The N54 mutant of $G\alpha_s$ has a conditional dominant negative phenotype which suppresses hormone-stimulated but not basal cAMP levels

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Abstract The phenotype of a Ser to Asn mutation at position 54 of the α subunit of G_s (N54-α_s) was characterized in transient transfection experiments in COS and HEK293 cells. Expression of either wild type or N54-α_s increased basal cAMP levels. In contrast, expression of wild type α_s potentiated agonist-stimulated cAMP levels, while expression of N54-α_s caused a decrease. Thus, the N54-α_s mutant possesses a conditional dominant negative phenotype, suppressing preferentially hormone-stimulated effects.

Key words: G protein; Hormone-stimulated cAMP level

1. Introduction

Heterotrimeric GTP binding proteins, or G proteins, transduce signals from receptors on the surface of the cell to various intracellular effector enzymes. The superfamily of GTP binding proteins, which includes among others the α subunits of heterotrimeric G proteins and the monomeric GTP binding proteins, share four conserved amino acid sequences that form the core of the GTP regulatory mechanism of these proteins [1–3]. The consensus sequence of the first conserved region of the guanine nucleotide binding site is $GX_4GK(S/T)$, which includes Ser-17 in Ras and the equivalent Ser-54 of $G\alpha_s$. These residues are involved in binding Mg^{2+} ion when it is complexed with nucleotide [4]. Ras with a Ser to Asn mutation at this site (S17N) has an increased preference for GDP over GTP, and a dominant negative phenotype [5].

Previously, the Ser to Asn mutation at position 54 of α_s (N54-α_s) was characterized in S49 cyc^- cells (which lack endogenous α_s) by measuring adenylyl cyclase activity in broken cell preparations [6]. Those studies suggest that it has an increased preference for GDP, especially in the presence of hormone stimulation, similar to the altered nucleotide binding of the N17-Ras protein [5]. Those studies did not establish, however, the phenotype of the N54 mutant in intact cells. To determine the phenotype of N54-α_s when expressed in intact cells with endogenous α_s present, COS cells and HEK293 cells were transiently transfected with either wild type or mutant α_s. These studies show that the N54-α_s mutant has a conditional dominant negative phenotype expressed preferentially in the presence of receptor activation of the protein.

2. Materials and methods

2.1. Materials

[^{3}H]Adenine, donkey anti-rabbit horseradish peroxidase (HRP) linked antibody, and ECL reagents were purchased from Amersham. Bovine thyroid stimulating hormone (TSH, lot AFP-5555B) was obtained from the National Hormone and Pituitary Program, NIDDK, and Dr. A.F. Parlow. All other materials were of the highest quality grade available.

2.2. Construction of vectors

cDNAs encoding the long form of $G\alpha_s$ and the N54 mutation of $G\alpha_s$ in pMV7 [6] were subcloned into the *Hin*dIII site of the mammalian expression vector pcDNA3 (Invitrogen) driven by the CMV promoter. The activated α_s mutant (Q212L) in the mammalian expression vector pCr/CMV was kindly provided by Dr. R. Iyengar. The rat TSH receptor (TSH-R) cDNA inserted in the SV40 promoter-driven expression vector pSG5 [7] was kindly provided by Dr. L. Kohn.

2.3. Transient transfections

COS-1 and HEK293 cells were grown in DMEM containing 10% FBS at 37°C and were transfected with lipofectamine (Gibco). cDNAs were mixed with lipofectamine in OPTI-MEM and added to either COS cells or HEK293 cells in 6 well dishes (1×10^5 or 4×10^5, respectively) and allowed to incubate for 5 h. Total cDNA was kept constant by addition of vector, pcDNA3. Transfections were stopped by the addition of 20% FBS in DMEM. The next day, cells were replated into 24 well plates for cAMP determinations or 12 well plates to provide samples for Western blots. Cells were used in an experiment 24 h later.

2.4. cAMP determination

cAMP was measured by use of the [^{3}H]adenine uptake assay [8]. Cells were labelled with 1 μCi/ml [^{3}H]adenine in 0.5 ml DMEM/10% serum for approximately 24 h prior to treatment with hormone. Cells were washed once with DMEM/20 mM Na-HEPES, followed by incubation in DMEM/20 mM Na-HEPES containing 500 μM IBMX for 10 min prior to hormone treatment. Cells were stimulated with indicated hormone (3 nM TSH or 10 μM isoproterenol) for 20 min at 32°C. Stimulation was terminated by the addition of 5% TCA solution containing 1 mM ATP and 1mM cAMP. [^{3}H]cAMP was separated from ^{3}H-labelled adenine nucleotides, principally [^{3}H]ATP, by sequential chromatography on Dowex and alumina columns. Both [^{3}H]cAMP and ^{3}H-labelled adenine nucleotides were recovered from the columns and data are expressed as the percent of total ^{3}H recovered as [^{3}H]cAMP. Values represent mean of triplicate data points ± S.E.M. Figures shown are representative of experiments conducted at least three times.

2.5. Immunoblotting

Samples were prepared by washing cells in PBS followed by addition of sample buffer containing 10% β-mercaptoethanol. Proteins were separated by SDS polyacrylamide gel electrophoresis according to Laemmli [9] and transferred [10] to nitrocellulose using a Bio-Rad semi-dry transfer apparatus. Proteins were incubated with anti-α_s antibody (ASC) produced according to the protocol of Spiegel [11] or with a common anti-β antibody (BC1) [12]. Bands were visualized by ECL after incubation with goat anti-rabbit HRP conjugate (New England Nuclear).

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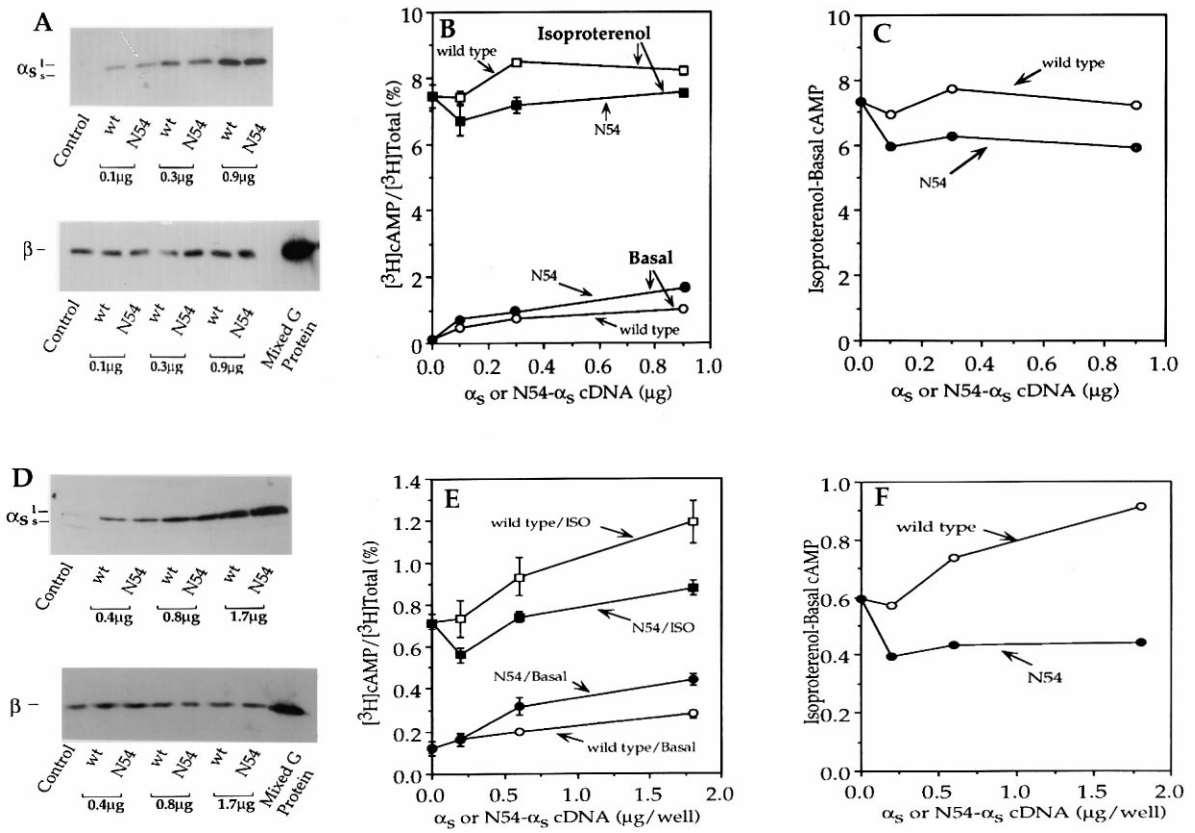

Fig. 1. A: Effect of expression of wild type α_s or N54-α_s in COS-1 cells on α_s and β immunoreactivity. B: Expression of wild type α_s or N54-α_s in COS-1 cells and their effects on basal and isoproterenol-stimulated cAMP levels. C: Effect of wild type or N54-α_s on isoproterenol-stimulated cAMP levels in COS-1 cells. The graph was generated by subtracting basal levels from isoproterenol-stimulated cAMP levels from B. D: Effect of expression of wild type α_s or N54-α_s in COS-1 cells on α_s and β immunoreactivity. E: Expression of wild type α_s or N54-α_s in HEK293 cells and their effects on basal and isoproterenol-stimulated cAMP levels. F: Effect of wild type or N54-α_s on isoproterenol-stimulated cAMP levels in HEK293 cells. The graph was generated by subtracting basal levels from isoproterenol-stimulated cAMP levels from E.

3. Results

3.1. *Effect of wild type α_s or N54-α_s on cAMP levels in COS-1 and HEK293 cells*

Transient transfection of COS-1 or HEK 293 cells with increasing amounts of either wild type α_s or N54-α_s cDNA resulted in comparable increases in α_s immunoreactivity with little or no change in Gβ subunit levels (Fig. 1A,D, respectively). This increased expression of α_s protein was associated with increased basal cAMP accumulation, and the mutant was slightly more effective than wild type α_s (Fig. 1B,E, respectively). In contrast, cellular cAMP levels were less affected by isoproterenol in the presence of the mutant than wild type α_s (Fig. 1B,E). Consequently, agonist-stimulated cAMP levels were slightly but consistently less in both cell lines transfected with the mutant than in cells transfected with wild type α_s (Fig. 1C,F).

These experiments suggested that N54-α_s selectively decreases agonist-stimulated cAMP levels while at the same time increasing basal cAMP. The ability to substantiate these conclusions seemed limited, however, in transient transfection assays using the endogenous β-adrenergic receptors of the transfected cell lines. The degree of effect of $G\alpha_s$ expression on agonist-stimulated cAMP levels appeared to be explained by the percentage of the cells that were transfected, as determined by immunofluorescence studies with green fluorescence protein (data not shown). To circumvent these problems, α_s was co-expressed in cells with exogenous receptors not normally found in these cells.

3.2. *Effect of wild type α_s or N54-α_s on stimulation of cAMP levels through expressed receptors coupled to G_s*

To study the interaction of α_s proteins with an exogenously expressed receptor, HEK293 cells were transfected with the rat TSH receptor (TSH-R) [7]. Basal and agonist-stimulated cAMP levels in HEK293 cells transfected with a fixed amount of TSH-R cDNA and increasing amounts of either wild type or mutant cDNA showed the same pattern of responses (Fig. 2) seen previously with the endogenous β-adrenergic receptor (Fig. 1). The mutant caused a slightly greater increase in basal cAMP levels than did wild type α_s, whereas the reverse was true for TSH-stimulated levels (Fig. 2A), and the difference in TSH-stimulated and basal activities increased for wild type α_s but decreased for the mutant (Fig. 2B). These same patterns were also seen when the TSH-R response was characterized in COS-1 cells (Fig. 2C,D).

3.3. *N54-α_s has inherent basal activity but less than that of a constitutively activated mutant*

There seemed to be two components to the phenotype of the N54-α_s mutant when expressed in intact cells. The first was a decreased ability of receptors to stimulate cAMP levels in response to agonists. The second was an increased basal level of cAMP production. The increased basal activity of the

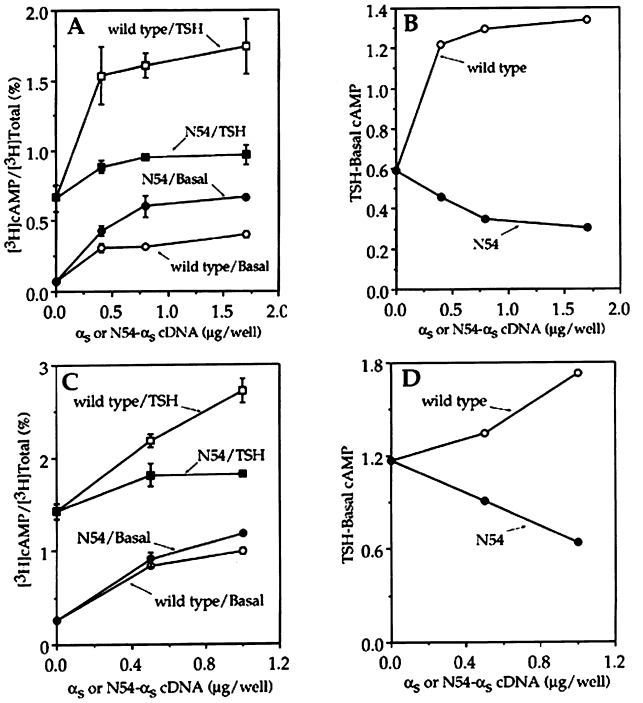

Fig. 2. A: Expression of wild type α_s or N54-α_s in HEK293 cells co-expressing TSH-R and their effects on TSH stimulation of cAMP levels. Cells were transfected with 0.8 μg TSH-R cDNA along with indicated amounts of either wild type or N54-α_s cDNA. B: Effect of wild type or N54-α_s on TSH-stimulated cAMP levels in HEK293 cells. The graph was generated by subtracting basal levels from TSH cAMP levels from A. C: Effect of co-expression of TSH-R with either wild type α_s or N54-α_s in COS-1 cells on basal and TSH-stimulated cAMP levels. Cells were transfected with 0.4 μg TSH-R cDNA along with indicated amounts of either wild type or N54-α_s cDNA. D: Effect of wild type α_s or N54-α_s on TSH-stimulated cAMP levels in COS-1 cells. The graph was generated by subtracting basal levels from TSH cAMP levels from C.

mutant might be explained by its increased nucleotide exchange rate for both GDP and GTP [6]. It was not clear, however, whether this activity was comparable to that of wild type α_s, or greater than that of the wild type protein and closer to that of a constitutively-active mutant. To determine the inherent activity of N54-α_s compared to that of an activated α_s* (Q212L-α_s designated α_s*), cAMP production was determined in COS-1 cells (Fig. 3A,B) and HEK293 cells (Fig. 3C,D) at approximately equal levels of protein expression for wild type, N54 and activated (α_s*) α_s.

Expression of comparable amounts of immunoreactive protein required about 10 times the amount of α_s* cDNA (450 ng) compared to either N54 or wild type cDNA (45ng) (Fig. 3A,C). This may be due to greater instability of the activated protein, although we have not attempted to verify this hypothesis. One difference in the two cell lines was that transfection of α_s*, which is the short form of the protein, in COS-1 cells resulted in an increase in the endogenous expression of the long form of α_s (Fig. 3A). In contrast, in HEK293 cells, expression of α_s* did not cause this unexplained increase/induction (Fig. 3C). Regardless, in both cell types and at equal levels of protein expression, cAMP levels in the presence of activated α_s* are approximately 10 times higher than those for either N54 or wild type α_s (Fig. 3B,D). These experiments indicate that even though basal activity is increased for N54-α_s, it does not have the same phenotype as an activating mutation.

4. Discussion

When expressed in COS and HEK293 cells the N54-α_s mutant has two observable phenotypes which might seem contradictory without the previous biochemical characterization of the mutant [6]. First, in the absence of hormone, the mutant activates adenylyl cyclase in a hormone-independent manner. This activity is slightly greater than that of wild type α_s,

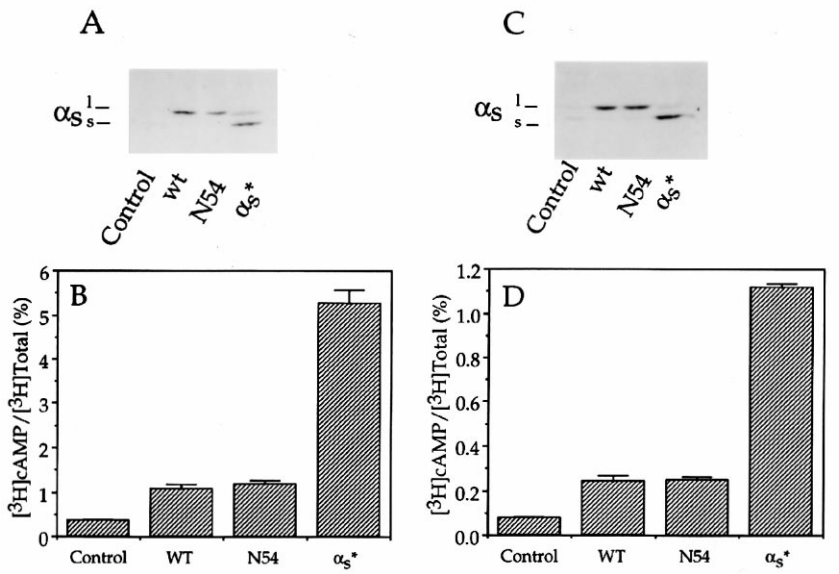

Fig. 3. The relationship between the inherent activity of wild type, N54-α_s, and α_s* when expressed at comparable levels in cells. A: Immunoreactivity of α_s proteins expressed in COS-1 cells. For both A and B, COS-1 cells were transfected with either 450 ng pcDNA3, 45 ng wild type α_s, 45 ng N54-α_s, or 450 ng α_s* cDNA. B: Effect of expression of α_s proteins on basal cAMP levels in COS-1 cells. C: Effect of expression of α_s proteins on immunoreactivity of α_s in HEK293 cells. For both C and D, HEK293 cells were transfected with either 1 μg pcDNA3, 100 ng wild type α_s, 100 ng N54-α_s, or 1 μg α_s* cDNA. D: Effect of expression of α_s proteins on basal cAMP levels in HEK293 cells.

particularly at higher levels of expression, but significantly less than that of strongly activating α_s mutations such as Q212L. This property is explainable by the increased nucleotide exchange rate of the mutant compared to wild type α_s and by the fact that the mutant can stimulate adenylyl cyclase [6].

The second observable phenotype of the mutant is its ability to interfere with receptor-stimulated cAMP levels even when the receptor uses endogenous α_s protein. This is seen most clearly for the TSH receptor expressed in HEK293 cells (Fig. 2A,B), but this phenotype is consistently observed with three different α_s-coupled receptors (β-adrenergic, TSH, VIP) (VIP-R data not shown) in three different cell lines (COS-1 and HEK293). In all of these cases the difference between agonist-stimulated and basal cAMP decreased in the presence of N54-α_s, but increased with expression of wild type α_s. The less than additive effect of basal and receptor-stimulated cAMP levels in cells expressing N54-α_s, could result from cAMP production being rate limiting (i.e. the cells could only produce so much cAMP, so that increased basal cAMP would decrease the amount of cAMP that could be stimulated by hormone). That this is not the case is indicated by the fact that greater effects on cAMP levels could be obtained by receptor activation in cells expressing wild type α_s. Instead, these results most likely indicate that N54-α_s interferes with receptor stimulation of cAMP levels even though it does not suppress basal cAMP levels. Thus, the N54-α_s mutant has a conditional dominant negative phenotype that specifically interferes with hormone stimulation of cAMP levels in cells. This effect is presumably related to the observation that receptor stimulation increases the preference of the mutant for GDP [6].

Whether the dominant negative effect of N54 α_s is upstream on receptor or downstream on adenylyl cyclase remains to be established. The mechanism of N17 ras is not completely clear either, with evidence for both upstream [13,14] and downstream [13] effects of that mutant. A clue to the mechanism of the mutant may be its high concentrations required relative to endogenous α_s and the fact that it only incompletely suppresses TSH stimulation. However, an additional factor for N54 α_s is βγ, which could modify either upstream or downstream effects of the mutant. Future studies will examine the mechanism of action of the mutant and the effect of βγ on its phenotype.

Bacterial toxins and mutations of α subunits that alter their GTPase activity can produce disease, underscoring the importance of the binding and hydrolysis of guanine nucleotide to G protein function. Both loss and gain of function mutations in α_s have been implicated in human disease [15]. Somatic mutation of either Arg-201 or Glu-227 results in loss of the GTPase activity of α_s, associated with hyperfunction and uncontrolled growth of thyroid and pituitary cells [15,16]. In contrast, in pseudohypoparathyroidism, loss of function α_s mutations result in resistance to hormones that stimulate G protein-coupled receptors that activate G_s [15,17,18]. The paradoxical activity of the N54-α_s conditional dominant negative mutant described here may define a new, potentially important pathological phenotype, one neither analogous to constitutively active nor to totally dysfunctional protein. Although cells expressing this mutant would have a blunted response to hormones, even if wild type α_s was also present, these cells would still exhibit normal or even elevated basal cAMP levels that could partly compensate for the lack of regulated levels. Future studies will need to define whether such mutations actually occur and if they cause specific disease processes.

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