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Mutagenesis in the Switch IV of the Helical Domain of the Human Gsα Reduces Its GDP/GTP Exchange Rate

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Abstract The Gα subunits of heterotrimeric G proteins are constituted by a conserved GTPase "Ras-like" domain (RasD) and by a unique α-helical domain (HD). Upon GTP binding, four regions, called switch I, II, III, and IV, have been identified as undergoing structural changes. Switch I, II, and III are located in RasD and switch IV in HD. All Gα known functions, such as GTPase activity and receptor, effector, and Gβγ interaction sites have been found to be localized in RasD, but little is known about the role of HD and its switch IV region. Through the construction of chimeras between human and *Xenopus* Gsα we have previously identified a HD region, encompassing helices αA, αB, and αC, that was responsible for the observed functional differences in their capacity to activate adenylyl cyclase (Antonelli et al. [1994]: FEBS Lett 340:249–254). Since switch IV is located within this region and contains most of the nonconservative amino acid differences between both Gsα proteins, in the present work we constructed two human Gsα mutant proteins in which we have changed four and five switch IV residues for the ones present in the *Xenopus* protein. Mutants M15 (hGsααS133N, M135P, P138K, P143S) and M17 (hGsααS133N, M135P, V137Y, P138K, P143S) were expressed in *Escherichia coli*, purified, and characterized by their ability to bind GTPγS, dissociate GDP, hydrolyze GTP, and activate adenylyl cyclase. A decreased rate of GDP release, GTPγS binding, and GTP hydrolysis was observed for both mutants, M17 having considerably slower kinetics than M15 for all functions tested. Reconstituted adenylyl cyclase activity with both mutants showed normal activation in the presence of AlF_4^-, but a decreased activation with GTPγS, which is consistent with the lower GDP dissociating rate they displayed. These data provide new evidence on the role that HD is playing in modulating the GDP/GTP exchange of the Gsα subunit. J. Cell. Biochem. 76:368–375, 2000.

Key words: G-protein; Gsα; adenylyl cyclase

Heterotrimeric G proteins belong to a family of proteins, whose signal transduction function depends on the binding of guanine nucleotides [Birnbaumer, 1990; Sprang, 1997a; Hamm, 1998]. Binding of GTP to the α subunit (Gα) "turns on" the system and causes dissociation of the heterotrimer Gαβγ in the active species Gα-GTP and Gβγ heterodimer [Neer, 1995]. Both active proteins can then regulate the activity of different effector molecules, such as adenylyl cyclase, phospholipase C_β, and ion channels [Sternweis, 1994; Neer, 1995; Wickman and Clapham, 1995; Hurley, 1999]. Signal transduction is "turned off" by the intrinsic GTPase activity of the Gαprotein, that hydrolyzes the bound GTP to GDP, inducing the reassociation of Gα-GDP with Gβγ [Sternweis,1994; Gautam et al., 1998]. In addition to the receptor and the intrinsic GTPase activity of Gα, the on/off G protein ratio can be regulated by other proteins known as guanine exchange factors (GEF), guanine dissociation inhibitors (GDI), and regulators of G protein signaling (RGS) [Geyer and Wittinghofer, 1997; Sprang, 1997b]. Recently, elucidation of the tertiary structure of Gtα, Giα, and Gsα subunits [Noel et al., 1993; Coleman et al., 1994; Lambright et al., 1994] has shown that these proteins are folded into two separate

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domains: a conserved domain called "GTPase or Ras-like" domain (RasD), and a unique domain called α-helical domain (HD), composed of six α-helices [Wall et al., 1995; Lambright et al., 1996]. RasD possesses GTP binding and GTPase activity and also interacts with the receptor, Gβγ and the effector [Sunahara et al., 1997], but the role of HD remains unknown. HDs of different Gαs display higher sequence variation than RasDs, suggesting some Gα-specific functions for this domain. In earlier studies, this domain was postulated to act as an "attenuator" of RasD function by inhibiting the basal GDP/GTP exchange and by contributing with an arginine residue to the GTPase activity of the Gαsubunit [Osawa et al., 1990a,b; Dhanasekaran et al., 1991; Markby et al., 1993]. Recently, the Gtα-HD was shown to participate in regulating the cGMP phosphodiesterase activity and to mediate the interaction of Gtα with RGS9 [Liu and Northup, 1998; Liu et al., 1998; Skiba et al., 1999], indicating that HDs may have multiple functions. Switch IV (SWIV), a region that, together with switch I, II, and III, shows the most important conformational changes induced by GTP binding, is located in HD [Mixon et al., 1995] and presents a high amino acid diversity among Gα subunits, which may be important in specific interactions with other regulatory proteins.

In our laboratory, we have cloned a *Xenopus laevis* Gsα [Olate et al., 1990], which in spite of being 92% identical to human Gsα, is unable to activate and physically interact with mammalian and *Xenopus* adenylyl cyclases [Antonelli et al., 1994; Torrejón et al., 1998]. Interestingly, of a total of 29 amino acid differences, 19 are located within HD, and 14 are clustered along SWIV [Antonelli et al., 1994]. By analysis of human-*Xenopus* Gsα chimeras, we also demonstrated that a region, encompassing HD helices αA, αB, SWIV, and αC, played an important role in the capacity of Gsα to activate adenylyl cyclase [Antonelli et al., 1994]. Since SWIV contains most of the amino acid differences between *Xenopus* and human Gsα proteins, and has been shown to undergo an important conformational change upon GTP binding, we decided to replace several amino acids of this region of the human Gsα with the corresponding amino acids present in the *Xenopus* Gsα subunit. Mutant M15 (hGsα; S133N, M135P, P138K, P143S) and M17 (hGsαα S133N, M135P, V137Y, P138K, P143S), which contain 4 and 5 of the SWIV *Xenopus* amino acids respectively, showed slower decreased kinetics of GDP dissociation and of GTPγS binding.

MATERIALS AND METHODS

Mutagenesis of the Human Gαs cDNA

A *Nco*I/*Xba*I fragment, containing the complete wild-type human Gsα coding sequence (short splice variant), was ligated into pAGA vector [Hsu et al., 1990] and used as template to perform site-directed mutagenesis by polymerase chain reaction (PCR), using the overlap extension method [Ho et al., 1989]. A hGsα cDNA fragment, flanked by an *Eco*RI/*Hin*dIII restriction site, was amplified to introduce a triple mutation and, over this mutated cDNA, a fourth mutation, and finally a fifth mutation. To create the quadruple mutant M15, the upstream primer 5′GCTCATAGAATTCGGGTGAGAAGTCAAAG3′ and downstream primer 5′CTTTGACTTCTCACCCGAATTCTATGAGC3′ were used. For the quintuple mutant M17, the M15 Gsα cDNA was used as template and the upstream primer 5′GTGCCGAACTACAAAGACTTTG3′ and downstream primer 5′CAAAGTCTTTGTAGTTCGGCAC3′ were used. The mutated cDNA fragments were digested with *Eco*RI and *Hin*dIII and exchanged with the corresponding wild-type region of the hGsα/pGEM15zf- recombinant vector. The complete mutated coding sequence of the Gsα cDNA was excised as an *Nco*I-*Hin*dIII fragment from pGEM15Zf- and subcloned into H_6-pQE60 vector to express the recombinant proteins with an hexa-histidine tag at their amino termini. All mutations were confirmed by DNA sequencing.

Expression and Purification of Recombinant Gsα Proteins

BL21(DE3) were co-transformed with the recombinant H_6-pQE60 plasmids containing the wild-type or mutated Gsα cDNAs and with the pREP4 plasmid that encodes for the *lac* repressor. Expression of the Gsα proteins was induced by the addition of IPTG to the culture medium and purified using affinity chromatography [Lee et al., 1994]. Purified Gsα proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and quantified from SYPRO® Orange-stained gels, by comparing the fluorescence of the purified protein with

different concentration of bovine serum albumin (BSA) used as the standard.

Trypsin Protection Assay

Trypsin protection analysis was performed essentially as described with some modifications [Graziano and Gilman, 1989]. In brief, wild-type and mutant Gsα proteins (2 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 $MgS0_4$ and 100 µM GTPγS. After 1-h incubation, trypsin was added to a final concentration of 6 µg/ml and incubated at room temperature for 15 min. The reaction was stopped by the addition of Laemmli buffer; trypsin-resistant products were visualized by SDS-PAGE analysis followed by Coomassie blue staining.

GTPγS Binding Assay

GTPγS binding by Gsα proteins was measured as described by Kleuss et al. [1994], with minor modifications. A typical binding reaction was carried out at 20°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 (30,000 cpm/pmol). At the indicated time points, aliquots were withdrawn and 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 through BA85 nitrocellulose filters under vacuum. Filters were dried and radioactivity was quantified by liquid scintillation counting.

GDP Dissociation

The time course of GDP dissociation from Gsα proteins was performed accord to Glick et al. [1996], with some modifications. Briefly, 50 pmol of purified Gsα protein was incubated at 20°C for 30 or 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 0.5 µM [^{3}H]GDP (26,000 cpm/pmol). Samples were then spiked with unlabeled GDP, such that the final concentration of GDP in the chase was 50 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 through BA85 nitrocellulose filters. Filters were dried and radioactivity quantified by liquid scintillation counting.

GTP Hydrolysis Analysis

Steady-state GTPase activity was measured incubating purified Gsα protein (50 pmol) at 20°C in a buffer containing 50 mM Na-Hepes, pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 0.1% lubrol, 1 µM [γ-^{32}P]GTP (24,000 cpm/pmol) and 10 mM $MgSO_4$. For the [^{32}P]PO_4^{3-} time-dependent release, the reaction was initiated by the addition of the Gsα protein to the mix that had been previously warmed up to the reaction temperature. At the indicated times, 50-µl aliquots were removed, mixed with 750 µl of 5% (w/v) Norit in 50 mM NaH_2PO_4 at 0°C, and vortexed. The charcoal was then removed by centrifugation at 3,000*g* for 10 min, and the amount of radioactivity in 500 µl of supernatant aliquot was determined by liquid scintillation counting. GTPase activity was determined by subtracting the rate of protein-independent [^{32}P]PO_4^{3-} formation [Graziano and Gilman, 1989].

Reconstitution of Adenylyl Cyclase Activity

The S49 cyc$^-$ reconstitution assay was performed essentially as described by Olate et al. [1988], with some modifications. Normally, 100 ng of purified Gαs protein was added to 20 µg of purified S49 cyc$^-$ plasma membranes in a final volume of 50 µl containing 0.12 mM [α-^{32}P]ATP (300–500 cpm/pmol) and 10 mM $MgCl_2$ in the absence or presence of the components indicated in the figure. Incubations were performed at 32°C for 15 min; cAMP was isolated and quantified according to Solomon et al. [1974]. Membranes were prepared from S49 cyc$^-$ cells as described [Ross et al., 1977].

Superposition of Wild-Type and M17 Gsα Protein Structures

Modeling of wild-type and mutant M17 Gsα were performed interactively with the Swiss-PDB Viewer program [Guex and Peistch, 1997]. The Gsα 1AZT Protein Data Bank (PDB) entry was used to introduce the five amino acid changes corresponding to mutant M17 and the eight residues corresponding to linker 1, not defined in the crystal Gsα structure. Both structures were conformational energy-minimized by conjugate gradient with an rms gradient of 0,001 kcal/Å mol as the termination condition, using the AMBER program [Weiner and Kollman, 1981].

RESULTS

Expression, Purification, and Trypsin Protection Assay of Recombinant Gsα Proteins

We have previously shown that functional differences between human and *Xenopus* Gsα are localized in HD and that most of the nonconservative changes are clustered in SWIV [Antonelli et al., 1994]. To further characterize the role of this region, we mutated S^{133}, M^{135}, V^{137}, P^{138} and P^{143} of the human Gsα to the corresponding amino acids present in the *Xenopus* subunit, creating Gsα M15 (S133N, M135P, P138K, P143S) and M17 (S133N, M135P, V137Y, P138K, P143S). To facilitate the purification of the proteins we subcloned the wild-type and mutated hGsα cDNAs into the H_6-pQE60 vector, which introduces an hexa-histidine tag at the N-terminus of the protein and facilitates purification with yields of 0.3–0.9 mg of purified protein from 1 L of bacterial culture (Fig.1). It is well known that upon GTP binding, Gα subunits undergo a conformational change that is detectable by the acquisition of a trypsin-resistant conformation [Sternweis and Robishaw, 1984] and the GTPγS activated protein form is cleaved near the N-terminus, accumulating a 39-kD product. By contrast, the Gsα-GDP form is readily proteolysed to small fragments not detectable in a normal SDS-PAGE analysis. Therefore, to determine whether the wild-type and mutated Gsα proteins were capable to experiment the conformational change that accompanies activation of the protein, we performed the trypsin-protection assay. All Gsα proteins were protected by GTPγS against trypsin digestion, indicating that not only the wild-type, but also M15 and M17 mutants, were able to undergo the GTPγS-induced conformational change (Fig. 2).

Binding, Dissociation, and Hydrolysis of GTP by Wild-Type and Mutant Gsα Subunits

In order to characterize the functional properties of M15 and M17 mutants, we measured their capacity to bind GTPγS and compared it with the wild-type protein. As shown in Figure 3A, both mutants displayed reduced apparent nucleotide binding kinetics, with M17 fivefold lower ($k$app = 0.042 min^{-1}) and M15 twofold lower ($k$app = 0.146 min^{-1}) than the wild-type Gsα ($k$app = 0.210 min^{-1}). These data indicate that mutations introduced in the SWIV of HD caused a perturbation in the nucleotide binding pocket of the protein that reduces its GTPγS binding kinetics.

Because the GTP binding kinetics is limited by the GDP dissociation rate [Ferguson et al., 1986], we determined the $k$off constant rates of [^{3}H]GDP for the wild-type and mutant Gsα subunits. As expected, both mutants showed again slower GDP dissociation kinetics (Fig. 3B), with $k$off constant values similar to the calculated GTPγS binding $k$apps. We conclude that the slower GTPγS binding activity observed for the mutant proteins is caused by a diminished GDP dissociation rate.

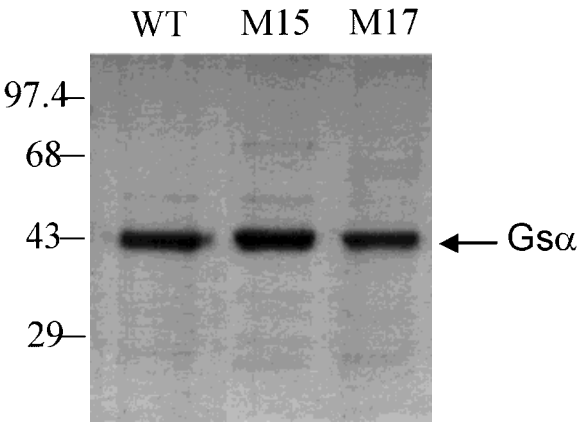


Fig. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of purified Gsα proteins expressed in *Escherichia coli*. A total of 500 ng of purified wild-type (wt), mutant 15 (M15), and mutant 17 (M17) was subjected to 10% SDS-PAGE and visualized by Coomassie blue staining. Arrow indicates the position of the purified hGsα proteins (short species, MW = 43 kDa).

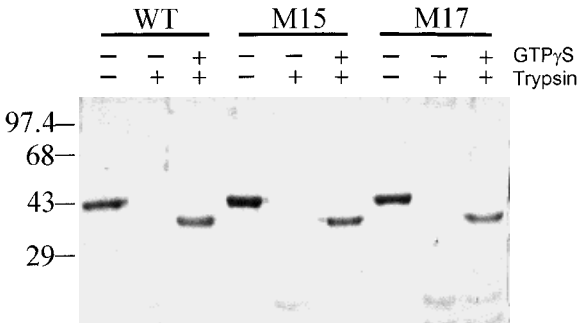
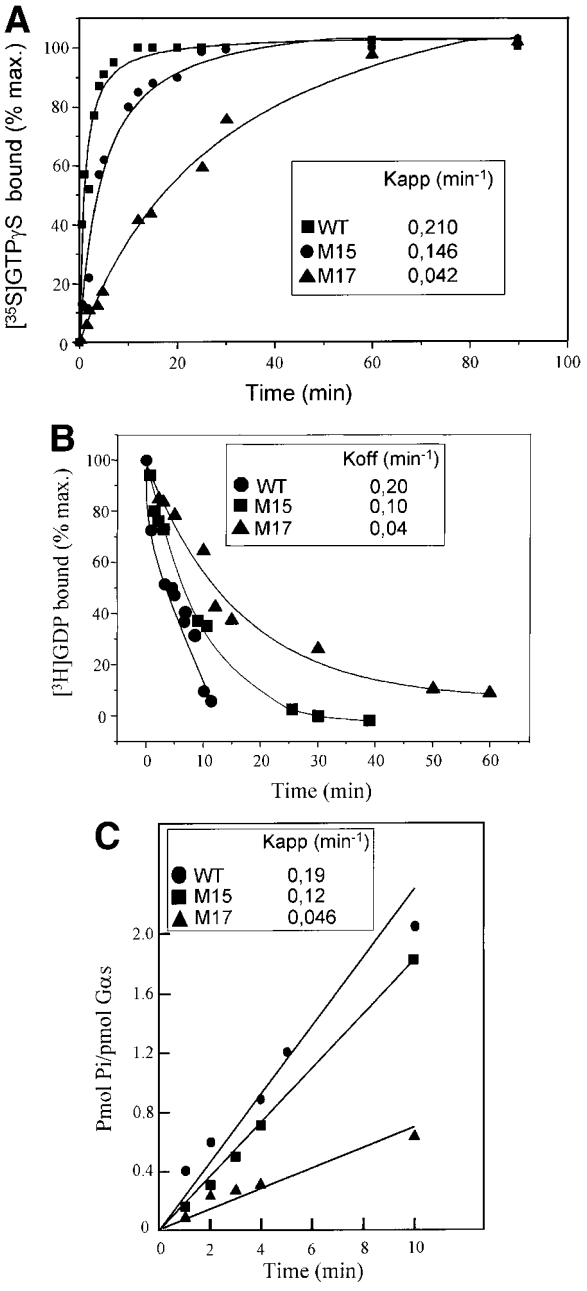


Fig. 2. Trypsin protection of wild-type and mutant Gsα proteins. A total of 500 ng of wild-type and mutant Gsα proteins was incubated with or without GTPγS and trypsin. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue staining.

Gsα steady-state GTPase activity is dependent on the GTP binding kinetics and consequently on the GDP dissociation rate [Ferguson et al., 1986]. For this reason, according to our GTPγS binding and GDP dissociation data, we expected that M15 and M17 mutants would also display lower GTPase activities. As shown in Figure 3C, both mutants displayed decreased steady-state GTPase activity, mutant M15 having a k_{app} value two fold lower and mutant M17 four fold lower than the wild-type protein.



Adenylyl Cyclase Stimulation by Wild-Type and Mutant Gsα Subunits

In order to determine whether mutations introduced in the HD SWIV region of the human Gsα were affecting other functional properties than the GDP dissociation rate, we examined the ability of these proteins to stimulate adenylyl cyclase by reconstitution experiments using S49 cyc⁻ membranes. When GTPγS was used as activator, both mutants were considerably less efficient than the wild-type protein in stimulating cAMP synthesis. However when AlF_4^- was tested, no significant differences among the three proteins were observed (Fig. 4). This result is in agreement with previous observations that indicate that AlF_4^- acts by binding to the GDP-bound form of the protein to reach the transition state conformation, without depending on GDP dissociation. On the contrary, when GTP or GTPγS are used as activators, GDP dissociation is a prerequisite for GTP binding and Gsαactivation. Therefore, we believe that the reduce adenylyl cyclase activity observed with the GTPγS-bound mutants is due to the decreased GDP dissociation rate that these proteins display.

DISCUSSION

Extending previous studies performed with human-*Xenopus* Gsα chimeras [Antonelli et al.,

Fig. 3. Binding, dissociation, and hydrolysis of GTP by wild-type and mutant Gsα subunits **A:** Time course of GTPγS binding to wild-type, M15, and M17 Gsα proteins. Recombinant Gsα proteins (100 ng) were incubated at 20°C with 2 μM of [35S]GTPγS (spec act 20,000 cpm/pmol) and 10 mM $MgSO_4$. Duplicate aliquots were withdrawn at the indicated times, filtered, and counted. Data shown represent the mean of duplicate determinations from a representative experiment. The values shown for *k*app were determined by fitting the data to equation $B = B_{eq}(1 - e^{-kt})$. The stoichiometry of binding of [35S]GTPγS to the wild-type and mutant Gsα preparations was between 0.5 and 0.8. **B:** Time course of [3H]GDP dissociation from wild-type, M15, and M17 Gsα proteins. A total of 50 pmoles of wild-type and mutant Gsα proteins was incubated at 20°C with 0.5 μM [3H]GDP (spec act 26,000 cpm/pmol). Dissociation of labeled bound GDP was assessed by adding 50 μM of unlabeled GDP. At the indicated times, aliquots were withdrawn and [3H]GDP binding was quantitated by filtration and counting. Data shown represent the mean of duplicate determinations from a representative experiment. **C:** Steady-state GTP hydrolysis by wild-type and mutant Gsα proteins. A total of 100 ng of wild-type and mutant proteins were incubated at 20°C with 10 mM $MgSO_4$ and 1 μM [γ-32P]-GTP (specific activity 24,000 cpm/pmol). At the indicated times aliquots were withdrawn and Pi release measured. Data shown represent the mean of duplicate determinations from a representative experiment.

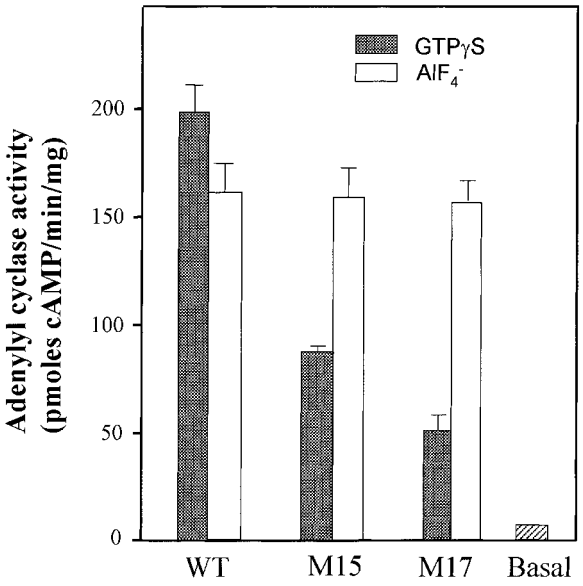


Fig. 4. Reconstitution of adenylyl cyclase activity in S49 cyc⁻ membranes. S49 cyc⁻ membranes (20 μg) were reconstituted with 100 ng of either wild-type, M15, or M17 Gsα, as described under Materials and Methods. Before reconstitution, Gsα proteins were quantified by [^{35}S]GTPγS binding and diluted to a concentration of 10 ng/μl. Adenylyl cyclase activity was assayed for 15 min at 32°C with either 10 μM GTPγS or 10 mM NaF (AlF_4^-).

1994], we have constructed two human Gsα mutants by replacing 4 and 5 SWIV amino acids that showed nonconservative differences with the *Xenopus* protein. We have focused our attention on SWIV because this region has clustered most of the amino acids differences and also undergoes an important conformational change upon Gsα activation. The amino acids that we have changed in the human Gsα, which are normally present in the wild-type *Xenopus* protein, predict an important torsion of the SWIV loop, as can be seen from the superposition of the wild-type and M17 modeled structures (Fig. 5). Important positional changes were observed for Val^{137} and Pro^{138}, with deviations of 3.3 and 4.3 Å, respectively. These changes caused the displacement of SWIV toward the solvent by 4.4 Å (Fig. 5) and of helix B by 2.5 Å (not shown). RasD did not show important structural changes, but positional differences of lateral groups of important amino acids that cause the disruption of three hydrogen bonds between switch II and III were detected (not shown). For protein modeling and structural comparisons we used M17, because it showed more dramatic differences in all the functions tested (Figs. 3, 4).

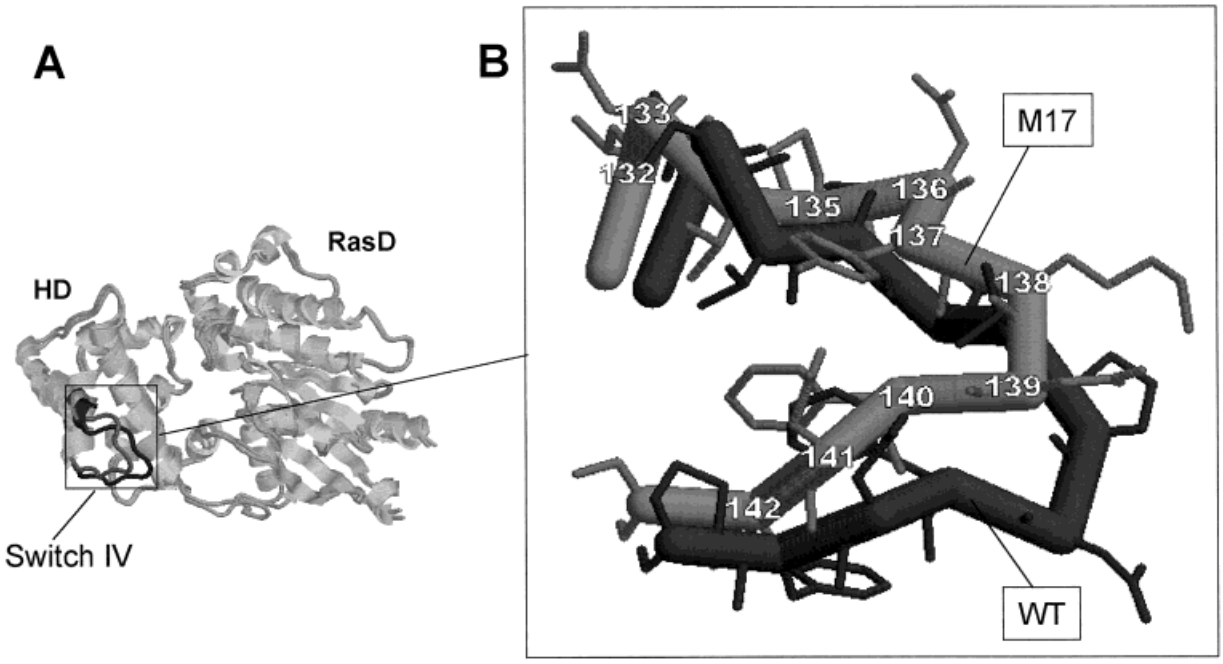


Fig. 5. Superposition of wild-type and M17 Gsα protein structures. **A:** Superposition of wild-type and M17 Gsα modeled protein structures. HD denotes helical domain and RasD denotes the GTPase domain. The switch IV region is delimited by a rectangle. **B:** Detailed view of wild-type (dark gray) and M17 (light gray) switch IV structure (residues 131–142). Numbers correspond to the amino acid position along the primary sequence.

Mutation of Val^{137} to Tyr is the amino acid change that more drastically affected the GDP/GTP exchange rate, as M17 has diminished its GTP binding and GDP dissociation kinetics fivefold and M15 (which does not include mutation of Val^{137}) only twofold. We do not know whether this single amino acid change is causing this important GDP dissociation effect or if it is a global effect produced by all the five mutations introduced in M17. To address the role of this single mutation, it would have been necessary to mutate it individually, and then look for its functional properties. From this study we can only conclude that mutation of these five hGsαSWIV residues is affecting the GDP/GTP exchange kinetics of the protein, by reducing the GDP dissociation rate, and therefore decreasing its GTP binding kinetics and adenylyl cyclase stimulation. We believe that the lower adenylyl cyclase activity displayed by the mutant proteins when GTPγS was used was due to a lower activation kinetics of Gsα, since similar cAMP levels were obtained with AlF_4^-, which does not require GDP dissociation for its activation. Isoproterenol, which activates Gsα through a receptor, also showed reduced adenylyl cyclase levels when M15 and M17 were tested (data not shown).

Although switch regions I, II, and III of RasD have received considerable attention, very little is known about the role of SWIV of HD. Yet a functional importance was predicted by Wilson and Bourne [1995], who found that mutating four amino acids (F^{142}, P^{144}, E^{145}, and F^{146}) located in HD, abolished the ability of Gsα to activate adenylyl cyclase. These investigators postulated that the conformation of this region, now known as SWIV, was functionally important for Gsα but no characterization of the nucleotide binding functions of the mutant was performed. Further evidence illustrating a possible functional role for the whole HD stems from studies of Giα-HD and Gsα/RasD chimeras. These studies showed that, contrary to expectations, the chimeras were better activators of adenylyl cyclase than the wild-type proteins [Osawa et al., 1990a; Dhanasekaran et al., 1991; Markby et al., 1993]. These results have led the authors to postulate the hypothesis that HD would be acting as an effector regulator or as an attenuator of RasD function, by modulating the GDP dissociation rate. Interestingly, Lan et al. [1998] recently showed that a Goα mutant, in which Trp 132 was replaced by Phe, caused a twofold reduction in the GDP dissociation rate of the protein. It is important to point out that Trp^{132} is located in helix C of HD, just 10 residues downstream of the SWIV region.

We conclude that substantial evidence is accumulating that involves the HD and its SWIV region as regulators of Gα function and our work highlights the importance that SWIV region has in the functional and regulatory properties of human Gsα. Because of the high sequence divergence of the SWIV region, as well as the conformational change that it undergoes upon GTP binding, it is tempting to speculate and propose this region as a potential regulatory domain of the Gα subunit family.

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