Communication

Substitution of Transducin Ser<sup>202</sup> by Asp Abolishes G-protein/RGS Interaction* (Received for publication, December 10, 1997) Michael Natochin and Nikolai O. Artemyev† From the Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

Known RGS proteins stimulate GTPase activity of G<sub>i</sub> and G<sub>q</sub> family members, but do not interact with G<sub>a</sub> and G<sub>q</sub>α. To determine the role of specific G<sub>a</sub> residues for RGS protein recognition, six RGS contact residues of chimeric transducin α-subunit (G<sub>a</sub>) corresponding to the residues that differ between G<sub>a</sub> and G<sub>a</sub>α have been replaced by G<sub>a</sub>α residues. The ability of human retinal G<sub>q</sub> (hRGSr) to bind mutant G<sub>a</sub>α subunits and accelerate their GTPase activity was investigated. Substitutions Thr<sup>178</sup> → Ser, Ile<sup>181</sup> → Phe, and Lys<sup>306</sup> → Arg of G<sub>a</sub>α did not alter its interaction with hRGSr. The Lys<sup>306</sup> → Leu mutant had the same affinity for hRGSr as G<sub>a</sub>α, but the maximal GTPase stimulation by hRGSr was reduced by ~2.5-fold. The substitution His<sup>209</sup> → Gln led to a 3-fold decrease in the affinity of hRGSr for the G<sub>a</sub>α mutant without significantly affecting the maximal GTPase enhancement. The Ser<sup>202</sup> → Asp mutation abolished G<sub>a</sub>α recognition by hRGSr. A counteracting replacement of Glu<sup>229</sup> by Ala in hRGSr did not restore the interaction of hRGSr with the G<sub>a</sub>α Ser<sup>202</sup> → Asp mutant. Our data suggest that the Ser residue at position 202 of G<sub>a</sub>α is critical for the specificity of RGS proteins toward G<sub>a</sub> and G<sub>a</sub>α families of G-proteins. Consequently, the corresponding residue, Asp<sup>229</sup> of G<sub>a</sub>α, is likely responsible for the inability of RGS proteins to interact with G<sub>a</sub>. –

Heterotrimeric GTP-binding proteins (G-proteins) are components of many major signaling systems that are used by cells to transduce a variety of signals from specific cell surface receptors to intracellular effector proteins. Regulation of G-protein GTPase activity represents an important mechanism for establishing proper signal duration. A novel class of proteins called RGS (regulators of G-protein signaling) is known to bind and stimulate the GTPase activity of G<sub>i</sub> and G<sub>q</sub>-like G-proteins by stimulating their GTPase activity (~6–10). Identification of RGS proteins has helped to solve a long standing discrepancy between the fast signal termination in vitro and relatively slow intrinsic GTPase rates typically observed under in vivo conditions (~6, 11). However, no RGS protein or other GTPase-activating protein (GAP) specific toward G<sub>a</sub>α has been described to date (~9, 10). The recently solved crystal structure of RGS4 bound to G<sub>a</sub> α-AlF<sub>4</sub> provides the first structural insights into the mechanism of G-protein GAP function and offers a starting point for studying the structural basis of the specificity of known RGS proteins (~12). RGS4 interacts with the switch regions of G<sub>a</sub>α that are likely to have a similar general conformation with the corresponding regions of G<sub>a</sub>. The incompetence of RGS proteins to bind and stimulate the GTPase activity of G<sub>a</sub>α therefore originates from the differences between amino acid residues of G<sub>a</sub>α contacting RGS4 and corresponding residues of G<sub>a</sub>. In this study we investigate molecular determinants of the specificity of RGS/G-protein interaction using transducin α-subunit (G<sub>a</sub>) as a prototypical member of the G<sub>a</sub> family and a human homologue (hRGSr) of mouse retinal mRGSr (~13, 14). We have carried out mutational analysis of specific amino acid residues of chimeric G<sub>a</sub>α corresponding to the RGS contact residues that are different between G<sub>a</sub>α and G<sub>a</sub>α to determine their specific role for RGS protein recognition.

EXPERIMENTAL PROCEDURES

Materials—GTP was a product of Boehringer Mannheim. [γ<sup>32</sup>P]GTP (>5000 Ci/mmol) was purchased from Amersham Corp. All other chemicals were acquired from Sigma.

Preparation of Rod Outer Segment (ROS) Membranes, G<sub>b</sub>γ and hRGSr—Bovine ROS membranes were prepared as described previously (~15). Urea-washed ROS membranes (uROS) were prepared according to protocol in Ref. 16. G<sub>b</sub>γ was prepared by the procedure described in Ref. 17. GST-hRGSr and hRGSr were prepared and purified as described previously (~14). The purified proteins were stored in 40% glycerol at ~20 °C or without glycerol at ~80 °C.

Site-directed Mutagenesis of Chimeric G<sub>a</sub>α—Mutagenesis of G<sub>a</sub>α residues was performed using the vector for expression of His<sub>6</sub>-tagged G<sub>a</sub>/G<sub>a</sub>α chimera 8 (Chim) as a template for PCR amplifications (~18). The G<sub>a</sub>/G<sub>a</sub>α Lys<sup>306</sup> → Leu and Thr<sup>178</sup> → Ser substitutions were introduced using 5′-primer 1 and 3′-primers 2 and 3, respectively, for PCR amplification (see below). The PCR products were digested with PstI and subcloned into the BamHI-digested pHis6Chim. Primer 3 also contained silent mutations creating the unique XbaI site that was used to make the Ile<sup>181</sup> → Phe mutant. The 5′-primer 4 and 3′-primer 5 were used to obtain the PCR product carrying the Ile<sup>181</sup> → Phe mutation. The product was cut with XbaI and HindIII and subcloned into the XbaI/HindIII-digested pHis6Chim Thr<sup>181</sup> → Ser. The Ser<sup>202</sup> → Asp and Lys<sup>306</sup> → Arg substitutions were introduced by PCR-directed mutagenesis using 5′-primer 6 and 3′-primers 7 and 8, respectively, followed by insertion of the NcoI/BamHI-digested PCR products into pHis6Chim. Mutation His<sup>209</sup> → Gln was generated using 5′-primer 9 and 3′-primer 5 and subcloning of the PCR product into the BamHI and HindIII sites of pHis6Chim. The sequences of all mutants were verified by automated DNA sequencing at the University of Iowa DNA Core Facility.

This paper is available online at http://www.jbc.org.

* This work was supported by National Institutes of Health Grant EY-10843. The services provided by the Diabetes and Endocrinology Research Center of the University of Iowa were supported by National Institutes of Health Grant DK-22925. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence and reprint requests should be addressed.

‡ The abbreviations used are: RGS proteins, regulators of G-protein signaling; hRGSr, human retinal G<sub>q</sub> protein; ROS, rod outer segment(s); uROS, urea-washed ROS membranes; GAP, GTPase-activating protein; G<sub>a</sub>/G<sub>a</sub>α, rod G-protein (transducin) α-subunit; G<sub>a</sub>/G<sub>a</sub>α, G<sub>a</sub>α, G<sub>a</sub>α, and G<sub>a</sub>α, α-subunits of G-proteins; GST, glutathione S-transferase; Chi8, chimera 8; PCR, polymerase chain reaction.
Mutational Analysis of RGS Contact Residues in Gαi/Gαs Chimera

RESULTS

Effects of hRGSr on GTPase Activity of Gα Mutants—Six residues directly interacting with RGS4 are different in Gαi, and Gαs (12). These residues correspond to Lys176, Thr178, Ile181, Ser202, and His209 of Gαi. Except for a conservative substitution, Gαi Ile181/Gαs Val185, these residues are identical in Gαi and Gαs. To analyze functional consequences of the replacement of these Gαi residues by corresponding Gαs residues we took advantage of the efficient expression of functional Gai/Gαs chimeras in E. coli (18). All the Gα mutants were made based on Chi8 that contains 80% of Gαs amino acid sequence, including all three Gαi switch regions (18). Analysis of Chi8 GTPase activity showed properties similar to native Gα. The GTP hydrolysis by Chi8 alone or in the presence of uROS was negligible (not shown). In the presence of both, the GTPase rate constants were calculated by fitting the experimental data to an exponential function: % GTP hydrolyzed = 100(k6t + k5). Where k6 is a rate constant for GTP hydrolysis.

Miscellaneous—Protein concentrations were determined by the method of Bradford (22) using IgG as a standard or using calculated extinction coefficients at 280 nm. SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (23) in 12% acrylamide gels. Rhodopsin concentrations were measured using the absorbance in 500 nm between “dark” and bleached ROS preparations. Fitting of the experimental data was performed with nonlinear least squares criteria using GraphPad Prism (version 2) software. The results are expressed as the mean ± S.E. of triplicate measurements.

Expression of Chi8 and all six Gα mutants, Lys176 → Leu, Thr178 → Ser, Ile181 → Phe, Ser202 → Asp, Lys205 → Arg, and His209 → Gln produced comparable amounts of fully soluble proteins (~5–7 mg/liter of culture). Mutants Lys176 → Leu, Ile181 → Phe, Lys205 → Arg, and His209 → Gln had EC50 values comparable with the EC50 value for Chi8 and transducin, suggesting that these mutations did not alter affinity of the G-protein-RGS interaction (Fig. 2, A and B). A 3-fold increase in the EC50 value was observed for the His209 → Gln mutant (EC50 337 ± 25 nm) (Fig. 2A).

Binding of Gα Mutants to GST-hRGSr—Binding between the Gα mutants and hRGSr was examined using precipitation of mutants by glutathione-agarose beads containing immobi-
Mutational Analysis of RGS Contact Residues in $G_{\alpha}/G_{\alpha}$ Chimera

...of Chimeric Gt

...hRGSr to interact and stimulate GTPase activity of $G_{\alpha}$ Ser$^{202} \rightarrow$ Asp. hRGSr Glu$^{129} \rightarrow$ Ala was fully active toward Chi8 and five of its mutants, but deficient of any GAP activity toward $G_{\alpha}$ Ser$^{202} \rightarrow$ Asp (not shown). Similarly, to the wild type hRGSr, the Glu$^{129} \rightarrow$ Ala mutant failed to bind $G_{\alpha}$ Ser$^{202} \rightarrow$ Asp, whereas its binding to Chi8 was intact (Fig. 3C).

**DISCUSSION**

Since its recent discovery, the family of RGS proteins has been rapidly growing. Those RGS proteins that have already been extensively characterized share a common specificity pattern. These RGS proteins interact with G-protein $\alpha$-subunits from $G_{i}$ and $G_{q}$ families but have no activity toward $G_{s}$ (6–8, 10) and $G_{12}$ (9). Both possibilities remain open: a member(s) of the RGS family capable of interaction with $G_{i\alpha}$ (G12$\alpha$) has not been yet identified or characterized, or none of the RGS proteins would be a GAP for $G_{i\alpha}$ (G12$\alpha$). The answer to this question lies in understanding the structural details and requirements for RGS/G-protein interaction.

The crystal structure of the complex of RGS4 with $G_{i\alpha}$-AlF$_4^-$ has revealed a structural basis for the inability of RGS4 to interact with $G_{i\alpha}$. Six amino acid residues from the RGS/G-protein interface are different between $G_{i\alpha}$ and $G_{q}\alpha$ (12). Three of these residues, corresponding to Thr$^{178}$, Ser$^{202}$, and His$^{209}$ in $G_{i\alpha}$ are conserved among the $G_{i\alpha}$, $G_{q}\alpha$, $G_{s}\alpha$, and $G_{12}\alpha$ subunits that are known to interact with RGS. Another two $G_{i\alpha}$ residues, Ile$^{181}$ and Lys$^{205}$, have homologous substitutions. Ile$^{181}$ is substituted by Val in $G_{i\alpha}$ and $G_{q}\alpha$, and Lys$^{205}$ is replaced by Arg in $G_{i\alpha}$. To identify the residue(s) critical for the failure of $G_{i\alpha}$ to interact with RGS proteins, we replaced the RGS contact residues in $G_{i\alpha}$ by corresponding residues in $G_{s}\alpha$ and examined the ability of hRGSr to stimulate GTPase activity of these mutants. hRGSr is a human homologue (hRGSr) of mouse retinal mRGS, which was originally thought to be a retina-specific RGS protein, but later it was found in other tissues as well (13, 25). Like other characterized RGS proteins, hRGSr interacts with $G_{i}$- and $G_{q}$-like $\alpha$-subunits, but does not bind $G_{s}\alpha$ (24). Substitutions Thr$^{178} \rightarrow$ Ser, Ile$^{181} \rightarrow$ Phe, and Lys$^{205} \rightarrow$ Arg did not significantly alter the activity of hRGSr toward these mutants. While this was not unexpected for the conservative replacement Lys$^{205} \rightarrow$ Arg, it was rather surprising for the Thr$^{178} \rightarrow$ Ser mutant. The corresponding $G_{i\alpha}$ Thr$^{185}$ residue interacts with seven invariant or highly conserved residues of RGS4 and, thus, even homologous substitution by Ser could have had a major impact on the $G_{i\alpha}$/RGS interaction (12). It appears that Ser may substitute Thr$^{178}$...
suitably in most of the RGS contacts. Another substitution that did not interfere with the affinity of Gα to hRGSr is Lys176 → Leu. This is consistent with the lack of conservation at this position between Gα, Gγ, and Gα. Interestingly, however, this mutation led to a substantial reduction in the GTPase V_max value elicited by hRGSr. Perhaps the lower stimulated GTPase activity of the Lys176 → Leu mutant reflects an intrinsic partial impairment of the catalytic site not evident from the basal GTPase activity. The adjacent Gα Thr177 residue is intimately involved in the GTP hydrolysis (26) and may not be fully stabilized in the RGS/Gα Lys176 → Leu complex. The Lys176 → Leu mutation highlights the possibility that Gα may have a limited ability for stimulation by RGS proteins assuming there is one that binds Gα. A modest decrease in the affinity for hRGSr without significantly affecting the maximal degree of the GTPase rate acceleration was observed for Gα His209 → Gln. The most severe outcome for the Gα/hRGSr interaction was caused by the Ser202 → Asp mutation. This mutation resulted in the loss of hRGSr binding. The crystal structure of Gα1 with RGS4 provides a rationale for such an outcome (12). A negative charge introduced by the Asp residue might be repelled by the negative charge of the counteracting Glu129 residue of hRGSr, which corresponds to the Glu 126 residue of RGS4. However, the Glu residue is not absolutely conserved in RGS proteins. A number of RGS proteins, RGS1, RGS6, and RGS7, have residues other than Glu at this position. Small uncharged residues such as the Ala residue in RGS7 might be the most accommodating residue for Asp. We found that the Glu129 → Ala substitution in hRGSr cannot rescue the ability of hRGSr to interact with Gα Ser202 → Asp. Perhaps, additional residue(s) such as Asn131 of hRGSr (Asn128 of RGS4) also interferes with the Asp side chain. RGS4 Asn128 makes a contact with Gα1 Ser206 (Ser202 of Gα). The RGS Asn residue is critical for the RGS/Gα interaction (12), and may only be substituted by Ser, though with a notable loss of the RGS affinity for Gα (20). Quite possibly, an interference of the Gα Asp residue with the network of interactions involving the hRGSr Asn131 residue is also responsible for the lack of interaction between hRGSr and Gα Ser202 → Asp.

The degree of impairment of the RGS/Gα interaction in the Ser202 → Asp mutant allows us to speculate that the corresponding Asp229 of Gα is mainly responsible for the inability of Gα to interact with characterized RGS proteins. Other differences in RGS contact residues between Gα and the Gγ-like α-subunits could be more easily accommodated by limited variability of different RGS domains. Our results do not support a likelihood that one of the currently identified RGS proteins may serve as a GAP for Gα. Nevertheless, they provide a direction toward identification of potential candidates for interaction with Gα among yet undiscovered RGS proteins.

Acknowledgments—We thank R. McEntaffer for technical assistance and Drs. H. Hamm and N. Skiba for providing us with the Gα/Gα expression vector.

REFERENCES

1. Koelle, M. R., and Horvitz, H. R. (1996) Cell 84, 115–125
2. De Vries, L., Mousli, M., Wurmser, A., and Farquhar, M. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 11916–11920
3. Druey, K. M., Blumer, K. J., Kang, V. H., and Kehrl, J. H. (1996) Nature 379, 742–746
4. Dohliman, H. G., and Thoerner, J. (1987) J. Biol. Chem. 262, 3871–3874
5. Koelle, M. R. (1997) Curr. Opin. Cell Biol. 9, 143–147
6. Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) Cell 86, 445–452
7. Watson, N., Linder, M. E., Druey, K. M., Kehrl, J. H., and Blumer, K. J. (1996) Nature 383, 172–175
8. Hunt, T. W., Fields, T. A., Casey, P. J., and Perrell, E. G. (1996) Nature 383, 175–177
9. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) J. Biol. Chem. 271, 27209–27212
10. Huang, C., Hepler, J. R., Gilman, A. G., and Mumby, S. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6159–6163
11. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
12. Tesmer, J. J. G., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) Cell 89, 251–261
13. Chen, C. K., Wieland, T., and Simon, M. I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 12885–12889
14. Natochin, M., Granovsky, A. E., and Artemyev, N. O. (1997) J. Biol. Chem. 272, 17444–17449
15. Papermaster, D. S., and Dreyer, W. J. (1974) Biochemistry 13, 2438–2444
16. Yamanaka, G., Eckstein, F., and Stryer, L. (1985) Biochemistry 24, 8094–8101
17. Kleuss, C., Pallast, M., Brendel, S., Rosenthal, W., and Scultz, G. (1987) J. Chromatogr. 407, 281–289
18. Skiba, N. P., Bae, H., and Hamm, H. E. (1996) J. Biol. Chem. 271, 413–424
19. Kleuss, C., Raw, A. S., Lee, E., Sprang, S. R., and Gilman, A. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9828–9831
20. Natochin, M., McEntaffer, R. L., and Artemyev, N. O. (1996) J. Biol. Chem. 273, in press
21. Arshavsky, V. Y., Gray-Keller, M. P., and Bownds, M. D. (1991) J. Biol. Chem. 266, 18530–18537
22. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
23. Laemmli, U. K. (1970) Nature 379, 742–746
24. Natochin, M., Lipkin, V. M., and Artemyev, N. O. (1997) FEBS Lett. 411, 179–182
25. Buckhinder, L., Velasco-Miguel, S., Chen, Y., Xu, N., Talbott, R., Helbert, L., Gao, J., Seizinger, B. R., Gutkind, J. S., and Kley, N. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7686–7692
26. Sondek, J., Lambricht, D. G., Noel, J. P., Hamm, H. E., and Sigler, P. B. (1994) Nature 372, 276–279
