Interdomain Interactions Regulate GDP Release from Heterotrimeric G Proteins†

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ABSTRACT: The role of interdomain contact sites in basal GDP release from heterotrimeric G proteins is unknown. $G_{\alpha o}$ and $G_{\alpha i1}$ display a 5-fold difference in the rate of GDP dissociation with half-times of 2.3 ± 0.2 and 10.4 ± 1.3 min, respectively. To identify molecular determinants of the GDP release rate, we evaluated the rate of binding of the fluorescent guanine nucleotide 2′(3′)-*O*-(*N*-methyl-3′-anthraniloyl)-guanosine 5′-*O*-(3-thiotriphosphate) (mGTPγS) to chimers of $G_{\alpha o}$ and $G_{\alpha i1}$. Although no one region of the G protein determined the GDP dissociation rate, when the C-terminal 123 amino acids in $G_{\alpha i1}$ were replaced with those of $G_{\alpha o}$, the GDP release rate increased 3.3-fold compared to that of wild-type $G_{\alpha i1}$. Within the C-terminal portion, modification of four amino acids in a coil between β4 and the α3 helix resulted in GDP release kinetics identical to those of wild-type $G_{\alpha o}$. Based on the $G_{\alpha i1}$-GDP crystal structure of this region, Leu^{232} appeared to form a hydrophobic contact with Arg^{144} of the helical domain. The role of this interaction was confirmed by $G_{\alpha i1}$ L232Q and $G_{\alpha i1}$ R144A which displayed 2−5-fold faster GDP release rates compared to wild-type $G_{\alpha i1}$ ($t_{1/2}$ 4.7 and 1.5 min, respectively), suggesting that interdomain bridging contacts partially determine the basal rate of GDP release from heterotrimeric G proteins.

Receptor-mediated activation of heterotrimeric GTP binding proteins is a common mechanism for transducing biological signals. Cell surface receptors regulate G proteins (composed of α, β, and γ subunits) by stimulating the release of GDP from the α subunit, allowing GTP to bind to and activate the G protein (for review, see ref *1*). The dissociated α-GTP and $\beta\gamma$ subunits interact with effector proteins to modulate cellular responses including second messenger metabolism and ion channel function *(2−4)*. G protein deactivation is mediated by an intrinsic α subunit GTPase activity, and α-GDP rebinding to the $\beta\gamma$ subunits. Because GDP dissociates slowly, the α subunit remains in the GDP-liganded inactive form until interaction with an agonist-bound receptor promotes the release of GDP and subsequent G protein activation. The spontaneous GDP release rate corresponds to a tonic activation rate in the absence of activated receptors.

The G protein α subunit, composed of 354 amino acids in $G_{\alpha i1}$ and $G_{\alpha o}$, is made up of 2 domains: a GTPase domain that resembles the protooncogene protein $p21^{ras}$, and a helical domain (spanning residues 63−174 in $G_{\alpha i1}$) consisting of α helices and connecting loops. The bound guanine nucleotide is buried between these two domains in crystal structures of the G proteins transducin ($G_{t\alpha}$) *(5)* and $G_{\alpha i1}$ *(6)*. The solvent inaccessibility of the guanine nucleotide suggests that conformational changes separating the two G protein domains must occur to exchange GDP for GTP. Although the importance of interdomain contact sites has been demonstrated in receptor- *(7)* and GDP/aluminum fluoride-mediated activation *(8)*, the role of interdomain contact sites in basal GDP release is unknown.

The rate of GDP release is a critical factor in determining activation of G proteins. It is particularly relevant to the spontaneous activity of G proteins in cells. We wanted to determine which portion of the G protein molecule determines the rate of GDP release. $G_{\alpha o}$ and $G_{\alpha i1}$ amino acid sequences are 72% identical with even higher homology, yet $G_{\alpha i1}$ has a 5-fold slower rate of GDP release. The amino acids that directly contact the bound guanine nucleotide are identical in $G_{\alpha i1}$ and $G_{\alpha o}$ *(5)*, suggesting that rate differences are the result of subtle differences in the position of residues with respect to nucleotide *(9)*. Both the N- and C-termini of Gα subunits have been implicated in the regulation of GDP release. A 14 amino acid C-terminal deletion mutant of $G_{\alpha o}$ resulted in a large decrease in affinity for GDP with little change in that for GTPγS[1] *(10)*. Both crystal structure and mutagenesis data identify C-terminal amino acids that contact the N-terminal region of the α subunit at the start of β strands 1 and 3 in the GTPase domain. Neer and colleagues have shown that the N-terminal halves of $G_{\alpha i2}$ and $G_{\alpha o}$ (amino acids 1−212) determine the apparent affinity for GDP *(9)*. Disruption of the C-terminal helix contacts with the N-terminus may result in decreased GDP affinity. In addition, mutation of Cys^{325} of $G_{\alpha o}$ to Ala resulted in a 10-fold

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[1] Abbreviations: DTT, dithiothreitol; mGTPγS, 2′(3′)-*O*-(*N*-methyl-3′-anthraniloyl)guanosine 5′-*O*-(3-thiotriphosphate); His_6G_o, hexa-histidine-tagged G_o protein α subunit; $His_6G_{\alpha i1}$, hexa-histidine-tagged $G_{\alpha i1}$ protein α subunit; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); $T_{50}\beta_{20}P_{0.1}$, 50 mM Tris-HCl, pH 8.0, 20 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride; HEDNML, 50 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$, 20 ppm deionized Lubrol; MANT, *N*-methyl-3′-anthraniloyl.

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decrease in GDP affinity relative to wild type, without changing the affinity for GTPγS *(11)*. Furthermore, an A366S mutation in G protein α_s, homologous to Ala^{326} in $G_{\alpha i1}$, resulted in accelerated release of GDP from the guanine nucleotide binding site and is responsible for the testotoxicosis and hypoarathydroidism phenotype *(12)*.

Chimers of $G_{\alpha o}$ and $G_{\alpha i1}$ were prepared using unique restriction sites in the cDNA at codons 59 and 232 in $G_{\alpha o}$ (codons 59 and 231 in $G_{\alpha i1}$). The N-terminal 55 amino acids of $G_{\alpha i1}$ are in the GTPase domain, and the helical domain spans residues 59−177. Thus, by evaluating the rate of GDP release from the $G_{\alpha o}$ and $G_{\alpha i1}$ chimers, we are able to evaluate whether the N-terminus, middle, and C-terminus determine the rate of GDP release.

Here we use the mGTPγS binding assay to evaluate the rates of GDP release from both wild-type $G_{\alpha o}$ and $G_{\alpha i1}$ and $G_{\alpha o}/G_{\alpha i1}$ chimers. The half-time for mGTPγS binding to wild-type $G_{\alpha o}$ was 5-fold faster than that for $G_{\alpha i1}$. All chimers displayed mGTPγS binding rates faster than that of wild-type $G_{\alpha i1}$, suggesting that multiple regions and contacts are responsible for the slow rate of GDP release from $G_{\alpha i1}$. When the C-terminal region of $G_{\alpha i1}$ was replaced with that of $G_{\alpha o}$, the rate of GDP dissociation increased 3.3-fold. Specifically, it appears that an interdomain hydrophobic contact site between Arg^{144} and Leu^{232} of $G_{\alpha i1}$ contributes to the slower rate of basal guanine nucleotide binding.

MATERIALS AND METHODS

Materials. Dithiothreitol (DTT) and guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) as well as antibodies AS/7 (directed against the $G_{\alpha i1}$ C-terminus) and GC/2 (directed against the $G_{\alpha o}$ N-terminus) were purchased from CalBiochem (San Diego, CA). [^{3}H]GDP, [^{35}S]GTPγS, and [γ-^{32}P]-GTP were obtained from New England Nuclear (Boston, MA). Antibodies against amino acids 94−105 of $G_{\alpha o}$ (antibody 2353) and amino acids 159−167 of $G_{\alpha i1}$ (antibody 3646) were kindly provided by Dr. David Manning (University of Pennsylvania) *(13)*. Ni^{2+}-NTA resin was from Qiagen (Chatsworth, CA). *N*-Methyl-3′-*O*-anthraniloyl-GTPγS was synthesized and purified as described *(14)*. The rat G_α expression vectors pQE/$G_{\alpha o}$ and the hexa-histidine-tagged pQE60/$G_{\alpha i1}$ were generously provided by Dr. M. E. Linder (Washington University).

Chimer Preparation and Mutagenesis. Chimers were prepared by splicing $G_{\alpha o}$ and $G_{\alpha i1}$ at unique restriction sites in the cDNA at codons 59 and 232 in $G_{\alpha o}$ (codons 59 and 231 in $G_{\alpha i1}$) using *Bst*XI and *Sex*AI restriction enzymes, respectively. His_6-tagged $G_{\alpha i1}$ and $G_{\alpha o}$ expression vectors, with an internal *Nco*I site removed from the $G_{\alpha o}$ sequence *(15)*, were cut with *Nco*I at the ATG start codon, *Bst*XI, *Sex*AI, or *Hin*dIII. All restriction enzyme digest reactions with *Sex*AI were performed on plasmids purified from the JM110 strain of bacteria which is *dcm*$^-$. The appropriate pieces were ligated to generate expression vectors coding for the WT and chimer Gα proteins shown in Figure 2. Additional chimers and mutations (Figure 3A) were made to the C-terminal portion of $G_{\alpha i1}$ by overlap−extension PCR using the following sense and antisense mutagenic primers, respectively:

chimer 1: $G_{\alpha i1}(232-240)G_{\alpha o}$ ($G_{\alpha i1}$L232Q/A235H/E239T/M240T)

5′-CAAGTACTACATGAGGACGAGACCACGA-ACCGGATGCATGAAAC-3′

5′-CGTGGTCTCGTCCTCATGTAGTACTTGGT-CATAGTCACTCAGGGCCACACT

chimer 2: $G_{\alpha i1}$ A291P

5′-CCAGGATCCAACACCTATGAAGAGGCGG-CTGCGTAT-3′

5′-TCATAGGTGTTGGATCCTGGATATTCTGG-ATAGCATATCGTGAG-3′

chimer 3: $G_{\alpha i1}(310-317)G_{\alpha o}$

5′-AGCAAGAACCGCTCACCTAACAAGGAA-ATTTACACCCACTTCAC-3′

5′-GTTAGGTGAGCGGTTCTTGCTTTCAAAC-TGACACTGGATATAC-3′

chimer 4: $G_{\alpha i1}(330-334)G_{\alpha o}$

5′-CAACATCCAGGTGGTCTTCGATGCTGTA-ACGGACGTCATCA-3′

5′-AAGACCACCTGGATGTTGTTCGTATCCG-TCGCGCAAGT-3′

$G_{\alpha i1}$ L232Q:

5′-CCAGGTTCTTGCTGAGGATGAAG-3′

5′-CTCAGCAAGAACCTGGTCATAGTCACTC-AGG-3′

$G_{\alpha i1}$ R144A:

5′-CAACAGATCCGCGGAGTACCAGCTGAACG-3′

5′-GGTACTCCGCGGATCTGTTGAAGCAGGC-3′

The sequence of all G_α mutants was confirmed by dideoxy DNA sequencing over the PCR-amplified region.

Expression and Purification of His$_6$-Tagged $G_{i1\alpha}$ and $G_{o\alpha}$ Chimers and Mutants. $His_6G_{\alpha i1}$ and $His_6G_{\alpha o}$ proteins were purified by a modification of the method of Lee et al. *(16)*. The cDNA of hexa-histidine-tagged G_α in the expression vector pQE60 was transformed into the *E. coli* strain BL21/DE3. The cells were grown in 1 L of enriched medium (2% tryptone, 1% yeast extract, 0.5% NaCl, 0.2% glycerol, and 50 mM KH_2PO_4, pH 7.2) in the presence of 50 μg/mL ampicillin at 30 °C up to an OD_{600} of 0.4 and then incubated for 18 h with IPTG (30 μM) and chloramphenicol (1 μg/mL). The bacterial cell pellet was frozen in liquid nitrogen, resuspended in $T_{50}\beta_{20}P_{0.1}$ (50 mM Tris-HCl, pH 8.0, 20 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride), and incubated with 0.1 mg/mL lysozyme, 0.02 mg/mL DNase, and 5 mM $MgSO_4$. The lysate was centrifuged at 4 °C for 30 min at 30 000 rpm (Beckman Ti 45 rotor). The supernatant was then applied to a 2 mL Ni^{2+}-NTA column (Qiagen) equilibrated with $T_{50}\beta_{20}P_{0.1}$ containing 100 mM NaCl. The column was washed with 80 mL of $T_{50}\beta_{20}P_{0.1}$ containing 500 mM NaCl and 10 mM imidazole. Proteins were eluted with $T_{50}\beta_{20}P_{0.1}$ containing 100 mM NaCl, 10% glycerol, and 100 mM imidazole. The protein in the peak fractions was further subjected to anion exchange chromatography on a Mono Q HR5/5 (Pharmacia Biotech) column

using a BioCAD SPRINT System (Perspective Biosystems, Inc., Framingham, MA). The fractions of His_6G_α proteins eluted in 25 mM Tris, 25 mM Bis Tris Propane, pH 8.0, and 20 mM NaCl buffer were concentrated by Centricon-30 (Amicon, Inc., Beverly, MA), and stored at −80 °C. GDP (1 μM) was added to purified proteins before freezing. The total amount of purified proteins was measured by Bradford assay *(17)*, and the amounts of active His_6G_α proteins were determined by [^{35}S]GTPγS binding *(18)*. Gα proteins were nearly 100% pure based on SDS gel electrophoresis and had specific activities of 9−18 pmol of [^{35}S]GTPγS binding/μg of protein with the exception of three of the mutants. The GiGoGi chimer had a specific activity of 1.2 pmol/μg. The two point mutants which did not express as well as the chimers had specific activities of 1.45 and 0.21 pmol/μg for Gi-R144A and Gi-L232Q, respectively. Even for partially purified preparations, the mant-GTPγS fluorescence method provides a highly reproducible measurement of nucleotide binding (ref *14* and see error bars in figures). The identities of the WT and chimer His_6G_α proteins were verified using antibodies recognizing the $G_{\alpha i1}$ C-terminus (AS/7) and the $G_{\alpha o}$ N-terminus (GC/2), as well as internal regions of the protein sequence using antibodies against amino acids 94−105 of $G_{\alpha o}$ (antibody 2353) and amino acids 159−167 of $G_{\alpha i1}$ (antibody 3646).

GDP Binding Affinity. Ten nanomolar His_6G_α proteins was incubated with 30−1500 nM [^{3}H]GDP in HEDNML buffer in the presence or absence of 2 μM GTPγS (to determine the nonspecific binding). Following incubation at 20 °C for 1 h, the samples were filtered onto BA85 nitrocellulose filters (Schleicher & Schuell, Keene, NH), and the filters were washed with ice-cold 20 mM Tris, 100 mM NaCl, and 25 mM MgCl, pH 8.0. The filters were counted in 4 mL of ScintiVerse scintillation cocktail (Fisher Scientific, Fair Lawn, NJ). The data were fit by nonlinear least-squares analysis with a hyperbolic binding function using GraphPad Prism (San Diego, CA).

Rate of GDP Dissociation. Purified G proteins have GDP bound to them, and GDP dissociation is the rate-limiting step for nucleotide binding *(19)*. We measured the rate of GDP release by observing the fluorescence increase observed upon the binding of *N*-methyl-3′-*O*-anthraniloyl-GTPγS (mGTPγS) to $G_{\alpha o}$ and $G_{\alpha i1}$. The *N*-methyl-3′-*O*-anthraniloyl moiety attached to the 2′ or 3′ position in the ribose ring is an environmentally sensitive fluorescent probe that displays increased fluorescence upon binding to heterotrimeric G proteins *(20)*. The large fluorescence increase is due, in part, to resonance energy transfer from tryptophans in the G protein to the bound guanine nucleotide *(15)*. The rate of mGTPγS fluorescence increase upon addition to Gα is identical to the GDP release rate *(20)*. Thus, this fluorescence assay, which is useful for detection of both purified and partially purified G proteins *(14)*, provides a rapid and accurate means to evaluate GDP release rates.

Fluorescence measurements were determined using a PTI ALPHASCAN fluorometer (Photon Technology Intl., Monmouth Junction, NJ) with a water-cooled 150 W xenon arc lamp as described *(14)*. His_6G_α proteins were diluted into buffer containing 50 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 10 mM $MgSO_4$, and 20 ppm of deionized Lubrol (HEDNML), placed in custom-made 5 mm round quartz cuvettes in temperature-controlled (30 °C) 4-place rotating sample holders, and continuously stirred. Following a 3 min incubation of 100 nM Gα and 100 nM GDP at 30 °C in HEDNML, 500 nM mGTPγS was added and fluorescence monitored (excitation 280 nm, emission 440 nm) for 25 min. The association of mGTPγS was detected by exciting His_6G_α tryptophans at 280 nm and detecting MANT emission at 440 nm. The concentration of mGTPγS was saturating (500 nM). The time-dependent fluorescence values were fit to a one-component exponential association curve using Prism (Graphpad Software, San Diego, CA).

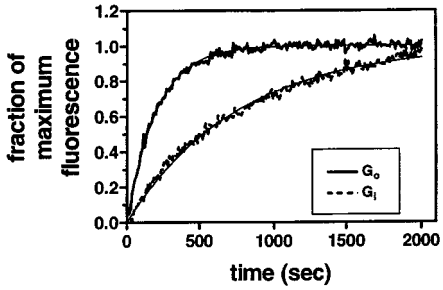

FIGURE 1: Measurement of GDP release rate from $G_{\alpha o}$ and $G_{\alpha i1}$ using mGTPγS. Following a 3 min incubation of 100 nM Gα and 100 nM GDP at 30 °C in HEDNML, 500 nM mGTPγS was added and fluorescence monitored (excitation 280 nm, emission 440 nm) for 25 min as described under Materials and Methods. Background fluorescence of protein and mGTPγS alone was subtracted; the data were normalized and fit to a monoexponential association curve for $G_{\alpha o}$ (solid line) and $G_{\alpha i1}$ (dashed line). Shown is a representative measurement repeated 12 times with half-times of 2.3 ± 0.2 and 10.4 ± 1.3 min for $G_{\alpha o}$ and $G_{\alpha i1}$, respectively.

RESULTS

The real-time acquisition of mGTPγS fluorescence increase provides a simple means to measure rates of nucleotide binding which reflects the rate of GDP release *(19, 20)*. The rate constants for the mGTPγS fluorescence increase at 30 °C for $His_6G_{o\alpha}$ and $His_6G_{i1\alpha}$ are 0.3 and 0.067 min^{-1}, respectively (half-times of 2.3 ± 0.2 and 10.4 ± 1.3 min, Figure 1). These values are similar to those previously reported by Ferguson et al. (ref *19*; see Discussion). We used this 5-fold difference in rate to identify regions of the G protein α subunit that determine the basal GDP release rate.

Go/Gi chimers allowed us to evaluate the following amino acid segments: 1−58, 59−231/230, and 232/231−354 in $G_{o\alpha}$ and $G_{\alpha i1}$, respectively. All of the chimers were able to bind GDP and GTPγS (Figure 2). The GDP binding affinity varied approximately 10-fold among the chimers but did not correlate with the apparent rate of GDP release, suggesting that the mutations altered both association and dissociation rates. Although there is no simple relation between the presence of a single specific region of the G protein and the GDP release rate, some general conclusions can be made. All mutants displayed a faster rate of GDP release than wild-type $G_{\alpha i}$, suggesting that multiple regions within $G_{\alpha i}$ are required to maintain the slow GDP dissociation rate. Both the GiSGo and GoSGi chimers have rates nearly as fast as $G_{\alpha o}$, suggesting that contacts between the amino- and carboxy-terminal portions of $G_{\alpha i}$ maintain the slow GDP release rate. This is further supported by the GoGiGo chimer, where replacement of the N-terminal 58 $G_{\alpha i}$ amino acids in GoBGi with $G_{\alpha o}$ amino acids actually slowed the rate compared to the GoBGi and GiSGo chimers.

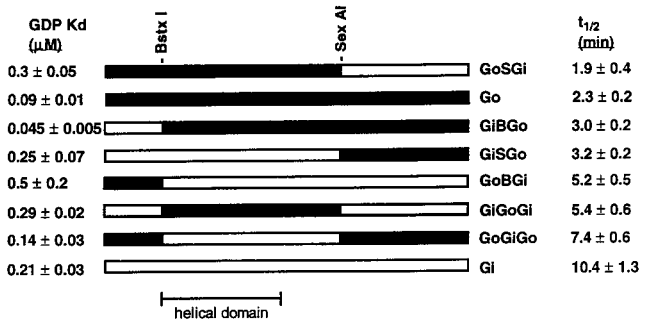

FIGURE 2: $G_{\alpha o}$ and $G_{\alpha i1}$ chimers and their GDP binding affinities and rates of GDP release. $G_{\alpha o}$ and $G_{\alpha i1}$ chimers were prepared as described under Materials and Methods, and the rates of mGTPγS binding were measured as described in the legend to Figure 1. Shown is a graphical representation of both wild-type and chimer G proteins where $G_{\alpha o}$ is represented by a filled bar and $G_{\alpha i1}$ is represented by an open bar. The $G_{\alpha o}$ and $G_{\alpha i1}$ sequence interfaces are at two restriction enzyme sites common to both G proteins: *Bst*XI at amino acid 58 in both $G_{\alpha o}$ and $G_{\alpha i1}$, and *Sex*AI at amino acid 232 in $G_{\alpha o}$ and amino acid 231 in $G_{\alpha i1}$. The helical domain is indicated below the bars. The G proteins are arranged in order of decreasing rate of mGTPγS binding. Rate measurements (n = 5−12) were made and half-times averaged, and the results (mean ± SEM) are listed on the right. All rates are significantly faster (P < 0.05) than that of wild-type $G_{\alpha i1}$. The equilibrium binding affinity of GDP was determined by saturation curves using [^{3}H]GDP as described under Materials and Methods. The K_d values from 2−4 experiments were averaged, and the mean and SEM are reported on the left side of the figure.

To further explore the specific residues involved in regulation of the GDP release rate, we noted that replacement of the C-terminal 123 amino acids of $G_{\alpha i}$ with those of $G_{\alpha o}$ (chimer GiSGo) resulted in an increase in the GDP release rate nearly to that of $G_{\alpha o}$. In this portion of the sequence (amino acids 232−354), rat $G_{\alpha i1}$ and $G_{\alpha o}$ have only 30 nonidentical amino acids of which 15 are in 4 clusters (Figure 3A). Thus, four additional chimers were prepared where the $G_{\alpha i}$ sequence was replaced with the $G_{\alpha o}$ sequence (Figure 3). We had anticipated that chimer 4, which changed amino acids 330−334 in the guanine nucleotide binding region to the $G_{\alpha o}$ sequence (chimer 4), would alter the GDP release rate, but it did not. In addition, the Ala 291 to Pro mutation, which could have strongly altered secondary structure, did not significantly increase the rate of GDP release. Replacement of the four divergent amino acids of $G_{\alpha i}$ with those of $G_{\alpha o}$ [chimer 1 ($G_{\alpha i1}$ L232Q/A235H/E239T/M240T)] resulted in a GDP dissociation rate that was identical to that of $G_{\alpha o}$! Thus, it appears that most of the differences in the GDP release rate regulated by the C-terminus are caused by one or all of the differences between the $G_{\alpha i}$ and $G_{\alpha o}$ sequences at residues $G_{\alpha i}$ Leu^{232}, Ala^{235}, Glu^{239}, or Met^{240}. We examined the location of these four amino acids in the crystal structure of GDP-liganded $G_{\alpha i1}$ *(6)*. They are in a loop of the GTPase domain between the β4 sheet and the α3 helix with direct contact to the helical domain. The main interdomain contact is between the δ-carbon methyl group of Leu^{232}, forming a hydrophobic contact with the β-carbon of Arg^{144} in the helical domain (Figure 4).

To test the importance of this potential interdomain contact site in the regulation of GDP release, we separately replaced the two contacting amino acids in $G_{\alpha i1}$ with those found in $G_{\alpha o}$ to generate $G_{\alpha i1}$ R144A and $G_{\alpha i1}$ L232Q. Both G_{i1} R144A and $G_{\alpha i1}$ L232Q had significantly faster GDP release rates than wild-type $G_{\alpha i1}$ (Figure 5). The L232Q mutation

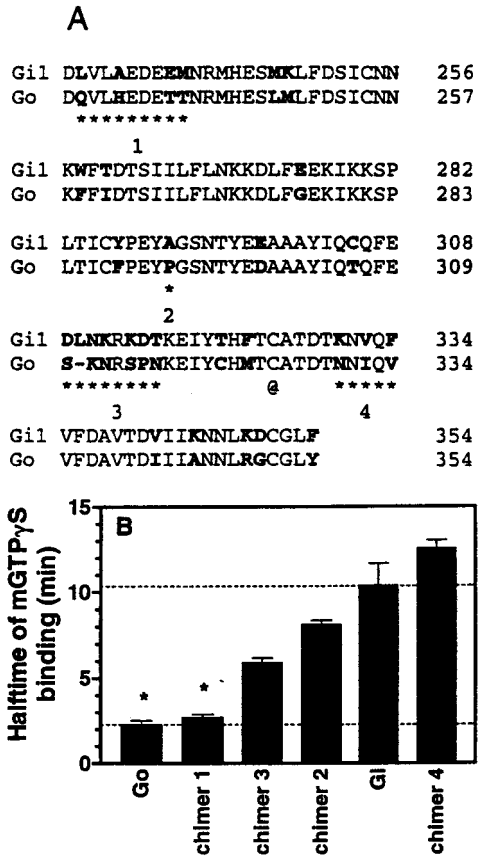

FIGURE 3: C-terminal region $G_{\alpha o}$ and $G_{\alpha i1}$ chimers and their rates of mGTPγS binding. (A) Shown is a sequence alignment of rat $G_{\alpha o}$ and $G_{\alpha i1}$ from amino acids 232/231 to 354. The amino acid sequence differences are indicated in boldface type. The locations of chimers 1−4 are labeled with asterisks where the $G_{\alpha i1}$ sequence was substituted with that from $G_{\alpha o}$. Chimer 4 is near the guanine binding region. Cys^{325} (indicated with the @), when mutated to an alanine, leads to an decrease in GDP affinity *(11)*. (B) The chimers were prepared and the rates of mGTPγS binding measured as described under Materials and Methods and in the Figure 1 legend. Shown are the mean and standard error of three rate determinations. The average rate of mGTPγS binding to the WT proteins is indicated with the dashed lines. Results of a two-tailed unpaired *t*-test indicate that chimers 2, 3, and 4 are all significantly different (P < 0.001) than $G_{\alpha o}$. Only the rates for chimer 1 (P < 0.01) and wild-type $G_{\alpha o}$ (P < 0.001) are significantly different from that of $G_{\alpha i1}$ (*) (for chimer 3, P = 0.07).

accounted for much but not all of the acceleration in the rate seen in chimer 1. Interestingly, the single point mutation R144A produced a GDP dissociation rate in $G_{\alpha i1}$ which is just as fast as that seen with $G_{\alpha o}$. These results strongly suggest that this specific hydrophobic contact between Arg^{144} and Leu^{232} is important for regulating GDP release.

DISCUSSION

In this report, we evaluate the rate of GDP dissociation from $G_{\alpha i1}$ and $G_{\alpha o}$ chimers and mutants to determine the specific amino acids that contribute to the ~5-fold slower rate of GDP dissociation from $G_{\alpha i1}$ compared to $G_{\alpha o}$. We show that the C-terminal 123 amino acids of Gα contain an important determinant of GDP release. We also propose that the molecular basis for the observed differences in $G_{\alpha i1}$ and $G_{\alpha o}$ GDP release rates is due to an interdomain hydrophobic contact between Leu^{232} and Arg^{144} in $G_{\alpha i1}$ which contributes to the relatively slow basal GDP exchange rate of $G_{\alpha i1}$.

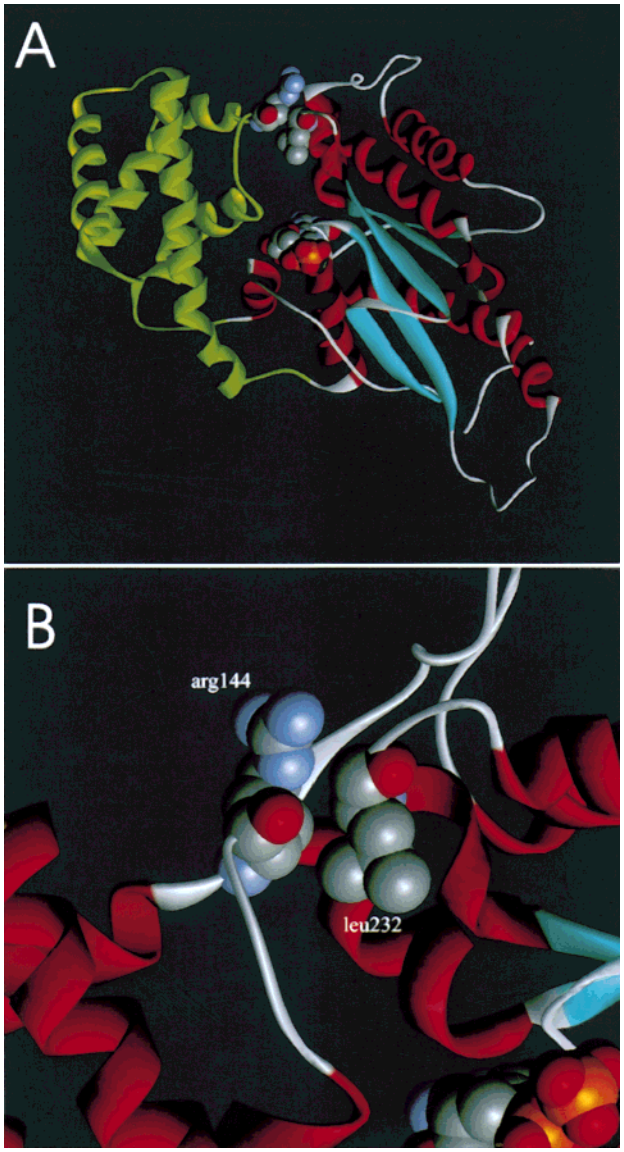

FIGURE 4: $G_{\alpha i1}$ helical and GTPase domain interface contact. (A) The helical domain (green) and GTPase domain (colored by 2° structure) of heterotrimeric G proteins form a clasp around the nucleotide binding pocket. Leu^{232} in the GTPase domain and Arg^{144} in the helical domain of $G_{\alpha i1}$ show substantial interdomain contact. The GDP, shown in space-fill, is bound between the two domains. (B) Detail of the molecular contact between Leu^{232} and the side chain of Arg^{144}. The δ-carbon methyl group of Leu^{232} forms a hydrophobic contact with the β-carbon of Arg^{144}. The GDP is partially shown in the lower right.

Role of the C-Terminus in GDP Dissociation. Mutations which alter GDP binding rates are fairly common and have been frequently identified in the carboxyl terminus of the G protein α subunits. For example, mutation of Cys^{325} in the GTPase domain of $G_{\alpha o}$ to alanine decreased GDP affinity by a factor of 10 *(11)*. The crystal structure of the analogous Cys^{321} in transducin indicates that it restricts the side chain of Thr^{323} which forms a favorable van der Waals contact with the guanine ring *(5)*. In addition, we have recently shown that mutation of Trp^{212} to Phe, which is close to the nucleotide binding site in $G_{\alpha o}$, accelerates GDP release. Based on additional kinetic data, it is likely that the Trp^{212} mutation results in a more open binding pocket *(15)*. The C-terminal chimer data described here support the hypothesis that the C terminus may block GDP dissociation by an

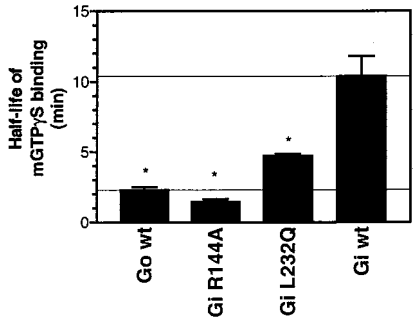

FIGURE 5: Rates of mGTPγS binding in $G_{\alpha i1}$ mutants R144A and L232Q. Two amino acids in the potential contact site between the helical and GTPase domains of $G_{\alpha i1}$ were mutated, and the rates of mGTPγS binding to the expressed proteins were measured as described under Materials and Methods and in the legend to Figure 1. Shown are the mean and SEM for three determinations of the half-time of mGTPγS binding to the mutant proteins and $n = 12$ for the WT proteins. The average rate of mGTPγS binding to the WT proteins is indicated with the dashed lines. Results from a two-tailed unpaired *t*-test indicate that both $G_{\alpha i1}$ R144A ($P = 0.002$) and $G_{\alpha i1}$ L232Q ($P = 0.029$) show significantly lower rates than WT $G_{\alpha i1}$ (asterisk).

interaction with the N terminus *(9, 10)*. First, the rates of GDP release from all of the chimers were faster than that from $G_{\alpha i1}$, suggesting that several interactions may impede GDP release. The equilibrium affinity for GDP did not correlate with the GDP dissociation rate, suggesting that the guanine nucleotide association rate also varied among the chimers. GDP release rates for chimers GoSGi and GiBGo are close to that of wild-type $G_{\alpha o}$ perhaps due to mismatch of the termini. A similar result was observed with a transducin (G_t)/$G_{\alpha i1}$ chimer where C-terminal amino acids 295−350 from transducin in a $G_{\alpha i1}$/G_t chimer were not sufficient to slow the time course of GTPγS binding as compared to wild-type $G_{\alpha i1}$ *(21)*. Replacement of the 58 N-terminal amino acids of GoSGi with those of $G_{\alpha i1}$ to create GiGoGi results in a protein with a slower GDP release rate. Possibly N- and C-termini interact to stabilize the GDP-bound state of the G protein *(9)*. If this were the only determinant of GDP release rates, however, one would predict that GoGiGo would display a $G_{\alpha o}$ phenotype, but this is not what is observed. Thus, multiple contact sites contribute to the rate of GDP release.

Regulation of GDP Release by Interdomain Contacts. The relative orientation of the helical and GTPase domains may account for differences in basal GDP release from different G proteins. $G_{\alpha i1}$ has a slightly more open structure than transducin *(6)*, which has an extremely slow rate of GDP exchange *(21)*. Although interdomain contact sites have been demonstrated to regulate G protein activation by guanine nucleotide and receptor, this is the first evidence that a hydrophobic contact between the GTPase domain and helical domain alters the basal rate of GDP dissociation. Presumably, a series of low-energy conformational changes are used to produce domain movements in proteins (for a review, see ref *22*). We found that the $G_{\alpha i1}$ mutation L232Q was mainly responsible for the fast GDP release phenotype observed for chimer 1. Based on the $G_{\alpha i1}$ crystal structure, in which this amino acid contacted Arg^{144} in the helical domain, we predicted that the mutation of Arg^{144} in $G_{\alpha i1}$ to Ala, to minimize hydrophobic contacts, would result in faster GDP release.

Berlot and colleagues constructed several $G_{\alpha s}$ and $G_{\alpha i2}$ chimers and mutants at the interface between the GTPase and helical domains and found that a $G_{\alpha s}$ and $G_{\alpha i2}$ chimer corresponding to our chimer 1 ($G_{\alpha i1}$ L232Q/A235H/E239T/M240T) resulted in reduced G protein activation by the β-adrenergic receptor *(7)*. Mutant $G_{\alpha s}$ with $G_{\alpha i2}$ residues on both sides of the domain interface restored receptor-initiated activation *(23)*. The interdomain contact sites in the G protein α subunit (containing Leu^{232} and Arg^{144} of $G_{\alpha i1}$) appear to regulate GDP release (shown here) as well as receptor-mediated G protein activation *(23)*.

Codina and Birnbaumer showed that a Asp−Lys salt bridge in $G_{\alpha s}$ between the helical and GTPase domains is required for the proper positioning of the two domains with respect to each other and that this positioning is essential for G protein activation by aluminum fluoride and GDP *(8)*. Our results raise the interesting possibility that faster GDP dissociation rates may account for the reduced ability of aluminum fluoride to activate G protein.

The regulation of GDP release in intact cells is a complex process involving, at a minimum, $\beta\gamma$ subunits and receptors in addition to the G_α subunit. Additional information on the effects of these mutations on $\beta\gamma$ and receptor-regulated release will be of interest. Unfortunately, the His_6-tagged proteins which lack an N-terminal myristoyl group have a 20−50-fold lower affinity for $\beta\gamma$ subunits[2] and do not couple to receptors at all,[3] so these questions cannot be addressed with these constructs.

Because GDP release is the rate-limiting step in G protein activation, the GDP dissociation rate should correspond to a basal activation state in the absence of receptors. This leads to the prediction that $G_{\alpha o}$ might exhibit basal coupling of calcium channel inhibition due to its relatively rapid GDP dissociation rate. Indeed, prepulse facilitation of N-type calcium channels, which is a hallmark of $\beta\gamma$-mediated channel inhibition, indicates that basal inhibition of calcium channels does occur in rat sympthetic neurons *(3)*. We have recently showed that RGS4 can increase Ca^{2+} currents in dorsal root ganglion cells (*24*), further supporting the idea of basal activity of $G_{\alpha o}$. Neither of these observations distinguishes between spontaneous GDP release from $G_{\alpha o}$ and constitutive activation of $G_{\alpha o}$ by receptors, but it raises the possibility that basal GDP release may have physiological consequences, lending added significance to our results on the structural determinants of basal GDP release.

[2] Sarvazyan and Neubig, in preparation.
[3] Lim and Neubig, unpublished observations.

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