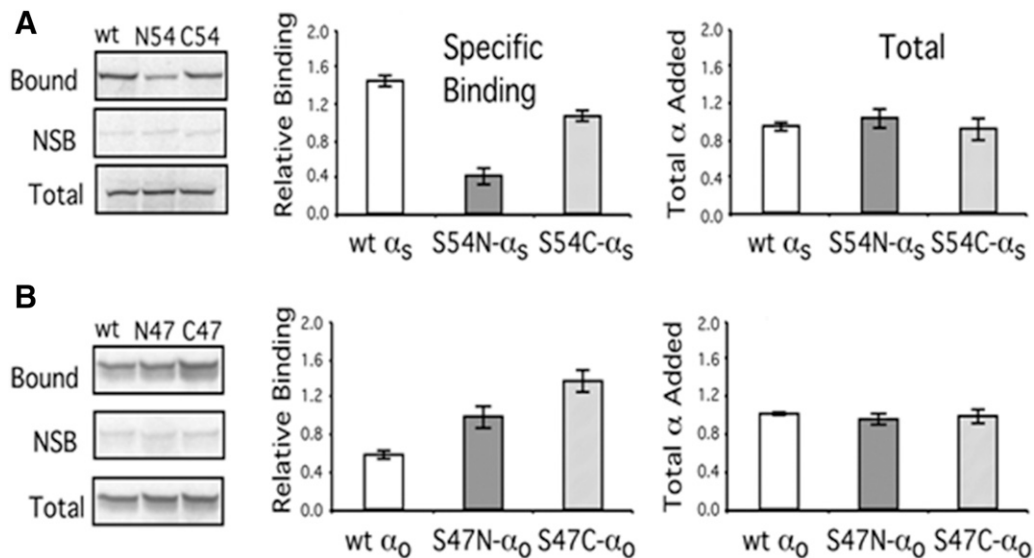


Fig. 4. The effect of Asn vs. Cys substitutions for Ser54- α_s and Ser47-of α_o . (A) Binding of α_s , N54- α_s , or C54- α_s to $b\beta\gamma$. (Left) Representative autoradiogram. As described in Fig. 2A, except that Total is 10% of the input sample. (Middle) Summary of binding data. Data are the mean \pm S.E.M. of protein bound to $b\beta\gamma$ streptavidin beads (Bound) minus nonspecific binding (NSB) for six experiments normalized as in Fig. 2B. (Right) Summary of Total data. Groups were significantly different for bound protein by one-way analysis of variance (ANOVA) ($F = 52.6$, $P = 0.0001$, $df = 2,15$), but not for total protein added ($F = 2.1$, $P = 0.162$). N54 and C54 Bound were different from wild type ($P < 0.01$) as determined by the method of least significant difference. (B) Binding of α_o , N47- α_o , or C47- α_o to $b\beta\gamma$. (Left) Representative autoradiogram. Total is 10% of input as in (A). (Middle) Summary of binding data. Normalized mean \pm S.E.M. of Bound minus NSB for three experiments. (Right) Summary of Total data. Groups were significantly different for bound protein by one-way ANOVA ($F = 15.3$, $P = 0.0044$, $df = 2,6$), but not for total protein ($F = 0.35$, $P = 0.715$). Bound N47 ($P < 0.05$) and C47 ($P < 0.01$) were significantly different from wild type by the method of least significant difference. wt, wild type.

(Figs. 3 and 4). Finally, a decreased affinity for $\beta\gamma$ explains, in part, some of the previously characterized properties of the N54- α_s mutant. This relates particularly to the paradoxical

increased basal activity of N54- α_s despite the fact that it is not activated by receptors (Hildebrandt et al., 1991; Cleator et al., 1999). This can now be explained by its spontaneous activation after dissociation of $\beta\gamma$ and by the fact that the GTP-bound protein does activate its downstream effector, adenylyl cyclase (Hildebrandt et al., 1991; Cleator et al., 1999).

Although dominant-negative GTP binding proteins are often the focus of cellular studies of signaling pathways (Feig, 1999; Barren and Artemyev, 2007), the molecular characterization of these proteins also offers opportunities to understand better the molecular mechanism of signaling through normal variants of these proteins (Wall et al., 1998). Thus, the results reported here may shed light on the mechanism by which guanine nucleotide exchange is coupled to subunit dissociation in the process of G protein activation by receptors, in that the N54- α_s mutant may constitute an analog of a discrete functional state of the G_s protein. Historically, the properties of point mutations have been used to infer, for example, the concepts of a two-state model for GPCR activation (Lefkowitz et al., 1993), where point mutants of selective GPCRs generate constitutively active receptors (Samama et al., 1993). For G proteins, a discrete “state” is inferred from point mutants inhibiting their GTPase activity that trap the $G\alpha$ subunit in its active state with GTP bound. In the case of N54- α_s , the properties of the state of this protein are that it has increased guanine nucleotide exchange rates for both GDP and GTP (Hildebrandt et al., 1991; Cleator et al., 1999), resulting in an increased preference for GDP over GTP binding, decreased affinity for $\beta\gamma$ (shown here), and an increased stable interaction with receptor accounting for its dominant-negative activity (Cleator et al., 2004). Although it is speculative that this would represent a discrete functional state of $G\alpha$, supporting evidence for this comes from the recent characterization of an α_t -related mutant (Pereira and Cerione, 2005). In this case, mutation of an entirely different site in Switch III, R238E, generates a mutant with a phenotype strikingly similar to that of N54- α_s . These similarities include increased nucleotide exchange rates and decreased affinity for guanine nucleotides; insensitivity to NaF, as previously shown for N54- α_s (Hildebrandt et al., 1991; Cleator et al., 1999); decreased binding of $\beta\gamma$, a dominant-negative phenotype related to receptor sequestration (Cleator et al., 2004); and an inferred interaction of the $\beta\gamma$ -free (or compromised) α -subunit as a stable complex with GPCR (Pereira and Cerione, 2005). Why would two unrelated mutations, in two different $G\alpha$ isoforms, one a component of the Mg^{2+} /guanine nucleotide binding site (α_s Ser54) and the other a part of Switch III (α_t Arg238), generate proteins with strikingly similar complexes of biochemical changes? One explanation would be that these residues participate in generating stable states of their respective proteins with specific functional properties related to their activation by receptors.

A long-standing idea about GPCR signaling is that G proteins are activated through a dual mechanism involving nucleotide exchange (GTP for GDP) and subunit dissociation. Figure 5A shows a classic interpretation of G protein activation whereby the receptor catalyzes nucleotide exchange prior to subunit dissociation. The idea that the N54 mutant identifies a discrete state of $G\alpha$ with low affinity for $G\beta\gamma$, high affinity for GPCR, and high affinity for GDP suggests an alternative activation sequence mediated by GPCR, one in which subunit dissociation precedes nucleotide exchange

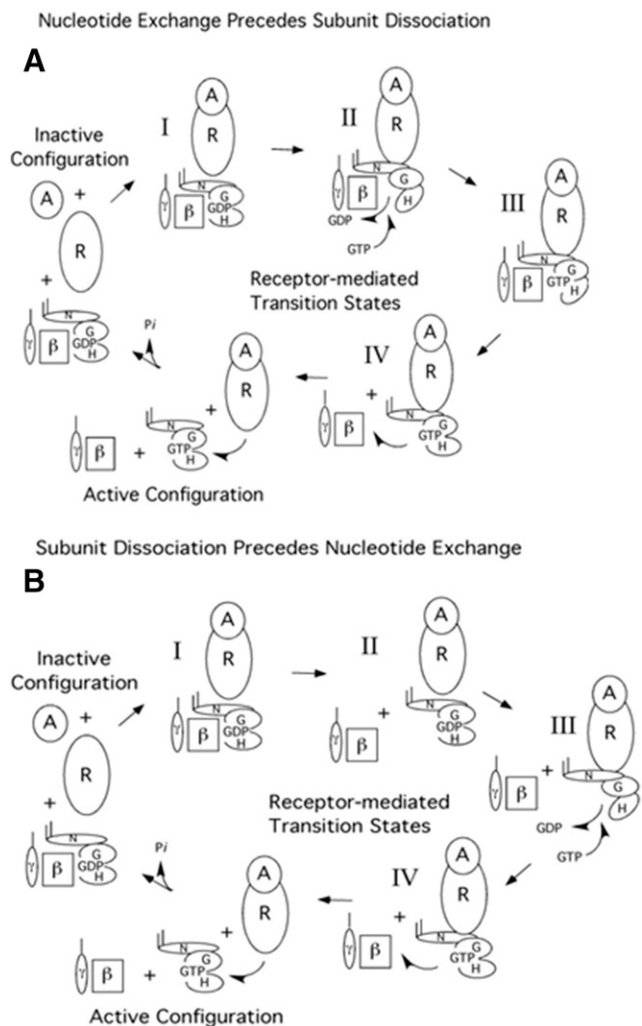


Fig. 5. (A) The classic model of GPCR activation of heterotrimeric G proteins focusing on the four intermediate complexes that would exist between GPCR and G protein during this activation process. In this classic model, nucleotide exchange precedes subunit dissociation. State I would be a recognition complex between receptor and G protein that would be quickly converted to a nucleotide-free intermediate activation complex (State II). This would be the complex characterized in the X-ray structure of the G_s - β_2 adrenergic receptor crystal structure (Dror et al., 2015). Binding of GTP to $G\alpha$ would then produce a transient state (State III) that promotes dissociation of $G\beta\gamma$ (State IV) and, finally, the release of the activated, GTP-bound free $G\alpha$. (B) An alternative model of GPCR activation of heterotrimeric G proteins in which subunit dissociation precedes nucleotide exchange based on the molecular characteristics of the 54N- α_s dominant-negative mutant. In this case, State I is still a recognition state, but State II is a complex between GPCR and α -GTP free of $G\beta\gamma$. State III would be the GPCR-nucleotide-free $G\alpha$ complex, and State IV would be the GTP-bound complex, which would rapidly dissociate as free activated $G\alpha$. The model in Fig. 5B would account for the mechanism by which N54 becomes a dominant-negative inhibitor of receptor activation of G protein even though, by itself, it has some constitutive activity. In this model, the G_s - β_2 adrenergic receptor crystal (Dror et al., 2015) would correspond to State III (i.e., the nucleotide-free state). This state does not, however, contain $G\beta\gamma$, as is seen in the crystal structure. This can be explained by the fact that the change in $G\beta\gamma$ affinity seen here for the N54 mutant is not large, on the order of 3-fold, and that the protein concentrations used to produce crystals are very high, permitting complexes with moderately reduced affinity to still form.

(Fig. 5B). In this model, the Gs- β_2 AR crystal (Dror et al., 2015) would correspond to State III (i.e., the nucleotide-free state). These ideas would also explain the curious observation that in the Gs- β_2 -adrenergic receptor crystal structure, G $\beta\gamma$ makes no contact with the receptor (Dror et al., 2015), even though G $\beta\gamma$ has long been thought to be required for GPCR activation of G α (Fig. 5, legend).

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Authorship Contributions

Participated in research design: Cleator, Wells, Dingus, Kurtz, and Hildebrandt.

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Contributed new reagents or analytic tools: Cleator, Dingus, and Kurtz.

Performed data analysis: Cleator, Wells, Dingus, and Hildebrandt.

Wrote or contributed to the writing of the manuscript: Cleator and Hildebrandt.

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