Binding of Transducin to Light-Activated Rhodopsin Prevents Transducin Interaction with the Rod cGMP Phosphodiesterase γ-Subunit†

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ABSTRACT: In photoreceptor cells of vertebrates, the GTP-bound α-subunit of rod G-protein, transducin ($G_{t\alpha}$), interacts with the cGMP phosphodiesterase inhibitory γ-subunit ($P\gamma$) to activate the effector enzyme. The GDP-bound $G_{t\alpha}$ can also bind the $P\gamma$ subunit, albeit with a lower affinity than $G_{t\alpha}$GTP. In this work, interactions between $G_{t\alpha}$GDP and $P\gamma$ or $P\gamma$-24−45Cys labeled with the fluorescent probe 3-(bromoacetyl)-7-(diethylamino)coumarin ($P\gamma$BC, $P\gamma$-24−45BC) have been investigated. Addition of $G_{t\alpha}$GDP to $P\gamma$BC produced approximately a 6-fold maximal increase in the probe fluorescence, while the fluorescence of $P\gamma$-24−45BC was enhanced by 2.3-fold. The K_d's for the $G_{t\alpha}$GDP binding to $P\gamma$BC and $P\gamma$-24−45BC were 75 ± 8 nM and 400 ± 110 nM, respectively. The $G_{t\beta\gamma}$ subunits had no notable effect on the binding of $G_{t\alpha}$GDP to $P\gamma$BC or $P\gamma$-24−45BC, suggesting that $P\gamma$ and $G_{t\beta\gamma}$ bind to $G_{t\alpha}$GDP noncompetitively. The $G_{t\alpha\beta\gamma}$ interaction with the fluorescently labeled $P\gamma$ was effectively blocked in the light-activated rhodopsin (R*)−$G_{t\alpha\beta\gamma}$ complex. Furthermore, addition of excess $P\gamma$ or $P\gamma$-24−45 prevented binding of $G_{t\alpha\beta\gamma}$ to R*, indicating that the R* and $P\gamma$ binding surfaces on $G_{t\alpha\beta\gamma}$ may overlap. It is likely that R* has a binding site within the α3−β5 region of $G_{t\alpha}$, which is a proposed site of $G_{t\alpha}$GDP binding to $P\gamma$-24−45. Alternatively, R* may induce conformational changes of the $G_{t\alpha}$ α3−β5 region such that the resulting structural changes alter the adjacent consensus sequence for the guanine ring binding of GDP/GTP(NKXD), and lead to a reduction in the affinity of G-protein for guanine nucleotides.

When visual receptor rhodopsin is activated by light, its chromophore, 11-*cis*-retinal, rapidly isomerizes into an *all-trans*-retinal. This isomerization is followed by the relaxation of the receptor through a number of transitional states. In the active Meta II conformation, rhodopsin (R*)[1] binds tightly to the holo G-protein, transducin ($G_{t\alpha\beta\gamma}$), causing the nucleotide-binding pocket on $G_{t\alpha}$ to open and GDP to dissociate. The exchange of GDP for GTP is favored because GTP binding induces a conformational change of $G_{t\alpha}$ that leads to dissociation of $G_{t\alpha}$GTP from R* and the $G_{t\beta\gamma}$ subunits. In the absence of GTP, the complex R*−$G_{t\alpha\beta\gamma}$ is stable and protects R* from decaying into opsin and 11-*trans*-retinal. $G_{t\alpha}$GTP activates cGMP phosphodiesterase (PDE) by relieving an inhibitory constraint imposed by two identical inhibitory subunits of PDE ($P\gamma$) on the enzyme $\alpha\beta$ catalytic subunits ($P\alpha\beta$). cGMP hydrolysis by active PDE results in closure of cGMP-gated channels in the plasma membrane and hyperpolarization of the cell [for a review, see Chabre and Deterre (1989), Hargrave et al. (1993), Yarfitz and Hurley (1994), and Stryer (1996)].

Evidence suggests that $G_{t\alpha}$GTP binds mainly to the $P\gamma$ subunits complexed with $P\alpha\beta$ (Hurley & Stryer, 1982; Deterre et al., 1988; Fung & Griswold-Prenner, 1989; Wensel & Stryer, 1990). Two major regions of $P\gamma$, a polycationic region ($P\gamma$-24−45) and the C-terminal region ($P\gamma$-63−76), have been implicated in the interaction with $G_{t\alpha}$GTP (Lipkin et al., 1988; Artemyev et al., 1992; Brown, 1992; Takemoto et al., 1992; Skiba et al., 1995; Slepak et al., 1995). Significant progress has been made in the identification of $P\gamma$−binding domains on transducin (Rarick et al., 1992; Artemyev et al., 1992, 1993; Faurobert et al., 1993; Cunnick et al., 1994; Erickson et al., 1995; Skiba et al., 1996; Liu et al., 1996). Recent studies have found that $G_{t\alpha}$GDP can also interact with $P\gamma$, although the affinity of this interaction is significantly lower than that of the $G_{t\alpha}$GTP−$P\gamma$ complex (Otto-Bruc et al., 1993; Yamazaki et al., 1990; Artemyev et al., 1993; Skiba et al., 1995). This study probes the interactions of $G_{t\alpha}$GDP or holo $G_{t\alpha\beta\gamma}$ with $P\gamma$, and investigates how $G_{t\alpha\beta\gamma}$ binding to R* affects its interaction with $P\gamma$.

EXPERIMENTAL PROCEDURES

Materials. GTP and GTPγS were products of Boehringer Mannheim. Blue-Sepharose CL-6B was obtained from Pharmacia. 3-(Bromoacetyl)-7-(diethylamino)coumarin was purchased from Molecular Probes, Inc. All other chemicals were acquired from Sigma.

Preparation of ROS Membranes, $G_{t\alpha\beta\gamma}$, $G_{t\alpha}$GTPγS, $G_{t\alpha}$GDP, and $G_{t\beta\gamma}$. Bovine ROS membranes were prepared by the method described in Papermaster and Dreyer (1974). Urea-washed ROS membranes were prepared according to Yamanaka et al. (1985) and were stored at −80 °C. Hydroxylamine-treated ROS membranes were prepared by incubating bleached urea-washed ROS membranes with 50 mM hydroxylamine in 20 mM HEPES buffer (pH 7.6), containing

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[1] Abbreviations: R*, photoexcited rhodopsin; $G_{t\alpha\beta\gamma}$, rod GTP-binding protein transducin; PDE, rod outer segment cGMP phosphodiesterase; Pα, Pβ, Pγ, subunits of PDE; $P\gamma$BC and $P\gamma$-24−45BC, $P\gamma$ and $P\gamma$-24−45Cys labeled with 3-(bromoacetyl)-7-(diethylamino)coumarin; $P\gamma$LY, $P\gamma$ labeled with the fluorescent probe lucifer yellow vinyl sulfone; GTPγS, guanosine 5′-*O*-(thiotriphosphate); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography.

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100 mM NaCl and 4 mM $MgCl_2$ (buffer A), for 30 min at room temperature. The membranes were then centrifuged for 10 min at 20000*g*, and the pellet was rinsed twice with 1 mL of buffer A and resuspended in the same buffer. Transducin, $G_{t\alpha\beta\gamma}$, was extracted from ROS membranes using GTP as described in Stryer et al. (1983). The $G_{t\alpha}GTP\gamma S$ and $G_{t\beta\gamma}$ subunits were extracted from ROS membranes using GTPγS and purified by chromatography on Blue-Sepharose CL-6B by the procedure of Kleuss et al. (1987). $G_{t\alpha}GDP$ was prepared and purified according to protocols in Yamazaki et al. (1988). The purified proteins were stored in 40% glycerol at −20 °C.

Preparation of Pγ, PγBC, and Pγ-24−45BC. Recombinant Pγ subunit was expressed in *E. coli* and purified on a SP-Sepharose fast flow column and on a C-4 HPLC column (Microsorb-MW, Rainin) as described in Skiba et al. (1995). To obtain PγBC, a 2-fold molar excess of 3-(bromoacetyl)-7-(diethylamino)coumarin in *N*′,*N*-dimethylformamide was added to 100 μM Pγ in buffer A (minus $MgCl_2$), and the mixture was incubated for 30 min at room temperature (23−24 °C). The PγBC was then passed through a PD-10 column (Pharmacia) equilibrated with buffer A and purified by RP HPLC on a C-4 column Microsorb-MW (Rainin) using a 0−100% gradient of acetonitrile, 0.1% TFA. Using $\epsilon_{445} = 53\,000$ for BC, the molar ratio of BC to Pγ was greater than 0.8 mol/mol. Pγ-24−45BC was prepared by labeling of peptide Pγ-24−45Cys and purified as described in Natochin and Artemyev (1996). A Pγ mutant, PγCys68→Ser (Artemyev et al., 1996), and peptide Pγ-24−45 that contains no cysteine were not derivatized with BC under similar conditions, suggesting the selectivity of the cysteine labeling.

Peptide Synthesis. Peptides Pγ-24−45 and Pγ-24−45Cys were synthesized by the solid-phase Merrifield method on an Applied Biosystems automated peptide synthesizer. The extra cysteine was added to the C-terminus of the Pγ-24−45 sequence as a site for the introduction of the environmentally sensitive fluorescent probe BC. The peptides were purified by RP HPLC on a preparative Aquapore Octyl column (25 × 1cm) (Applied Biosystems). The purity and chemical formula of each peptide were confirmed by fast-atom-bombardment mass spectrometry, and analytical reverse-phase HPLC.

Fluorescent Assays. Fluorescent assays were performed on a F-2000 fluorescence spectrophotometer (Hitachi) in 1 mL of buffer A at room temperature (23−24 °C). The fluorescence of PγBC or Pγ-24−45BC was monitored with excitation at 445 nm and emission at 495 nm. Concentrations of PγBC and Pγ-24−45BC were determined using $\epsilon_{445} = 53\,000$.

Binding of $G_{t\alpha\beta\gamma}$ to ROS Membranes. $G_{t\alpha\beta\gamma}$ (1 μM) and urea-washed ROS membranes containing 20 μM rhodopsin were mixed in 100 μL of buffer A. Where indicated, buffer A contained Pγ or Pγ-24−45. The mixture was then illuminated with a White light transilluminator lamp (Fisher) for 5 min at room temperature (23−24 °C). ROS membranes were centrifuged for 10 min at 20000*g*, and the pellets were rinsed with 300 μL of buffer A. Bound $G_{t\alpha\beta\gamma}$ was then extracted using 5 μM GTPγS and analyzed by SDS−PAGE.

Gel Filtration. Gel filtration of $G_{t\alpha\beta\gamma}$, Pγ, Pγ-24−45, or a mixture of $G_{t\alpha\beta\gamma}$ with Pγ or Pγ-24−45 was carried out on a Superose 12HR (1.0 × 30 cm) column (Pharmacia) using a Bio-Rad 2800 HPLC system. Samples (200 μL) containing $G_{t\alpha\beta\gamma}$ (2 μM) and/or Pγ (3, 10 μM) or Pγ-24−45 (20, 50 μM) were injected onto the column equilibrated with buffer A containing 1 mM β-mercaptoethanol and 0.005% polyoxyethylene ether W-1. Proteins were eluted at 0.4 mL/min. Fractions were collected and analyzed by SDS−PAGE.

Analytical Methods. Protein concentrations were determined by the method of Bradford (1976) using IgG as a standard or using calculated extinction coefficients at 280 nm. SDS−PAGE was performed by the method of Laemmli (1970) in 10−12% acrylamide gels. Rhodopsin concentrations were measured using the difference in absorbance at 500 nm between "dark" and bleached ROS preparations. Coomassie-stained gels were scanned using an HP ScanJet II CX/T scanner and analyzed using NIH Image software. A $K_{1/2}$ for the $G_{t\alpha}GDP-P\gamma$ interaction was calculated from the competition curve using eq 1 derived in Linden (1982):

$$K_{1/2} = \frac{IC_{50}}{1 + H_f/K_d + (R_T/K_d)[(K_d + H_f/2)/(K_d + H_f)]} \quad (1)$$

where IC_{50} is the concentration of Pγ which reduces the relative fluorescence increase by 50%, H_f is the free PγBC concentration in the absence of Pγ, R_T is the total concentration of $G_{t\alpha}GDP$, and K_d is the dissociation constant for the $G_{t\alpha}GDP-P\gamma BC$ complex.

Fitting of the experimental data was performed with nonlinear least-squares criteria using GraphPad Prizm Software.

RESULTS

Interaction of $G_{t\alpha}GDP$ with Pγ. To study the interaction between $G_{t\alpha}GDP$ and Pγ, the Pγ subunit has been labeled with the environmentally sensitive fluorescent probe 3-(bromoacetyl)-7-(diethylamino)coumarin at a single cysteine (Cys68). Previously, Pγ labeled with lucifer yellow vinyl sulfone (PγLY) has been employed to monitor the binding of $G_{t\alpha}GTP\gamma S$ to Pγ (Artemyev et al., 1992). However, we found that binding of $G_{t\alpha}GTP\gamma S$ to PγLY resulted in a maximal fluorescence increase of approximately 3-fold (Artemyev et al., 1992), while the maximal fluorescence increase due to $G_{t\alpha}GTP\gamma S$ binding to PγBC was almost 7-fold (not shown). The calculated affinity of the $G_{t\alpha}GTP\gamma S-P\gamma BC$ interaction was around 4 nM. The K_d for this interaction is nearly 10-fold lower than the K_d for $G_{t\alpha}GTP\gamma S$ binding to PγLY(36 nM) (Artemyev et al., 1992). Moreover, the labeling of Pγ with LY appears to reduce its affinity for $G_{t\alpha}GTP\gamma S$. The K_d of Pγ binding to $G_{t\alpha}GTP\gamma S$ calculated from earlier competition experiments is 10 nM (Slepak et al., 1995). The elevated fluorescence increase coupled with the higher affinities lead us to employ PγBC, especially in light of the weaker interaction between $G_{t\alpha}GDP$ and Pγ.

The binding of $G_{t\alpha}GDP$ to PγBC as measured by the fluorescence increase of PγBC is shown in Figure 1A. The curve displays a single class of binding sites with a K_d of 75 ± 8 nM and a maximal fluorescence enhancement $F/F_o = 5.8 \pm 0.2$. Unlabeled Pγ competed with PγBC, resulting in a decrease in fluorescence (Figure 1B). A K_d of 110 nM for Pγ binding to $G_{t\alpha}GDP$ was calculated from the competition curve (Figure 1B). The peak of the fluorescence emission of PγBC (λ = 498 nm) shifted maximally to a

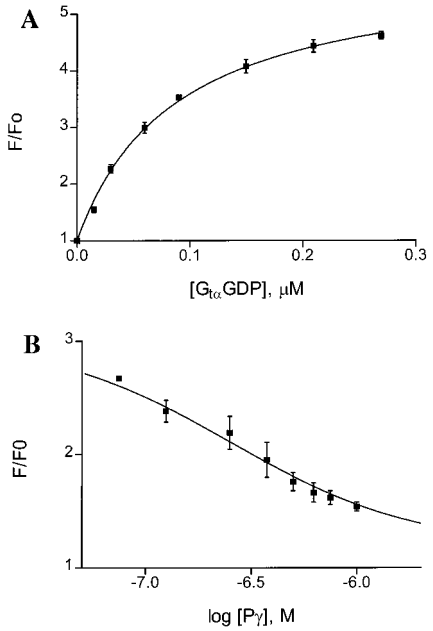

FIGURE 1: (A) Binding of $G_{t\alpha}$GDP to PγBC. The relative increase in fluorescence (F/F_o) of PγBC (25 nM) (excitation at 445 nm, emission at 495 nm) was determined after addition of increasing concentrations of $G_{t\alpha}$GDP. The binding curve (K_d = 75 ± 8 nM, maximum F/F_o = 5.8 ± 0.2) fits the data with r = 0.99. (B) Competition between PγBC and Pγ for binding to $G_{t\alpha}$GDP. Fluorescence of PγBC (25 nM) in the presence of $G_{t\alpha}$GDP (50 nM) was measured before and after addition of increasing concentrations of Pγ. The fluorescent change (F/F_o) is plotted as a function of Pγ concentration. The competition curve (IC_{50} = 230 nM) fits the data with r = 0.96.

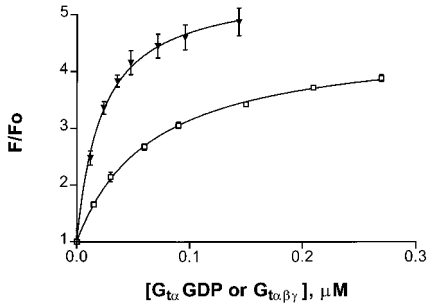

FIGURE 2: Binding of $G_{t\alpha\beta\gamma}$ to PγBC. The relative increase in fluorescence (F/F_o) of PγBC (25 nM) in the presence of 0.5 μM $G_{t\beta\gamma}$ was determined after addition of increasing concentrations of $G_{t\alpha}$GDP (open squares). The relative increase in fluorescence (F/F_o) of PγBC (25 nM) was determined after addition of increasing concentrations of holo-transducin, $G_{t\alpha\beta\gamma}$ (filled triangles). The binding curves (squares: K_d = 64 ± 4 nM, maximum F/F_o = 4.6 ± 0.1; triangles: K_d = 28 ± 3 nM, maximum F/F_o = 5.5 ± 0.2) fit the data with r values of 0.99 and 0.98, respectively.

shorter wavelength (λ = 490 nm) when complexed with $G_{t\alpha}$-GDP, indicative of a more hydrophobic environment for the fluorescent probe.

Interaction of $G_{t\alpha\beta\gamma}$ with Pγ. Addition of $G_{t\beta\gamma}$ (up to 0.5 μM final concentration) to PγBC did not change the PγBC fluorescence. The binding of $G_{t\alpha}$GDP to PγBC in the presence of excess $G_{t\beta\gamma}$ is shown in Figure 2. The binding curve (K_d = 64 ± 4 nM, maximal F/F_o = 4.6 ± 0.1) is analogous to the binding curve in the absence of $G_{t\beta\gamma}$. Holo-transducin interacted with PγBC with even higher affinity (K_d = 28 ± 3 nM) and produced comparable maximal fluorescence enhancement of PγBC (maximal F/F_o = 5.5 ± 0.2) (Figure 2). The somewhat higher affinity of the $G_{t\alpha\beta\gamma}$−PγBC interaction may reflect a greater stability of

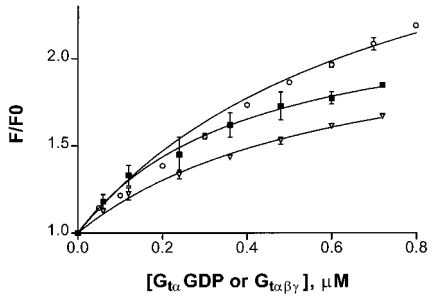

FIGURE 3: Interactions of $G_{t\alpha}$GDP and $G_{t\alpha\beta\gamma}$ with Pγ-24−45BC. The relative increase in fluorescence (F/F_o) of Pγ-24−45BC (50 nM) alone (filled squares) or in the presence of 0.5 μM $G_{t\beta\gamma}$ (open triangles) was determined after addition of increasing concentrations of $G_{t\alpha}$GDP. The relative increase in fluorescence (F/F_o) of Pγ-24−45BC (50 nM) was determined after addition of increasing concentrations of holo-transducin, $G_{t\alpha\beta\gamma}$ (open circles). The binding curve characteristics are (squares) K_d = 400 ± 110 nM, maximum F/F_o = 2.3 ± 0.2, r = 0.96; (triangles) K_d = 610 ± 90 nM, maximum F/F_o = 2.2 ± 0.1, r = 0.99; (circles) K_d = 720 ± 30 nM, maximum F/F_o = 3.2 ± 0.1, r = 0.98.

$G_{t\alpha\beta\gamma}$ than $G_{t\alpha}$GDP after protein purification. The data suggest that $G_{t\beta\gamma}$ and PγBC do not compete for binding to $G_{t\alpha}$GDP.

Interaction of $G_{t\alpha}$GDP and $G_{t\alpha\beta\gamma}$ with Pγ-24−45. A synthetic peptide, Pγ-24−45Cys, was labeled with the BC probe and used to elucidate the interaction between the polycationic region of Pγ and $G_{t\alpha}$GDP or $G_{t\alpha\beta\gamma}$. Figure 3 shows a single class of binding sites for the complex $G_{t\alpha}$-GDP−Pγ-24−45BC with a K_d of 400 ± 110 nM and maximal fluorescence enhancement F/F_o = 2.3 ± 0.2. Similar results were obtained using $G_{t\alpha}$GDP in the presence of excess $G_{t\beta\gamma}$ (K_d = 610 ± 90 nM, F/F_o = 2.2 ± 0.1) and holo-transducin (K_d = 720 ± 30 nM, F/F_o = 3.2 + 0.1) (Figure 3). This indicates that $G_{t\beta\gamma}$ does not affect binding of $G_{t\alpha}$GDP to Pγ-24−45.

Binding to R Blocks Transducin Interaction with PγBC.* Next, the effects of $G_{t\alpha\beta\gamma}$ binding to R* on the interaction between $G_{t\alpha\beta\gamma}$ and PγBC were investigated. In these experiments, $G_{t\alpha\beta\gamma}$ was mixed with "dark" urea-washed ROS membranes that were depleted of active components of the visual transduction cascade except for rhodopsin. The rhodopsin to $G_{t\alpha\beta\gamma}$ molar ratio (25:1) was sufficient to bind almost all transducin added, with less than 10% of added $G_{t\alpha\beta\gamma}$ remaining in the supernatant following centrifugation of the bleached ROS membranes. In control experiments, urea-washed ROS membranes were substituted with hydroxylamine treated ROS membranes. Addition of urea-washed ROS membranes containing 2 μM rhodopsin or an equivalent concentration of hydroxylamine-treated ROS membranes to PγBC did not change the PγBC fluorescence. After illumination to allow tight binding of $G_{t\alpha\beta\gamma}$ to R*, the $G_{t\alpha\beta\gamma}$−R* complexes were added to an assay buffer containing PγBC for the fluorescence measurements. The interaction between $G_{t\alpha\beta\gamma}$ and PγBC was blocked by binding of $G_{t\alpha\beta\gamma}$ to R*, as measured by the fluorescence decrease (Figure 4A). Addition of hydroxylamine-treated ROS, that did not contain R*, produced no decrease in the fluorescence of the $G_{t\alpha\beta\gamma}$−PγBC complex (Figure 4A). $G_{t\alpha\beta\gamma}$ retained low-affinity binding to hydroxylamine-treated or "dark" ROS membranes under the conditions of the fluorescence assay. Approximately 20% and 25% of transducin were bound to hydroxylamine-treated and "dark" ROS membranes, respectively (Figure 4B). Presumably, this binding was due to the

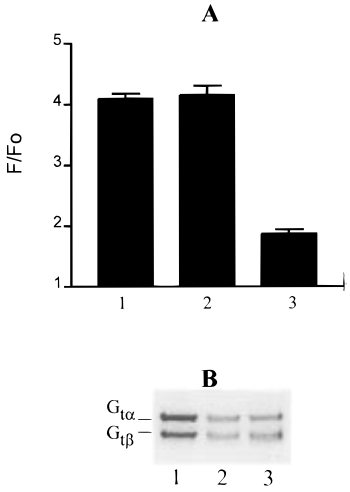

FIGURE 4: Effect of binding to R* on transducin interaction with PγBC. (A) Samples of $G_{t\alpha\beta\gamma}$ in 40 μL of buffer A alone (1), $G_{t\alpha\beta\gamma}$ mixed with hydroxylamine-treated ROS membranes in 40 μL of buffer A (2), and $G_{t\alpha\beta\gamma}$ mixed with urea-washed ROS membranes in 40 μL of buffer A (3) were illuminated for 5 min and then added to 1 mL of buffer A containing PγBC for the fluorescence measurements. Final concentrations of rhodopsin, $G_{t\alpha\beta\gamma}$, and PγBC were 2 μM, 80 nM, and 80 nM, respectively. The bars represent mean ±SE for three independent measurements. (B) SDS−polyacrylamide gel stained with Coomassie Blue. Binding of $G_{t\alpha\beta\gamma}$ to urea-washed ROS membranes (1), hydroxylamine-treated ROS membranes (2), and "dark" ROS membranes (3) was carried out as described in panel A, except "dark" ROS membranes were not illuminated. The ROS membranes were pelleted from 1 mL of buffer A, and the membrane-bound $G_{t\alpha\beta\gamma}$ was extracted using hypotonic buffer (buffer A without 100 mM NaCl) containing 5 μM GTPγS and analyzed by SDS−PAGE.

interaction of $G_{t\alpha\beta\gamma}$ with the disk membranes, and it had no effect on the $G_{t\alpha\beta\gamma}$−PγBC interaction (Figure 4A).

Pγ and Pγ-24−45 Block Binding of Transducin to R.* An earlier study has shown that Pγ at high concentrations dissociated $G_{t\alpha\beta\gamma}$ into $G_{t\alpha}$GDP and $G_{t\beta\gamma}$ and comigrated with $G_{t\alpha}$GDP over a gel filtration column (Otto-Bruc et al., 1993). Therefore, concentrations of Pγ and Pγ-24−45 that may interfere with the $G_{t\alpha}$−$G_{t\beta\gamma}$ interaction were determined first. The gel filtration experiments were performed essentially as described in Otto-Bruc et al. (1993). Gel filtration of $G_{t\alpha\beta\gamma}$ alone already showed slight dissociation of the holotransducin into $G_{t\alpha}$GDP and $G_{t\beta\gamma}$ (Figure 5A,D). Addition of 3 μM Pγ had no notable effect on the elution profile of $G_{t\alpha\beta\gamma}$ (not shown). In the presence of 10 μM Pγ, the degree of $G_{t\alpha\beta\gamma}$ dissociation was increased (Figure 5B,D). However, only trace amounts of Pγ were detected in fraction 4 (Figure 5B), suggesting that the affinity of the $G_{t\alpha}$−$G_{t\beta\gamma}$ interaction might be higher than that of the $G_{t\alpha}$GDP(or $G_{t\alpha\beta\gamma}$)−Pγ interaction. It appears that Pγ has additional low affinity nonspecific site(s) on $G_{t\alpha}$. Binding of Pγ to this (these) site(s) may, competitively or noncompetitively, cause dissociation of $G_{t\alpha}$ and $G_{t\beta\gamma}$ subunits. Pγ-24−45 (up to 50 μM) had no effect on the elution profile of $G_{t\alpha\beta\gamma}$ on a Superose 12HR (Pharmacia) (not shown).

To test if binding of $G_{t\alpha\beta\gamma}$ to R* could be blocked in the presence of excess Pγ, $G_{t\alpha\beta\gamma}$ was mixed with increasing concentrations of Pγ prior to addition of "dark" urea-washed ROS membranes. Following illumination, the ROS membranes were pelleted by centrifugation, and the membrane-bound transducin was extracted using GTPγS and analyzed with SDS−PAGE. Figure 6 shows that addition of Pγ significantly reduced the amount of $G_{t\alpha\beta\gamma}$ bound to R*. Approximately 55% of transducin was bound to bleached ROS membranes in the presence of 3 μM Pγ (Figure 6A,C). High concentrations of Pγ (>6 μM) disproportionally reduced amounts of $G_{t\beta\gamma}$ in $G_{t\alpha\beta\gamma}$ bound to ROS membranes (not shown). A peptide, Pγ-24−45, had a lower affinity for $G_{t\alpha\beta\gamma}$ than Pγ. Addition of this peptide effectively decreased the binding of $G_{t\alpha\beta\gamma}$ to R* in a dose-dependent manner (Figure 6B,C). Approximately 25% of transducin was bound to bleached ROS membranes in the presence of 50 μM Pγ-24−45 (Figure 6B,C). Taking into account the relatively small size of Pγ-24−45, the data would suggest that the peptide directly, rather than sterically, competes with R* for binding to $G_{t\alpha\beta\gamma}$.

DISCUSSION

Recent studies have demonstrated that $G_{t\alpha}$GDP interacts with Pγ, though at a lower affinity than $G_{t\alpha}$GTP (Otto-Bruc et al., 1993; Yamazaki et al., 1990; Artemyev et al., 1993; Skiba et al., 1995). The structural details and functional significance of the $G_{t\alpha}$GDP−Pγ interaction are not well understood. In an inactive GDP-bound conformation, G-protein α-subunits form tight complexes with the $G_{\beta\gamma}$ subunits. As heterotrimeric proteins, they interact with the corresponding ligand-activated seven transmembrane domain receptors. In this study, effects of $G_{t\beta\gamma}$ and R* on the $G_{t\alpha}$-GDP interaction with Pγ were investigated.

Results reported here suggest that the interaction between $G_{t\alpha}$GDP and Pγ is not affected by $G_{t\beta\gamma}$. The affinity of the $G_{t\alpha}$GDP−Pγ interaction was similar to that when holotransducin or excess $G_{t\beta\gamma}$ was present. This finding does not support the model that the $G_{t\beta\gamma}$ subunits are necessary to release Pγ from the Pγ−$G_{t\alpha}$GDP complex (Yamazaki et al.,1990). Lack of competition between Pγ and $G_{t\beta\gamma}$ for binding to $G_{t\alpha}$GDP is in agreement with studies on the $G_{t\alpha}$−Pγ interaction (Artemyev et al., 1993; Skiba et al., 1996) in a view of the crystal structure of $G_{t\alpha\beta\gamma}$ (Lambright et al., 1996). An earlier study reported cross-linking of Pγ-24−45 to both $G_{t\alpha}$GTPγS and $G_{t\alpha}$GDP (Artemyev et al., 1993). The cross-linking site on $G_{t\alpha}$GTPγS was localized to the α4/β6 loop, suggesting that Pγ-24−45 has a binding site in the vicinity of this loop (Artemyev et al., 1993). Skiba et al. (1996) have demonstrated that $G_{t\alpha}$GDP interacts with Pγ predominantly through the α3/β5 region of $G_{t\alpha}$. Analysis of the $G_{t\alpha}$ effector interface using $G_{t\alpha}$/$G_{i\alpha}$ chimeras has indicated that a region of $G_{t\alpha}$ (aa 237−270), which contains the α3 helix, α3/β5 loop, and β5 sheet, interacts with the N-terminal segment, Pγ-1−45, a region that contains the peptide sequence Pγ-24−45 (Skiba et al., 1996). The crystal structure of heterotrimeric transducin shows that the α3/β5 region of $G_{t\alpha}$ in the $G_{t\alpha\beta\gamma}$ complex is readily accessible, especially for the relatively small Pγ molecule. The α3 helix and the α3/β5 loop do not undergo conformational changes upon GTP hydrolysis (Lambright et al., 1994), and $G_{t\alpha}$GDP subunits may remain loosely bound to Pγ after reassociation with $G_{t\beta\gamma}$. However, the binding of $G_{t\beta\gamma}$ to $G_{t\alpha}$GDP could disrupt the interaction of $G_{t\alpha}$GDP with Pγ if the latter is bound to the large catalytic PDE subunits. $G_{t\alpha}$GDP has been reported to activate PDE at very high concentrations ($K_a \sim$ 50 μM), and the activation was reversed by the $G_{t\beta\gamma}$ subunits (Kutuzov & Pfister, 1994). This suggests that $G_{t\beta\gamma}$ sterically interferes with $G_{t\alpha}$GDP binding to Pγ complexed with P$\alpha\beta$.

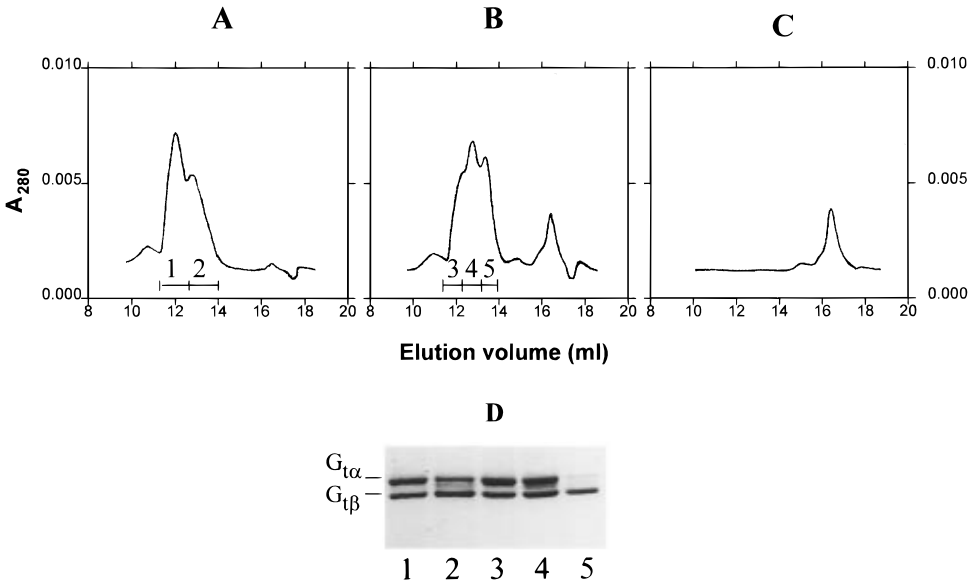

FIGURE 5: Effects of Pγ on the gel filtration elution profile of $G_{t\alpha\beta\gamma}$. Gel filtration of 200 μL samples of (A) 2 μM $G_{t\alpha\beta\gamma}$ alone, (B) 2 μM $G_{t\alpha\beta\gamma}$ in the presence of 10 μM Pγ, or (C) 10 μM Pγ alone was performed as described under Experimental Procedures. (D) SDS−polyacrylamide gel stained with Coomassie Blue. (1−5) Fractions after the HPLC gel filtration as indicated in panels A and B.

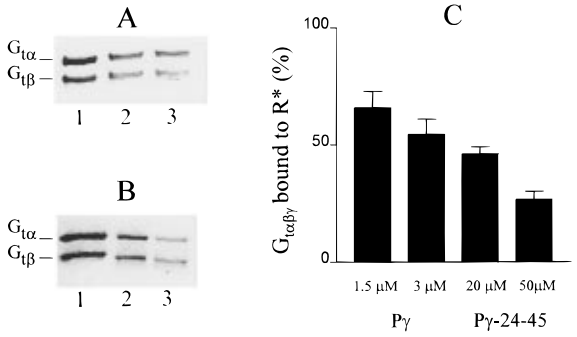

FIGURE 6: Pγ and Pγ-24−45 block $G_{t\alpha\beta\gamma}$ binding to R*. (A, B) SDS−polyacrylamide gels stained with Coomassie Blue. Binding of $G_{t\alpha\beta\gamma}$ to R* was performed as described under Experimental Procedures. $G_{t\alpha\beta\gamma}$ binding to R* (A, B; lane 1) in the presence of 1.5 μM (A, lane 2) and 3 μM (A, lane 3) Pγ or 20 μM (B, lane 2) and 50 μM (B, lane 3) Pγ-24−45. (C) Effects of Pγ and Pγ-24−45 on $G_{t\alpha\beta\gamma}$ binding to R*. The bars represent mean ±SE for the scans of three gels.

Tryptophan 207 in $G_{t\alpha}$ has been implicated as a critical residue in effector binding (Faurobet et al., 1993). This residue is located within the switch II region that is covered by the β-propeller domain of $G_{t\beta}$ in $G_{t\alpha\beta\gamma}$ (Lambright et al., 1996). Therefore, it appears that Trp207 is not essential for the interaction between $G_{t\alpha}$GDP and Pγ. Binding of GTP to $G_{t\alpha}$ leads to dissociation of the $G_{t\beta\gamma}$ subunits and exposes the switch II region (Lambright et al., 1996). However, the GTPγS-induced conformational change of $G_{t\alpha}$ brings the exposed side chains of the conserved residues Arg201, Arg204, and Trp207 into contacts with conserved residues in the α3 helix. Trp207 is then buried between the side chains of Leu245 and Ile249 (Lambright et al., 1994). Possibly, a failure of the Trp207Phe mutant of $G_{t\alpha}$ to form important contacts with the α3 helix, rather than direct interaction of Trp207 with Pγ, is responsible for the mutant inability to effectively bind and activate PDE (Faurobet et al., 1993).

Two lines of experimental evidence suggest that the R*-binding surface on $G_{t\alpha\beta\gamma}$ may overlap with the Pγ-binding sites. First, binding to R* effectively blocked transducin interaction with the fluorescently labeled Pγ. Second, addition of excess Pγ or Pγ-24−45 prevented binding of $G_{t\alpha\beta\gamma}$ to R*. However, the possibility that conformational changes occur on the Pγ-binding sites of $G_{t\alpha\beta\gamma}$ upon formation of the R*−$G_{t\alpha\beta\gamma}$ complex cannot be excluded. Based on results of this study and the evidence that Pγ and Pγ-24−45 most probably interact with the α3−β5 region on $G_{t\alpha}$GDP (Cunnick et al., 1994; Skiba et al., 1996), it seems likely that R* either has a binding site or induces conformational change within the α3−β5 region of $G_{t\alpha}$. This conclusion is consistent with the proposed surface for $G_{t\alpha\beta\gamma}$ interaction with R* (Lambright et al., 1996). The α3−β5 domain is positioned on the same face of $G_{t\alpha\beta\gamma}$ as the myristoylated N-terminus of $G_{t\alpha}$, the farnesylated C-terminus of $G_{t\gamma}$, and the regions $G_{t\alpha}$-311−328 and $G_{t\alpha}$-340−350 that were previously implicated in the binding to R* (Hamm et al., 1988). In fact, from the crystal structure of $G_{t\alpha}$GDP the shortest distance between the α3−β5 region (Ser259) and the $G_{t\alpha}$-340−350 region (Ile340) is only ∼10 Å. Arg310 of $G_{t\alpha}$ is protected from tryptic cleavage upon $G_{t\alpha\beta\gamma}$ binding to R* (Mazzoni & Hamm, 1996) and is only ∼10 Å away from the Thr257 in the α3−β5 region as determined using the RasMol program (v. 2.6-beta-2). Furthermore, regions of $G_{t\alpha}$, $G_{t\alpha}$-310−329, and the α4/β6 loop may represent additional sites for competitive binding of Pγ and R*. $G_{t\alpha}$-310−329 overlaps with the $G_{t\alpha}$-293−314 domain that appears to participate in PDE activation by $G_{t\alpha}$GTP (Rarick et al., 1992; Artemyev et al., 1992; Spickofsky et al., 1994; Skiba et al., 1996). In a recent study, Liu et al. (1996) have investigated the $G_{t\alpha}$GTPγS−Pγ interaction using cross-linking of $G_{t\alpha}$GTPγS to Pγ. Two out of three identified cross-linked residues (Met308 and Arg310) are situated within the α4/β6 loop of $G_{t\alpha}$ (Liu et al., 1996). However, it is not clear if $G_{t\alpha}$-293−314 is involved in $G_{t\alpha}$GDP interaction with Pγ.

The α3−β5 region in heterotrimeric G-proteins is adjacent to the consensus sequence NKXD for the guanine ring binding of GDP or GTP. Binding of activated receptors to the α3−β5 region of G_{α} subunits or a receptor-induced conformational change within this domain would explain the

drastically reduced affinity of G-proteins for GDP in the receptor/G-protein complex.

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