Inhibition of GDP/GTP Exchange on Gα Subunits by Proteins Containing G-Protein Regulatory Motifs†

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ABSTRACT: A novel Gα binding consensus sequence, termed G-protein regulatory (GPR) or GoLoco motif, has been identified in a growing number of proteins, which are thought to modulate G-protein signaling. Alternative roles of GPR proteins as nucleotide exchange factors or as GDP dissociation inhibitors for Gα have been proposed. We investigated the modulation of the GDP/GTP exchange of $Gi\alpha_1$, Goα, and Gsα by three proteins containing GPR motifs (GPR proteins), LGN-585-642, Pcp2, and RapIGAPII-23-131, to elucidate the mechanisms of GPR protein function. The GPR proteins displayed similar patterns of interaction with $Gi\alpha_1$ with the following order of affinities: $Gi\alpha_1GDP \gg Gi\alpha_1GDPAlF_4^- \geq Gi\alpha_1$-GTPγS. No detectable binding of the GPR proteins to Gsα was observed. LGN-585-642, Pcp2, and RapIGAPII-23-131 inhibited the rates of spontaneous GTPγS binding and blocked GDP release from $Gi\alpha_1$ and Goα. The inhibitory effects of the GPR proteins on $Gi\alpha_1$ were significantly more potent, indicating that Gi might be a preferred target for these modulators. Our results suggest that GPR proteins are potent GDP dissociation inhibitors for Giα-like Gα subunits in vitro, and in this capacity they may inhibit GPCR/Gi protein signaling in vivo.

G-protein-coupled receptors (GPCRs)[1] respond to extracellular stimuli by activating heterotrimeric GTP binding proteins at the intracellular surface of the plasma membrane. The activation involves the GDP/GTP exchange on Gα subunits and release of GαGTP and Gβγ to interact with their effectors (*1*−*3*). Multiple mechanisms regulate the signal output at different stages of the G-protein cascades. The possibility for novel G-protein regulatory mechanisms has emerged with the identification of a new group of Gα interacting proteins. These proteins, including human mosaic protein, LGN (*4*), *Drosophila* RGS protein, LOCO (*5*), Purkinje cell protein-2 (Pcp2) (*6*), Rap1GAP (*7*), and the activator of G-protein signaling 3 (AGS3) (*8*), share conserved sequence repeats termed the G-protein regulatory (GPR) (*8*) or GoLOCO motifs (*9*) that mediate the binding to Gα subunits. The mechanism and function of the GPR-motif-containing proteins (GPR proteins) in regulation of G-protein signaling remain largely obscure. Known GPR proteins appear to selectively bind Gα subunits from the Gi family. Pcp2 and Rap1GAP were shown to interact with Goα and Giα but not Gsα or Gqα (*6*, *7*), whereas AGS3 preferentially targeted Giα and Gtα (*8*, *10*−*12*). The conformational specificity of GPR proteins remains unclear given that different GPR proteins were reported to bind favorably either to GDP-bound (*4*, *7*, *8*) or to activated Gα subunits (*13*, *14*).

The initial study on the regulation of a G-protein by a GPR protein has opened up the intriguing possibility that GPR proteins act as the guanine nucleotide exchange factors (GEFs). Pcp2 was reported to stimulate GDP release from Goα (*6*). On the other hand, the GPR domain of AGS3 (AGS3GPR) and the GPR consensus peptide were later shown to inhibit spontaneous GTPγS binding to Giα by blocking the GDP release (*10*−*12*). These results indicated that different GPR proteins might have opposite effects on the guanine nucleotide exchange. The activity of certain GPR proteins as GDP dissociation inhibitors (GDIs) and others as GEFs might have been determined by the difference in sequences within or outside the GPR motifs. It also could have been linked to the differential selectivity of GPR proteins for active/inactive conformations of Gα subunits. To address these issues, we have examined the binding and regulation of guanine nucleotide exchange of $Gi\alpha_1$, Goα, and Gsα by three GPR proteins, LGN-585-642, Pcp2, and RapIGAPII-23-131. Our results suggest that all three GPR proteins preferentially interact with the GDP-bound conformations of Giα and Goα and serve exclusively as GDP dissociation inhibitors of these Gα subunits.

EXPERIMENTAL PROCEDURES

Materials. [^{35}S]GTPγS (1160 Ci/mmol) and [α-^{32}P]GTP (>3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. 3-(Bromoacetyl)-7-diethylaminocoumarin (BC) was from Molecular Probes. Restriction enzymes were

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[1] Abbreviations: GPCR, G-protein-coupled receptor; GPR motif, G-protein regulatory motif; Giα, Goα, and Gsα, G-protein α-subunits; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); Pcp2h, GST-fusion protein of human Pcp2; Pcp2m, mouse Pcp2 GST-fusion construct; AGS3, activator of G-protein signaling 3; GDI, GDP dissociation inhibitor; GEF, guanine nucleotide exchange factor; BC, 3-(bromoacetyl)-7-diethylaminocoumarin.

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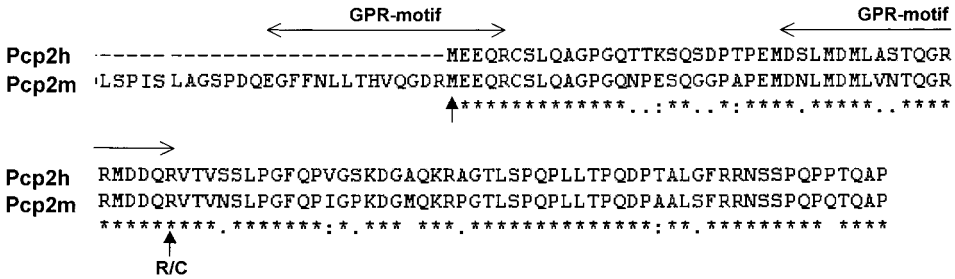

FIGURE 1: Sequence alignment of human and mouse Pcp2 proteins. The sequence of Pcp2h was obtained on the basis of the sequence of the PCR-amplified human Pcp2 cDNA. Except for the substitution Glu3/Gly, a matching sequence was obtained upon translation of the human EST clone GI 11130097. The sequence of mouse Pcp2 (*21*) follows the starting Met residue indicated by the arrow. The sequence upstream of the Met residue is present in the GST-Pcp2m construct (*6*). A second arrow indicates the Arg45/Cys substitution in the original GPR motif of GST-Pcp2m.

from New England Biolabs. AmpliTaq DNA polymerase was from Perkin-Elmer. Cloned Pfu DNA polymerase was from Stratagene. All other chemicals were from Sigma or Fisher. Transducin G$\beta\gamma$ subunit (G$\beta_1\gamma_1$) was purified according to Kleuss et al. (*15*).

Preparation of Gα Subunits. cDNA coding for the rat Goα was excised from the NpT7-5 vector (*16*) using *Hin*dIII and *Nco*I (partial digest). The insert was purified using agarose gel and ligated into the pHis$_6$ vector digested with *Nco*I and *Hin*dIII. Rat Goα, rat Giα$_1$, and bovine Gsα (short splice form) cDNAs (*17*, *18*) subcloned into pHis$_6$-tagged vector were expressed in *Escherichia coli* BL21(DE3) and purified as described (*18*, *19*).

Cloning and Expression of GPR Proteins. The human retinal cDNA λgt10 library (*20*) was used as a template for all PCR amplifications. cDNAs corresponding to residues 585−642 of human LGN protein and residues 23−131 of human Rap1GAPII were PCR-amplified using primers synthesized on the basis of reported cDNA sequences of LGN and Rap1GapII (*4*, *13*). LGN-585-642 contains two of the four LGN GPR motifs, GPR-III (aa 588−606), and GPR-IV (aa 622−640). Rap1GAPII-23-131 contains a single GPR motif (aa 27−45). Pcp2 cDNA (*21*) was PCR-amplified using primers designed on the basis of the human EST cDNA clone GI 111300097 that is similar to mouse Pcp2 cDNA. The DNA sequence of the PCR product was 98% identical to the corresponding sequence of the EST clone. The amino acid sequence of putative human Pcp2 was 82% identical to mouse Pcp2 (Figure 1). The PCR DNA fragments were digested with *Xba*I and *Xho*I (*Hin*dIII for the Rap1GAPII cDNA) and ligated into the pGEX-KG vector (*22*) digested with the same enzymes. Sequences of all the constructs were confirmed by automated DNA sequencing at the University of Iowa DNA Core Facility. The mouse PGEX4-Pcp2 construct (Pcp2m) was kindly provided by Dr. B. Denker (Harvard Medical School) (*6*). Expression of the GST-GPR proteins in BL21 cells was induced with 100 μM IPTG for 4 h at 30 °C. The yields of recombinant proteins purified using glutathione−agarose were 10−25 mg/L of culture.

To obtain the His$_6$-tagged Pcp2h, the GPR cDNA was PCR-amplified with primers containing the *Nde*I and *Bam*HI sites and subcloned into pET-15b (Novagen). The expression of His$_6$-Pcp2h was induced with 100 μM IPTG for 4 h at 30° C. His$_6$-Pcp2h was purified using columns with a His·Bind resin (Novagen) and DEAE-Sephacel. The yield of His$_6$-Pcp2h was ∼1 mg/L of culture of 95% pure protein.

Binding of the GST-GPR Fusion Proteins to Gα Subunits. Giα$_1$, Goα, and Gsα (1 μM) were incubated for 1 h at 25 °C in 20 mM Tris-HCl (pH 8.0), 130 mM NaCl, and 10 mM $MgSO_4$ (buffer A) containing 5 μM GTPγS, 5 μM GDP, or 5 μM GDP, 10 mM NaF, and 30 μM $AlCl_3$. Afterward, the GST fusion proteins LGN-585-642, Pcp2h, Pcp2m, and RapIGAPII-23-131 (2 μM each) were added, and the incubation was continued for 20 min. Glutathione−agarose (10 μL bed volume) was added and mixed with the proteins for another 20 min at 25 °C. Samples containing GαGTPγS were washed four times with 1 mL of buffer A. Samples containing GαGDP or Gα activated with AlF_4^- were washed four times with 1 mL of buffer A containing 5 μM GDP or 5 μM GDP, 10 mM NaF, and 30 μM $AlCl_3$. The bound proteins were eluted with SDS−PAGE sample buffer. Gα subunits bound to LGN-585-642, Pcp2h, and RapIGAPII-23-131 were visualized by Coomassie Blue staining of SDS gels. Giα$_1$ and GST-Pcp2m have identical electrophoretic mobility; hence a Western blot analysis was performed. The proteins were transferred to a nitrocellulose membrane BA85 (Schleicher and Schuell), and Giα$_1$ was probed with rabbit Giα$_{1-3}$-specific antibodies (Santa Cruz Biotechnology) at a 1:5000 dilution. The antibody−antigen complexes were detected using goat anti-rabbit IgG conjugated to horseradish peroxidase and ECL reagent (Amersham Pharmacia Biotech).

Fluorescence Assay of Pcp2 Binding to Gα Subunits. His$_6$-Pcp2h was labeled with the environmentally sensitive fluorescent probe, BC, at a single cysteine residue (Pcp2Cys6). A 2-fold molar excess of BC was added from a stock 5 mM solution in *N*,*N*-dimethylformamide to 100 μM His$_6$-Pcp2h in 20 mM HEPES buffer (pH 7.6). The mixture was incubated for 30 min at 25 °C. The labeled protein, Pcp2hBC, was then passed through a PD-10 column (Pharmacia) equilibrated with 20 mM HEPES buffer (pH 7.6) containing 100 mM NaCl. Using $\epsilon_{445} = 53\,000$ for BC, the incorporation of BC into Pcp2h was greater than 0.8 mol/mol. Fluorescent assays were performed on a F-2000 fluorescence spectrophotometer (Hitachi) in 1 mL of 20 mM HEPES buffer (pH 7.6) containing 100 mM NaCl and 5 mM $MgCl_2$. Where indicated, the buffer contained 30 μM $AlCl_3$ and 10 mM NaF. Fluorescence of Pcp2hBC (40 nM) was monitored prior to and following the additions of increasing concentrations of Gα subunits using excitation at 445 nm and emission at 495 nm. Concentration of Pcp2hBC was determined using $\epsilon_{445} = 53\,000$. The specificity of binding of Pcp2hBC to Gα subunits was confirmed by the competitive displacement with unlabeled Pcp2h.

GTPγS Binding Assay. $Gi\alpha_1$ (0.1 μM), Goα (0.1 μM), and Gsα subunits (1 μM) alone or Giα1 mixed with 0.2 μM $Gt\beta\gamma$ were incubated for 3 min at 25 °C in 50 μL of buffer A containing 10 mM DTT and 10 μM GST, with or without the addition of varying concentrations of the GST fusion proteins LGN-585-642, Pcp2m, Pcp2h, and RapIGAPII-23-131. Binding reactions were started by the addition of 5 μM [^{35}S]GTPγS (5 Ci/mmol). Aliquots of 10 μL were withdrawn at the indicated times and passed through Whatman cellulose nitrate filters (0.45 μm). The filters were then washed three times with 1 mL of ice-cold buffer A and counted in a liquid scintillation counter after dissolution in 3a70B cocktail (RPI Corp.). A background GTPγS binding in the absence of Gα was subtracted from all samples. The GTPγS binding data were fit to the equation GTPγS bound (%) = $100\%(1 - e^{-kt})$.

GDP Dissociation Assay. $Gi\alpha_1$ and Goα complexed with [α-^{32}P]GDP (1 μM) were prepared by incubating 1 μM Gα with 2 μM [α-^{32}P]GTP in buffer A for 1.5 h at 25 °C. Excess of unlabeled GTP (1 mM) was added to monitor dissociation of [α-^{32}P]GDP from Gα subunits in the absence or the presence of the GST-GPR proteins. Aliquots were withdrawn at the indicated times and passed through Whatman cellulose nitrate filters (0.45 μm). The dissociation rate constants (k_{off}) were calculated by fitting the experimental data to a single-exponential decay function: % GDP bound = $100\%(e^{-kt})$.

Other Methods. Protein concentrations were determined by the method of Bradford (*23*) using IgG as a standard. SDS−PAGE was performed according to the method of Laemmli (*24*). The experimental data were fit with nonlinear least squares criteria using GraphPad Prizm (v.2) software. Data are shown as the mean ± SE of three or more experiments.

RESULTS

Cloning of a Human Pcp2 Protein. In addition to LGN-585-642 and RapIGAPII-23-131 GST fusion proteins, two recombinant Pcp2 constructs, Pcp2m and Pcp2h, were utilized in the analysis of GPR protein function. Pcp2m is the GST-Pcp2 fusion protein of mouse Pcp2 that was shown earlier to act as GEF for Goα (*6*). The Pcp2m cDNA that was subcloned into the PGEX4-2 vector (*6*) contained a DNA fragment located upstream of the codon for initiator Met. This fragment included six codons from the 3′ end of exon 1 and twenty codons from the 5′ end of exon 2 of the mouse Pcp2 gene, which are normally not translated (*25*). Inadvertently, translation of this fragment in the context of GST-Pcp2m resulted in the introduction of a second artificial GPR motif positioned N-terminally to the genuine Pcp2 GPR motif (Figure 1). A second potentially serious problem with Pcp2m is that it has a substitution of an important Arg45 by Cys within the original GPR motif. Mutation of the corresponding residue in AGS3GPR has disrupted its binding to Giα (*8*, *12*). A human GST-Pcp2h protein has been generated to correct for these errors. The amino acid sequence of Pcp2h shown in Figure 1 is derived from the sequencing of the PCR-amplified human cDNA. An analogous sequence with only a single substitution can be obtained upon translation of the human EST clone GI 11130097. Pcp2h has an 82% sequence identity to the mouse Pcp2 (*21*).

Conformational Selectivity of the GPR Proteins. The binding of GST fusion proteins LGN-585-642, Pcp2h, Pcp2m, and Rap1GAPII-23-131 to $Gi\alpha_1$ in different conformations was analyzed by coprecipitation of $Gi\alpha_1$ with the GPR protein using glutathione−agarose beads. The amounts of coprecipitated $Gi\alpha_1$ were assessed with Coomassie Blue stained gels. The Western blot analysis was used in the case of GST-Pcp2m because it comigrates with $Gi\alpha_1$ on an SDS gel. The results of the GST pull-down experiments in Figure 2 demonstrate that all three GPR proteins bind tightly $Gi\alpha_1$-GDP. Very small amounts if any of $Gi\alpha_1GTP\gamma S$ and $Gi\alpha_1GDPAlF_4^-$ were coprecipitated with the GPR constructs. Thus, LGN-585-642, Pcp2, and Rap1GAPII-23-131 display similar patterns of interaction with $Gi\alpha_1$ with the following order of affinities: $Gi\alpha_1GDP \gg Gi\alpha_1GDPAlF_4^- \geq Gi\alpha_1$-$GTP\gamma S$. $Gi\alpha_1$ did not coprecipitate with glutathione−agarose in the absence of GST-GPR proteins (not shown). LGN-585-642 (Figure 2E), Pcp2, and Rap1GAPII-23-131 (not shown) did not bind Gsα.

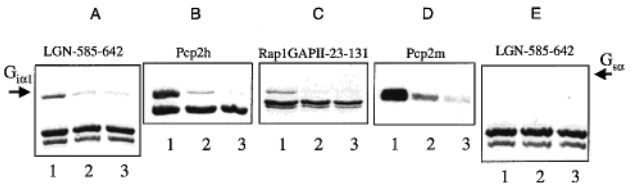

FIGURE 2: Binding of the GPR proteins to Gα. SDS gels stained with Coomassie Blue (A, B, C, and E) and Western blot analysis (D). GαGDP (lanes 1), $G\alpha GDPAlF_4^-$ (lanes 2), and $G\alpha GTP\gamma S$ (lanes 3) bound to GST fusion proteins LGN-585-642, Pcp2h, Pcp2m, and Rap1GAPII-23-131 were pulled down using glutathione−agarose as described under Experimental Procedures. The bound Giα (A, B, C, and D) and Gsα (E) were analyzed using Coomassie Blue stained SDS gels (A, B, C, and E) or Western blot analysis (D).

Binding of Gα Subunits to Fluorescently Labeled Pcp2h. To establish a quantitative assay of binding of Pcp2h to Gα subunits, His_6-Pcp2h was labeled with the environmentally sensitive fluorescent probe, BC, at Pcp2Cys6 (Figure 1). We found that binding of $Gi\alpha_1GDP$ to Pcp2hBC led to a large fluorescence increase (maximum F/F_0 of 9.1) (Figure 3A). The K_d value of 0.37 μM was calculated from the binding curve (Figure 3A). The specificity of binding of Pcp2hBC to $Gi\alpha_1GDP$ was confirmed by competitive displacement with unlabeled Pcp2h (not shown). Furthermore, the fluorescence change of Pcp2hBC was sensitive to the conformational state of $Gi\alpha_1$. By comparison to $Gi\alpha_1GDP$, additions of $Gi\alpha_1GDPAlF_4^-$ and $Gi\alpha_1GTP\gamma S$ caused only small changes in the fluorescence of Pcp2hBC. The binding curves for $Gi\alpha_1GDPAlF_4^-$ and $Gi\alpha_1GTP\gamma S$ (Figure 3A) suggest low affinity of activated $Gi\alpha_1$ for Pcp2h, which is consistent with the results of the GST pull-down experiments (Figure 2). Next, the fluorescence binding assay was applied to examine the interaction of Pcp2h with Goα. Additions of GoαGDP to Pcp2hBC resulted in an approximately 5-fold maximal increase in the probe fluorescence (Figure 3B). The affinity of GoαGDP for Pcp2hBC (K_d of 1.6 μM) was notably lower than that of $Gi\alpha_1$ (Figure 3B). The AlF_4^-- and GTPγS-bound conformations of Goα showed no significant binding to Pcp2hBC (Figure 3B), confirming the preference of Pcp2 for the GDP-bound Gα subunits of the Gi family. The lower affinity of Pcp2h for Goα in comparison to Giα was corroborated by the GST pull-down experiments (Figure 3B, inset). No binding of Pcp2h to all conformations of Gsα was detected using the fluorescence assay.

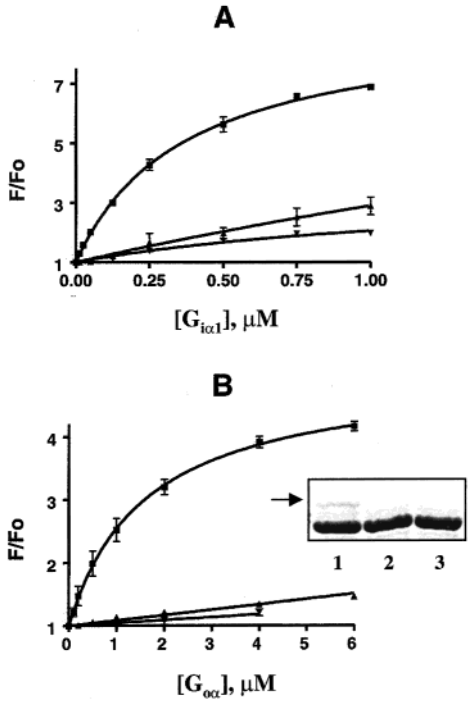

FIGURE 3: Binding of Giα$_1$ and Goα to Pcp2hBC. The relative increase in fluorescence (F/F_0) of Pcp2hBC (40 nM) (excitation at 445 nm, emission at 495 nm) was determined following the addition of increasing concentrations of Giα$_1$ (A) or Goα (B) in the GDP- (■), GDPAlF$_4^-$- (▲), or GTPγS-bound (▼) conformations. The binding curve characteristics are (A, ■) $K_d = 0.37 \pm 0.02$ μM, maximum $F/F_0 = 9.1$ and (B, ■) $K_d = 1.6 \pm 0.2$ μM, maximum $F/F_0 = 5.0$. (B, inset) The GST pull-down experiment. GoαGDP (1), GoαGDPAlF$_4^-$ (2), and GoαGTPγS (3) bound to GST-Pcp2h. The arrow indicates Goα.

Effects of the GPR Proteins on GTPγS Binding to Gα Subunits. The effects of GST fusion proteins of LGN-585-642, Pcp2h, Pcp2m, and Rap1GAPII-23-131 on guanine nucleotide exchange of Giα$_1$ and Goα were first tested using the GTPγS binding assay. Giα and Goα bound GTPγS due to spontaneous nucleotide exchange with initial rates of 0.12 and 0.14 mol of GTPγS/(min·mol of Gα), respectively (Figures 4 and 5). LGN-585-642, Pcp2h, and Rap1GAPII-23-131 all effectively inhibited the initial rates of GTPγS binding to Giα$_1$ with IC$_{50}$ values of 0.52, 0.17, and 0.12 μM, respectively (Figure 4E). An equivalent IC$_{50}$ value was obtained for the Pcp2h inhibition of Giα$_1$ using His$_6$-Pcp2h (not shown), indicating that the GST tag does not interfere with the GPR function. The IC$_{50}$ value for Pcp2h is comparable with the K_d value of 0.37 μM for the Pcp2BC/Giα$_1$ interaction from the fluorescence binding assay, suggesting the correlation between the GPR binding and the inhibitory effect. The inhibition of GTPγS binding to Giα$_1$ by LGN-585-642 was cooperative with a Hill coefficient of 1.7, perhaps reflecting the presence of two GPR motifs in the LGN construct. The lower effectiveness of Pcp2m (IC$_{50}$ 1.7 μM) might be attributed to the Arg45Cys substitution in the original GPR motif. In fact, it is possible that this mutation rendered the GPR motif completely inactive, and the observed effect was due to presence of an artificial upstream GPR repeat in Pcp2m. The major difference between the effects of the GPR proteins on the kinetics of GTPγS binding to Giα$_1$ and Goα was the considerably lower potency toward Goα. The IC$_{50}$ values for the inhibition of Goα GTPγS binding varied from 4.1 μM for RapIGAPII-23-131 to 23.7 μM for Pcp2m (Figure 5). Moreover, the IC$_{50}$ value for Pcp2h (5.6 μM) exceeded the K_d value for the Pcp2BC/Goα interaction from the fluorescence binding assay (1.6 μM). Not surprisingly, LGN-585-642, Pcp2h, Pcp2m, and Rap1GAPII-23-131 had no effect on GTPγS binding to Gsα even when added at high concentrations (Figure 6). These results suggest that GPR proteins may specifically and potently modulate Gi-mediated signaling.

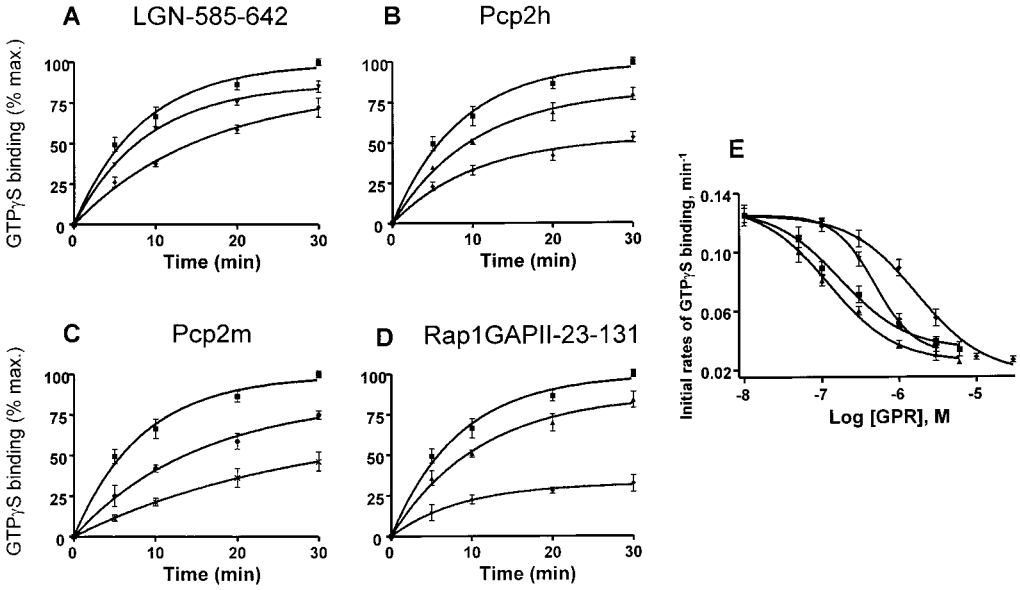

FIGURE 4: Effects of the GPR proteins on the kinetics of GTPγS binding to Giα$_1$. (A, B, C, and D) The binding of GTPγS to Giα$_1$ (0.1 μM) in the absence (■) or presence of GST fusion proteins LGN-585-642, Pcp2h, Pcp2m, and Rap1GAPII-23-131 [0.1 μM (▲), 0.3 μM (▼), 1 μM (◆), 3 μM (●), 10 μM (×)] was initiated by the addition of 5 μM [^{35}S]GTPγS. Giα-bound GTPγS was counted by withdrawing aliquots at the indicated times and passing them through cellulose nitrate filters (0.45 μm). The calculated initial rates of GTPγS binding [mol of GTPγS/(min·mol of Gα)] are as follows: 0.12 ± 0.01 (■), (A) 0.095 ± 0.008 (▼), 0.053 ± 0.005 (◆); (B) 0.073 ± 0.008 (▲), 0.045 ± 0.004 (◆); (C) 0.065 ± 0.008 (●), 0.037 ± 0.005 (×); and (D) 0.082 ± 0.008 (▲), 0.037 ± 0.006 (◆). (E) The initial rates of GTPγS binding to Giα$_1$ are plotted as functions of LGN-585-642 (▼), Pcp2h (■), Pcp2m (◆), or Rap1GAPII-23-131 (▲) concentrations. The IC$_{50}$ values (μM) are 0.52 ± 0.05 (▼), 0.17 ± 0.01 (■), 1.7 ± 0.1 (◆), and 0.12 ± 0.01 (▲).

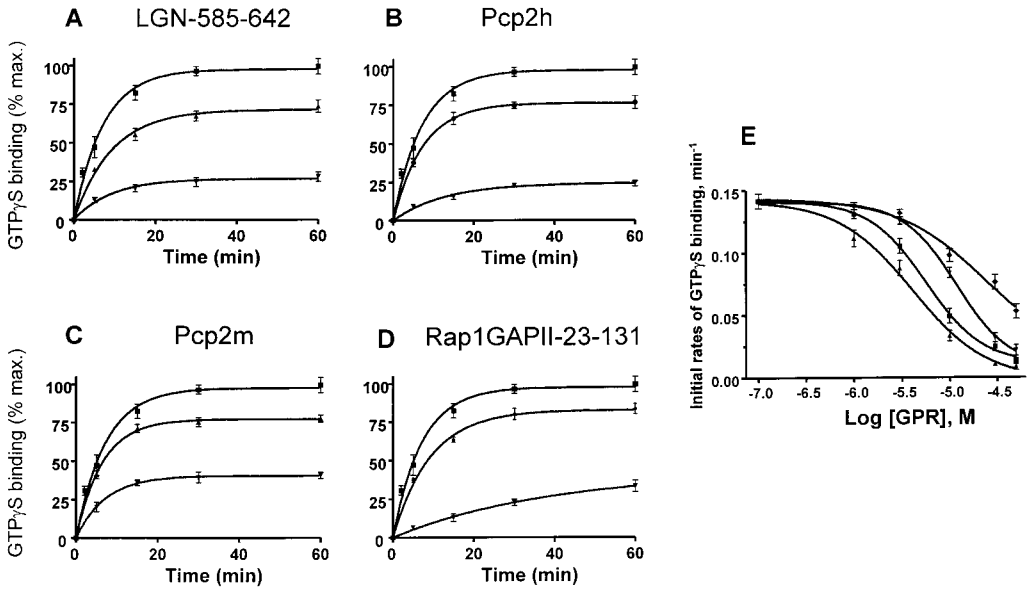

FIGURE 5: Effects of the GPR-proteins on the kinetics of GTPγS binding to Goα. A, B, C, D. The binding of GTPγS to Goα (0.5 μM) in the absence (■) or presence of GST-fusion proteins LGN-585−642, Pcp2h, Pcp2m, and Rap1GAPII-23−131 (3 μM ●, 10 μM ▲, 30 μM ▼). The initial rates of GTPγS binding [mol of GTPγS/min·mol of Gα)] are as follows: 0.14 ± 0.01 (■); (A) 0.084 ± 0.006 (▲), 0.032 ± 0.003 (▼); (B) 0.11 ± 0.01 (●), 0.025 ± 0.003 (▼); (C) 0.13 ± 0.01 (▲), 0.076 ± 0.006 (▼); and (D) 0.09 ± 0.01 (●), 0.010 ± 0.001 (▼) (E) The initial rates of GTPγS binding to Goα are plotted as functions of LGN-585-642 (▼), Pcp2h (■), Pcp2m (◆), or Rap1GAPII-23-131 (▲) concentrations. The IC_{50} values (μM) are 11.7 ± 1.4 (▼), 5.6 ± 1.1 (■), 23.7 ± 3.1(◆), and 4.1 ± 0.9 (▲).

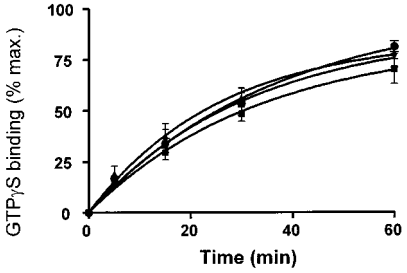

FIGURE 6: Binding of GTPγS to Gsα in the presence of GPR proteins. The binding of GTPγS to Gsα (1 μM) in the absence (■) or presence of 30 μM GST fusion proteins LGN-585-642 (▲), Pcp2h (●), and Rap1GAPII-23-131 (▼). The initial rates of GTPγS binding [mol of GTPγS/(min·mol of Gα)] are 0.030 ± 0.004 (■), 0.038 ± 0.007 (▲), 0.028 ± 0.004 (●), and 0.032 ± 0.006 (▼).

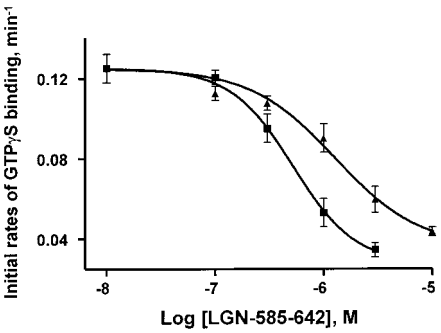

FIGURE 7: Effect of G$\beta_1\gamma_1$ on the inhibition of GTPγS binding to Giα_1 by LGN-585-642. The initial rates for GTPγS binding to Giα_1 (0.1 μM) in the absence (■) or presence (▲) of G$\beta_1\gamma_1$ (0.2 μM) are plotted as functions of LGN-585-642 concentration. The IC_{50} values (μM) are 0.52 ± 0.05 (■) and 1.3 ± 0.1 (▲).

The G$\beta_1\gamma_1$ Subunit Competes with the GPR Proteins for Binding to GαGDP. Gα subunits complexed with GDP associate with G$\beta\gamma$ subunits to form heterotrimeric G-proteins. The preferential recognition of GαGDP by GPR proteins suggests the potential competition with G$\beta\gamma$ for binding to Gα. We have tested how G$\beta\gamma$ affects the inhibition of GTPγS binding to Gα by a GPR protein using Giα_1, G$\beta_1\gamma_1$, and LGN-585-642. G$\beta\gamma$ subunits may themselves inhibit the binding of GTPγS to Gα subunits by stabilizing the GDP-bound conformations. We have previously demonstrated that G$\beta_1\gamma_1$ effectively inhibits GTPγS binding to Gsα (*26*). G$\beta_1\gamma_1$ had no effect on the rate of GTPγS binding to Giα_1 (Figure 7). The lack of the G$\beta_1\gamma_1$ effect is probably due to the use of nonmyristoylated Giα_1 (*27*). This simplified the test of the GPR effect on the Gα nucleotide exchange in the presence of G$\beta\gamma$. Reconstitution of Giα_1 with G$\beta_1\gamma_1$ led to a rightward shift of the dose-dependent inhibition of GTPγS binding by LGN-585-642 (Figure 7). These results indicate that GPR proteins and G$\beta\gamma$ subunits compete for binding to GiαGDP.

The GPR Proteins Act as GDP Dissociation Inhibitors. To examine the effects of GPR proteins on the release of GDP from Giα_1 and Goα, the Gα subunits were loaded with [α-^{32}P]GDP, and the rates of GDP dissociation were measured in the absence or presence of LGN-585-642, Pcp2h, Pcp2m, and Rap1GAPII-23-131. The GDP release is generally believed to be a rate-limiting step in the nucleotide exchange by Gα subunits (*16*, *28*). However, rates of GDP dissociation exceeding GTPγS binding rates have also been reported (*29*). The GDP dissociation rates from Giα_1 (k_{off} 0.38 min^{-1}) and Goα (k_{off} 0.47 min^{-1}) were somewhat higher than the corresponding GTPγS binding rates under our experimental conditions (Figure 8). The GDP dissociation from Giα_1 (Figure 8A) was largely blocked with additions of 10 μM each of the GPR proteins. Similar effects of the GPR proteins were observed on the release of GDP from Goα at 30 μM GPR concentration (Figure 8B). Although the finding that Pcp2m inhibits the GDP release from Goα contrasts the previous observation (*6*), it correlates well with

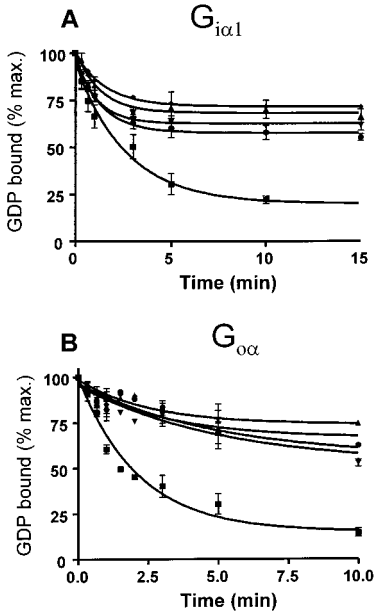

FIGURE 8: Effects of the GPR proteins on the dissociation of GDP from Giα$_1$ and Goα. Giα$_1$ and Goα complexed with [α-^{32}P]GDP were obtained as described under Experimental Procedures. GTP (1 mM) and the GST fusion proteins LGN-585-642 (▼), Pcp2h (▲), Pcp2m (●), and Rap1GAPII-23-131 (◆) (A, 10 μM; B, 30 μM) were added to 0.5 μM Giα$_1$GDP (A) or Goα (B). Aliquots were withdrawn at the indicated times and passed through cellulose nitrate filters (0.45 μm). The k_{off} (min^{-1}) values in the absence of GPR proteins are 0.38 ± 0.07 for Giα$_1$ and 0.47 ± 0.06 for Goα (■).

the inhibitory effects of all tested GPR proteins in the GDP release and GTPγS binding assays.

DISCUSSION

Recently, a novel G-protein α-subunit binding motif has been recognized (*8*, *9*). Several proteins including Pcp2, Rap1GAP, and RGS12 contain a single copy of such G-protein regulatory (GPR) motif. Other proteins, such as AGS3 and LGN, contain four GPR repeats (*4*, *8*, *9*, *30*). Initial biochemical studies consistently demonstrate that GPR proteins interact with Gα subunits from the Gi family (*6*−*10*). However, the conformational specificity of GPR proteins is still poorly understood. AGS3 was shown to bind tightly with the GDP-bound Giα and Gtα subunits (*8*, *10*, *11*). Similarly, Rap1GAP interacted with the GDP-bound conformation of Goα but not with the constitutively active Q205L mutant (*7*). On the contrary, Rap1GAP bound exclusively the GTPγS- or AlF_4^--activated forms of Gzα (*14*). Furthermore, an isoform of Rap1GAP, Rap1GAPII, was also shown to preferentially bind the Q → L mutant of Giα (*13*). In addition, Pcp2 bound equally the GDP and GTPγS conformations of Goα (*6*). Together, this evidence indicates that different GPR proteins may selectively recognize active/inactive conformations of Gα subunits, and, perhaps, the identity of a Gi-family member contributes to the selectivity. Even more striking differences between individual GPR proteins have been implied by recent studies on regulation of Gα guanine nucleotide exchange by GPR proteins. Pcp2 was reported to promote GDP release from Goα (*6*), whereas the GPR domain of AGS3 potently inhibited GDP release and GTPγS binding of Giα (*10*, *11*). The inhibitory effect of AGS3 on the Giα nucleotide exchange has been recently confirmed (*31*).

In view of potential role of GPR proteins as important modulators of G-protein signaling, we have examined three GRP proteins, LGN-585-642, Pcp2, and Rap1GAPII-23-131 for their conformational selectivity and effects on Gα guanine nucleotide exchange. One of the central findings of this study is that all tested GPR proteins have relatively uniform properties. The GST pull-down experiments indicated that LGN, Pcp2, and Rap1GAPII bind with high affinity to GiαGDP and significantly lower affinities to the active GTPγS-bound or AlF_4^--induced conformations. Likewise, Pcp2h displayed preferential binding to the GDP-bound Giα and Goα using the fluorescence binding assay. The latter assay also indicated that Pcp2 binds Giα stronger than Goα. None of the GPR proteins bound Gsα. No major differences were found in the effects of different GPR proteins on Giα guanine nucleotide exchange. LGN-585-642, Pcp2h, and Rap1GAPII-23-131 inhibited GTPγS binding to Giα with similar potencies comparable to that of the inhibitory effect of AGS3GPR (*10*). A lower effectiveness of Pcp2m might have been caused by the Arg45Cys mutation in the GPR motif. Notably, the GPR proteins were significantly less potent in inhibiting binding of GTPγS to Goα. Similarly, an AGS3 GPR consensus peptide had very little effect on the GTPγS binding to Goα (*12*). Together, these data suggest that GPR proteins may selectively target Giα. The dose-dependent inhibition of GTPγS binding to Giα by LGN-585-642 was right-shifted in the presence of $G\beta_1\gamma_1$, indicating the competition between the GPR protein and Gβγ. This is in agreement with the recently shown competition of AGS3GPR with Gβγ for binding to GαGDP (*11*). Following the finding that LGN-585-642, Pcp2, and Rap1GAPII-23-131 can block GTPγS binding to Giα$_1$ and Goα, the inhibitory effect of these GPR proteins on the GDP release was not unexpected. The activity of the GPR proteins as GDIs of Giα is consistent with that of AGS3GPR (*10*, *12*, *31*). A recent study concluded that AGS3 binds but does not inhibit guanine nucleotide exchange of Goα (*31*). Our results suggest that GPR proteins, including AGS3,[2] act as GDIs on Goα as well as on Giα, although the inhibitory effect on Goα is seen at significantly higher concentrations of GPRs. We were unable to confirm the GEF-like activity of Pcp2 toward Goα (*6*). It is possible that additional factors mediate the interactions of GPR proteins with Gα subunits in vivo, introducing some of the reported differences in specificities and functions of various GPR proteins. Nonetheless, on the basis of the results of this study, we hypothesize that GPR proteins generally inhibit signaling from G-protein-coupled receptors to the Gi family of G-proteins by acting as GDIs. Initial evidence for such a functional role of AGS3 has been newly developed (*10*, *12*).

[2] M. Natochin, K. G. Gasimov, and N. O. Artemyev, unpublished observation.

REFERENCES

1. Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615−649.
2. Bourne, H. R. (1997) *Curr. Opin. Cell Biol. 9*, 134−142.
3. Hamm, H. E. (1998) *J. Biol. Chem. 273*, 669−672.
4. Mochizuki, N., Cho, G., Wen, B., and Insel, P. A. (1996) *Gene 181*, 39−43.

5. Granderath, S., Stollewerk, A., Greig, S., Goodman, C. S., O'Kane, C. J., and Klambt, C. (1999) *Development 126*, 1781−1791.
6. Luo, Y., and Denker, B. M. (1999) *J. Biol. Chem. 274*, 10685−10688.
7. Jordan, J. D., Carey, K. D., Stork, P. J., and Iyengar, R. (1999) *J. Biol. Chem. 274*, 21507−21510.
8. Takesono, A., Cismowski, M. J., Ribas, C., Bernard, M., Chung, P., Hazard, S., III, Duzic, E., and Lanier, S. M. (1999) *J. Biol. Chem. 274*, 33202−33205.
9. Siderovski, D. P., Diverse-Pierluissi, M. A., and De Vries, L. (1999) *Trends Biochem. Sci. 24*, 340−342.
10. Natochin, M., Lester, B. R., Peterson, Y. K., Bernard, M. L., Lanier, S. M., and Artemyev, N. O. (2000) *J. Biol. Chem. 275*, 40981−40985.
11. Bernard, M. L., Peterson, Y. K., Chung, P., Jourdan, J., and Lanier, S. M. (2001) *J. Biol. Chem. 276*, 1585−1593.
12. Peterson, Y. K., Bernard, M. L., Ma, H., Hazard, S., III, Graber, S. G., and Lanier, S. M. (2000) *J. Biol. Chem. 275*, 33193−33196.
13. Mochizuki, N., Ohba, Y., Kiyokawa, E., Kurata, T., Murakami, T., Ozaki, T., Kitabatake, A., Nagashima, K., and Matsuda, M. (1999) *Nature 400*, 891−894.
14. Meng, J., Glick, J. L., Polakis, P., and Casey, P. J. (1999) *J. Biol. Chem. 274*, 36663−36669.
15. Kleuss, C., Pallast, M., Brendel, S., Rosenthal, W., and Schultz, G. (1987) *J. Chromatogr. 407*, 281−289.
16. Linder, M. E., Ewald, D. A., Miller, R. J., and Gilman, A. G. (1990) *J. Biol. Chem. 265*, 8243−8251.
17. Natochin, M., Lipkin, V. M., and Artemyev, N. O. (1997) *FEBS Lett. 411*, 179−182.
18. Skiba, N. P., Bae, H., and Hamm, H. E. (1996) *J. Biol. Chem. 271*, 413−424.
19. Natochin, M., and Artemyev, N. O. (1998) *Biochemistry 37*, 13776−13780.
20. Nathans, J., Thomas, D., and Hogness, D. S. (1986) *Science 232*, 193−202.
21. Nordquist, D. T., Kozak, C. A., and Orr, H. T. (1988) *J. Neurosci. 8*, 4780−4789.
22. Guan, K., and Dixon, J. E. (1991) *Anal. Biochem. 192*, 262−267.
23. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
24. Laemmli, U. K. (1970) *Nature 227*, 680−685.
25. Vandaele, S., Nordquist, D. T., Feddersen, R. M., Tretjakoff, I., Peterson, A. C., and Orr, H. T. (1991) *Genes Dev. 5*, 1136−1148.
26. Natochin, M., Muradov, K. G., McEntaffer, R. L., and Artemyev, N. O. (2000) *J. Biol. Chem. 275*, 2669−2675.
27. Linder, M. E., Pang, I. H., Duronio, R. J., Gordon, J. I., Sternweis, P. C., and Gilman, A. G. (1991) *J. Biol. Chem. 266*, 4654−4659.
28. Ferguson, K. M., Higashijima, T., Smigel, M. D., and Gilman, A. G. (1986) *J. Biol. Chem. 261*, 7393−7399.
29. Carty, D. J., Padrell, E., Codina, J., Birnbaumer, L., Hildebrandt, J. D., and Iyengar, R. (1990) *J. Biol. Chem. 265*, 6268−6273.
30. Siderovski, D. P., Strockbine, B., and Behe, C. I. (1999) *Crit. Rev. Biochem. Mol. Biol. 34*, 215−251.
31. De Vries, L., Fischer, T., Tronchere, H., Brothers, G. M., Strockbine, B., Siderovski, D. P., and Farquhar, M. G. (2000) *Proc. Natl. Acad. Sci. U.S.A. 97*, 14364−14369.

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