JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

TITLE PAGE

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.

by

John H. Cleator*†, Christopher A. Wells, Jane Dingus, David T. Kurtz, and John D. Hildebrandt

from

Department of Pharmacology, Medical University of South Carolina, Charleston, SC 29425

*Current Address, Vanderbilt University Medical Center, Division of Cardiovascular Medicine

RUNNING TITLE PAGE

Running Title: The N54-α_s mutant has decreased affinity for $\beta\gamma$

†. Corresponding author and contact information: John H. Cleator

Vanderbilt University

Department of Medicine and Pharmacology, 346B PRB

Nashville, TN 37232

(615)-936-0330, (615)-936-1872 (fax)

john.cleator@vanderbilt.edu

Number of text pages:25 (30 total including figures)

Number of figures:5

Number of references:38

Number of words in Abstract:249

Number of words in Introduction:741

Number of words in Discussion:982

A list of nonstandard abbreviations:

GPCR; G protein-coupled receptor, GEF; Guanine nucleotide Exchange Factor, PLCβ; phospholipase Cβ, α_{1B}-AR; α_{1B}-adrenergic receptor, IP3;Inositol triphosphate, PE; phenylephrine, b$\beta\gamma$; biotinylated-$\beta\gamma$, NSB; Nonspecific binding.

Recommended section assignment: Cellular and Molecular

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

Ser54 of $G_s\alpha$ 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 $G\alpha$ subunits. Unexpectedly, wild type or N54-α_s co-expressed with α_{1B}-adrenergic receptor in HEK293 cells decreased receptor stimulation of IP3 production by a cAMP-independent mechanism; but wt-t-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 PLCβ2. 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 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 exchange of GDP for GTP on Gα, leading to 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 non-productively, preventing GPCR signaling to other G proteins (Cleator et al., 2004). The analogous N17-Ras mutant also has dominant negative properties based on non-productive interaction with guanine nucleotide exchange factor (GEF), its equivalent of GPCR (Farnsworth and Feig, 1991), and this is true of many small G proteins (Feig, 1999). Homologous mutations of other Gα subunits also often have similar dominant negative properties to that of N54-α_s. For example, studies of analogous S43C or S43N mutants of α_t suggest they are also dominant negative proteins by directly binding GPCR, in this case rhodopsin (Natochin et al., 2006).

Mutation of the Ser54 homolog in Gα proteins does not always, however, result in proteins with identical characteristics, or mechanism 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; Slepak et al., 1995), but the mechanism in this case is different

from that 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; Slepak et al., 1995). 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 effects of a GPCR, such mutants can block downstream signaling by sequestering receptor-generated $\beta\gamma$.

Just recently, it was discovered that dominant-negative Gα_{i3} subunits are involved in Auriculo-condylar syndrome (ACS) a rare condition that impairs craniofacial development. ACS is caused by interference of the endothelin type A receptor ET_AR/PLCβ 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_AR, and the downstream effector PLCβ4 (Rieder et al., 2012; Gordon et al., 2013b), but not G_q or G_{11}. Unexpectedly, mutations are found in Gα_{i3} and are all clustered around the nucleotide-binding pocket of Gα_{i3}. One of the mutations fully characterized was the Gα_{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 Gα_{i3} S47R mutant preferentially binds GDP and sequesters its receptor, ET_AR, from activating G_q (Marivin et al., 2016), very similar to the way in which N54-α_s sequesters the 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 non-productively to 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.

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Materials. [^{3}H]inositol, [^{3}H]adenine, and [^{35}S]methionine were from Amersham.

Construction of vectors. cDNAs for α_s, N54-α_s, activated α_s (α_s*) and α_{1B}-adrenergic receptor were as described (Cleator et al., 1999; Cleator et al., 2004). Gβ and Gγ cDNAs were recently described (Dingus et al., 2005). A rat α_o cDNA in pRC/CMV from Dr. Randall Reed was transferred into pcDNA3.1(+) as an EcoRI-XbaI fragment. The βARK-minigene in the pRK construct was obtained from Robert J. Lefkowitz as described (Koch et al., 1994). The S47N and S47C mutants were generated from this construct using the QuikChange site-directed mutagenesis kit (Stratagene). The sense primer for S47C-α_o was 5"GGAGAATCAGGAAAATGCACCATTGTGAAGCAG, 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"GCTTCACAATGGTGCATTTGCCAGACTCTCCAG, changing codon 54 from AGC to TGC.

Cell Transfections and Inositol Phosphate Determination. HEK293 cells were grown and transfected with lipofectamine as described (Cleator et al., 1999; Cleator et al., 2004). Previous studies validated that wild type 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; Cleator et al., 2004). IP3 production was measured using a [^{3}H]inositol uptake assay (Cleator et al., 2004). Cells were labelled with 2 µCi/ml myo-[^{3}H] inositol 24 hr prior to experiments. Data are presented as the percent of Total [^{3}H]inositol recovered as [^{3}H]inositol phosphates.

***In vitro* Transcription/Translation and Biotinylated $\beta\gamma$ Binding Assay**. Wild type or mutant α was synthesized in 50 µl containing 1µg cDNA, 10µCi [^{35}S]Met, 1 mM cold Met and 40 µl Promega TNT Quick Coupled Transcription/Translation System, as described (Dingus et al., 2005). The b$\beta\gamma$ binding assay was as previously described (Dingus et al., 1994). Protein was translated at 30^{o}C for 90 min (by which time there is little or no additional synthesis) and then frozen at -80^{o}C until used in an experiment. Binding of α_s or α_o to b$\beta\gamma$ was in 200µl 20 mM

Hepes, pH 7.5, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 100μM GDP, 0.5 μCi translation mixture and 10 μl of UltrLink Neutravidin beads with 0.1μg bβγ. Controls included [^{35}S]-α incubated with beads lacking bβγ. Samples were incubated at 4^{o}C for 2 hours on a rotary shaker. Beads were collected in a Picofuge microfuge for 10 s and labeled proteins in the pellet separated on 11% SDS-PAGE gels. Fixed, dried gels were exposed 1-2 days on a Molecular Dynamics phosphoimaging 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.

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Effect of wild type or N54-α_s on signaling to phospholipase Cβ2 (PLC-β2) through the α_{1B}-Adrenergic Receptor (α_{1B}-AR) in HEK 293 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 TSH, VIP and β-adrenergic receptors, but not those coupled primarily to G_q, such as the α_{1B}-AR. For the TSH receptor, 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 IP3 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 IP3 levels through the α_{1B}-AR, wild type α_s actually suppressed agonist-stimulated IP3 levels (Cleator et al., 2004). This effect was even more prominently in HEK 293 cells, where expression of wild type α_s suppressed PE-stimulated IP3 levels by as much as 60% (Fig 1). In this case, N54-α_s also decreased PE-stimulated IP3 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 TSH, VIP and β-adrenergic receptors 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); while here, N54-α_s is less effective than wild type α_s. Because there was a difference in efficacy of wild type 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 HEK 293 cells to examine the possibility that increases in cAMP levels caused by wild type or N54-α_s accounted for their ability to decrease α_{1B}-AR-mediated stimulation of IP3 levels (Fig 1B). Co-expression 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 expression of α_s suppressing free $\beta\gamma$ levels, as proposed for C47 mutants of the α_i-related proteins (Slepak et al., 1993; Slepak et al., 1995). Such an effect would be consistent with, for example, studies showing that expression of α_o (Yu et al., 1997) or α_t (Lustig et al., 1993) block $\beta\gamma$-mediated effects in cells. This hypothesis also implied, however, that the N54-α_s mutant has decreased affinity for $\beta\gamma$ compared to wild type α_s, since it was less effective in suppression of PE stimulation of IP3.

Wild type or N54-α_s inhibition of $\beta_2\gamma_2$ stimulation of PLCβ2.

Wild type or N54-α_s inhibition of $\beta_2\gamma_2$ stimulation of PLCβ2 was utilized 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 PLCβ2, has been previously used to study in vivo binding of α_o (Yu et al., 1997). $\beta_2\gamma_2$ co-expressed with PLCβ2 in HEK293 cells caused a 4-5 fold increase in PI turnover (Fig 2A), similar to previously 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 PLCβ2 (Fig. 2A).

Two possible mechanisms for inhibition effects of α_s subunits on $\beta\gamma$ stimulation of PLCβ2, one mediated by α_s subunits complexing $\beta\gamma$ and the other resulting from downregulation of PLCβ2 following its phosphorylation by PKA (Liu and Simon, 1996). Cyclic AMP has been shown to downregulate PLCβ2 through phosphorylation mediated by PKA (Liu and Simon, 1996). Activated α_s* could be used to explore the contributions of cAMP-mediated inhibition of PLCβ2, while the ability of the βARK minigene to bind-sequester $\beta\gamma$ could be employed to investigate the contributions of α_s complexing $\beta\gamma$ in inhibiting PLCβ2. Exploring the relative contributions of these two mechanisms, we found that α_s* decreased $\beta\gamma$ activation of PLCβ2 to a similar extent as the βARK minigene, which scavenges $\beta\gamma$ (Koch et al., 1994). Neither α_s* nor the βARK minigene was as effective as wild type or N54-α_s. One possible reason for the greater

effect of wild type or N54-α_s to inhibit $\beta\gamma$–mediated PLCβ2 activation is that wild type 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 PLCβ2 by cAMP, α_s* was co-expressed with wild type α_s and N54-α_s and $\beta_2\gamma_2$–mediated PLCβ2 stimulation was measured (Fig 2B). This strategy was used to measure binding of wild type 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 PLCβ2 stimulation compared to N54-α_s suggesting that N54-α_s binds $\beta\gamma$ with less affinity than wild type α_s.

Binding of wild type or N54-α_s to biotinylated-$\beta\gamma$ (b$\beta\gamma$).

To directly examine $\beta\gamma$ interactions, wild type and N54-α_s were synthesized using an *in vitro* rabbit reticulocyte system and assayed for their ability to bind to b$\beta\gamma$ immobilized on streptavidin beads (Dingus et al., 1994). Both proteins specifically bound b$\beta\gamma$ (Fig 3A). However, significantly less N54-α_s than wild type α_s bound to b$\beta\gamma$ (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 b$\beta\gamma$ were hindered by the relatively low amounts of protein synthesized (data not shown). At low concentrations, however, particularly at levels below the 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 biotinylated $\beta\gamma$ being at least 3 fold lower than that of wild type α_s (footnote b).

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

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

temperature-dependent denaturation prior to assaying bβγ binding at 4ºC (Fig 3C). Thus aliquots of previously synthesized wild type and mutant N54-α_s were then incubated at different temperatures for 30 min and then evaluated in the bβγ binding assay. The expectation of this experiment was that, if N54-α_s is more temperature sensitive, less N54-α_s would bind to bβγ in a subsequent binding assay because of a reduced functional concentration. Although both proteins were sensitive to denaturation at 39ºC (essentially a positive control for the denaturation protocol), and the mutant somewhat more so, both proteins were stable to temperatures up to 30ºC, indicating that differences in stability did not explain the differences in apparent affinity of wild type and N54-α_s (Figs 3B).

Binding of S54-α_s and S47-α_o, mutants to bβγ.

The decreased binding of N54-α_s to Gβγ, compared to wild type α_s, suggests differences in βγ binding properties of α_s mutants compared to the analogous mutants of α_o and α_i (S47C), which have increased βγ binding (Slepak et al., 1993; Slepak et al., 1995). A possible explanation for this could be that substitution of the conserved Ser by Cys results in a different phenotype than substitution with Asn. To test this idea, Ser54 in α_S was mutated to Cys (C54-α_s), while Ser47 of α_o was mutated to Asn or Cys (N47-α_o and C47-α_o respectively) and βγ binding evaluated. C54-α_S bound to bβγ 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 βγ, 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.

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

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 GEF and it does not activate downstream effectors (Feig, 1999). In contrast, the phenotype of N54-α_s is more complex, which has limited its use 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, N54-α_S also works upstream by binding receptor non-productively, preventing activation of endogenous α_S, and in the case of the TSH receptor, 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 $\beta\gamma$ (Slepak et al., 1993; Slepak et al., 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 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). Secondly, wild type-α_s more effectively than N54-α_s suppresses stimulation of PLCβ2 by $\beta_2\gamma_2$ co-expressed in HEK 293 cells (Figure 2). Thirdly, under similar conditions, less N54-α_s binds to b$\beta\gamma$ than wild type-α_s, consistent with a 3-fold or greater decrease in affinity of N54-α_s

for $\beta\gamma$ (Figs 3 and 4). Finally, a decreased affinity for $\beta\gamma$ explains, in part, some of previously characterized properties of the N54-α_s mutant. This relates particularly to the paradoxical increased basal activity of N54-α_s in spite of 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 Gα 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 speculative that this would represent a discrete functional state of Gα, 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 Gα isoforms, one a component of the Mg^{2+}/guanine nucleotide-binding site (α_s Ser54) and the other 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 longstanding idea about GPCR signaling is that G proteins are activated through a dual mechanism involving nucleotide exchange (GTP for GDP) and subunit dissociation. Fig 5A shows a classical interpretation of G protein activation whereby receptor catalyzes nucleotide exchange prior to subunit dissociation. The idea that the N54 mutant identifies a discrete state of Gα 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 5B). In this model, the Gs-β_2AR 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-$\beta_2$adrenergic receptor crystal structure, G$\beta\gamma$ makes no contacts with receptor (Dror et al., 2015) even though G$\beta\gamma$ has long been thought to be required for GPCR activation of Gα (see legend to Fig 5).

Acknowledgements

The authors thank Alicia Arnold, Roneka Ravenell and Brook White for expert technical assistance, Dr. Ravi Iyengar for the PLCβ2 and Q212L-α_s cDNA, Dr. Diane Perez for the α_{1B}-adrenergic receptor cDNA, Dr. Robert J. Lefkowitz for the βARK minigene, and Dr. Randell Reed for the α_o cDNA.

Author contributions

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

Conducted experiments: Cleator, Dingus, and Hildebrandt

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

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

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

References

Allgeier A, Offermanns S, Van Sande J, Spicher K, Schultz G and Dumont J (1994) The Human Thyrotropin Receptor Activates G-proteins Gs and Gq/11. *JCellBiol* **269**:13733-13735.

Barren B and Artemyev NO (2007) Mechanisms of dominant negative G-protein alpha subunits. *J Neurosci Res* **85**:3505-3514.

Birnbaumer L (1990) G proteins in signal transduction. *AnnuRevPharmacolToxicol* **30**:675-705.

Cleator JH, Mehta ND, Kurtz DT and Hildebrandt JD (1999) The N54 mutant of G alpha s has a conditional dominant negative phenotype which suppresses hormone-stimulated but not basal cAMP levels. *FEBSLett* **243(2)**:205-208.

Cleator JH, Ravenell R, Kurtz DT and Hildebrandt JD (2004) A Dominant Negative G s Mutant That Prevents Thyroid-stimulating Hormone Receptor Activation of cAMP Production and Inositol 1,4,5-Trisphosphate Turnoverr: Competition by different G proteins for activation by a common receptor. *J Biol Chem* **279**:36601-36607.

Dettlaff-Swiercz DA, Wettschureck N, Moers A, Huber K and Offermanns S (2005) Characteristic defects in neural crest cell-specific Galphaq/Galpha11- and Galpha12/Galpha13-deficient mice. *Dev Biol* **282**:174-182.

Dingus J, Wells CA, Campbell L, Cleator JH, Robinson K and Hildebrandt JD (2005) G protein bg dimer formation: Gb and Gg differentially determine efficiency of in Vitro dimer formation. *Biochemistry* **44**:11882-11890.

Dingus J, Wilcox MD, Kohnken RE and Hildebrandt JD (1994) Synthesis and Use of Biotinylated beta/gamma Complexes Prepared form Bovine Brain G Proteins. *Methods Enzymol* **237**:457-471.

Dror RO, Mildorf TJ, Hilger D, Manglik A, Borhani DW, Arlow DH, Philippsen A, Villanueva N, Yang Z, Lerch MT, Hubbell WL, Kobilka BK, Sunahara RK and Shaw DE (2015) SIGNAL

TRANSDUCTION. Structural basis for nucleotide exchange in heterotrimeric G proteins. *Science* **348**:1361-1365.

Farnsworth CL and Feig LA (1991) Dominant inhibitory mutations in the Mg2+ binding site of RasH prevents its activation by GTP. *MolCellBiol* **11**:4822-4829.

Feig LA (1999) Tools of the trade.

Fung BK (1983) Characterization of transducin from bovine retinal rod outer segments. I. Separation and reconstitution of the subunits. *JBiolChem* **258**:10495-10502.

Gilman AG (1987) G proteins: transducers of receptor-generated signals. *AnnuRevBiochem* **56**:615-649.

Gordon CT, Petit F, Kroisel PM, Jakobsen L, Zechi-Ceide RM, Oufadem M, Bole-Feysot C, Pruvost S, Masson C, Tores F, Hieu T, Nitschke P, Lindholm P, Pellerin P, Guion-Almeida ML, Kokitsu-Nakata NM, Vendramini-Pittoli S, Munnich A, Lyonnet S, Holder-Espinasse M and Amiel J (2013a) Mutations in endothelin 1 cause recessive auriculocondylar syndrome and dominant isolated question-mark ears. *Am J Hum Genet* **93**:1118-1125.

Gordon CT, Vuillot A, Marlin S, Gerkes E, Henderson A, AlKindy A, Holder-Espinasse M, Park SS, Omarjee A, Sanchis-Borja M, Bdira EB, Oufadem M, Sikkema-Raddatz B, Stewart A, Palmer R, McGowan R, Petit F, Delobel B, Speicher MR, Aurora P, Kilner D, Pellerin P, Simon M, Bonnefont JP, Tobias ES, Garcia-Minaur S, Bitner-Glindzicz M, Lindholm P, Meijer BA, Abadie V, Denoyelle F, Vazquez MP, Rotky-Fast C, Couloigner V, Pierrot S, Manach Y, Breton S, Hendriks YM, Munnich A, Jakobsen L, Kroisel P, Lin A, Kaban LB, Basel-Vanagaite L, Wilson L, Cunningham ML, Lyonnet S and Amiel J (2013b) Heterogeneity of mutational mechanisms and modes of inheritance in auriculocondylar syndrome. *J Med Genet* **50**:174-186.

Hamm HE (1998) The many faces of G protein signaling. *JBiolChem* **273**:669-672.

Hildebrandt JD (1997) Role of subunit diversity in signaling by heterotrimeric G proteins. *BiochemPharmacol* **54**:325-339.

Hildebrandt JD, Day R, Farnsworth CL and Feig LA (1991) A mutation in the putative Mg2+-binding site of Gs a prevents its activation by receptors. *MolCellBiol* **11**:4830-4838.

Koch WJ, Hawes BE, Inglese J, Luttrell LM and Lefkowitz RJ (1994) Cellular expression of the carboxyl terminus of a G protein-coupled receptor kinase attenuates G beta gamma-mediated signaling. *J Biol Chem* **269**:6193-6197.

Lefkowitz RJ, Cotecchia S, Samama P and Costa T (1993) Constituitive activity of receptors coupled to guanine nulceotide regulatory proteins. *TrendsPharmacolSci* **14**:303-307.

Liu M and Simon MI (1996) Regulation by cAMP-dependent protein kinease of a G-protein-mediated phospholipase C. *Nature* **382**:83-87.

Lustig KD, Conklin BR, Herzmark P, Taussig R and Bourne HR (1993) Type II adenylyl cyclase integrates coincident signals from Gs, Gi, and Gq. *JBiolChem* **268**:13900-13905.

Marivin A, Leyme A, Parag-Sharma K, DiGiacomo V, Cheung AY, Nguyen LT, Dominguez I and Garcia-Marcos M (2016) Dominant-negative Galpha subunits are a mechanism of dysregulated heterotrimeric G protein signaling in human disease. *Sci Signal* **9**:ra37.

Natochin M, Barren B and Artemyev NO (2006) Dominant negative mutants of transducin alpha that block activated receptor. *Biochemistry* **45**:6488-6494.

Neer EJ (1995) Heterotrimeric G proteins: Organizers of transmembrane signals. *Cell* **80**:249-258.

Offermanns S, Zhao LP, Gohla A, Sarosi I, Simon MI and Wilkie TM (1998) Embryonic cardiomyocyte hypoplasia and craniofacial defects in G alpha q/G alpha 11-mutant mice. *EMBO J* **17**:4304-4312.

Pereira R and Cerione RA (2005) A switch 3 point mutation in the alpha subunit of transducin yields a unique dominant-negative inhibitor. *J Biol Chem* **280**:35696-35703.

Ramachandran S and Cerione RA (2011) A dominant-negative Galpha mutant that traps a stable rhodopsin-Galpha-GTP-betagamma complex. *J Biol Chem* **286**:12702-12711.

Rieder MJ, Green GE, Park SS, Stamper BD, Gordon CT, Johnson JM, Cunniff CM, Smith JD, Emery SB, Lyonnet S, Amiel J, Holder M, Heggie AA, Bamshad MJ, Nickerson DA, Cox TC, Hing AV, Horst JA and Cunningham ML (2012) A human homeotic transformation resulting from mutations in PLCB4 and GNAI3 causes auriculocondylar syndrome. *Am J Hum Genet* **90**:907-914.

Samama P, Cotecchia S, Costa T and Lefkowitz RJ (1993) A mutation-induced activated state of the beta-2 adrenergic receptor. Extending the ternary complex model. *JBiolChem* **268**:4625-4636.

Slepak VZ, Katz A and Simon MI (1995) Functional analysis of a dominant negative mutant of G alpha i2. *JBiolChem* **270**:4037-4041.

Slepak VZ, Quick MW, Aragay AM, Davidson N, Lester HA and Simon MI (1993) Random mutagenesis of G protein a subunit Goa: Mutations altering nucleotide binding. *JBiolChem* **268**:21889-21894.

Spiegel AM (1992) Heterotrimeric GTP-binding proteins: An expanding family of signal transducers. *MedResRev* **12**:55-71.

Sprang SR (1997) G protein mechanisms: Insights from structural analysis. *AnnuRevBiochem* **66**:639-678.

Wall MA, Posner BA and Sprang SR (1998) Structural basis of activity and subunit recognition in G protein heterotrimers. *Structure* **6**:1169-1183.

Wittinghofer A and Pai EF (1991) The structure of Ras protein: a model for a universal molecular switch. *Trends BiochemSci* **16**:382-387.

Yasuda H, Lindorfer MA, Woodfork KA, Fletcher JE and Garrison JC (1996) Role of the prenyl group on the G protein gamma subunit in coupling trimeric G proteins to A1 adenosine receptors. *JBiolChem* **271**:18588-18595.

Yu B, Slepak VZ and Simon MI (1997) Characterization of a Go alpha mutant that binds xanthine nucleotides. *JBiolChem* **272**:18015-18019.

Footnotes

a) JHC and CAW were supported by the Medical Scientist Training Program at MUSC. JHC was funded by the Vanderbilt Clinical and Translational Research Award. In addition, this work was supported in part by National Institute of Health grants NS38534 and DK37219 (JDH).

b) The argument for a 3-fold decrease in apparent affinity of the mutant for G$\beta\gamma$ 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 x (B_{max}/K_D)], or directly proportional to K_A [i.e., Bound = Free x Bmax x K_A]. Thus, if binding of the mutant is only 33% of binding of wildtype, and the two have the same Bmax, as designed in the experiment, then the K_A of the mutant is 33% or less of that of mutant (i.e., wildtype 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.

Figure Legends

Fig 1. IP3 levels in HEK 293 cells transfected with α_{1B}-AR (A). Coexpression of α_{1B}-AR with α_s or N54-α_s. Cells were transfected with 0.2µg α_{1B}-AR cDNA and the indicated amounts of wild type or N54-α_s cDNA, labeled with [^{3}H]-inositol as described in Methods and stimulated 24 h latter with 100µM phenylephrine (PE) for 40 min. **(B). Coexpression of α_s* with the α_{1B}-AR.** Cells were transfected with 0.2µg α_{1B}-AR and 0.8µg of α_s*, wild type α_s or N54-α_s. Cells were stimulated with 100µM PE for 40 min. at 35^{o}C. In each case, data representative of 3 experiments.

Fig. 2. Inhibition of βγ stimulation of PLCβ2 in HEK 293 cells by α_s and βARK. (A). Cells were transfected with 0.4µg PLCβ2, 0.5µg β_2, 0.5µg γ_2, and 0.25µg of either wild-type α_s, N54-α_s, α_s*, or βARK cDNA as indicated. Inositol phosphates were allowed to accumulate for 30 min at 35^{o}C after addition of LiCl. **(B). Dose response of wild type or N54-α_s inhibition of βγ stimulation of PLCβ2 in HEK 293 cells.** Cells were transfected with 0.4µg PLCβ2, 0.5µg β_2, 0.5µg γ_2 0.25µg α_s* cDNA along with indicated amounts of either wild type or N54-α_s cDNA. Inositol phosphates were allowed to accumulate for 30 min at 35^{o}C after addition of LiCl. Control cells transfected with PLCβ2 and α_s* cDNA possessed a value of 2.137+/-0.168.

Fig 3. Binding of α_s or N54-α_s to biotinylated βγ. (**A**). **Representative binding data.** Results of a typical experiment in duplicate measuring binding of [^{35}S]-α_s to bβγ. Total added [^{35}S]-α_s and protein bound to bβγ streptavidin beads (Bound), as described in Methods. Nonspecific binding (NSB) was [^{35}S]-α_s bound to beads lacking bβγ. (**B**). **Summary of binding data.** Data are the mean ± SEM from 3 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 NSB. Graph on the right is Total input to each sample. Values normalized in an experiment by dividing by the average values for wild type and N54-α_s

samples. **(C).** Stability **of α_s and N54-α_s to heat denaturation.** Wild type and N54-α_s were synthesized by *in vitro* translation, as described in Methods, and then, in a subsequent treatment, incubated for 30 min at 4º, 22º, 30º or 39º C. Following this incubation, binding to b$\beta\gamma$ was determined at 4º C as above. Data are mean $\pm$ SEM 4 experiments.

Fig 4. The effect of Asn versus 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 in Fig 2A, except that Total is 10% of input sample. (**Middle). Summary of binding data**. Mean $\pm$ SEM of Bound minus NSB for 6 experiments normalized as in Fig 2B. (**Right). Summary of Total data.** Groups were significantly different for bound protein by one-way 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) 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 $\pm$ SEM of Bound minus NSB for 3 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) significantly different from wild type by the method of Least Significant Difference.

Fig 5. Panel A represents the classical 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 classical 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 Gs-$\beta_2$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, release of the activated, GTP-

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

bound, free G alpha. **Panel B** represents 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 Gα complex, and State IV the GTP bound complex, which would rapidly dissociate as free activated Gα. The model in Fig 6B would account for the mechanism by which N54 becomes a dominant negative inhibitor of receptor activation of G protein even though it, itself, has some constitutive activity. In this model, the Gs-$\beta_2$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.

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

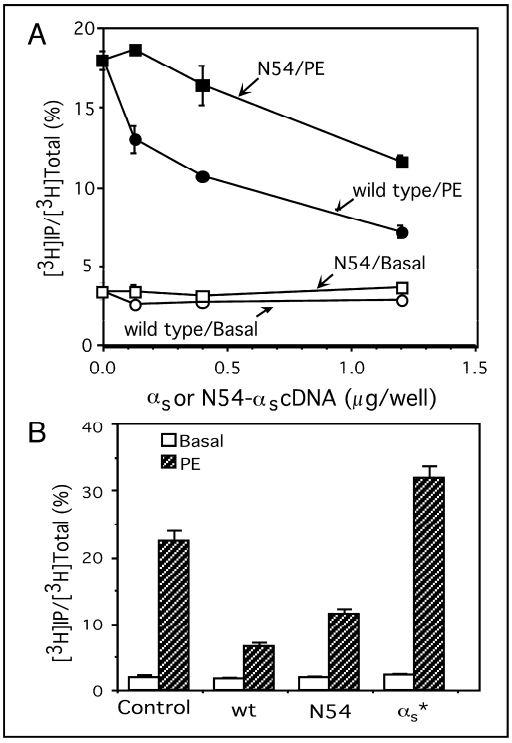

Fig 1

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

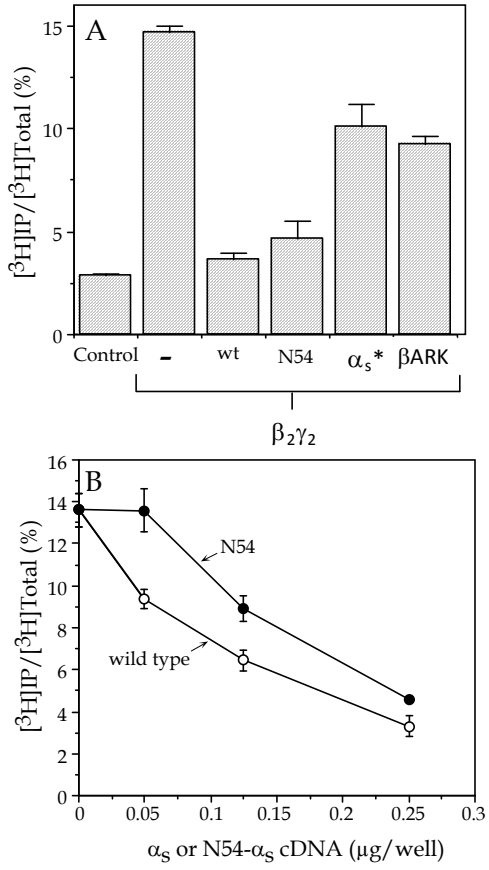

Fig 2

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

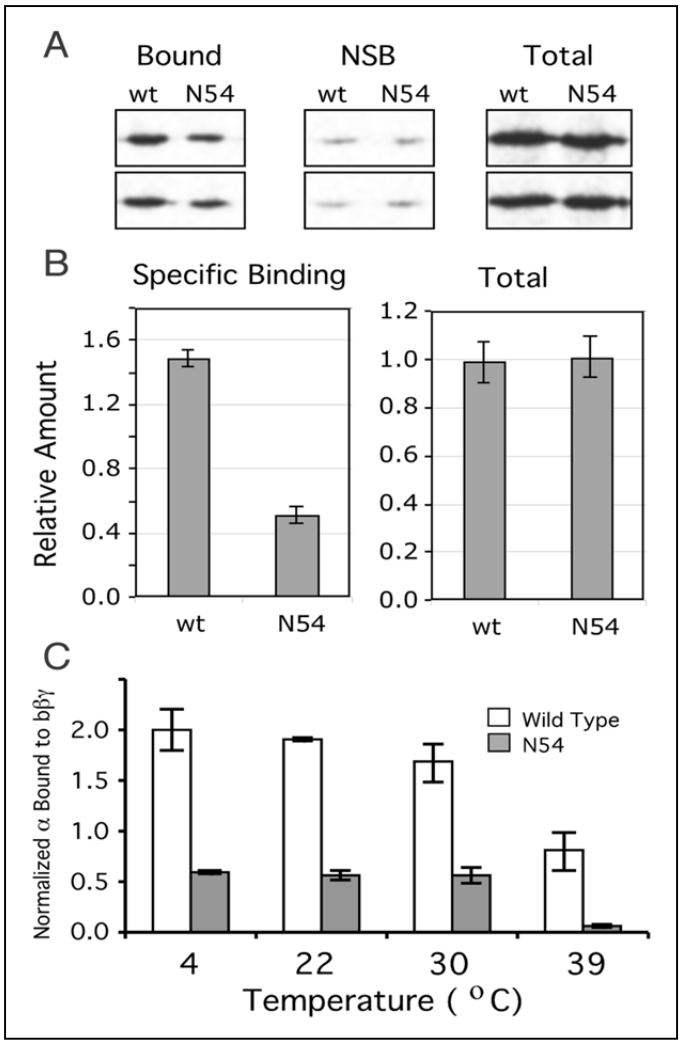

Fig 3

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

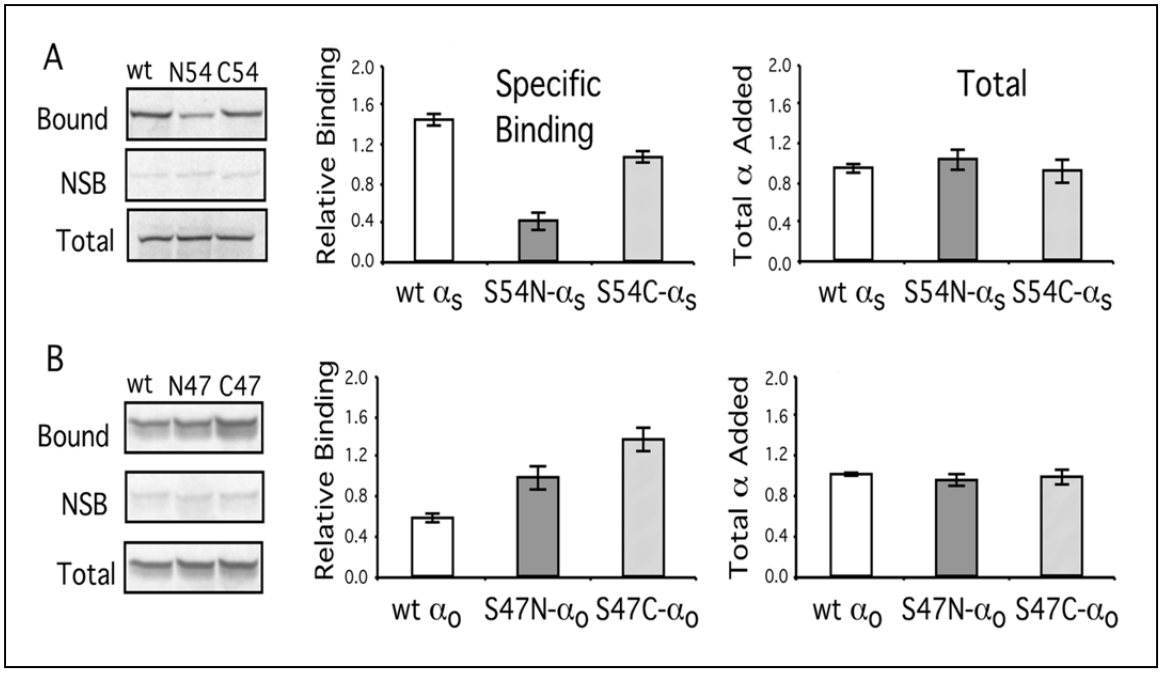

Fig 4

JPET Fast Forward. Published on February 28, 2018 as DOI: 10.1124/jpet.117.245779
This article has not been copyedited and formatted. The final version may differ from this version.

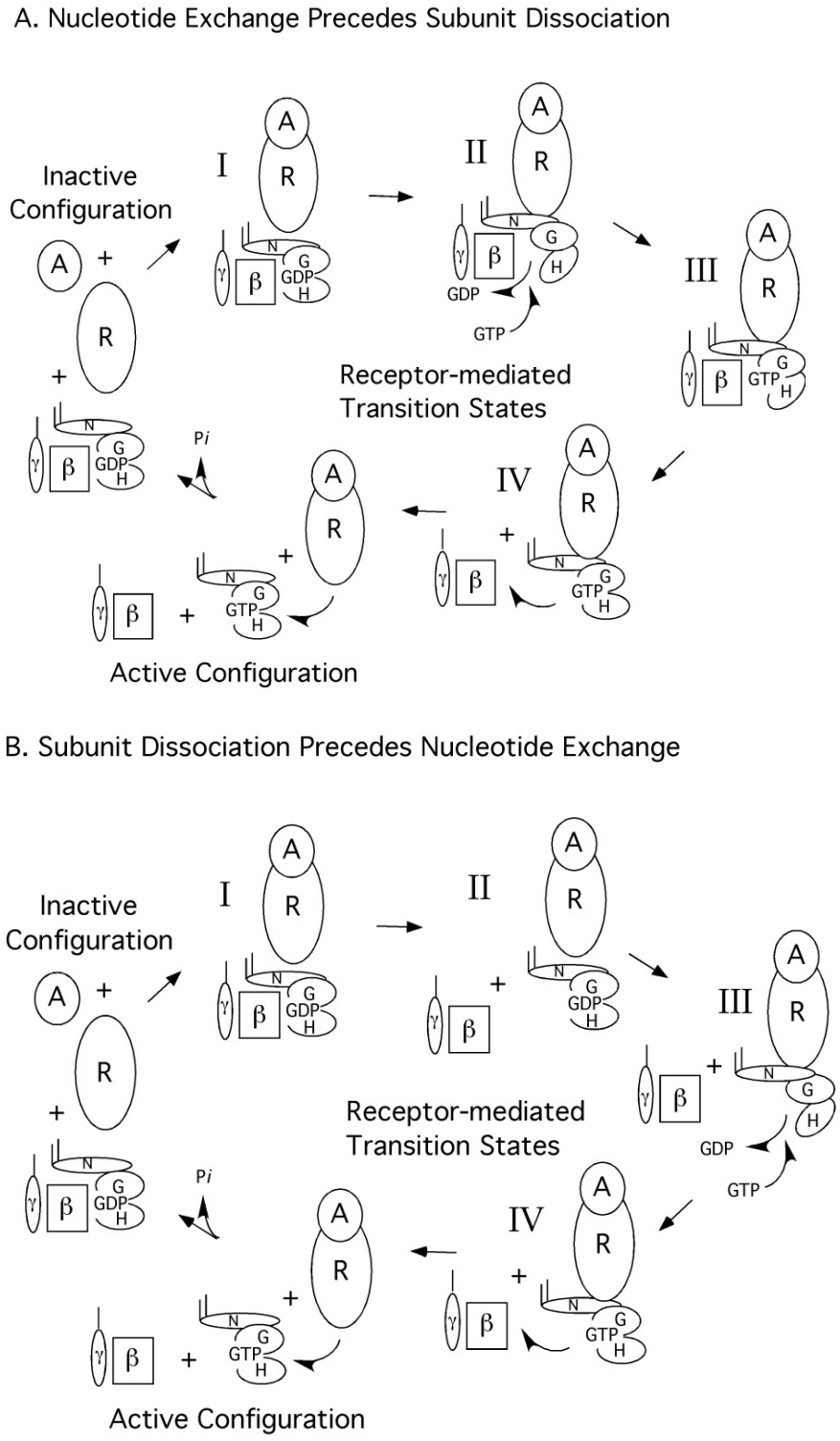

Fig 5