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$S^{111}N$ Mutation in the Helical Domain of Human Gsα Reduces Its GDP/GTP Exchange Rate

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Abstract G-protein α subunits consist of two domains: a Ras-like domain also called GTPase domain (GTPaseD), structurally homologous to monomeric G-proteins, and a more divergent domain, unique to heterotrimeric G-proteins, called helical domain (HD). G-protein activation, requires the exchange of bound GDP for GTP, and since the guanine nucleotide is buried in a deep cleft between both domains, it has been postulated that activation may involve a conformational change that will allow the opening of this cleft. Therefore, it has been proposed, that interdomain interactions are playing an important role in regulating the nucleotide exchange rate of the α subunit. While constructing different Gsα quimeras, we identified a Gsα random mutant, which was very inefficient in stimulating adenylyl cyclase activity. The introduced mutation corresponded to the substitution of Ser^{111} for Asn ($S^{111}N$), located in the carboxi terminal end of helix A of the HD, a region neither involved in AC interaction nor in the interdomain interface. In order to characterize this mutant, we expressed it in bacteria, purified it by niquel-agarose chromatography, and studied its nucleotide exchange properties. We demonstrated that the recombinant $S^{111}N$ Gsα was functional since it was able to undergo the characteristic conformational change upon GTP binding, detected by the acquisition of a trypsin-resistant conformation. When the biochemical properties were determined, the mutant protein exhibited a reduced GDP dissociation kinetics and as a consequence a slower GTPγS binding rate that was responsible for a diminished adenylyl cyclase activation when GTPγS was used as activator. These data provide new evidence that involves the HD as a regulator of Gsα function, in this case the αA helix, which is not directly involved with the nucleotide binding site nor the interdomain interface. J. Cell. Biochem. 85: 615–620, 2002.

Key words: G-protein; Gsα; adenylyl cyclase

Heterotrimeric G-proteins transmit signals from cell surface receptors to effector proteins that modulate a wide variety of cellular processes [Bourne et al., 1990; Hamm, 1998]. The hormone-receptor complex promotes the exchange of GDP by GTP in the inactive GαGDP/βγ form, which results in the binding of GTP to the Gα subunit and dissociation of GαGTP/βγ in the active species GαGTP and Gβγ [Sprang, 1997; Hamm, 1998]. Both proteins can subsequently regulate the activity of different effector systems such as adenylyl cyclase, phospholipase C, and ionic channels [Clapham and Neer, 1993, 1997; Sunahara et al., 1996; Skiba and Hamm, 1998]. The active GαGTP is then turned off by its intrinsic GTPase activity, which is highly regulated by some effectors or by G-protein signaling proteins (RGS), and reassociation with Gβγ returns the G-protein to its inactive heterotrimeric state. High resolution structural analysis of several forms of G alphas [Noel et al., 1993; Coleman et al., 1994; Lambright et al., 1994, 1996] has allowed the identification of two domains: a GTPase domain (GTPaseD), homologous to monomeric G-proteins, which contains the guanine nucleotide binding site as well as the receptor and effector interaction sites, and the helical domain (HD), composed by six α helices that display the highest sequence variations between the different Gα subunits. Although the function of the HD is not completely understood, it has been postulated to be involved in several different processes like recognition of the receptor and

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effector system, stimulation of GTPase activity, regulation of the transition state between the active and inactive form, and regulation of Gα oligomerization [Osawa et al., 1990a,b; Dhanasekaran et al., 1991; Markby et al., 1993; Liu and Northup, 1998; Liu et al., 1998; Krieger-Brauer et al., 1999; Skiba et al., 1999]. Because the guanine nucleotide is buried in a deep cleft between both domains, nucleotide exchange has been postulated to involve a conformational change that would allow the opening of this cleft. Interdomain interactions have been, therefore, considered to be important in modulating the rate of nucleotide exchange. Accumulating evidence has shown that interactions between residues of the interdomain interfaces are playing an important role in regulating the activation of the Gα subunit. The main regions participating in the constitution of the interfaces of the cleft are the αD/αE loop of the HD and the β4/α3 (or switch III) and αG/α4 loops of the GTPaseD. Interactions between residues of these loops have been described to be crucial in regulating the nucleotide exchange properties of different Gαs [Codina and Birnbaumer, 1994; Grishina and Berlot, 1998].

We recently reported that mutations in the switch IV (αB/αC loop) of Gsα, a region exposed to the outside of the HD and very distant from the interdomain interface, affects the nucleotide exchange rate of Gsα diminishing the GDP dissociation and GTP binding kinetics five-fold [Echeverría et al., 2000]. Here, we describe the effect of a single mutation, the substitution of Ser^{111} for Asn, localized in the C-terminal end of the αA helix, which is on the opposite side of switch IV in the HD (Fig. 1). This mutant also shows a decreased GDP/GTP exchange rate, and as a consequence of that, a reduced ability to stimulate adenylyl cyclase.

MATERIALS AND METHODS

Mutagenesis of Gsα

While performing PCR amplifications of hGsα, a random mutation was introduced into the cDNA that changed G^{278} for A. This mutation produced the substitution of Ser^{111} for Asn, a residue located in the C-terminal end of the αA helix of the HD. The mutated cDNA was subcloned into the H_6pQE60 vector as an NcoI-HindIII fragment, to express the recombinant protein with a hexa-histidine tag at its amino termini. In this study, we worked with the short form of Gsα, but the residues were numbered according to the long form of the gen.

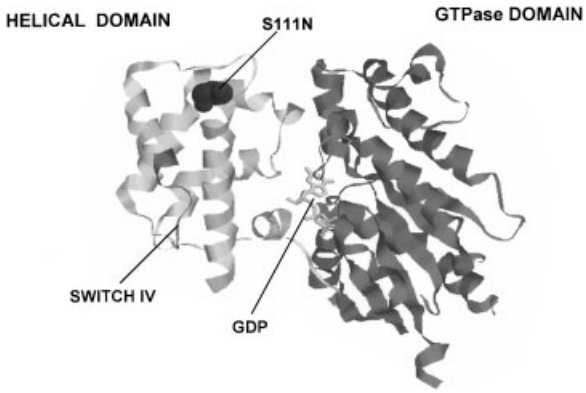

Fig. 1. Crystal structure of Gsα. The Gsα tertiary structure shown in the figure corresponds to the Protein Data Bank entry 1AZT and was modeled by RasMol. The GTPase domain is to the right shown in dark gray and the helical domain to the left in light gray. Ser^{111}, the residue mutated in this study, is shown in black and the switch IV in dark gray. Buried inside the cleft formed between both domains is the guanine nucleotide (GDP, in sticks).

Expression and Purification of Recombinant Gsα Proteins

BL21(DE3) bacteria were co-transformed with the recombinant plasmid H_6pQE60 containing the wild type or mutated hGsα cDNAs and with vector pRP4 that encodes for the lac repressor. Expression of Gsα was induced with IPTG as described by Lee et al. [1994] and purified using niquel-agarose chromatography according to Echeverría et al. [2000].

Trypsin Protection Assay

The trypsin protection analysis was performed essentially as described by Graziano and Gilman, [1989] and modified by Echeverría et al. [2000]. Essentially, mutant and wild type Gsα proteins (2.5 μg/ml) were incubated at 30°C in a buffer containing 50 mM Na-HEPES, pH 8.0, 1 mM EDTA, 5 mM DTT, 10 mM $MgSO_4$, and 100 μM GTPγS or 10 mM AlF^{4-} for 1 h. Trypsin was then added to a final concentration of 10 μg/ml and incubated at room temperature for 15 min. Laemmli buffer was then added to stop the reaction, and trypsin-resistant products were visualized by SDS–PAGE analysis followed by Coomassie blue staining.

GTPγS Binding Assay

GTPγS binding to Gsα proteins was performed as described by Echeverría et al. [2000].

Briefly, the reaction was carried out at 26°C in a mixture containing 50 mM Na-HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$, 0.1% Lubrol, and 2 μM [^{35}S]GTPγS (45,000 cpm/pmol). Aliquots were withdrawn at the indicated time points, stopped by the addition of 2 ml ice-cold buffer (20 mM Tris-HCl, pH 8.0, 25 mM $MgCl_2$, 100 mM NaCl, and 0.1 mM GTP), and filtered and washed with 20 ml of the same buffer under vacuum. Radioactivity was quantified by liquid scintillation counting of the dried filters.

GDP Dissociation

GDP dissociation time course from Gsα proteins was performed according to Echeverría et al. [2000]. A typical reaction consisted in the incubation of the purified Gsα protein (50–60 pmol) at 20°C for 60 min in a buffer containing 50 mM HEPES, pH 7.6, 1 mM EDTA, 1 mM DTT, 0.1% Lubrol, 10 mM $MgSO_4$, and 2 μM [^{3}H]GDP (10,000 cpm/pmol). Samples were then diluted with unlabeled GDP to bring them to a final concentration of 200 μM. At the indicated time points, aliquots were mixed with ice-cold buffer (20 mM Tris-HCl, pH 7.6, 100 mM NaCl, 25 mM $MgCl_2$) and filtered and washed with the same buffer through BA85 nitrocellulose filters under vacuum. Filters were dried and radioactivity quantified by liquid scintillation counting.

Reconstitution of Adenylyl Cyclase Activity

S49 cyc^- reconstitution assays were performed essentially as described by Echeverría et al. [2000]. Normally, 100–200 ng of purified Gsα protein was added to 25 μg of cyc^- membranes in a final volume of 50 μl containing 0.10 mM [α-^{32}P]ATP (1,000–2,000 cpm/pmol) and 10 mM $MgCl_2$ in the absence or presence of different activators as indicated in the figure. Incubations were performed at 32°C for 20 min and cAMP was isolated and quantified according to Salomon et al. [1974]. S49 cyc^- membranes were prepared as described by Ross et al. [1977].

RESULTS

Expression, Purification, and Trypsin Protection Analysis of Wild Type and $S^{111}N$ Gsα

During PCR amplification of Gsα, a random mutation was introduced that produced a change of Ser^{111} to Asn in the protein. This Gsα mutant showed, in preliminary in vitro expression studies, a decreased capacity to stimulate adenylyl cyclase. Since this aminoacid is located in the HD-αA helix, a structure very distant from the nucleotide binding site and also from the cleft interface, we became interested in characterizing it further. To do so, we expressed the wild type and the $S^{111}N$ Gsα in bacteria with an hexa-histidine tag at its N-terminus and purified it through Ni-agarose chromatography as described in Materials and Methods. A highly pure recombinant protein was obtained with yields of 0.5–0.9 mg/L for the wild type protein and of 0.3–0.5 mg/L for the mutant Gsα. In order to determine if the mutant protein was properly folded and able to undergo the activating conformational change, we performed the trypsin-protection analysis. This assay has been widely used to differentiate between the inactive GDP bound conformation, in which case the Gsα is completly digested, and the active GTP bound state, that can be recognized by the appearance of a smaller fragment that has lost a short N-terminal peptide. As shown in Figure 2, both proteins were completly digested when incubated with trypsin (lanes 5 and 9), but preincubation with GTPγS or AlF^{4-} protected them from the digestion, indicating that not only the wild type but also the mutant was able to bind GTPγS and to aquire the transition state conformation (lanes 3, 4, 7, and 8).

Substitution of Ser^{111} to Asn Leads to a Decreased Nucleotide Exchange Kinetics in Gsα

To characterize the functional properties of the $S^{111}N$ Gsα mutant, we first measured its

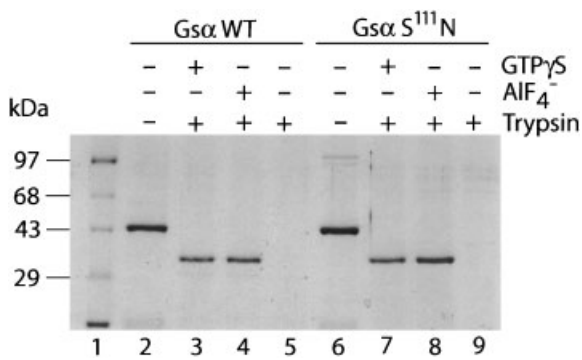

Fig. 2. Trypsin protection analysis of wild-type and $S^{111}N$ Gsα. One microgram of wild-type or mutant Gsα was incubated for 60 min in the abscense or presence of 100 μM GTPγS or 10 mM AlF^{4-} and followed by trypsin digestion. Samples were analyzed by SDS–PAGE and visualized by Coomassie blue staining.

capacity to bind GTPγS and compared it with the wild type Gsα. As shown in Figure 3, the $S^{111}N$ mutant displayed a reduced nucleotide binding kinetics ($\kappa = 0.045\ min^{-1}$), that was 4–5 times lower than the wild type protein ($\kappa = 0.19\ min^{-1}$).

Since the GTP binding kinetics is limited by the GDP dissociation rate, we continue determining the Koff constant rates of [^{3}H]GDP for the wild type and mutant Gsα. As expected, the $S^{111}N$ mutant showed again a slower GDP dissociation time course that was 5–6 times lower than the wild type subunit (Fig. 4).

Therefore, we conclude that substitution of Ser^{111} produces a long distance conformational change in the interdomain cleft or in the nucleotide binding pocket that reduces the GDP dissociation rate of the protein.

$S^{111}N$ Gsα Mutant Shows Impaired Adenylyl Cyclase Activation With GTPγS But Normal Receptor and AlF^{4-} Activation

To evaluate whether the $S^{111}N$ mutation was affecting other functional properties of the Gα subunit, we determined its capacity to stimulate adenylyl cyclase by reconstitution experiments using S49 cyc^- membranes. Figure 5 shows that when GTPγS was used as activator, the mutant protein was considerably less efficient than the wild type in activating its effector, which is consistent with the lower basal nucleotide exchange activity displayed. However, when AlF^{4-} and isoproterenol were used, no significant differences between both proteins were observed. These results indicate that the adenylyl cyclase and receptor interaction sites have not been affected by the $S^{111}N$ mutation, which is in accordance with several structural and site directed mutagenesis studies that have shown to be located in the GTPaseD.

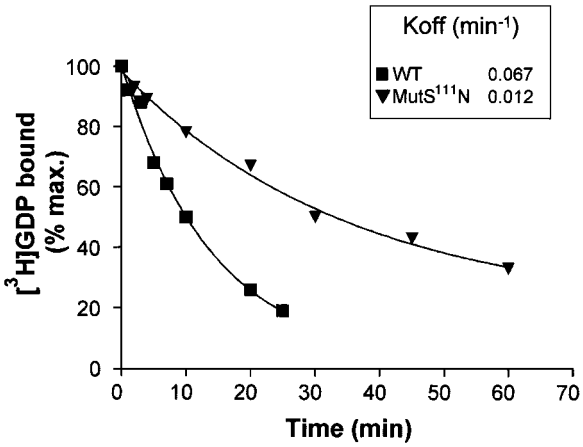

Fig. 4. Dissociation of GDP from purified wild-type and $S^{111}N$ Gsα. Fifty picomoles of wild-type and $S^{111}N$ Gsα were incubated at 20°C with 2 μM [^{3}H]GDP (10,000 cpm/pmol) for 60 min. Dissociation of labeled bound GDP was assessed by adding 200 μM of unlabeled GDP, and at the indicated times aliquots were withdrawn, and [^{3}H]GDP binding was quantitated by filtration and counting.

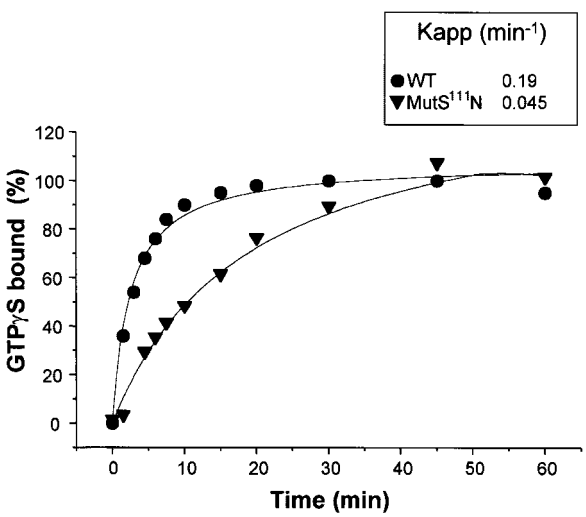

Fig. 3. Time course of GTPγS binding to purified wild-type and $S^{111}N$ Gsα. Recombinant wild-type or $S^{111}N$-Gsα proteins were incubated at 26°C with 2 μM [^{35}S]GTPγS (20,000 cpm/pmol) for varying times, and the amount of bound GTPγS was determined. The apparent on rates for GTPγS (κ_{app}) are shown.

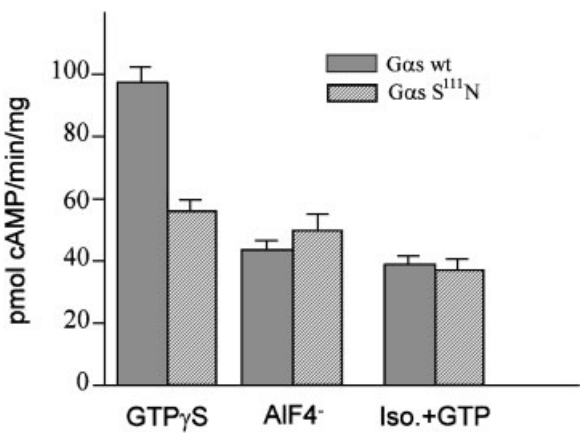

Fig. 5. Adenylyl cyclase stimulation by recombinant wild-type and $S^{111}N$ Gsα. S49 cyc^- membranes were reconstituted with 200 ng of either wild-type or $S^{111}N$ Gsα. Adenylyl cyclase activity was measured at 32°C for 20 min with either GTPγS, AlF^{4-} or isoproterenol and GTP. Before reconstitution, functional Gsα proteins were quantitated by [^{35}S]GTPγS binding, and equal amount of functional protein was added to each assay.

DISCUSSION

In this report, we show the characterization of a Gsα mutant that has a single point mutation in residue Ser^{111}, located in the carboxi terminal portion of helix A of the HD. Since this region has not been implicated in any of the Gsα functions described so far, like receptor or effector interaction or nucleotide binding, we were very surprised to discover that this mutant displayed impaired adenylyl cyclase stimulation activity. Biochemical characterization of the mutant protein revealed that the defect was due to a slower GDP dissociation rate (Fig. 4), which was responsible for a slower basal nucleotide exchange activity. Accordingly, we only observed impaired adenylyl cyclase activation, when GTPγS was used as activator (that requires basal GDP dissociation) and not when AlF^{4-} or isoproterenol were added (Fig. 5). Interestingly, the same effect was exhibited by another Gsα mutant, in which five residues of the switch IV that correspond to the αB/αC loop of the HD, were changed [Echeverría et al., 2000].

Another example that shows a decreased GDP dissociation effect due to a HD mutation, was described by Lan et al. [1998], who showed that a Gαo mutant, in which Trp132 was replaced by Phe, caused a two-fold reduction in its nucleotide exchange rate. It is important to point out that Trp132 is located in helix C of HD, just ten residues downstream of the switch IV.

Analysis of Gsα crystal structure reveals two direct contacts between HD and GTPaseD that are established through two salt bridges formed between residues located in the αD/αE loop of the HD (Gln170 and Asp173) and residues of the switch III (Arg258) and the β5/αG loop (Lys293) of the GTPaseD. Mutation of any of these residues, that cause the disruption of one of the ion-counterion interactions, has been shown to provoke the opposite effect as the one described before, which is to produce a mutant protein with an accelerated GDP dissociation rate and nucleotide exchange activity. Therefore, the HD has been postulated to act as a "lid" over the cleft that contains the guanine nucleotide that is kept tightly associated to the GTPaseD through several interdomain interactions, and disturbance of any of these interactions would produce the opening of the cleft and facilitate its GDP release [Remmers et al., 1999].

The reduced GDP dissociation rate displayed by the Ser^{111}, Trp132, and switch IV Gsα mutations is more difficult to explain, since these residues are located in different positions of the HD and distant from the GTPaseD interface. Thus, we believe that small conformational changes introduced in the HD by this mutations, are altering the proper positioning of the two domains with respect to each other, closing the interdomain cleft and reducing the basal nucleotide exchange rate of the protein.

Ser^{111}, the residue that was substituted by Asn in our mutant, is included in the epitope recognized by the K-20 antibody (residues 100–119), which was proposed to constitute a novel Gsα-receptor contact site by Krieger-Brauer et al. [1999], however our data show that receptor-mediated stimulation was unaffected in the mutant protein (Fig. 5). This result does not rule out this region as a receptor-interaction-site, because, we do not know if the Ser to Asn substitution will affect the putative receptor interaction site.

In summary, we can conclude that substantial evidence is accumulating that involves the HD in controlling the basal nucleotide exchange activity of Gα and that small structural perturbances can produce important changes in the functional properties of this protein.

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