Structural Basis for the Selectivity of the RGS Protein, GAIP, for Ga Family Members

IDENTIFICATION OF A SINGLE AMINO ACID DETERMINANT FOR SELECTIVE INTERACTION OF Ga SUBUNITS WITH GAIP

Donna S. Woulfe‡ and Jeffrey M. Stadel†

From the Department of Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the Department of Cardiovascular Pharmacology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

GAIP is a regulator of G protein signaling (RGS) that accelerates the rate of GTP hydrolysis by some G protein α subunits. In the present studies, we have examined the structural basis for the ability of GAIP to discriminate among members of the Ga family. Ga11, Ga12, and Ga13 interacted strongly with GAIP, whereas Ga12 interacted weakly and Ga13 did not interact at all. A chimeric G protein composed of a Ga2 N terminus and a Ga12 C terminus interacted as strongly with GAIP as native Ga12, whereas a chimeric N-terminal Ga13 with a Ga2 C terminus did not interact. These results suggest that the determinants responsible for GAIP selectivity between these two Ga subunits reside within the C-terminal GTPase domain of the G protein. To further localize residues contributing to G protein-GAIP selectivity, a panel of 15 site-directed Ga1 and Ga13 mutants were assayed. Of the Ga1 mutants tested, only that containing a mutation at aspartate 229 located at the N terminus of Switch 3 did not interact with GAIP. Furthermore, the only Ga1 variant that interacted strongly with GAIP contained a replacement of the corresponding Ga2 Switch 3 residue (Ala229) with aspartate. To determine whether GAIP showed functional preferences for Ga subunits that correlate with the binding data, the ability of GAIP to enhance the GTPase activity of purified α subunits was tested. GAIP catalyzed a 3–5-fold increase in the rate of GTP hydrolysis by Ga11 and Ga13(A230D) but no increase in the rate of Ga12 and less than a 2-fold increase in the rate of Ga12(D229A) under the same conditions. Thus, GAIP was able to discriminate between Ga11 and Ga13 in both binding and functional assays, and in both cases residue 229/230 played a critical role in selective recognition.

Heterotrimeric G proteins associate with the cytoplasmic surfaces of 7-transmembrane spanning receptors and function to transduce signals from receptors activated by extracellular ligands to intracellular effectors (1). One of the most recent developments in the study of G protein regulation is the identification of a novel family of proteins known as regulators of G protein signaling or RGS proteins (2). RGS proteins are characterized by the presence of an RGS domain that is structurally conserved across evolution (3, 4). These molecules function to desensitize G protein-coupled responses in organisms from yeast to man by directly interacting with the α subunit of heterotrimeric G proteins and increasing their rate of GTP hydrolysis (5). Direct interaction between G protein α subunits and RGS molecules was first demonstrated by DeVries et al. (6), who isolated the cDNA for the RGS GAIP (G alpha interacting protein) using a yeast two-hybrid screen for Gαi3-interacting proteins. A number of studies quickly followed revealing GAP (GTPase-activating protein) activity to be the mechanism by which RGSs turned off G protein activation (7–10). Both the structural interaction between RGS and Ga subunits and the mechanism of RGS GAP activity were further elucidated by the co-crystallization of RGS4 with Ga11 (11). However, much remains to be revealed about the function of individual members of the RGS family, their specificities for interacting proteins, and the structural determinants that define these interactions.

Most of the initially described RGS proteins showed both binding and functional selectivity for the Ga family of G proteins (7–9, 12). More recently, a number of RGS molecules have demonstrated binding or functional interactions with Ga4 and/or Ga signaling pathways (13–17), and p115RhoGEF was shown to be a functional RGS for the Ga12/Ga13 family of G proteins (18–20). However, there has been little information about the ability of any RGS to discriminate among the closely related members of the Ga family. Evidence for some specificity of RGS binding to distinct Ga family members was demonstrated by DeVries et al. (6), who showed strong interaction of GAIP with Ga11, Ga13, and Ga12 but weak interaction with Ga12 and no interaction with Ga1. The differential binding characteristics of Ga11 and Ga12 are particularly intriguing because these two G proteins are highly homologous, having an amino acid sequence identity of 88%. Differences in RGS binding may reveal structural differences in these two G proteins that have implications for their ability to differentially activate divergent downstream signaling pathways.

To evaluate the structural basis for the selectivity of the RGS GAIP for individual members of the Ga family, we have expressed native, chimeric, and mutant Ga proteins and compared their abilities to bind GAIP and act as substrates for GAIP GAP activity. The results show a preference of GAIP for Ga11 over Ga12 in both binding assays and GAP assays. This preference was reversed by mutating residue Asp229 in Ga12 to alanine and making the reciprocal mutation (A230D) in Ga11. Interestingly,

* 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 should be addressed: University of Pennsylvania, 421 Curie Blvd., BRB-2, Rm. 913, Dept. Medicine, Philadelphia, PA 19104; E-mail: woulfe@pharm.med.upenn.edu.
† Deceased.

1 The abbreviations used are: GAP, GTPase-activating protein; PCR, polymerase chain reaction; bp, base pair; GST, glutathione S-transferase; GTPγS, guanosine 5′-O-(thiotriphosphate).

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

Vol. 274, No. 25, Issue of June 18, pp. 17718–17724, 1999
Printed in U.S.A.
the selectivity of GAIP for Gα3 over Gα2 was lost when GTPase-deficient mutants of these two G proteins were tested for GAIP binding. Thus, the structural preference of GAIP for Gα1 versus Gα2 in their ground (presumably GDP-bound) states has functional consequences in their respective GAP activities.

**EXPERIMENTAL PROCEDURES**

**Generation of Yeast Two-hybrid Fusion Constructs**—Rat G protein α subunits were PCR amplified with oligonucleotides containing 5’ EcoRI restriction sites and 3’ SalI restriction sites. PCR products were then subcloned into the pGAD vector (Invitrogen, Carlsbad, CA) and sequenced to ensure fidelity to the template. Inserts were excised with EcoRI and SalI and subcloned into the pGBK7 Gal 4 DNA-binding domain fusion vector (CLONTECH).

Human GAIP was PCR amplified from a human heart cDNA library using oligonucleotides containing a 5’ NarI restriction site and a 3’ SalI restriction site. PCR products were subcloned and sequenced as above, then removed from pGAD with NarI and SalI, and subcloned into the pGAD Gal 4 activation domain fusion vector (CLONTECH).

**Generation of G Protein α Subunit Chimeras**—The Gα3Gα chimera was generated by removing the N-terminal 635-bp fragment of Gαs cDNA via site-directed mutagenesis (see below) and then ligating the BamHI-digested N-terminal 700-bp fragment of Gαs to the 436-bp C-terminal fragment of pGBK7 Gal 4-cDNA. The Gα3Gα chimera was generated by ligating the N-terminal 630-bp Gα1 fragment to the C-terminal 516-bp fragment of the same digestion. Both chimeras were subcloned into the pGBK7 vector and characterized with BamHI and EcoRI as well as with BamHI and SalI digestions to ensure correct constructions.

Gα1Gα2 and Gα2Gα1 chimeras were made by engineering a BamHI site into the GαTαDNA at the same site as a naturally occurring BamHI in Gαs. Gα2Gα and mutant GααCDNAs were digested with BamHI and the N-terminal 635-bp fragment of Gα1 was ligated to the C-terminal 433-bp fragment of Gα2 to generate Gα1Gα2. Similarly, Gα2Gα consists of the N-terminal BamHI fragment of Gα2 ligated to the C-terminal BamHI fragment of Gαs.

**Site-directed Mutagenesis of G Protein α Subunits**—Site-directed mutants of Gαs and Gα2 were made using Stratagene QuickChange site-directed mutagenesis kit according to the manufacturer’s protocols. Template pGAD-Gαs or pGBK7-Gα2 was amplified for 14 cycles of 12-min extensions, each using overlapping forward and reverse primers encoding the applicable mutation. All mutants were sequenced throughout the entire coding region to ensure desired mutagenesis as well as to screen against unwanted PCR-induced mutations.

**Transformation of Competent Yeast**—Saccharomyces cerevisiae strain HF7c (see below) was transformed with pGAD containing the Gal 4 DNA-binding domain and pGBK7 containing the Gal 4 activation domain, respectively. Yeast transformants were grown overnight at 30 °C for 1 h, 100 μl of the same, and lysed by four freeze/thaw cycles. Four colonies of each construct were streaked on -Leu-Trp selective dropout agar medium to grow for 3 days at 30 °C. Four 15-min, then collected with a quick spin, and plated on -Leu-Trp minimal agar and grown overnight to high cell density. 50 μl of lysate was loaded per lane onto SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with rabbit antibody common to G protein α subunits (Calbiochem, La Jolla, CA) at 1:500 dilution in Tris-buffered saline/5% milk. Immunoreactivity was detected with horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody (1:2000 dilution) and developed using ECL reagents according to the manufacturer’s protocols (Amersham Pharmacia Biotech).

**Liquid β-Galactosidase Assays**—Single colonies of transformed cells were inoculated into 5 ml of SC-Leu-Trp agar and grown overnight to an A600 of 0.8. Cells were collected by centrifugation, washed once in Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCl, 1 mM MgSO4), resuspended in 300 μl of the same, and lysed by four freeze/thaw cycles. To start the assay, 100 μl of this cell lysate was suspended in 0.7 ml of Z buffer containing 0.27% β-mercaptoethanol and then added to 0.16 ml of Z buffer containing 4 mg/ml o-nitrophenyl β-D-galactopyranoside substrate. Suspensions were vortexed and incubated for 2 h at 30 °C. Color reactions were stopped with 0.4 ml of Na2CO3 and read at A420 after spinning out cell debris. β-Galactosidase units (21) were calculated according to the manufacturer’s protocols (CLONTECH), as follows: β-galactosidase units = 1000 X A420/ t × v × A600, where t is 120 min of incubation, v is 0.1 ml of reaction volume-concentration factor, and A600 was 0.8 for the culture.

**Histidine Growth Assays**—5-ml cultures of yeast transformants were grown to an A600 of 1.0 and then 3 μl of 1:10 serial dilutions of confluent growths were spotted on either SC-Leu-Trp or SC-Leu-Trp-His agar plates and allowed to grow at 30 °C for 3 days.

**Protein Expression and Purification**—Full-length G protein α subunits Gαs, Gα2, and Gα1α2α3 (D229A), and Gαα1α2α3 (A230D) and full-length GAIP were expressed as GST fusion proteins by subcloning cDNAs downstream of the GST tag using EcoRI/SalI sites of the vector pGEX-6P-1 (Amersham Pharmacia Biotech). Each plasmid construct was transformed into bacterial strain BL21, grown overnight, and induced to express protein with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Cells were harvested by centrifugation, sonicated in TE containing 0.1 M NaCl.
Fig. 2. Relative interaction of Gα chimeras with GAIP. Liquid β-galactosidase assays were conducted as described in the legend to Fig. 1A. Two clones of each transformant were assayed. The results shown are the means ± S.E. for n = 2–18 in triplicate. A, interaction-dependent release of β-galactosidase from clones expressing chimeras of Gαi3 and Gαs. A schematic diagram of the chimeras is shown at the bottom. B, interaction-dependent release of β-galactosidase from clones expressing chimeras of Gαi1 and Gαs. A schematic diagram of these chimeras is shown at the bottom, where the asterisk indicates the position of Gαi1 Asp229.

Fig. 3. A, relative interaction of C-terminal point mutants with GAIP. B, relative interaction of N-terminal point mutants with GAIP. Liquid β-galactosidase assays were conducted as described in the legend to Fig. 1A. Two clones of each transformant were assayed. The results shown are the means ± S.E. for n = 2–18 in triplicate.

RESULTS

Interactions of G Protein Fusions with GAIP—To explore the structural basis for the differences in GAIP binding by the different members of the Gα family, we engineered a panel of Gα protein chimeras and mutants. As a first step, native and engineered G proteins were assayed for the ability to bind GAIP using the yeast two-hybrid system. To make use of this system, Gα protein cDNA constructs were subcloned downstream of a Gal4-binding domain cDNA and coexpressed with a GAIP-Gal 4 activation domain fusion in the S. cerevisiae strain HF7Cα. All fusions were immunoblotted to control for relative expression levels. An anti-G protein α subunit antibody raised against the internal GTP-binding sequence common to all heterotrimeric G protein α subunits recognized a protein of the appropriate molecular mass (about 65 kDa) for a G protein α subunit fused to the Gal4-binding domain in each of the clones transformed with a G protein fusion (data not shown). All of the clones expressed comparable levels of G protein fusion, and no protein of the same size was seen in clones transformed with pGBT9-binding domain alone.

Given such a similar background of G protein fusion expression, a measure of the strength of interaction between various G proteins and GAIP can be evaluated at 6 °C. A panel of mutants and chimeras were generated from the relative activation of Gal4-dependent reporters. The yeast strain HF7Cα was stably transformed with cDNAs encoding both β-galactosidase and histidine reporters downstream of a Gal4 promoter. In this system, the promoter is activated in proportion to the degree of interaction between the Gal4 binding domain and activation domain fusions (23). Thus, two different reporters

mm phenylmethylsulfonyl fluoride and 1 mm β-mercaptoethanol, and solubilized with 1% Triton X-100. Lysates were cleared by centrifugation at 12,000 × g for 10 min, and supernatants were applied to pre-washed glutathione-Sepharose columns (Amersham Pharmacia Biotech). Columns were washed with TE containing phenylmethylsulfonyl fluoride and β-mercaptoethanol and GST fusion proteins eluted with 10 mm glutathione. Purified proteins were buffer exchanged into TED buffer (20 mm Tris-HCl, pH 8, 1 mm EDTA, 1 mm dithiothreitol, 10% glycerol), concentrated to 1 mg/ml in Nanosep spin columns (Pall Filtron Corp.), and stored at −80 °C. Size and homogeneity of purified proteins were verified using Coomassie-stained SDS-polyacrylamide gel electrophoresis, and in-frame translation of G proteins was verified via immunoblot using a Gαi/Gαs-selective antibody (kind gift of Dr. David Manning, University of Pennsylvania, Philadelphia, PA).

GTP-S Competition Curves—100 nm purified GST-tagged G protein α subunits were shaken for 4 h at 30 °C in the presence of 100 nm [35S]GTP-S and serial dilutions of 1–100 μM competing unlabeled GTP-S in 50 μl of binding buffer (50 mm HEPES, pH 8, 1 mm EDTA, 2 mm β-mercaptoethanol, 10 mm MgSO4, 2 mm ATP, 30% glycerol, 1 mg/ml bovine serum albumin) (22). Reactions were filtered over BAS5 nitrocellulose filters and washed three times with 2 ml of cold GTP-SA STOP buffer (20 mm Tris-Cl, pH 8, 150 mm MgCl2, 100 mm NaCl). Filters were immersed overnight in scintillation fluid before counting to determine amount of [35S]GTP-S bound.

GTPase Assays—100 nm purified GST-tagged G protein α subunits were loaded with 1 μM [γ-32P]GTP (8000 cpm/pmol) for 20 min at 30 °C in 600 μl of GTPase buffer (0.1% Lubrol PX, 50 mm HEPES, pH 7.5, 1 mm dithiothreitol, 5 mm EDTA). Reactions were chilled at 4 °C for 10 min, and assays were conducted at 30 °C. A 50-μl aliquot was removed immediately before initiating the reaction and quenched with 750 μl of 5% Norit activated charcoal in 50 mm NaPO4, pH 3. To initiate the reaction, 100 μl cold GTP and 15 mm MgSO4 (final concentrations) were added to reaction mixtures, and 50-μl aliquots were removed after 10 s, 20 s, 40 s, 1 min, 2 min, 3 min, 4 min, and 5 min and stopped as just described. Charcoal was precipitated by centrifugation for 15 min at 12,000 × g, and 400-μl free phosphate-containing supernatants were counted to determine the amount of P_i released per reaction.
were used to measure the relative strength of the interaction between the G protein-binding domain fusion and the GAIP activation domain fusion.

According to both histidine and β-galactosidase reporter systems, robust interaction of GAIP was seen with Ga_11, Ga_13, and Ga_α, whereas the interaction with Ga_22 was weak, and the interaction with Ga_β was undetectable (Fig. 1). These results are consistent with those obtained by DeVries et al. (6). Due to the quantitative nature of the assays, liquid β-galactosidase assays were used for interaction comparisons henceforth. Because Ga_11 gave a strong interaction with GAIP in its native conformation, which was statistically indistinguishable from that of Ga_22 and Ga_α, and because this G protein was tested in every assay conducted, this level of interaction was designated as 100% for comparison with all other G protein constructs. 100% interaction was used for interaction comparisons henceforth. Because Ga_11 mutants C-terminal to the BamHI site were tested, several impaired binding to GAIP, but only D229A abolished it (Fig. 3A).

Even more significantly, the reciprocal mutation in the corresponding residue in Ga_22 (Ga_α(A230D)) produced a variant Ga_α that bound to GAIP as strongly as Ga_11 (Fig. 3A). Thus, Ga_11(D229) appears to be particularly important for GAIP interaction.

In addition to the C-terminal mutants shown in Fig. 3A, three N-terminal Ga_11 mutants and the corresponding reciprocal Ga_α mutants were also assayed for β-galactosidase activity. Consistent with the results of the Ga_11/2 chimeras, all of the N-terminal Ga_11 mutants bound to GAIP, and none of the corresponding Ga_α mutants bound GAIP as strongly as Ga_11 (Fig. 3B). Thus, none of these residues appears to be a necessary determinant for GAIP binding.

**GTPase-deficient Mutants**—To determine whether different nucleotide-dependent conformations of these G proteins affected their relative GAIP affinities, GTPase-deficient mutants of Ga_11 and Ga_22 were generated to “trap” the α subunits in their GTP-bound forms and assayed for binding to GAIP. In contrast to the wild type proteins, the “activated” forms of both Ga_11 and Ga_22 interacted at least as strongly with GAIP as wild type Ga_11 (Fig. 4). Both Ga_11(Q204L) and Ga_22(Q205L) generated a 4-fold increase in GAIP binding activity relative to that seen with wild type (nonactivated) Ga_11, so that the selectivity of GAIP for Ga_11 over Ga_α appears to be restricted to the interaction with their ground state (presumably GDP-bound) conformations. Two additional GTPase-deficient mutants, Ga_11(R178C) and Ga_α(R179C), were also tested and interacted very strongly with GAIP although less strongly than the Q204L/Q205L mutants.

**Nucleotide Binding Affinity**—To explore the mechanism of the selectivity of GAIP for Ga_11 over Ga_22 in their GDP-bound states, the position of Ga_11 aspartate 229 in relation to the bound RGS4 molecule in the published crystal structure was examined (Fig. 5). In the AlF_4-activated state in which this G protein was crystallized, Asp_229 appears closer to the nucleotide-binding site than to the RGS-binding site of this G protein. Therefore, we examined the relative GTP_yS affinities of both Ga_11 and Ga_22 to determine whether there were differences in nucleotide binding affinity that in turn might affect their affinities for GAIP. Recombinant full-length Ga_11, Ga_α, Ga_11(D229A), and Ga_22(A230D) were GST-tagged, expressed in bacteria, and purified to homogeneity over glutathione affinity columns. The ability of unlabeled GTP_yS to displace [35S]GTP_yS from each of the proteins was measured over a range of GTP_yS concentrations. The IC_50 for [35S]GTP_yS dis-
FIG. 5. Position of Go1 Asp229 in relation to bound RGS4 and GDP-Mg2+ -AlF4− molecules. PDB 1AGR (2) showing the cocrystallization of Go1 with RGS4 was downloaded from the Brookhaven National Labs Protein Data Bank and viewed using RasMol. The Go1 subunit is shown in dark blue bound to a cyan RGS4 molecule. GAIP binding specificity determinant Go1(Asp229) is pictured in yellow at the top of the pink Switch 3 region of Go1. The bound GDP-AlF4− is the adjacent structure in green. Go1 residues Arg778 and Gln794 are highlighted in red.

FIG. 6. Competition between GTPγS and [35S]GTPγS for binding to native and mutant Go protein α subunits. Purified GST-tagged Go protein α subunits (100 nM) were incubated with 100 nM [35S]GTPγS (200,000 cpm/50 μl assay volume) and indicated concentrations of unlabeled GTPγS and filtered as described under “Experimental Procedures.” Each value is the mean ± S.E. of three experiments performed in triplicate.

Placement was the same for all four proteins (Fig. 6), so differences in GAIP binding are not reflective of differences in nucleotide binding affinities.

GAP Activity—Finally, to determine whether any functional differences might correlate with selective binding capacity, we tested the ability of GAIP to catalyze the GTPase activities of Go1, Go2, Goαi1(D229A), and Goαi3(A230D). GAIP catalyzed a 5-fold increase in the rate of GTP hydrolysis by Go1 (Fig. 7A) but caused no increase in the GTPase rate of Go2 (Fig. 7B) under the same conditions. In addition, GAIP only slightly increased the GTPase activity of Goαi1(D229A) (from Kobs of 2.1 in the absence of GAIP to Kobs of 3.7 in the presence of GAIP) (Fig. 7C). Of particular interest, the rate of GTP hydrolysis seen for this mutant form of Goαi1 in the presence of GAIP is similar to the GTPase rate in the presence of Go1 (Kobs = 4.2). Similarly, Goαi3(A230D) now behaves more like Go1 in that there is a significant increase in GAIP activation, and the GTPase rate seen in the presence of GAIP is similar to that seen for Go1 (in the presence of GAIP (Kobs = 5.2 for the former and 5.6 for the latter) (Fig. 7D). Therefore, the ability of GAIP to act as a GAP for these two Go proteins and their reciprocal mutants correlates with its affinities for these proteins in their “ground states” as measured in the yeast two-hybrid assay.

DISCUSSION

RGS proteins are a family of G protein regulators that down-regulate G protein-coupled responses by stimulating the GTPase activity of the Go subunits to which they bind (3, 5). Both the G protein binding and GAP activity of RGS molecules have been localized to a 130-amino acid domain (RGS domain) that is conserved among all RGS proteins (6, 10, 24). Within this domain, a number of residues have been shown to serve as contact points for Go protein binding (11, 25, 26).

Elucidation of the sites on G proteins with which RGS proteins interact and the selectivity of RGS proteins for different forms of Go have important implications for the mechanism by which RGSs stimulate α subunit GTPase activity. The observation that RGS4 binds more strongly to the AlF4−-GDP-Mg2+-bound state of Go1 than to the GDP or GTP-bound states suggests that RGSs exhibit GAP activity by stabilizing the transition state for GTP hydrolysis by Go1 (7–9, 27, 28). The crystal structure of AlF4−-GDP-Mg2−-Go1 bound to RGS4 further reveals that the RGS interacts directly with the Switch regions of Go1, reducing their flexibility in this transition state mimic and thus further supporting this proposed GAP mechanism (11). It has also been observed that the sites on Go1 to which RGS proteins bind may interfere with the binding of the effector PLCβ1, suggesting another possible mechanism for Go1 down-regulation by RGSs (13).

The sites on G protein α subunits responsible for the selectivity with which RGS proteins bind have been less well studied. DeVries et al. (29) showed a significantly reduced GAIP interaction with a 10-amino acid truncation of Go1αi3 but a chimeric Go1αi containing the last 10 residues of Go1αi did not bind to GAIP, indicating that other determinants remain to be identified. More recently, Lan et al. (30) showed that a G184S mutation in Go1αi and the equivalent mutation in Go1αi prevents both binding to and activation by RGS4, extending the observation by DiBello et al. (31) that a mutant Gpa1 prevented a functional interaction with the yeast RGS sst2. However, because this glycine is a highly conserved Switch 1 residue, it appears to be required for all Go interactions with RGS molecules rather than a determinant for specificity. Finally, Natochin and Artemyev (32) showed that the interaction of Go1αi...
with human retinal RGS could be abolished by mutating serine 202 to the corresponding Gα aspartate, providing one candidate Gα site that might interfere with RGS binding. They recently extended this finding by showing that mutation of this Gα aspartate (Gα Asp229) to the serine which occurs in Gai family members at the corresponding Switch 1 position promotes binding to an RGS (33).

To extend the characterization of RGS/G protein specificities and their structure/function relationships, we sought to identify regions in the Gai subunit that contributed to GAIP binding selectivity by testing the relative interaction strengths of GAIP with a number of native G protein α subunits, mutants, and chimeras using the yeast two-hybrid system. In this system, GAIP interacts equally strongly with native forms of Gαi1, Gαi3, and Gαo but very weakly with Gαi2 and not at all with Gαz. Both Gαi1D and Gαi1D/αo chimeras disrupted GAIP binding, indicating either that both the N and C termini of the Gai subunit contain determinants required for binding or that divergent sequences in the Gai protein relative to Gαo may interfere with GAIP interaction points. Gαi1D and Gαi1D/αo mutants gave more interpretable results, indicating that the C-terminal domain of Gαi1 is required for GAIP binding. This region constitutes most of the GTPase domain of the G protein, which is consistent with reports showing that GAIP binds in a groove within this domain (11). By comparison, the failure of either Gαo chimera to bind may indicate that N-terminal inserts in the Gαo sequence (such as amino acids 72–86) relative to Gai interfere with the Gαo-bound binding surface or that divergent residues in the Gαo N-terminal portion interfere with GRS contact. The interfering aspartate (Gαi residue 229) proposed by Natochin and Artemyev (32, 33) is in fact in the N-terminal portion of our chimeras, consistent with this possibility.

To further localize the region in the G protein C terminus responsible for GAIP selectivity, site-directed mutants were generated in which residues in Gαi1 and Gαo were swapped. Candidate residues were chosen on the basis of their conservation in Gai1 and Gαo and divergence in Gαo. The mutation of aspartate 229 of Gαi1 to the alanine present in Gαo nearly abolished GAIP binding. Conversely, when aspartate was substituted for the alanine normally present at the same site in Gαo, the mutant Gαo bound GAIP to the same extent as native Gαi1. These results reveal the importance of aspartate 229 for the binding of Gai subunits in their native state to GAIP and potentially suggest a site of physical contact with GAIP. Yet, upon inspection of the Gai-RGS4 crystal structure, this aspartate appears quite far from the sites of RGS4 interaction. Due to the location of Gai1 aspartate 229 at the far N terminus of Switch 3, it is possible that the position of this amino acid in the AlF4 transition state analogue in which it was co-crystallized with RGS4 differs from its position in the nonactivated state in which the Gai8 show selectivity for binding to GAIP. That is, it may be that in its GDP-bound (ground state) conformation, Gai8 Asp229 is in closer proximity to GAIP than in its AlF4-GDP-bound conformation.

Closer inspection of the RGS4-Gai1 crystal structure presents an alternative explanation. In this structure, aspartate 229 appears to be involved in a relay system that connects its carbonyl through a water molecule to lysine 270, which in turn maintains a hydrophobic interaction with GDP in the RGS4-Gai1 crystal structure. We hypothesized that removal of the carbonyl group at this position by mutation to an alanine might disrupt this relay system, destabilizing the binding of nucleotide and hence the binding of RGS, because its binding is dependent on the nucleotide-bound state of the G protein. To test this possibility, IC50 values for the ability of GTPγS to compete [35S]GTPγS binding by Gai1, Gai3, Gai1(D229A), and Gai3(A230D) were compared. The displacement curves were identical in all cases, implying that differences in nucleotide binding capacities do not account for RGS binding differences.

Finally, to determine whether there is also selectivity by GAIP for Gai1 versus Gai3 in their GTP-bound forms, GTPase-deficient mutants of both Gai1 and Gai3 were engineered and tested for GAIP binding in the yeast two-hybrid system. Interestingly, both Gai1(Q204L) and Gai3(Q205L) exhibited similarly high binding affinities to GAIP (about four times the native Gai1 interaction), consistent with an inability by GAIP to discriminate between the two proteins in their GTP-bound states. The Gai1(R178C) and Gai3(R179C) GTPase-deficient mutants interacted less strongly than the Q204L/Q205L mutants, although still more strongly than their native counter-
parts. This may reflect the ability of RGS proteins to partially restore the GTPase activity of R178C mutants, but not Q204L mutants (7), such that Q204L mutants remain in their GTP-bound state, but R178C mutants may reflect a mixture of conformations. These data also bring up an alternative explanation for the preferential binding of GAIP to nonmutated Goα1 over Goα2, namely that there is a greater population of GTP-bound Goα1 than GTP-bound Goα2 in the yeast cell. This could result from different rates of GTP/GDP exchange or GTP turnover by the two α subunits. Formally, that remains a possibility. However, because mammalian Go proteins do not couple to yeast G protein-coupled receptors (34) and because G proteins remain GDP-bound in the absence of receptor stimulation (35), we find it more likely that there is a structural difference between the two Goαs that is recognized by GAIP only in their nonactivated states.

To determine whether the ability of GAIP to discriminate between Goα1 and Goα2 only in their GDP-bound states has any functional significance, we measured the GAP activity of GAIP with each of these proteins and their mutants. GAIP enhanced the rate of GTP hydrolysis of Goα1 but not Goα2 under similar conditions. Furthermore, as predicted by the binding studies, Goα1(D229A) was a poor substrate for GAIP GAP activity compared with native Goα1, and Goα2(A230D) was comparable with Goα1 as a substrate for GAIP GAP activity. Although Berman et al. (7) showed GAIP-catalyzed increases in GTPase activity of both Goα1 and Goα2, Heximer et al. (17) also showed a greater enhancement by GAIP of Goα1 over Goα2 GTPase activity. Our results indicate that GAIP preferentially enhances Goα1 over Goα2 GTPase activity and that this activity correlates with the binding selectivity shown for Goαs in their ground state conformations. In addition, because GTPase-deficient mutants of both α1 and α2 subunits bind tightly to GAIP, these results may imply that GAP binding is not sufficient for GAP catalytic activity. Indeed, differential effects on Go binding versus GAP activity were discerned by Chen et al. (25) using various RGS mutants, consistent with this idea. It may be that the difference in the binding affinities for GTP-bound versus GDP-bound Goα conformations drives GTP hydrolysis, so that binding to the activated G protein conformation is not the only indicator of RGS functional selectivity.

The functional selectivity displayed by GAIP and other RGS proteins for G protein partners in vivo remains to be explored. The contributions of additional interacting partners, including C-terminal tails of GPCRs (36) and additional effector proteins (18–20), and post-translational modifications (37) will have to be considered to determine how individual RGS proteins modulate specific G protein signaling pathways.

Acknowledgments—We sincerely thank Skip Brass for much help with the manuscript, Dave Manning and Eliot Ohlstein for encouragement and advice, Katie Freeman for yeast expertise and many helpful discussions, and Cathy Peishoff for help with structural interpretations.

REFERENCES
1. Stadel, J. M., Wilson, S., and Bergsma, D. J. (1997) Trends Pharmacol. Sci. 11, 190–194
2. Koelle, M. R. (1997) Curt. Opin. Cell Biol. 9, 143–147
3. Dohlman, H. G., and Thorner, J. (1997) J. Biol. Chem. 272, 3871–3874
4. Druy, K. M., Blumer, K. J., Kang, V. H., and Kehrl, J. H. (1996) Nature 379, 742–746
5. Berman, D. M., and Gilman, A. G. (1998) J. Biol. Chem. 273, 1269–1272
6. DeVries, L., Mousli, M., Wurmser, A., and Farquhar, M. G. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 31916–32020
7. Berman, D. M., Wilkie, T. M., and Gilman, A. G. (1996) Cell 86, 445–452
8. Watson, N., Liner, M. E., Druy, K. M., Kehrl, J. H., and Blumer, K. J. (1996) Nature 383, 172–175
9. Hunt, T. W., Fields, T. A., Casey, P. J., and Peralta, E. G. (1996) Nature 383, 176–177
10. Faurobert, E., and Hurley, J. B. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2945–2950
11. Tesmer, J. G., Berman, D. M., Gilman, A. G., and Sprang, S. R. (1997) Cell 89, 251–261
12. Saitoh, O., Kuno, Y., Miyatani, Y., Asano, T., and Nakata, H. (1997) Nature 390, 525–529
13. Hepler, J. R., Berman, D. M., Gilman, A. G., and Kozasa, T. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 428–432
14. Shuey, D. J., Betty, M., Jones, P. G., Khavaja, Z. Z., and Cockett, M. I. (1998) J. Neurochem. 70, 1964–1972
15. Chatterjee, T. K., Kapan, A. K., and Fisher, R. A. (1997) J. Biol. Chem. 272, 15481–15487
16. Neill, J. D., Duck, L. W., Sellers, J. C., Musgrove, L. C., Scheshenka, A., Druy K. M., and Kehrl, J. H. (1997) Endocriology 138, 843–846
17. Heximer, S. P., Watson, N., Liner, M. E., Blumer, K. J., and Hepler, J. R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14389–14393
18. Kozasa, T., Jiang, X., Hart, M. J., Sternweis, P. M., Singer, W. D., Gilman, A. G., Bollag, G., and Sternweis, P. C. (1998) Science 280, 2109–2111
19. Hart, M. J., Jiang, X., Kozasa, T., Racoe, W., Singer, W. D., Gilman, A. G., Sternweis, P. C., and Bollag, G. (1998) Science 2112–2114
20. Hall, A. (1998) Science 280, 2077–2075
21. Miller, J. H. (1972) Experiments in Molecular Genetics, pp. 352–355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Markby, D. W., Onrust, R., and Bourne, H. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 2582–2590
23. Drury, K. M., and Kehrl, J. H. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 12851–12856
24. Berman, D. M., Kozasa, T., and Gilman, A. G. (1996) J. Biol. Chem. 271, 27209–27212
25. Natochin, M., Granovsky, A. E., and Artemyev, N. O. (1997) J. Biol. Chem. 272, 14447–14449
26. DeVries, L., Elenko, E., Hubler, L., Jones, T. L. Z., and Farquhar, M. G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 15203–15208
27. Lan, K-L., Sarvazyan, N. A., Taussig, R., Mackenzie, R. G., DiBello, P. R., Dohlman, H. G., and Neubig, R. R. (1998) J. Biol. Chem. 273, 12784–12797
28. Doolittle, P. R., Garrison, T. R., Apanovitch, D. M., Hoffman, G., Shuey, D. J., Mason, R., Cockett, M. I., and Dohlman, H. G. (1998) J. Biol. Chem. 273, 5780–5784
29. Natochin, M., and Artemyev, N. O. (1998) J. Biol. Chem. 273, 4300–4303
30. Natochin, M., and Artemyev, N. O. (1998) Biochemistry 37, 15776–15780
31. Kang, Y.-S., Kane, J., Kurjan, J., Stadel, J. M., and Tipper, D. J. (1999) Mol. Cell. Biol. 19, 2589–2599
32. Hamm, H. E. (1998) J. Biol. Chem. 273, 669–672
33. Snow, B. E., Hall, R. A., Krumins, A. M., Brothers, G. M., Bouchard, D., Brothers, C. A., Chuang, S., Mangion, J., Gilman, A. G., Leffowitz, R. J., and Siderovski, D. P. (1998) J. Biol. Chem. 273, 17749–17755
34. Tu, Y., Wang, J., and Ross, E. M. (1997) Science 278, 1132–1135