Mutational Analysis of the Asn Residue Essential for RGS Protein Binding to G-proteins*

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Members of the RGS family serve as GTPase-activating proteins (GAPs) for heterotrimeric G-proteins and negatively regulate signaling via G-protein-coupled receptors. The recently resolved crystal structure of RGS4 bound to Gαz suggests two potential mechanisms for the GAP activity of RGS proteins as follows: stabilization of the Gαz switch regions by RGS4 and the catalytic action of RGS4 residue Asn128. To elucidate a role of the Asn residue for RGS GAP function, we have investigated effects of the synthetic peptide corresponding to the Gαz-binding domain of human retinal RGS (hRGSr) containing the key Asn at position 131, and we have carried out mutational analysis of Asn131. Synthetic peptide hRGSr-(123-140) retained its ability to bind the AlF4−-complexed transducin α-subunit, GαzAlF4−, but failed to elicit stimulation of Gtα GTase activity. Wild-type hRGSr stimulated Gαz GTase activity by ~10-fold with an EC50 value of 100 nM. Mutant hRGSr proteins with substitutions of Asn131 by Ser and Gln had a significantly reduced affinity for Gtα. Mutants hRGSr-Leu131, hRGSr-Ala131, and hRGSr-Asp131 were able to accelerate Gtα GTase activity only at very high concentrations (>10 μM) which appears to correlate with a further decrease of their affinity for transducin. Two mutants, hRGSr-His131 and hRGSr-Asp131, had no detectable binding to transducin. Mutational analysis of Asn131 suggests that the stabilization of the G-protein switch regions rather than catalytic action of the Asn residue is a key component for the RGS GAP action.

The intensity and duration of signaling via heterotrimeric G-proteins is regulated at multiple levels. The key reaction in termination of G-protein-mediated signaling is the intrinsic GTase activity of Gα subunits that convert the active GTP-bound conformation of G-protein α subunits (Gα-GTP) to the inactive Gα-GDP conformation. The GTase activity of two G-proteins, Gαi and transducin, is stimulated by their effectors, phospholipase Cβ and cGMP phosphodiesterase (PDE), respectively (1-3). A novel class of GTase-activating proteins (GAPs) for heterotrimeric G-proteins called RGS has been identified (4-6). Strong evidence suggests that members of this family, GAIP, RGS4, RGS1, RGS10 and others, negatively regulate G-protein signaling by stimulating GTase activity of G-proteins, particularly those from Gαi and Gαz families (7-9). RGS proteins from yeast to mammals share a highly conserved RGS domain that provides relatively broad specificity of different RGS proteins toward members of the two G-protein classes in vitro. Tissue expression patterns and diverse domains outside the RGS segment may play an important role in determining specificity of RGS proteins in vivo (10-12). Precise mechanisms of RGS GAP activity are not yet clear. The transition state during GTP hydrolysis is thought to be mimicked by the AlF4−-bound conformation of Gα subunits (13, 14). It has been demonstrated that many RGS proteins interact preferentially with the AlF4−-bound conformation of Gα subunits and thus may accelerate GTP hydrolysis through stabilization of the transitional state of G-proteins (8, 15, 16).

Recently, the crystal structure of RGS4 bound to Gαi2AlF4− has been solved at a resolution of 2.8 Å (17). This structure provides the first structural insights into the mechanism of RGS protein action. The conserved RGS core forms three distinct sites of interaction with the three switch regions of Gαi1 suggesting that stabilization of the switch regions and Gz residues directly involved in GTP hydrolysis may be a major component of RGS GAP activity (17). Furthermore, RGS proteins could also contribute catalytic residues to the active site and thus enhance the GTase rate constant. The conserved residue Asn128 of RGS4 makes a contact with the side chain of Gln204 of Gαi1 which stabilizes and orients the hydrolytic water molecule in the transitional state of Gαi1 (17). Asn128 also may be localized within hydrogen-bonding distance of the hydrolytic water molecule for nucleophilic attack on the GTP γ-phosphate (17).

In this study we evaluate a potential catalytic role of the Asn residue for Gαi GTase acceleration by RGS proteins using the interaction between human retinal RGS (hRGSr) protein and transducin as a model system and mutational analysis of Asn131 of hRGSr which is equivalent to Asn128 of RGS4.

EXPERIMENTAL PROCEDURES

Materials—GTP and GDP were products of Boehringer Mannheim, Blue-Sepharose and PD-10 Sephadex G-25 columns were obtained from Pharmacia Biotech Inc. [γ-32P]GTP (>5000 Ci/mmol) was purchased from Amersham Corp. [35S]GTPγS (1250 Ci/mmol) was obtained from NEN Life Science Products. All other chemicals were acquired from Sigma.

Preparation of Rod Outer Segment (ROS) Membranes, Gαi-GTPγS, Gαi-GDP, and hRGSr—Bovine ROS membranes were prepared as described previously (18). Hypotonically washed ROS membranes (dROS) depleted of PDE subunits were prepared as described in Ref. 2. Transducin, Gαi-GTPγS, was extracted from ROS membranes using GTP as de-
scribe in Ref. 19. The Gα-GTP/S was extracted from ROS membranes using GTP·S and purified by chromatography on Blue-Sepharose CL-6B by the procedure described in Ref. 20. Gα-GDP was prepared and purified according to protocols in Ref. 21. hRGSr was purified and purified as described previously (22). The purified proteins were stored in 40 mM Tris-HCl at −20 °C or without glycerol at −80 °C.

Site-directed Mutagenesis of hRGSr—Mutagenesis of Asn131 of Human Retinal RGS

hRGSr was performed using PCR amplifications from the pGEX-KG-hRGSr template (22) with 3’antisense primer ATGCTCTGAGACTCAGGTTGTTGAGG (unique XhoI site is underlined) and the 5’primers: XXXTATTGACCATGAGACCGCGACG. XXX indicates nucleotides that generate substitutions of Asn131 of hRGSr cDNA by the following amino acid residues: Ala (GCC), Asp (GAT), His (CAT), Leu (CTG), Gln (CAG), Ser (AGC), and deletion mutant (—). PCR reactions were performed in 100 μl of reaction mixture containing 1 ng of the pGEX-KG-hRGSr plasmid, 3 units of AmpliTaq DNA polymerase (Perkin-Elmer), 25 mM Tris-(hydroxymethyl)-methylamino propane sulfonic acid, pH 9.3, 2 mM MgCl2, 1 mM 2-mercaptoethanol, 200 μM of dNTPs, and 0.5 μM primers. Conditions for PCR were as follows: 94 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at 50 °C, and 30 s at 72 °C for 3 min. The PCR products (~220 base pairs) were blunt-ended with Klenow fragment and digested with XhoI. Wild-type hRGSr cDNA was subcloned into the XhoI/XhoI sites of pBluescript polylinker. The resulting construct was digested with HindIII and XhoI and ligated with the XhoI-digested PCR products carrying mutations. The mutant sequences were verified by automated DNA sequencing at the DNA Facility of Iowa DNA Core Facility using the T7 primer and subcloned into the XhoI/XhoI sites of pGEX-KG vector for protein expression. Mutant GST-hRGSr proteins were expressed in DH5a Escherichia coli cells, and the GST portion was removed as described earlier (22). Typical yields of purified hRGSr and hRGSr mutants, except for a mutant with deletion of Asn131, were 5–6 mg/liter of culture. Deletion of Asn131 led to an ~4–5-fold reduction in expression of soluble recombinant protein suggesting that the residue at position 131 may be important to the stability and proper folding of RGS proteins.

Binding of Transducin to GST-hRGSr and Mutants—Gα-GTP/S or Gα-GDP (10 μg) were incubated with hRGSr or its mutants (50 μg) immobilized on glutathione-agarose in 100 μl of 20 mM Tris-HCl buffer (pH 8.0), containing 100 mM NaCl, 2 mM MgSO4, 6 mM 2-mercaptoethanol, and 5% glycerol (buffer A). Where indicated, the buffer contained 30 μM AlCl3 and 10 mM NaF. After incubation for 20 min at 25 °C, the agarose beads were washed twice with buffer A and 28 °C for 30 s, and a final extension at 72 °C for 3 min. The PCR products (~220 base pairs) were blunt-ended with Klenow fragment and digested with XhoI. Wild-type hRGSr cDNA was subcloned into the XhoI/XhoI sites of pBluescript polylinker. The resulting construct was digested with HindIII and XhoI and ligated with the XhoI-digested PCR products carrying mutations. The mutant sequences were verified by automated DNA sequencing at the DNA Facility of Iowa DNA Core Facility using the T7 primer and subcloned into the XhoI/XhoI sites of pGEX-KG vector for protein expression. Mutant GST-hRGSr proteins were expressed in DH5a Escherichia coli cells, and the GST portion was removed as described earlier (22). Typical yields of purified hRGSr and hRGSr mutants, except for a mutant with deletion of Asn131, were 5–6 mg/liter of culture. Deletion of Asn131 led to an ~4–5-fold reduction in expression of soluble recombinant protein suggesting that the residue at position 131 may be important to the stability and proper folding of RGS proteins.

Single Turnover GTPase Assay—Single turnover GTPase activity measurements were carried out in suspensions of dROS membranes containing 5 μM rhodopsin and 0.4 μM transducin essentially as described in Refs. 22 and 23. Transducin concentration of 0.4 μM was determined using the preliminary testing of the catalytic role of Asn131 of hRGSr, causing half-maximal stimulation of the GTPase activity, the GTPase activity (Fig. 1). Because the competition experiments were carried out at a concentration of hRGSr ~0.98 μM rhodopsin and 0.4 μM transducin, dROS membranes lacked intrinsic catalytic PDEβα and inhibitory PDEγ subunits. Use of such ROS avoided interference of PDEγ effects with effects of GRS protein or GRS peptide (22, 27, 28). The peptide at concentrations of up to 2 mM had no effect on GTPase activity of transducin (not shown). To determine if hRGSr-(123–140) is capable of binding to transducin, we investigated effects of the hRGSr peptide on the stimulation of GTPase activity of transducin by hRGSr. Fig. 1 shows that hRGSr-(123–140) was able to compete with hRGSr for binding to Gα resulting in a dose-dependent (IC50 ~ 1.6 ± 0.5 mM) decrease of the stimulated GTPase activity of transducin. hRGSr-(123–140) in the same range of concentrations had no notable effect on the basal transducin GTPase activity (Fig. 1). Because the competition experiments were carried out at a concentration of hRGSr causing half-maximal stimulation of the GTPase activity, the affinity of hRGSr-(123–140) for Gα can be estimated as 0.8 mM. In control experiments, four unrelated peptides (24) corresponding to residues 123–140 of hRGSr were custom made by Genosys Biotechnology Inc. The N and C termini of the peptide were acetylated and amidated, respectively. The peptide was purified by reverse-phase high performance liquid chromatography on a preparative Dynamax-300A column (Rainin). The purity and chemical formula of the peptide were confirmed by fast atom bombardment-mass spectrometry and analytical high performance liquid chromatography. Preparation of synthetic peptides corresponding to residues 21–31, 461–491, 492–516, and 517–541 of rat hRGSr was described previously (24).
evaluated the interaction between hRGSr mutants with substitutions of Asn
131 by Ser, Gln, Ala, Leu, His, Asp as well as the mutant with deletion of Asn
131 and transducin using precipitation of Gtα with the GST-hRGSr mutant proteins immobilized on glutathione-agarose beads. Mutations hRGSr-Ser131 and hRGSr-Gln131 led to a reduction in affinity of the corresponding Gt fusion proteins for Gtα-AlF4 (Fig. 2A). Mutants hRGSr-Leu131, hRGSr-Asp131, and hRGSr-Ala131 showed a more significant decrease in their affinity for the Gtα conformation (Fig. 2A). hRGSr-Asp131 and hRGSr-Δ131 failed to co-precipitate Gtα-AlF4. Mutations of Asn131 could potentially alter hRGSr interaction with Gtα-GTPγS and Gtα-GDP since the RGS4 Asn residue makes contact with the switch I and II regions of Gtα (17). We have tested this possibility by preincubating mutant GST-hRGSr containing beads with both conformations of Gtα. None of the seven hRGSr mutants has demonstrated enhanced affinity for either conformation of Gtα compared with the native GST-hRGSr (Fig. 2, B and C).

**Stimulation of GTPase Activity of Transducin by Mutant hRGSr**—Effects of hRGSr mutants with substitutions of Asn131 were tested in dROS membranes containing 5 μM rhodopsin and 0.4 μM transducin. Under these conditions, the calculated rate of GTP hydrolysis by transducin was 0.025 ± 0.004 s−1 (Fig. 3). The rates of transducin GTPase activity were then determined in the presence of increasing concentrations of hRGSr or individual hRGSr mutants and plotted as a function of their concentration. Wild-type hRGSr purified after cleavage of GST-hRGSr with thrombin stimulated GTPase activity of transducin by ∼10-fold to a maximal rate k = 0.27 ± 0.01 s−1 with an EC50 value of 101 ± 14 nM (Fig. 3). All hRGSr mutants had substantially reduced ability to stimulate the GTPase activity of transducin. The tested mutants can be arbitrarily separated into three groups. Two of the mutants, hRGSr-Ser131 and hRGSr-Gln131, were relatively potent, and saturation of their GAP effect could be achieved at 10−40 μM concentration of mutant. hRGSr-Ser131 mutant was the most effective and stimulated Gtα GTPase activity with an EC50 value of 1.34 ± 0.17 μM and Vmax ~ 80% (k = 0.22 ± 0.01 s−1). The mutant hRGSr-Gln131 was capable of accelerating the Gtα GTPase activity to Vmax of 60% (k = 0.16 ± 0.01 s−1) with an EC50 value of 3.9 ± 1.1 μM (Fig. 3). Three mutants, hRGSr-Leu131, hRGSr-Ala131, and hRGSr-Asp131, began to cause acceleration of Gtα GTPase activity only at very high concentrations (>10 μM) (Fig. 3). We were unable to practically achieve saturation of the GAP activity by these mutants due to the very high protein concentrations required. Two mutants, hRGSr-His131 and hRGSr-Δ131, did not show GAP activity at the concentration tested (40 μM). Interestingly, the potency of hRGSr mutants in stimulating Gtα GTPase activity (Fig. 3) appears to correlate well with their ability to bind and precipitate Gtα-AlF4 (Fig. 2A).

**Competition between hRGSr and hRGSr Mutants in Stimulation of Gtα GTPase Activity**—Experiments in Fig. 2 have suggested that hRGSr mutants with substitutions of Asn131 have impaired binding to Gtα-AlF4. The binding assay may, however, not be sufficiently sensitive to detect relatively weak interactions. To determine if the drastically reduced ability of some RGS mutants to stimulate the GTPase activity of transducin correlates with the lack of mutant binding to transducin, we carried out competition experiments. The hRGSr mutants incapable of accelerating Gtα GTPase activity were examined for their ability to block stimulation of GTPase activity of transducin by hRGSr. Fig. 4 demonstrates that none of the tested mutants, hRGSr-Ala131, hRGSr-His131, and hRGSr-Δ131, at 5 μM concentration, had any effect on stimulation of GTPase activity of transducin by 50 nM hRGSr. These data suggest that the hRGSr mutants that produced no stimulation of Gtα GTPase activity lost their binding to Gtα.

**DISCUSSION**

Molecular mechanisms of RGS protein action as GAP for heterotrimeric GTP-binding proteins are not well understood. Studies on the Ras-specific p120GAP suggest that the Ras GAP donates conserved Arg729 residue to the Ras catalytic site (29, 30) thus providing the catalytic mechanism for p120GAP activity. The crystal structure of RGS4 bound to Gtα-AlF4 has suggested two mechanisms for RGS GAP activity toward heterotrimeric G-proteins (17). Interaction of RGS protein with the G-protein switch regions indicates that the mechanism of the GTPase activation by RGS may primarily be a reduction in the free energy of the transitional state via stabilization of Gtα switch regions and residues directly involved in GTP hydrolysis (17). An additional putative mechanism for the RGS GAP activity would be a donation of the catalytic residue to the active site of Ga. The only residue that RGS4 introduces into the active site of Gtα is Asn128. Although Asn228 in contrast to the Ras GAP Arg729 or an intrinsic Arg in Ga subunits, does not directly interact with GDP and AlF4− (13, 14, 17, 30), it makes a contact with the side chain of Gln204 of Gtα, which stabilizes...
Functional Role of Asn$^{131}$ of Human Retinal RGS

GTPase activity nearly as well as native hRGSr (V$_{max}$ of hRGSr-His131, and hRGSr of transducin were determined in suspensions of dROS membranes (5 μM rhodopsin and 0.4 μM transducin) with and without addition of 50 nM hRGSr and 5 μM each of the following mutants: hRGSr-Ala$^{131}$, hRGSr-His$^{131}$, and hRGSr-$\Delta$$^{131}$).

and orients the hydrolytic water molecule in the transitional state of Gαt. Conceivably, Asn$^{128}$ is within hydrogen-bonding distance of the hydrolytic water molecule and may bind and orient it for nucleophilic attack of the γ-phosphate of GTP (17).

To probe the role of Asn$^{131}$ of hRGSr for the mechanism of RGS protein GAP activity, we initially synthesized a peptide of hRGSr corresponding to the region of interaction between Gαt:AlF$_4^-$ and RGS4 containing Asn$^{128}$. We reasoned that if the catalytic role of the Asn residue is a major component of RGS GAP activity, then perhaps a peptide containing the catalytic residue would alone be capable of eliciting the stimulation of GTPase activity. Our data demonstrated that hRGSr peptide-(123-140) containing catalytic Asn$^{131}$ retained the ability to bind hRGSr but failed to accelerate the GTPase activity of transducin. This indicates that the interaction of at least two and likely all three Gα binding regions of RGS protein is required to stimulate Gα GAP activity. Consistent with this conclusion is the recent finding that even short deletions within the RGS domain of RGS4 destroyed its GAP activity (31).

Further analysis of the role of Asn$^{131}$ of hRGSr was carried out using mutational substitutions of this residue. The major result from testing all hRGSr mutants is that replacement of Asn with other residues dramatically decreases the affinity of mutant hRGSr binding to Gαt. Substitution of Asn$^{131}$ by Ser was intriguing because the Asn residue is not absolutely conserved in RGS proteins, and some RGS proteins, including GAIP, have a Ser at this position (5, 17, 31). Serine has proven to be the best substitution for Asn with respect of retaining the GAP activity of hRGSr protein. The hRGSr-Ser$^{131}$ mutant had to be the best substitution for Asn with respect of retaining the catalytic role of the Asn residue as the key component of the RGS GAP activity. The hRGSr-Leu$^{131}$ and hRGSr-Ala$^{131}$ mutants at very high concentrations started to have a stimulatory effect on Gαt GTPase activity even though these residues are not expected to form hydrogen bonds which are made by the Asn residue. Our mutational analysis suggests that although Asn$^{131}$ of hRGSr may play a catalytic role in the RGS GAP activity, stabilization of the switch regions of G-protein and reduction of the energy of the transition state appear to be the major components of the RGS GAP function. The Asn residue is absolutely essential for the stabilization of the transition state for GTP hydrolysis because its replacement or deletion leads to a drastic reduction in hRGSr affinity for Gαt.

In addition to their role as GAPs, RGS proteins may act as antagonists for some G-protein effectors, particularly for phospholipase Cβ. RGS4 has been shown to block activation of phospholipase Cβ by GαtGTPγS (33). In another study, RGS4 inhibited inositol phosphate synthesis activated by AlF$_4^-$ in COS-7 cells overexpressing Gα (34). Tesmer et al. (17) have suggested that the RGS proteins lacking the Asn residue may better serve as inhibitors of effector binding than as GAPs. This would appear to be a likely scenario if replacements of the Asn residue resulted in a loss of GAP activity without a concurrent reduction of the G protein affinity for activated Gα subunits. The results of this work suggest that the main consequence of Asn replacement is an impairment of binding between mutated hRGSr protein and Gα. Furthermore, none of the hRGSr mutants have shown enhanced affinity to the active GαtGTPγS conformation which could be indicative of the potential of such a mutant to serve as an antagonist for the G-protein effector.

This study only begins to address the questions, introduced by the first crystal structure between G-protein and RGS protein, about the mechanism of RGS protein GAP activity (17). Further biochemical analysis coupled with resolution of other crystal structures between activated Gα subunits and RGS proteins would ultimately define a role of the critical Asn residue.

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