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Mutation of the Highly Conserved Arg^{165} and Glu^{168} Residues of Human Gsα Disrupts the αD–αE Loop and Enhances Basal GDP/GTP Exchange Rate

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Abstract G protein signalling regulates a wide range of cellular processes such as motility, differentiation, secretion, neurotransmission, and cell division. G proteins consist of three subunits organized as a Gα monomer associated with a Gβγ heterodimer. Structural studies have shown that Gα subunits are constituted by two domains: a Ras-like domain, also called the GTPase domain (GTPaseD), and an helical domain (HD), which is unique to heterotrimeric G-proteins. The HD display significantly higher primary structure diversity than the GTPaseD. Regardless of this diversity, there are small regions of the HD which show high degree of identity with residues that are 100% conserved. One of such regions is the α helixD–α helixE loop (αD–αE) in the HD, which contains the consensus aminoacid sequence R*-[RSA]-[RSAN]-E*-[YF]-[QH]-L in all mammalian Gα subunits. Interestingly, the highly conserved arginine (R*) and glutamic acid (E*) residues form a salt bridge that stabilizes the αD–αE loop, that is localized in the top of the cleft formed between the GTPaseD and HD. Because the guanine nucleotide binding site is deeply buried in this cleft and those interdomain interactions are playing an important role in regulating the basal GDP/GTP nucleotide exchange rate of Gα subunits, we studied the role of these highly conserved R and E residues in Gα function. In the present study, we mutated the human Gsα R^{165} and E^{168} residues to alanine (A), thus generating the R^{165} → A, E^{168} → A, and R^{165}/E^{168} → A mutants. We expressed these human Gsα (hGsα) mutants in bacteria as histidine tagged proteins, purified them by niquel-agarose chromatography and studied their nucleotide exchange properties. We show that the double R^{165}/E^{168} → A mutant exhibited a fivefold increased GTP binding kinetics, a higher GDP dissociation rate, and an augmented capacity to activate adenylyl cyclase. Structure analysis showed that disruption of the salt bridge between R^{165} and E^{168} by the introduced mutations, caused important structural changes in the HD at the αD–αE loop (residues 160–175) and in the GTPaseD at a region required for Gsα activation by the receptor (residues 308–315). In addition, other two GTPaseD regions that surround the GTP binding site were also affected. J. Cell. Biochem. 93: 409–417, 2004. © 2004 Wiley-Liss, Inc.

Key words: G-protein; Gsα; adenylyl cyclase

Heterotrimeric G-proteins, formed by α, β, and γ subunits, are widely distributed proteins that transmit signals in all eucaryotic cells. They act as on-off switches, regulating the hormonal transmition from cell surface receptors to effector proteins that modulate diverse cellular processes. [Neer, 1995; Cabrera-Vera et al., 2003; Offermanns, 2003]. The ligand–receptor complex, acting as a guanine exchange factor (GEF), promotes the exchange of GDP for GTP in the inactive GαGDP/βγ form, which results in the dissociation of the heterotrimer GαGTP/βγ to free GαGTP and Gβγ active species [Hildebrandt, 1997; Sprang, 1997; Cherfils and Chabre, 2003]. Both species can modulate the activity of effector systems such as adenylyl cyclase, phospholipase C, and ionic channels [Sunahara et al., 1996; Clapham and Neer, 1997; Skiba and Hamm, 1998; Dascal, 2001; Albert and Robillard, 2002]. The GαGTP specie remains active until it is turned off by the intrinsic GTPase activity of Gα, which can be regulated additionally by a new group of modulator proteins known as regulators of G protein signaling (RGS)

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[De Vries et al., 2000; Zhong and Neubig, 2001]. Reassociation of GαGDP with Gβγ regenerates the heterotrimer to its inactive state. Crystallographic analysis of different forms of Gα [Noel et al., 1993; Coleman et al., 1994; Lambright et al., 1994, 1996; Coleman and Sprang, 1999], have shown the presence of two domains. One is called the GTPase domain (GTPaseD), which is similar in structure to the monomeric G protein c-ras and contains the guanine nucleotide binding site and the receptor and effector interaction sites. The other domain, known as the helical domain (HD), is unique to heterotrimeric G-proteins and is formed by six α helices. The exactly function of the HD is not known, but recent studies indicate that it could be involved in: regulation of the GTPase activity of Gα [Markby et al., 1993], stabilization of the transition state between the active and inactive form of Gα [Liu and Northup, 1998; Liu et al., 1998], receptor interaction [Krieger-Brauer et al., 1999], interaction with some RGS isoforms [Skiba et al., 1999; Slep et al., 2001], and regulation of the basal GDP/GTP exchange rate [Osawa et al., 1990a,b; Dhanasekaran et al., 1991; Remmers et al., 1999; Echevería et al., 2000; Brito et al., 2002].

In heterotrimeric Gα subunits, the guanine nucleotide is buried in a deep cleft between both domains and therefore the GDP/GTP exchange process (needed for Gα activation) requires that the protein suffers a conformational change to allow the opening of this cleft. For this reason, interdomain interactions are considered to be important in modulating the nucleotide exchange rate of these proteins and several groups have shown that interactions between residues of these interfaces are playing an important role in regulating the activation of the Gα subunit [Codina and Birnbaumer, 1994; Grishina and Berlot, 1998; Marsh et al., 1998; Echevería et al., 2000; Brito et al., 2002].

The HD possesses an overall high primary structure diversity compared to the GTPaseD, reflecting probably the different functions attributed to this domain. One highly diverse region is that comprised between helixes αA and αB, which has been proposed to interact with the amino terminus of Gγ subunits that also displays a very variable aminoacid sequence [Cherfils and Chabre, 2003]. In the GTPaseD on the contrary, it is known that highly conserved aminoacids along the primary structure are essential to preserve important Gα functions, such as the G_1–G_5 motifs that constitute the guanine nucleotide binding site [Sprang, 1997]. In this regard, we searched for conserved aminoacid sequences in the HD, comparing all Gα subfamilies from different species (G_s, G_{o1}, G_{o2}, G_{i1}, G_{i2}, G_{i3}, G_{t1}, G_{t2}, G_q, G_z, G_{11}, G_{12}, G_{13}, G_{14}, and G_{15}). This analysis showed three regions with significant aminoacid sequence similarity: the R-[RSA]-[RSAN]-E-[YF]-[QL], the Y-F-L-X-X-L-[DE]-[RK]-[IVL] and the Y-X-P-[ST]-X-Q-D-[VIL]-L motifs. Interestingly, by inspection of the Gα terciary structure we found that the 100% conserved R^{165} and E^{168} residues of the R-[RSA]-[RSAN]-E-[YF]-[QL] motif, were forming a salt bridge in the αD–αE loop, which is localized at the top of the cleft formed between the GTPaseD and HD. Based on the absolute conservation of these residues in all Gα subunits and their strategical position in the HD-GTPase interfase, we postulated that they are playing an important role in Gα subunit structure and function.

In the present study, we mutated the hGsα R^{165} and E^{168} residues to alanine, generating the $R^{165} \rightarrow A$, $E^{168} \rightarrow A$, and $R^{165}/E^{168} \rightarrow A$ mutants. In agreement with our hypothesis, all three mutant proteins exhibited a considerably increased in GDP dissociation and GTP binding kinetics, and as a consequence of that an augmented capacity to activate the adenylyl cyclase.

MATERIALS AND METHODS

Construction of Human Gsα (hGsα) Mutants

The hGsα cDNA, subcloned into the H_6-pQE60 vector, was used as template to introduce the corresponding mutations using the PCR based overlap extension method [Ho et al., 1989]. The $R^{165} \rightarrow A$ mutant was obtained with the sense 5′-CCTGCTACGAAGCCTCCAACGAGTA-3′ and antisense 5′-ATCTCGTTGGAGGCTTCGTAGCAGG-3′ primers, the $E^{168} \rightarrow A$ mutant with the sense 5′-CGCTCCAACGCGTACCAGCTG-3′ and antisense 5′-CAGCTGGTACGCGTTGGAGCG-3′ primers, and the double $R^{165}E^{168} \rightarrow A$ mutant with the sense 5′-GCCTGCTACGAAGCCTCCAACGCGTACCAGCTG-3′ and antisense 5′-CAGCTGGTACGCGTTGGAGGCTTCGTAGCAGGC-3′ primers. All the introduced mutations were confirmed by automatized DNA sequencing. In this study, we worked with the short form of Gsα, but the residues were numbered according to the long

form of the protein to facilitate comparative analysis with previous reports.

Expression and Purification of Recombinant Gsα Proteins

The expression and purification of the hGsα wild type and R/E → A mutant proteins were performed as previously described [Lee et al., 1994; Brito et al., 2002].

GTPγS Binding Assay

GTPγS binding to hGsα proteins was performed essentially as described by Brito et al. [2002]. Briefly, the reaction was carried out at 22°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 times, the binding reaction 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 hGsα proteins was performed according to Echevería et al. [2000]. A typical reaction consisted in the incubation of the purified hGsα 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 times, 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 Brito et al. [2002]. Before reconstitution, functional hGsα recombinant proteins were quantitated by [^{35}S]GTPγS binding and diluted to a concentration of 10 ng/μl. Normally 80–150 ng of functional hGsα 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 the presence of 10 μM GTPγS or 10 mM NaF. 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].

Structural Analysis of the hGsα R/E → A Mutants

The atomic coordinates of hGsα were obtained from the Protein Data Bank (PDB code 1AZT) and the corresponding amino acid replacements were done using the Swiss-PDB Viewer program [Guex and Peistch, 1997]. The structures were conformational energy-minimized by conjugate gradient in successive steps, with a rms gradient of 0.001 kcl/Å mol as the termination condition, using the Amber program [Weiner and Kollman, 1981] included in the HyperChem 6.2 package (Hypercube, Inc., Ontario, Canada). Molecular graphics images were produced using the UCSF Chimera package [Huang et al., 1996] from the Computer Graphics Laboratory, University of California, San Francisco (http://www.cgl.ucsf.edu/chimera).

RESULTS

Guanine Nucleotide Exchange Kinetics of Wild Type hGsα and R/E → A Mutants

Previous to the kinetical studies, the purified hGsα R/E → A mutants were subjected to trypsin digestion analysis to demonstrate their functional integrity. As expected, all mutants proteins followed the expected conformational change induced by GTP binding, that is reflected by the appearance of a 37 kDa protein fragment after trypsin digestion (data not shown). To characterize the functional properties of the R/E → A mutant proteins, we first measured their ability to bind GTPγS compared with that of the wild type hGsα. As shown in Figure 1A, all three mutant proteins displayed an enhanced nucleotide binding kinetics, showing the double R^{165}/E^{168} → A mutant a kapp fivefold higher compared to the wild type protein (kapp 0.1401 min^{-1} vs. 0.0263 min^{-1}). The two single mutants (R^{165} → A and E^{168} → A) had very similar GTP binding kapp, twofold higher than that of the wild type protein. These differences are better reflected when the GTPγS binding capacities of the mutants are compared at short periods of incubation time. After 10 min, the R^{165}/E^{168} → A double mutant shows over

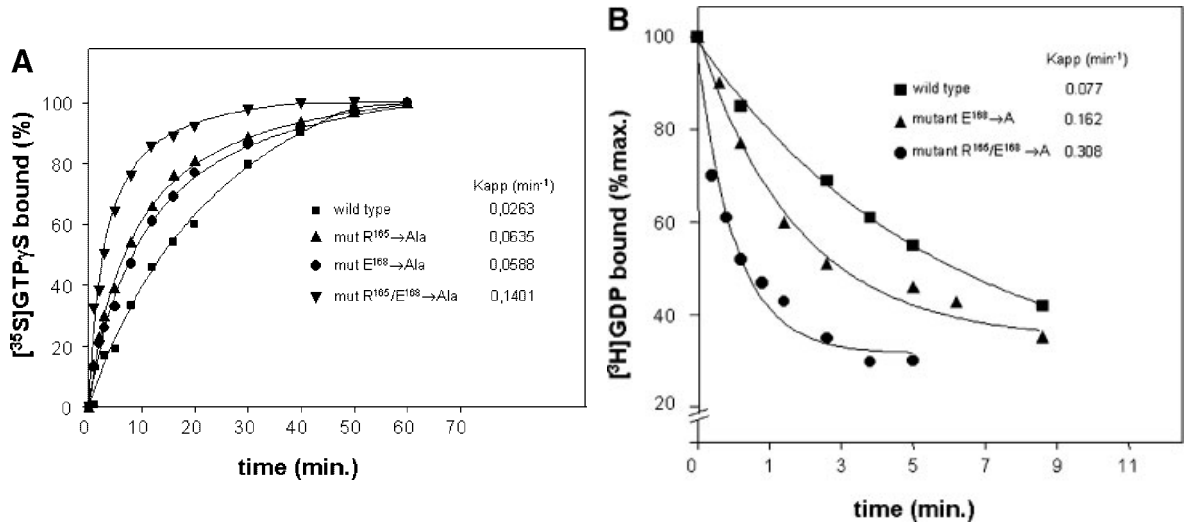

Fig. 1. GTP binding and dissociation kinetics of wild-type and mutant hGαs subunits. **A**: Purified recombinant wild-type, $R^{165} \rightarrow A$, $E^{168} \rightarrow A$, and $R^{165}E^{168} \rightarrow A$ mutants were incubated with [^{35}S]GTPγS as indicated in ''Materials and Methods.'' Data shown represents the mean of triplicate determinations from a representative experiment. Values of kapp were obtained by fitting the data to equation $B = Beq(1 - e^{-kt})$. **B**: Time course of [^{3}H]GDP dissociation from wild type, $E^{168} \rightarrow A$, and $R^{165}E^{168} \rightarrow A$ mutants was performed as described in ''Materials and Methods.'' Data shown represents the mean of triplicate determinations from a representative experiment and koff values were determined using the formula described previously in A.

80% of nucleotide binding, while the single mutant protein reached around 60% and the wild type protein showed less than 40% (Fig. 1A).

Because the GTP binding kinetics is limited by the GDP dissociation rate, we also analized the koff constant rates for [^{3}H]GDP dissociation. As expected, the double $R^{165}/E^{168} \rightarrow A$

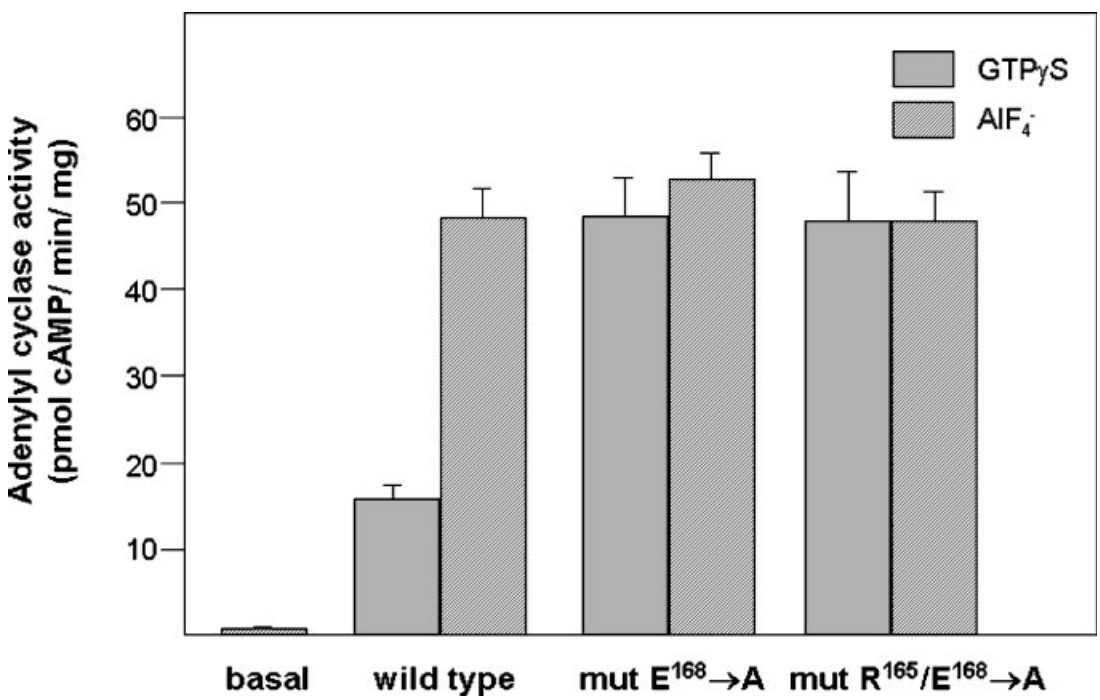

Fig. 2. Reconstitution of adenylyl cyclase activity in S49cyc^{-} membranes. S49cyc^{-} membranes were reconstituted with equal functional amounts of wild type hGαs or $E^{168} \rightarrow A$ and $R^{165}E^{168} \rightarrow A$ mutants as described in ''Materials and Methods.'' Adenylyl cyclase activity was measured during 15 min incubation at 32°C with either GTPγS or NaF(AlF_4^-). Data shown represents the mean of triplicate determinations.

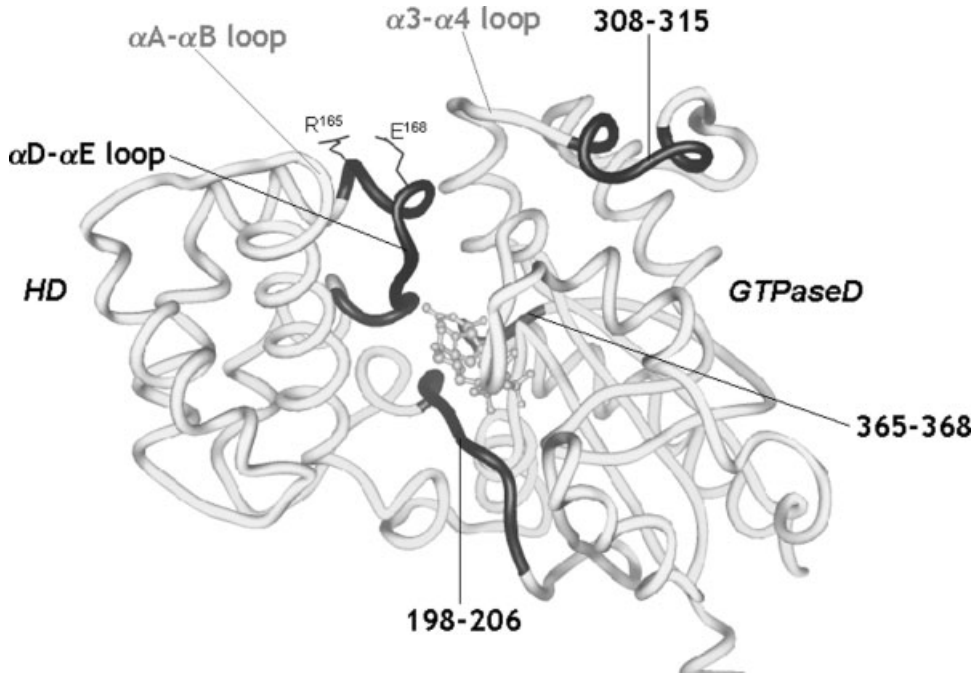

Fig. 3. Structure of the hGαs-GTP-Mg complex based on the X-ray crystal data (PDB code 1AZT). The helical domain (HD, left) and the GTPase domain (GTPaseD, right) are indicated in italics. In light gray, at the center of the molecule, is shown the nucleotide GTP as ball and stick model. In black thick sticks are indicated the position of mutated R^{165} and E^{168} residues. The black ribbons represent the regions that underwent structural changes due to the R^{165} and E^{168} mutations (αD–αE loop, 198–206, 308–315, and 365–368 regions). Loops αA–αB and α3–α4 are indicated by light gray lines. This molecular graphic image was produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco.

mutant protein showed the highest GDP dissociation time course (koff 0.308), which was fourfold higher than the wild type subunit (koff 0.077) (Fig. 1B). The single $E^{168} \rightarrow A$ mutant protein displayed an intermediate dissociation constant value (koff 0.162).

Adenylyl Cyclase Stimulation by the hGsα Wild Type, hGsα $E^{168} \rightarrow A$, and hGsα $R^{165}/E^{168} \rightarrow A$ Mutants

To evaluate whether the R/E → A mutations were affecting other functional properties of hGsα, we examined their capacity to stimulate the effector system adenylyl cyclase by reconstitution experiments using S49 cyc-cell membranes. As shown in Figure 2, when AlF^{4-} was used as activator, all Gsα proteins produced approximately the same level of adenylyl cyclase activation, indicating that the introduced mutations were not altering their functional interaction with this effector. In contrast, when GTPγS was added (which requires previous GDP dissociation to bind), both mutants were considerably more efficient in activating the AC, which is consistent with the higher basal nucleotide exchange activity displayed by the mutant proteins. Mutant $R^{165} \rightarrow A$ was not considered in this study due to its similar functional properties with $E^{168} \rightarrow A$.

Structural Analysis of the hGsα $R^{165}/E^{168} \rightarrow A$ Mutants

Using the hGsα atomic coordinates (PDB entry 1AZT) we modeled the mutant protein

TABLE I. Hydrogen Bonds Formed by Arg^{165} and Glu^{168} Residues in Wild Type hGαs and R/E → A Mutants

	Donnor-atom	Acceptor-atom
Wild type	Arg^{165}-NE	Glu^{168}-OE1
	Arg^{165}-N	Cys^{162}-O
	Arg^{165}-NH1	Pro^{115}-O
	Arg^{165}-NH2	Glu^{168}-OE1
	Glu^{168}-N	Arg^{165}-O
	Lys^{305}-NZ	Glu^{168}-OE2
	Arg^{165}-NH2	Val^{114}-O
$R^{165} \rightarrow A$	Ala^{165}-N	Ala^{161}-O
	Glu^{168}-N	Ala^{165}-O
	Lys^{168}-NZ	Glu^{168}-OE1
$E^{168} \rightarrow A$	Arg^{165}-NH1	Pro^{115}-O
	Arg^{165}-NH2	Val^{114}-O
	Ala^{168}-N	Arg^{165}-O
	Arg^{165}-N	Ala^{161}-O
$R^{165}/E^{168} \rightarrow A$	Ala^{168}-N	Arg^{165}-O
	Ala^{165}-N	Ala^{161}-O

structures and found local and distant structural changes (Fig. 3). The local changes (residues 164–175), induced by the R^{165} and E^{168} substitutions, result in the loss of several hydrogen bonds (Table I, Fig. 4A,B) that affected the stabilization and spatial orientation of the αD–αE loop with respect to the neighboring αA–αB and α3–α4 loops (Fig. 5A). R^{165} and E^{168} residues form seven hydrogen bonds in the wyld-type protein (Fig. 4A), which are reduced to two in the R/E → A mutant (Fig. 4B). A structural comparison between the αD–αE loop of wild type and R/E → A mutant shows an important displacement of the polypeptide chain toward the α3–α4 loop (Fig. 5A). The distant structural changes comprehend aminoacids between positions 198–206, 308–315, and 365–368 (Figs. 3 and 5B). It is noteworthy to mention that the first stretch (198–206) overlaps the Switch I region and the last two (308–315 and 365–368) correspond to regions previously described as involved in receptor contact and therefore necessary for receptor-mediated activation of Gαs [Grishina and Berlot, 1998; Marsh et al., 1998].

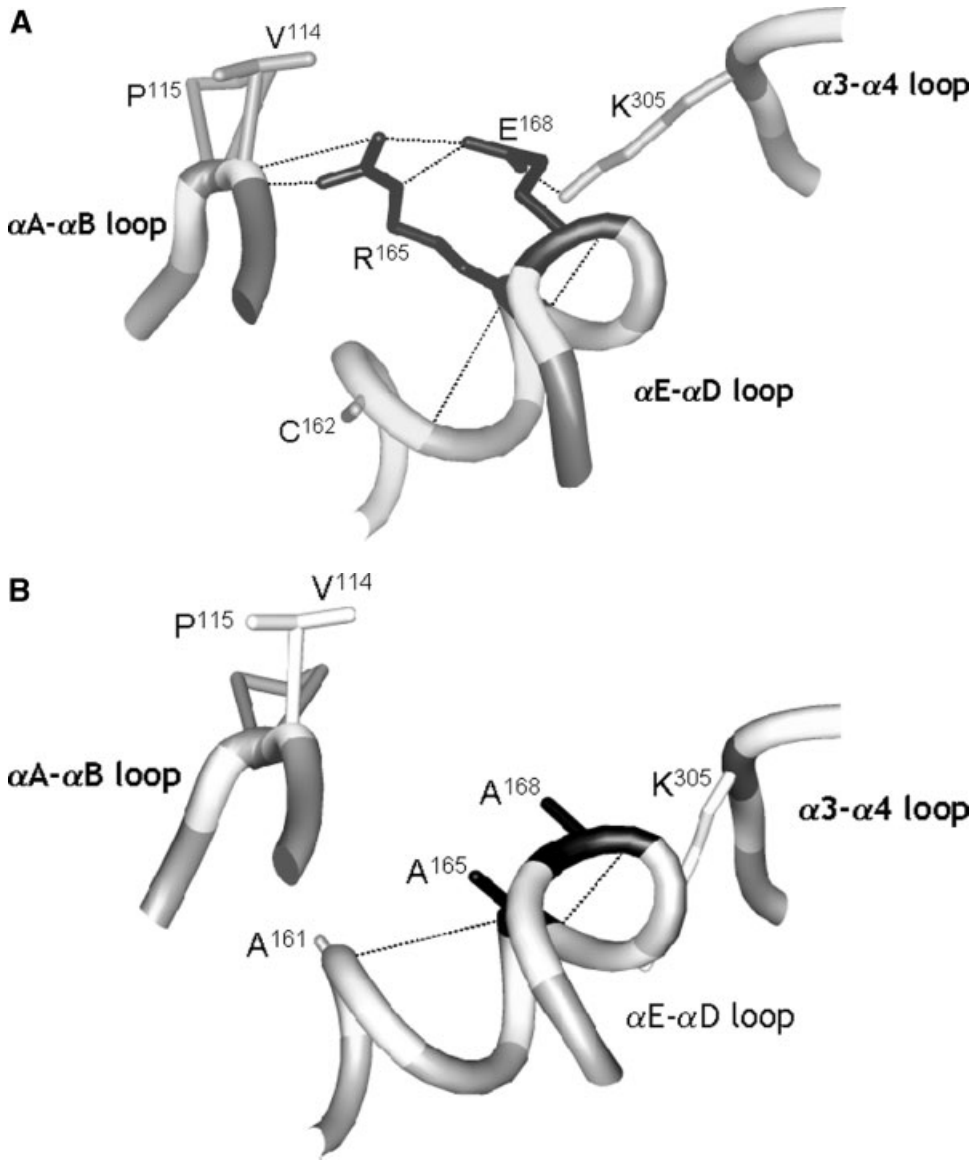

Fig. 4. Close up-view of the molecular interactions in which R^{165} and E^{168} are participating. **A**: Wild-type αE–αD loop structure. Broken lines represent the hydrogen bonds formed by R^{165} and E^{168} residues. **B**: Structure of the RE → A mutated αE–αD loop region. Broken lines represent the hydrogen bonds formed by A^{165} and A^{168} residues. The molecular graphic image was produced using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco.

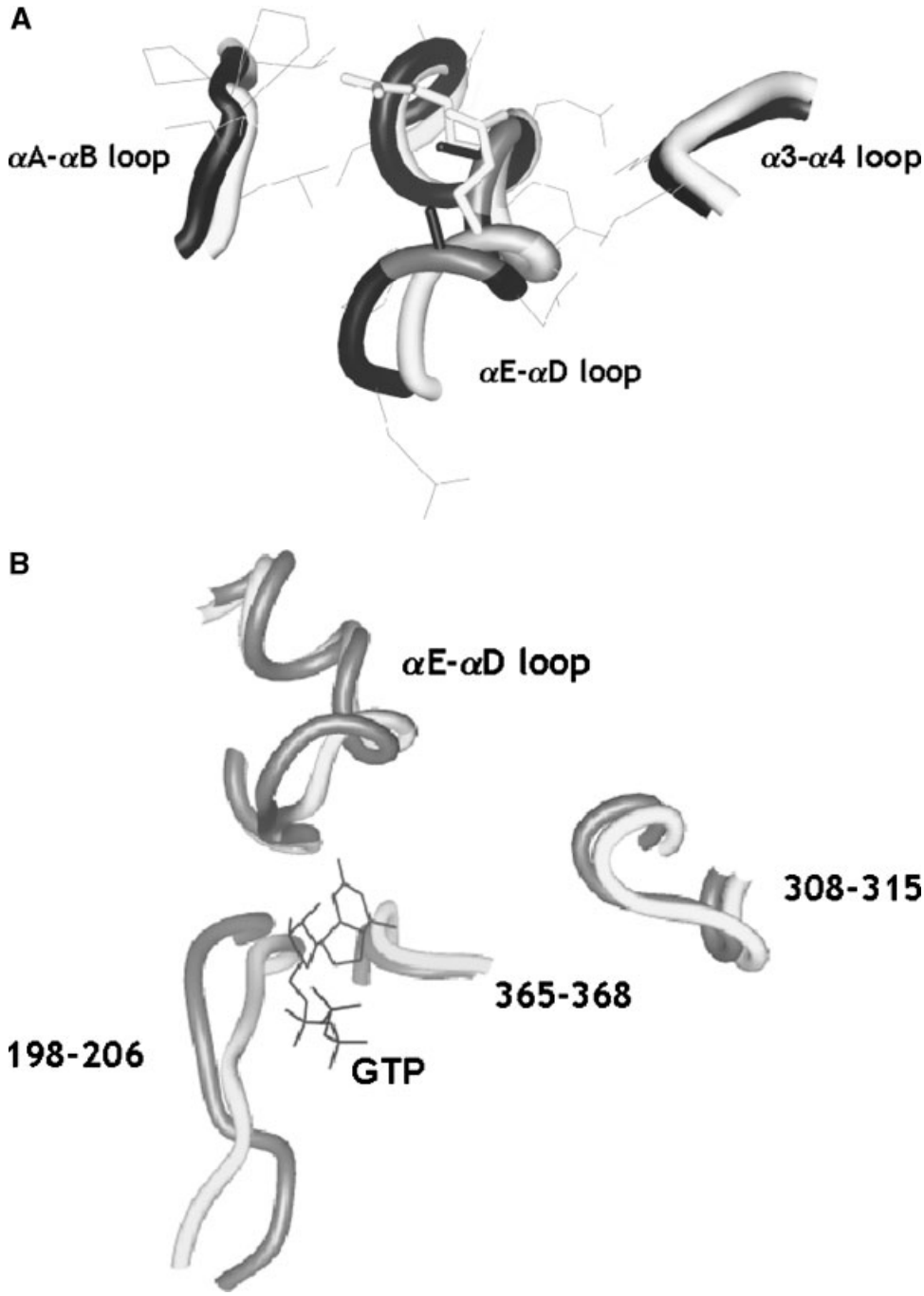

Fig. 5. Comparison of the local and distant structural changes produced in hGαs by the $R^{165}E^{168} \rightarrow A$ mutations. **A**: Superposition of the loops αE–αD, αA–αB, and α3–α4 of the wild-type (light gray ribbon) and $R^{165}E^{168} \rightarrow A$ double mutant (black ribbon). Wild-type R^{165} and E^{168} side chain residues are shown as thick white sticks and A^{165} and A^{168} mutated residues as thick black sticks. **B**: Close-up view of the local and distant hGαs structural changes.Wild-type hGαs is shown as a white ribbon and the $RE \rightarrow A$ mutant is shown as a dark gray ribbon. GTP is shown in a black stick model. This molecular graphic image was drawn using the UCSF Chimera package from the Computer Graphics Laboratory, University of California, San Francisco.

DISCUSSION

It has been shown that mutations affecting the interface between GTPaseD and HD, produce significant alterations on the guanine nucleotide binding capacity of Gα subunits and in receptor-induced activation [Codina and Birnbaumer, 1994; Grishina and Berlot, 1998;

Marsh et al., 1998; Warner et al., 1998; Remmers et al., 1999; Marin et al., 2001a,b; Thomas et al., 2001]. In the Gα subunits, the αD–αE loop is positioned at the top of the HD/GTPaseD interface and has the highly conserved motif R*-[RSA]-[RSAN]-E*-[YF]-[QH]-L, which is present in all these proteins. Within this region, R* and E* residues are 100% conserved in higher eukaryotes, but not in the yeasts *S.cerevisiae*, *S. pombe*, and *K. lactis*; in the fungi *N. crassa*; in the amoeba *D. discoideum*; in the worm *C. elegans*, and in the plant *L. esculentum* (tomato), indicating an important role during evolution. In fact, R^{165} and E^{168} are establishing diverse molecular contacts within the αD–αE loop and with other two regions localized near the GTPaseD and HD interface (αA–αB and α3–α4 loops, see Fig. 4A). Because the guanine nucleotide binding site is deeply buried in the cleft formed between both domains, interdomain interactions are playing an important role in regulating the basal GDP/GTP nucleotide exchange rate of Gα subunits. We considered relevant to study the role of these highly conserved R and E residues in Gα function.

Biochemical characterization of the R/E → A mutants, mainly the double $R^{165}/E^{168} \rightarrow$ A mutant, revealed a hGsα protein with a higher GDP/GTP basal exchange rate and GDP dissociation kinetics (Fig. 1A,B). Interestingly, these mutations did not affect hGsα regions involved in adenylyl cyclase interaction, because its activation was very similar with the wild-type and mutant proteins, when $AlF4^-$ was used as activator (see Fig. 2). To better understand the effect of the introduced mutations in hGsα function, we modeled the mutant proteins using as template the wild type Gsα crystal structure. In the wild-type subunit, Arg^{165} and E^{168} residues form an important intraloop salt bridge and several hydrogen bonds that are required to maintain the αD–αE loop structured in its right conformation (Fig. 4A). The R/E → A mutations disrupt these bonds and release both residues from these interactions, altering the structure of the αD–αE loop (Figs. 4B and 5A). As shown in Figure 5B, this perturbation also caused important distant structural changes in the GTPaseD. In summary, our results indicate that the R^{165}–E^{168} molecular interactions mantain the right space orientation of the αD–αE loop, which is playing an essential role in controlling the entrance of the nucleotide to the cleft. Structure comparison between the wild type and R/E → A mutant αD–αE loop showed a displacement of the polypeptide chain towards the αA–αB loop by 2Å (see Fig. 5A), producing the opening of the upper part of the nucleotide binding cleft between αE–αD and α3–α4 loops.

Besides the R^{165}–E^{168} salt bridge, there is another important interdomain interaction between D^{173} and K^{293} residues, which when disrupted caused an augmented response to GTPγS [Codina and Birnbaumer, 1994], confirming the important role that residues situated in the αD–αE loop of the HD are playing in mantaining the proper positioning of the HD-GTPaseD interfase and in the conformation of the GDP/GTP binding site.

Our structural analysis also show important conformational changes in GTPaseD regions that affect the Gα subunit function. Residues 198–206, which correspond to Switch I, moved away from the guanine nucleotide binding site (see Fig. 5B). In addition aminoacids 308–315 and 365–368, which correspond to the regions 2 and 3 described by Marsh et al. [1998] and that are implicated in receptor-mediated activation, also underwent spatial reorientation. Based on these observations, we propose that the $R^{165}/E^{168} \rightarrow$ Ala mutations emulate the receptor induced conformational change of Gα and thereby induces an active hGsα form, enhancing its basal GDP/GTP exchange rate.

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