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Structural Basis for the Selectivity of the RGS Protein, GAIP, for $G\alpha_i$ Family Members

IDENTIFICATION OF A SINGLE AMINO ACID DETERMINANT FOR SELECTIVE INTERACTION OF $G\alpha_i$ SUBUNITS WITH GAIP*

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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 $G\alpha_i$ family. $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_o$ interacted strongly with GAIP, whereas $G\alpha_{i2}$ interacted weakly and $G\alpha_s$ did not interact at all. A chimeric G protein composed of a $G\alpha_{i2}$ N terminus and a $G\alpha_{i1}$ C terminus interacted as strongly with GAIP as native $G\alpha_{i1}$, whereas a chimeric N-terminal $G\alpha_{i1}$ with a $G\alpha_{i2}$ C terminus did not interact. These results suggest that the determinants responsible for GAIP selectivity between these two $G\alpha_i$s 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 $G\alpha_{i1}$ and $G\alpha_{i2}$ mutants were assayed. Of the $G\alpha_{i1}$ 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 $G\alpha_{i2}$ variant that interacted strongly with GAIP contained a replacement of the corresponding $G\alpha_{i2}$ Switch 3 residue (Ala^{230}) with aspartate. To determine whether GAIP showed functional preferences for $G\alpha$ 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 $G\alpha_{i1}$ and $G\alpha_{i2}$(A230D) but no increase in the rate of $G\alpha_{i2}$ and less than a 2-fold increase in the rate of $G\alpha_{i1}$(D229A) under the same conditions. Thus, GAIP was able to discriminate between $G\alpha_{i1}$ and $G\alpha_{i2}$ 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 $G\alpha_{i3}$-interacting proteins. A number of studies quickly followed revealing GAP (GTPase-activating protein)[1] activity to be the mechanism by which RGSs turned off G protein activation (7–10). Both the structural interaction between RGS and $G\alpha$ subunits and the mechanism of RGS GAP activity were further elucidated by the co-crystallization of RGS4 with $G\alpha_{i1}$ (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 $G\alpha_i$ family of G proteins (7–9, 12). More recently, a number of RGS molecules have demonstrated binding or functional interactions with $G\alpha_q$ and/or $G\alpha_s$ signaling pathways (13–17), and p115RhoGEF was shown to be a functional RGS for the $G\alpha_{12}/G\alpha_{13}$ 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 $G\alpha_i$ family. Evidence for some specificity of RGS binding to distinct $G\alpha_i$ family members was demonstrated by DeVries *et al.* (6), who showed strong interaction of GAIP with $G\alpha_{i1}$, $G\alpha_{i3}$, and $G\alpha_o$ but weak interaction with $G\alpha_{i2}$ and no interaction with $G\alpha_s$. The differential binding characteristics of $G\alpha_{i1}$ and $G\alpha_{i2}$ 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 $G\alpha_i$ family, we have expressed native, chimeric, and mutant $G\alpha$ proteins and compared their abilities to bind GAIP and act as substrates for GAIP GAP activity. The results show a preference of GAIP for $G\alpha_{i1}$ over $G\alpha_{i2}$ in both binding assays and GAP assays. This preference was reversed by mutating residue Asp^{229} in $G\alpha_{i2}$ to alanine and making the reciprocal mutation (A230D) in $G\alpha_{i1}$. Interestingly,

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[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).

the selectivity of GAIP for Gα$_{i1}$ over Gα$_{i2}$ was lost when GTPase-deficient mutants of these two Gα$_i$s were tested for GAIP binding. Thus, the structural preference of GAIP for Gα$_{i1}$ *versus* Gα$_{i2}$ 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′ *Eco*RI restriction sites and 3′ *Sal*I restriction sites. PCR products were then subcloned into the pCRII vector (Invitrogen, Carlsbad, CA) and sequenced to ensure fidelity to the template. Inserts were excised with *Eco*RI and *Sal*I and subcloned into the pGBT9 Gal 4 DNA-binding domain fusion vector (CLONTECH).

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

Generation of G Protein α Subunit Chimeras—The Gα$_{s/i3}$ chimera was generated by removing the N-terminal *Bam*HI site in the Gα$_s$ cDNA via site-directed mutagenesis (see below) and then ligating the *Bam*HI-digested N-terminal 700-bp fragment of Gα$_s$ to the 430-bp C-terminal fragment of *Bam*HI-digested Gα$_{i3}$ cDNA. The Gα$_{i3/s}$ chimera was generated by ligating the N-terminal 630-bp Gα$_{i3}$ fragment to the C-terminal 516-bp Gα$_s$ fragment of the same digestions. Both chimeras were subcloned into the pGBT9 vector and characterized with *Bam*HI and *Eco*RI as well as with *Bam*HI and *Sal*I digestions to ensure correct constructions.

Gα$_{i1/i2}$ and Gα$_{i2/i1}$ chimeras were made by engineering a *Bam*HI site into the Gα$_{i1}$ cDNA at the same site as a naturally occurring *Bam*HI in Gα$_{i2}$. Gα$_{i2}$ and mutant Gα$_{i1}$ cDNAs were digested with *Bam*HI, and the N-terminal 635-bp fragment of Gα$_{i1}$ was ligated to the C-terminal 433-bp fragment of Gα$_{i2}$ to generate Gα$_{i1/i2}$. Similarly, Gα$_{i2/i1}$ consists of the N-terminal *Bam*HI fragment of Gα$_{i2}$ ligated to the C-terminal *Bam*HI fragment of Gα$_{i1}$.

Site-directed Mutagenesis of G Protein α Subunits—Site-directed mutants of Gα$_{i1}$ and Gα$_{i2}$ were made using Stratagene QuickChange site-directed mutagenesis kit according to the manufacturer's protocols. Template pGBT9-Gα$_{i1}$ or pGBT9-Gα$_{i2}$ 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* of strain HF7cα were co-transformed with pGBT9 (containing Trp marker) and pGAD (containing Leu marker) vector constructions by standard lithium acetate procedures (CLONTECH Matchmaker two-hybrid system). Briefly, single yeast colonies were grown overnight at 30 °C with continuous shaking to an A_{600} of 0.6. Cells were harvested by centrifugation for 10 min at 3000 rpm, washed once in sterile H_20, and resuspended in 2 ml of cold 100 mM lithium acetate. After shaking at 30 °C for 1 h, 100 μl of competent cells was added to 1–2 μg of transforming DNA in the presence of 5 μg of carrier salmon sperm DNA and 0.7 ml of 40% polyethylene glycol. Cells were heat shocked at 42 °C for 15 min, then collected with a quick spin, and plated on -Leu-Trp selective dropout agar medium to grow for 3 days at 30 °C. Four colonies of each construct were streaked on -Leu-Trp agar to propagate for assay.

Immunoblotting—Yeast transformants were grown overnight to high density in 4-ml cultures, harvested, and resuspended in binding buffer (0.2 M Tris, pH 8.0, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 20 μg/ml pepstatin A). Cells were lysed by vortexing three times for 1 min in the presence of glass beads at 4 °C and spun for 10 min at 12,000 × *g* to remove cell debris. 50 μg 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 innoculated into 5 ml of SC-Leu-Trp agar and grown overnight to an A_{600} of 0.8. Cells were collected by centrifugation, washed once in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2P0_4, 10 mM KCl, 1 mM $MgSO_4$),

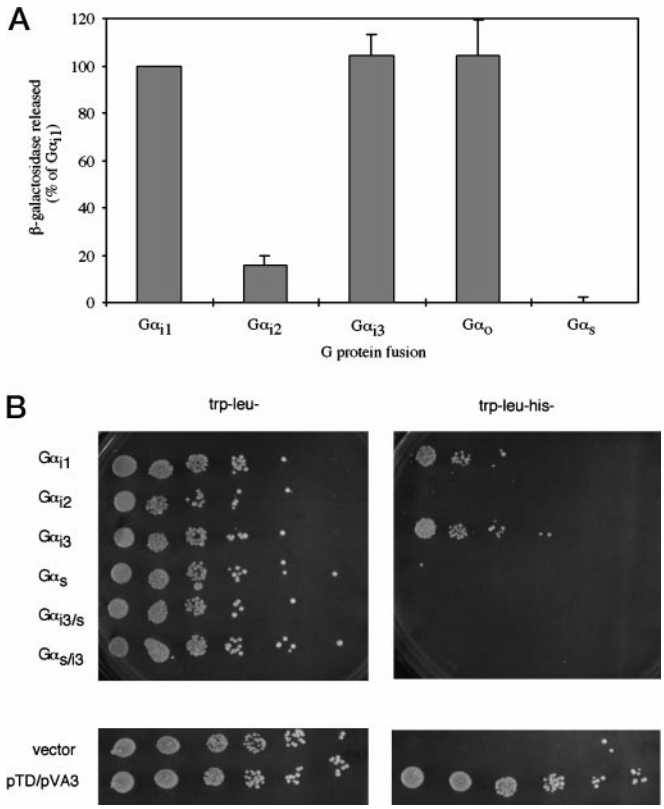

FIG. 1. *A*, liquid β-galactosidase assays of native G protein α subunit interactions with GAIP. Yeast clones co-expressing indicated Gal 4-binding domain-Gα fusions with activation domain-GAIP fusions were assayed for the interaction-dependent activation of a *lacZ* reporter. The amount of β-galactosidase released was measured colorimetrically using the substrate ONPG. Two clones of each transformant were assayed and normalized to the interaction of GAIP with Gα$_{i1}$ defined as 100%. The results shown are the mean ± S.E. for n = 4–18 in triplicate. *B*, histidine-minus growth assays of native *versus* mutant G protein α subunit interactions with GAIP. Yeast clones co-expressing indicated binding domain-Gα fusions with activation domain-GAIP were assayed for their interaction-dependent activation of a histidine reporter. Each clone was grown and plated as detailed under "Experimental Procedures." Plates on the *left* show limiting dilutions of clones grown on tryptophan- and leucine-lacking agar medium to control for noninteraction dependent growth. Plates on the *right* show identical dilutions of clones grown on tryptophan-minus, leucine-minus, and histidine-lacking medium to assay for interaction-dependent histidine reporter expression. This assay has been performed twice with identical results.

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 Na_2CO_3 and read at A_{420} after spinning out cell debris. β-Galactosidase units (21) were calculated according to the manufacturer's protocols (CLONTECH), as follows: β-galactosidase units = $1000 \times A_{420}/(t \times v \times A_{600})$, where t is 120 min of incubation, v is 0.1 ml of reaction volume·concentration factor, and A_{600} was 0.8 for the culture.

Histidine Growth Assays—5-ml cultures of yeast transformants were grown to an A_{600} 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 Gα$_{i1}$, Gα$_{i2}$, Gα$_{i1}$(D229A), and Gα$_{i2}$(A230D) and full-length GAIP were expressed as GST fusion proteins by subcloning cDNAs downstream of the GST tag using *Eco*RI/*Sal*I 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

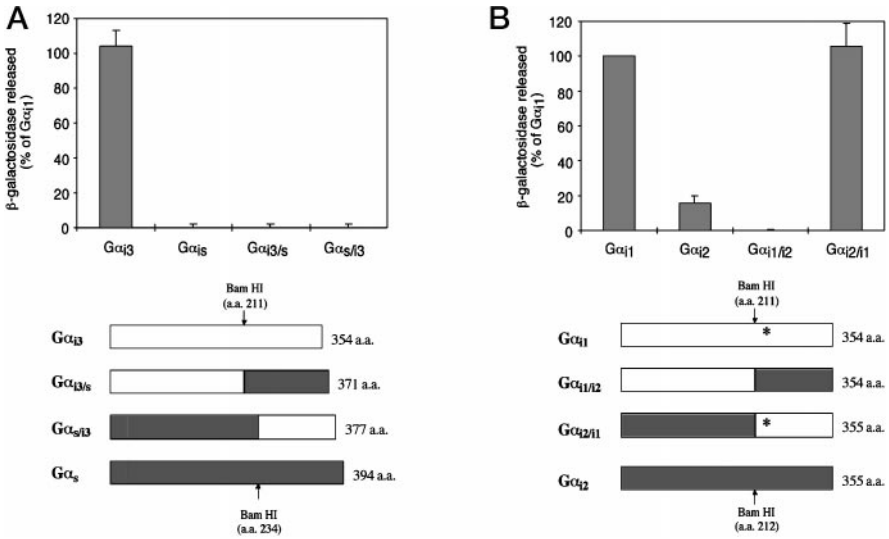

FIG. 2. **Relative interaction of Gα chimeras with GAIP.** Liquid β-galactosidase assays were conducted as described in the legend to Fig. 1*A*. Two clones of each transformant were assayed. The results shown are the means ± S.E. for n = 2–18 in triplicate. *A*, interaction-dependent release of β-galactosidase from clones expressing chimeras of Gα$_{i3}$ and Gα$_s$. A schematic diagram of the chimeras is shown at the *bottom*. *B*, interaction-dependent release of β-galactosidase from clones expressing chimeras of Gα$_{i1}$ and Gα$_{i2}$. A schematic diagram of these chimeras is shown at the *bottom*, where the *asterisk* indicates the position of Gα$_{i1}$ Asp^{229}.

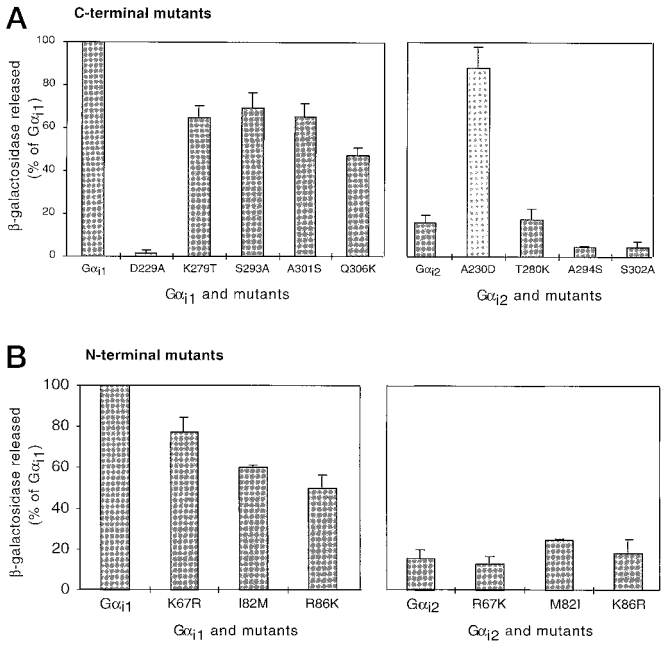

FIG. 3. *A*, relative interaction of C-terminal point mutants with GAIP. *B*, relative interaction of N-terminal point mutants with GAIP. Liquid β-galactosidase assays were conducted as described in the legend to Fig. 1*A*. Two clones of each transformant were assayed. The results shown are the means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

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 via Coomassie-stained SDS-polyacrylamide gel electrophoresis, and in-frame translation of G proteins was verified via immunoblot using a Gα$_{i1}$/Gα$_{i2}$-selective antibody (kind gift of Dr. David Manning, University of Pennsylvania, Philadelphia, PA).

GTPγS 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 [^{35}S]GTPγS and serial dilutions of 1–100 μM competing unlabeled GTPγS in 50 μl of binding buffer (50 mM HEPES, pH 8, 1 mM EDTA, 2 mM β-mercaptoethanol, 10 mM $MgSO_4$, 2 mM ATP, 30% glycerol, 1 mg/ml bovine serum albumin) (22). Reactions were filtered over BA85 nitrocellulose filters and washed three times with 2 ml of cold GTPγS STOP buffer (20 mM Tris-Cl, pH 8, 25 mM $MgCl_2$, 100 mM NaCl). Filters were immersed overnight in scintillation fluid before counting to determine amount of [^{35}S]GTPγS bound.

GTPase Assays—100 nM purified GST-tagged G protein α subunits were loaded with 1 μM [γ-^{32}P]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 6 °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 $NaPO_4$, pH 3. To initiate the reaction, 100 μM cold GTP and 15 mM $MgSO_4$ (final concentrations) in the presence *versus* absence of 500 nM GST-tagged GAIP 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.

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$_i$ 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 Gal 4-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 Gal 4-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 estimated from the relative activation of Gal 4-dependent reporters. The yeast strain HF7Cα was stably transformed with cDNAs encoding both β-galactosidase and histidine reporters downstream of a Gal 4 promotor. In this system, the promotor is activated in proportion to the degree of interaction between the Gal 4-binding domain and activation domain fusions (23). Thus, two different reporters

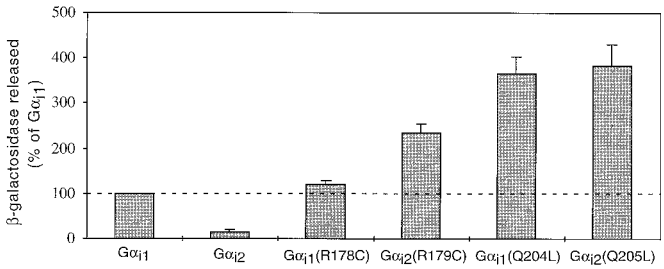

FIG. 4. **Relative interaction of GTPase-deficient mutant *versus* native Gα subunits with GAIP.** Liquid β-galactosidase assays were conducted as described in the legend to Fig. 1*A*. Two clones of each transformant were assayed. The results shown are the means ± S.E. for $n = 6–18$ in triplicate.

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 Gα$_{i1}$, Gα$_{i3}$, and Gα$_o$, whereas the interaction with Gα$_{i2}$ was weak, and the interaction with Gα$_s$ 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 Gα$_{i1}$ gave a strong interaction with GAIP in its native conformation, which was statistically indistinguishable from that of Gα$_{i3}$ and Gα$_o$, 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 in these assays corresponds to 1.4 β-galactosidase units (21). The β-galactosidase activity generated by GAIP co-transfected with pGBT9 vector alone (0.12 β-galactosidase units) was considered background and was subtracted from all values for G protein-GAIP interactions before normalization.

Chimeras—Because Gα$_{i3}$ interacted strongly with GAIP, whereas Gα$_s$ did not interact at all, chimeras of Gα$_{i3}$ with Gα$_s$ were generated in an attempt to localize the regions of Gα$_i$ required for GAIP binding. A *Bam*HI site that cuts both cDNAs roughly two-thirds into the length of the coding region was used to generate both chimeras (Fig. 2). This *Bam*HI site conveniently separates all of the N-terminal α-helical domain from most of the GTPase domain (a small part of which is encoded at the very N terminus of the cDNA). The binding characteristics of these chimeras could thus substantiate the relative importance of these two domains in GAIP binding. However, neither chimera bound to GAIP (Fig. 2*A*). These results potentially indicate that both domains of Gα$_{i3}$ contribute important determinants for RGS binding, but the divergence in α$_s$ sequence from that of the α$_i$ family presents a number of other possible interpretations.

To discriminate among these possibilities, chimeras composed of the initial two-thirds of α$_{i1}$ fused to the distal one-third of α$_{i2}$ and the reciprocal Gα$_{i2/i1}$ chimera were prepared using an engineered *Bam*HI site (Fig. 2*B*). Gα$_{i2}$ is highly homologous to Gα$_{i1}$, yet its interaction with GAIP is negligible compared with Gα$_{i1}$. The Gα$_{i2/i1}$ chimera interacted with GAIP just as strongly as native Gα$_{i1}$, whereas the reverse Gα$_{i1/i2}$ chimera, like wild type Gα$_{i2}$, showed little binding to GAIP (Fig. 2*B*). These results suggest that the Gα$_{i1}$ C terminus is required for GAIP interaction. The results may also imply that the determinants contributing to GAIP binding are entirely contained within the GTPase domain of the G protein, but there may be additional determinants that are conserved between the N termini of Gα$_{i1}$ and Gα$_{i2}$ that remain to be identified.

Site-directed Mutants—As a next step, site-directed mutagenesis of Gα$_{i1}$ and Gα$_{i2}$ was used to further localize determinants contributing to the selectivity of GAIP interaction. Because Gα$_{i1}$ and Gα$_{i2}$ are 88% identical at the amino acid level but show vastly different GAIP binding capacities in the yeast two-hybrid system, the primary sequences of the two proteins were compared with identify candidate residues that might contribute to differential GAIP binding. Of the amino acids that differed between Gα$_{i1}$ and Gα$_{i2}$, reciprocal mutants were generated at eight different positions in the primary sequence based on the likelihood that a given position would affect RGS binding given its location in the three-dimensional crystal structure of Gα$_{i1}$ bound to RGS4 (11). The effects of C-terminal mutants were of particular interest due to the results of the chimeras, but a number of N-terminal mutants were also studied because they appeared to be close to potential RGS contact sites in the crystal structure (11). Of the five Gα$_{i1}$ mutants C-terminal to the *Bam*HI site that were tested, several impaired binding to GAIP, but only D229A abolished it (Fig. 3*A*). Even more significantly, the reciprocal mutation in the corresponding residue in Gα$_{i2}$ (Gα$_{i2}$(A230D))[2] produced a variant Gα$_{i2}$ that bound to GAIP as strongly as Gα$_{i1}$ (Fig. 3*A*). Thus, Gα$_{i1}$(D229) appears to be particularly important for GAIP interaction.

In addition to the C-terminal mutants shown in Fig. 3*A*, three N-terminal Gα$_{i1}$ mutants and the corresponding reciprocal Gα$_{i2}$ mutants were also assayed for β-galactosidase activity. Consistent with the results of the Gα$_{i1/i2}$ chimeras, all of the N-terminal Gα$_{i1}$ mutants bound to GAIP, and none of the corresponding Gα$_{i2}$ mutants bound GAIP as strongly as Gα$_{i1}$ (Fig. 3*B*). 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 Gα$_{i1}$ and Gα$_{i2}$ 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 Gα$_{i1}$ and Gα$_{i2}$ interacted at least as strongly with GAIP as wild type Gα$_{i1}$ (Fig. 4). Both Gα$_{i1}$(Q204L) and Gα$_{i2}$(Q205L) generated about a 4-fold increase in GAIP binding activity relative to that seen with wild type (nonactivated) Gα$_{i1}$, so that the selectivity of GAIP for Gα$_{i1}$ over Gα$_{i2}$ appears to be restricted to the interaction with their ground state (presumably GDP-bound) conformations. Two additional GTPase-deficient mutants, Gα$_{i1}$(R178C) and Gα$_{i2}$(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 Gα$_{i1}$ over Gα$_{i2}$ in their GDP-bound states, the position of Gα$_{i1}$ 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 GTPγS affinities of both Gα$_{i1}$ and Gα$_{i2}$ to determine whether there were differences in nucleotide binding affinity that in turn might affect their affinities for GAIP. Recombinant full-length Gα$_{i1}$, Gα$_{i2}$, Gα$_{i1}$(D229A), and Gα$_{i2}$(A230D) were GST-tagged, expressed in bacteria, and purified to homogeneity over glutathione affinity columns. The ability of unlabeled GTPγS to displace [^{35}S]GTPγS from each of the proteins was measured over a range of GTPγS concentrations. The IC$_{50}$ for [^{35}S]GTPγS dis-

[2] A one-residue insertion at amino acid 117 in the primary sequence of Gα$_{i2}$ with respect to Gα$_{i1}$ is responsible for the difference in numbering between these two α subunits.

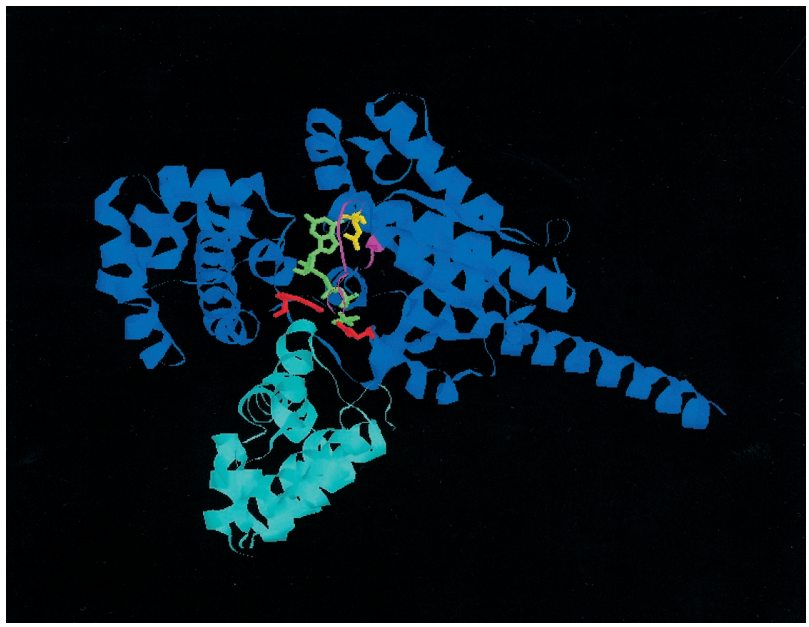

FIG. 5. **Position of Gα^{i1} Asp^{229} in relation to bound RGS4 and GDP-Mg^{2+}-AlF$_4$ molecules.** PDB 1AGR (2) showing the cocrystallization of Gα$_{i1}$ with RGS4 was downloaded from the Brookhaven National Labs Protein Data Bank and viewed using RasMol. The Gα$_{i1}$ subunit is shown in *dark blue* bound to a *cyan* RGS4 molecule. GAIP binding specificity determinant Gα$_{i1}$(Asp^{229}) is pictured in *yellow* at the top of the *pink* Switch 3 region of Gα$_{i1}$. The bound GDP-AlF$_4$ is the adjacent structure in *green*. Gα$_i$ residues Arg^{178} and Gln^{204} are highlighted in *red*.

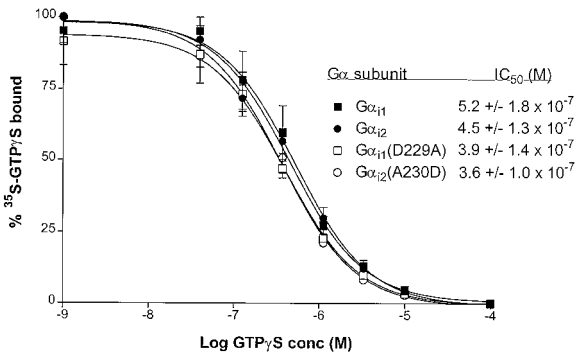

FIG. 6. **Competition between GTPγS and ^{35}S-GTPγS for binding to native and mutant G protein α subunits.** Purified GST-tagged G protein α subunits (100 nM) were incubated with 100 nM [^{35}S]GTPγS (200,000 cpm/50 μl assay volume) and indicated concentrations of unlabeled GTPγS and filtered as described 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 Gα$_{i1}$, Gα$_{i2}$, Gα$_{i1}$(D229A), and Gα$_{i2}$(A230D). GAIP catalyzed a 5-fold increase in the rate of GTP hydrolysis by Gα$_{i1}$ (Fig. 7*A*) but caused no increase in the GTPase rate of Gα$_{i2}$ (Fig. 7*B*) under the same conditions. In addition, GAIP only slightly increased the GTPase activity of Gα$_{i1}$(D229A) (from K_{obs} of 2.1 in the absence of GAIP to K_{obs} of 3.7 in the presence of GAIP) (Fig. 7*C*). Of particular interest, the rate of GTP hydrolysis seen for this mutant form of Gα$_{i1}$ in the presence of GAIP is similar to the GTPase rate of Gα$_{i2}$ in the presence of GAIP (K_{obs} = 4.2). Similarly, Gα$_{i2}$(A230D) now behaves more like Gα$_{i1}$ 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 Gα$_{i1}$ in the presence of GAIP (K_{obs} = 5.2 for the former and 5.6 for the latter) (Fig. 7*D*). Therefore, the ability of GAIP to act as a GAP for these two Gα$_i$ 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 Gα 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 Gα 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 Gα have important implications for the mechanism by which RGSs stimulate α subunit GTPase activity. The observation that RGS4 binds more strongly to the AlF$_4$-GDP-Mg^{2+}-bound state of Gα$_i$ than to the GDP or GTP-bound states suggests that RGSs exhibit GAP activity by stabilizing the transition state for GTP hydrolysis by Gα (7–9, 27, 28). The crystal structure of AlF$_4$-GDP-Mg^{2+}-Gα$_i$ bound to RGS4 further reveals that the RGS interacts directly with the Switch regions of Gα$_i$, 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 Gα to which RGS proteins bind may interfere with the binding of the effector PLCβ1, suggesting another possible mechanism for Gα$_i$ 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 Gα$_{i3}$, but a chimeric Gα$_q$ containing the last 10 residues of Gα$_{i3}$ 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 Gα$_o$ and the equivalent mutation in Gα$_{i1}$ 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 Gα interactions with RGS molecules rather than a determinant for specificity. Finally, Natochin and Artemyev (32) showed that the interaction of Gα$_t$

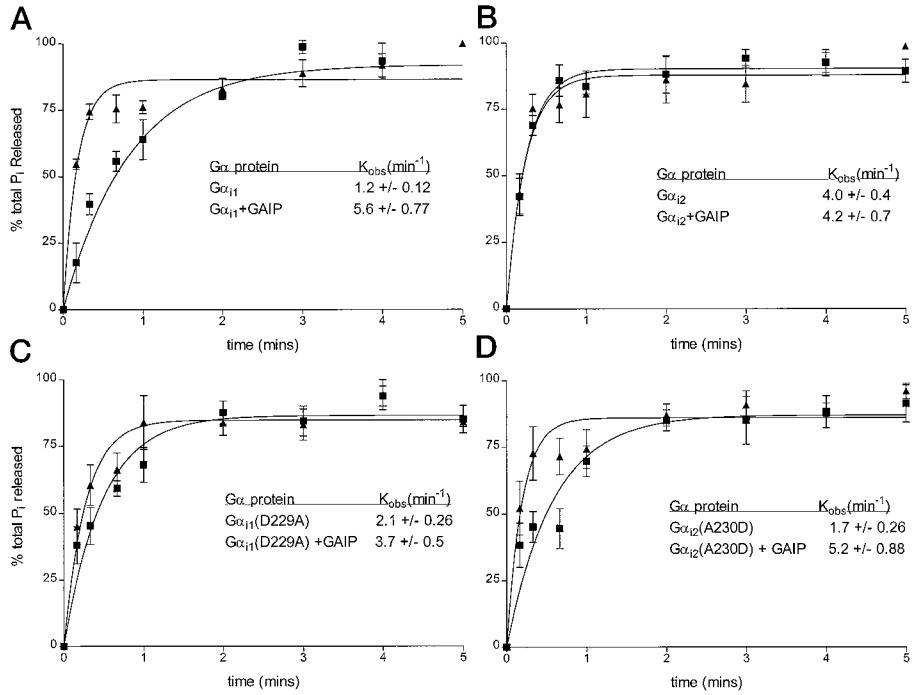

FIG. 7. **Effect of GAIP on single turnover GTPase activity of purified GST-tagged native and mutant Gα subunits.** *Squares*, G protein alone; *triangles*, G protein in the presence of GAIP. GST-GAIP (500 nM) was added to 100 nM [^{32}P]GTP-loaded Gα subunits in the presence of Mg^{2+} and excess unlabeled GTP to initiate reactions. *A*, Gα$_{i1}$; *B*, Gα$_{i2}$; *C*, Gα$_{i1}$(D229A); *D*, Gα$_{i2}$(A230D). Aliquots were removed at the indicated times, and free $^{32}P_i$ released was measured. An average of 470 fmol $^{32}P_i$ was released per assay, which was normalized to 100%. Values given are the means of four experiments for *A*, *B*, and *C* and the means of six experiments for *D*. The observed rate constants (K_{obs}) for each reaction were calculated based on an exponential association curve fit using GraphPad Prism.

with human retinal RGS could be abolished by mutating serine 202 to the corresponding Gα$_s$ aspartate, providing one candidate Gα$_s$ site that might interfere with RGS binding. They recently extended this finding by showing that mutation of this Gα$_s$ aspartate (Gα$_s$ Asp^{229}) to the serine which occurs in Gα$_i$ 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 Gα 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 Gα$_{i1}$, Gα$_{i3}$, and Gα$_o$ but very weakly with Gα$_{i2}$ and not at all with Gα$_s$. Both Gα$_{s/i3}$ and Gα$_{i3/s}$ chimeras disrupted GAIP binding, indicating either that both the N and C termini of the Gα$_i$ subunit contain determinants required for binding or that divergent sequences in the Gα$_s$ protein relative to Gα$_i$ may interfere with GAIP contact points. Gα$_{i1/i2}$ and Gα$_{i2/i1}$ mutants gave more interpretable results, indicating that the C-terminal domain of Gα$_{i1}$ 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 Gα$_s$ chimera to bind may indicate that N-terminal inserts in the Gα$_s$ sequence (such as amino acids 72–86) relative to Gα$_i$ interfere with the RGS-Gα binding surface or that other divergent residues in the Gα$_s$ N-terminal portion interfere with RGS contact. The interfering aspartate (Gα$_s$ 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 Gα$_{i1}$ and Gα$_{i2}$ were swapped. Candidate residues were chosen on the basis of their conservation in Gα$_{i1}$ and Gα$_{i3}$ and divergence in Gα$_{i2}$. The mutation of aspartate 229 of Gα$_{i1}$ to the alanine present in Gα$_{i2}$ nearly abolished GAIP binding. Conversely, when aspartate was substituted for the alanine normally present at the same site in Gα$_{i2}$, the mutant Gα$_{i2}$ bound GAIP to the same extent as native Gα$_{i1}$. These results reveal the importance of aspartate 229 for the binding of Gα$_i$ subunits in their native state to GAIP and potentially suggest a site of physical contact with GAIP. Yet, upon inspection of the Gα$_{i1}$-RGS4 crystal structure, this aspartate appears quite far from the sites of RGS4 interaction. Due to the location of Gα$_{i1}$ aspartate 229 at the far N terminus of Switch 3, it is possible that the position of this amino acid in the AlF_4 transition state analogue in which it was co-crystallized with RGS4 differs from its position in the nonactivated state in which the Gα$_i$s show selectivity for binding to GAIP. That is, it may be that in its GDP-bound (ground state) conformation, Gα$_{i1}$ Asp^{229} is in closer proximity to GAIP than in its AlF_4-Mg^{2+}-GDP-bound conformation.

Closer inspection of the RGS4-Gα$_{i1}$ 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-Gα$_{i1}$ 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, IC_{50} values for the ability of GTPγS to compete [^{35}S]GTPγS binding by Gα$_{i1}$, Gα$_{i2}$, Gα$_{i1}$(D229A), and Gα$_{i2}$(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 Gα$_{i1}$ *versus* Gα$_{i2}$ in their GTP-bound forms, GTPase-deficient mutants of both Gα$_{i1}$ and Gα$_{i2}$ were engineered and tested for GAIP binding in the yeast two-hybrid system. Interestingly, both Gα$_{i1}$(Q204L) and Gα$_{i2}$(Q205L) exhibited similarly high binding affinities to GAIP (about four times the native Gα$_{i1}$ interaction), consistent with an inability by GAIP to discriminate between the two proteins in their GTP-bound states. The Gα$_{i1}$(R178C) and Gα$_{i2}$(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 states, 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 $G\alpha_{i1}$ over $G\alpha_{i2}$, namely that there is a greater population of GTP-bound $G\alpha_{i1}$ than GTP-bound $G\alpha_{i2}$ 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 Gα 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 $G\alpha_i$s that is recognized by GAIP only in their nonactivated states.

To determine whether the ability of GAIP to discriminate between $G\alpha_{i1}$ and $G\alpha_{i2}$ 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 $G\alpha_{i1}$ but not $G\alpha_{i2}$ under similar conditions. Furthermore, as predicted by the binding studies, $G\alpha_{i1}$(D229A) was a poor substrate for GAIP GAP activity compared with native $G\alpha_{i1}$, and $G\alpha_{i2}$(A230D) was comparable with $G\alpha_{i1}$ as a substrate for GAIP GAP activity. Although Berman *et al.* (7) showed GAIP-catalyzed increases in GTPase activity of both $G\alpha_{i1}$ and $G\alpha_{i2}$, Heximer *et al.* (17) also showed a greater enhancement by GAIP of $G\alpha_{i1}$ over $G\alpha_{i2}$ GTPase activity. Our results indicate that GAIP preferentially enhances $G\alpha_{i1}$ over $G\alpha_{i2}$ GTPase activity and that this activity correlates with the binding selectivity shown for $G\alpha_i$s in their ground state conformations. In addition, because GTPase-deficient mutants of both α_{i1} and α_{i2} subunits bind tightly to GAIP, these results may imply that GAP binding is not sufficient for GAP catalytic activity. Indeed, differential effects on Gα 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 Gα 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.

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Structural Basis for the Selectivity of the RGS Protein, GAIP, for $G\alpha_i$ Family Members: IDENTIFICATION OF A SINGLE AMINO ACID DETERMINANT FOR SELECTIVE INTERACTION OF Gαi SUBUNITS WITH GAIP

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