Rhodopsin-Interacting Surface of the Transducin γ Subunit†

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ABSTRACT: The visual signaling pathway is initiated by photoactivation of the GPCR rhodopsin, which activates nucleotide exchange on the heterotrimeric G-protein transducin (Gt). Domains on both Gtα and Gt$\beta\gamma$ subunits participate in coupling to rhodopsin. Previously, we have shown by high-resolution NMR that the farnesylated C-terminal peptide of Gtγ(60−71), DKNPFKELKGGC, assumes an amphipathic helical conformation during interaction with metarhodopsin II [Kisselev, O. G., and Downs, M. A. (2003) *Structure 11*, 367−373]. This conformation was docked to the structure of holo-Gt to create a model of rhodopsin−Gt interaction. Here we test this model by mutational analysis of Gt. To evaluate the contribution of specific amino acids of the Gtγ C-terminal region involved in binding and GTP-dependent release of transducin from native rhodopsin membranes, we have systematically substituted each of the amino acids in the C-terminal region of Gtγ for alanine. The mutants were co-expressed with six-histidine-tagged Gtβ subunits in Sf9 insect cells. The Gtβ-6-His-γ mutant proteins were purified and assayed in the presence of Gtα for the GTP-dependent interactions with light-activated rhodopsin. Several of the alanine mutants, N62A, P63A, and F64A, exhibited significant functional defects at the level of R*−Gt complex formation. These data show that the conserved N-terminal end of the helical domain in the Gtγ(60−71) region has the most significant effect on rhodopsin−Gt interactions, which places important constraints on the model of the rhodopsin−Gt complex.

Transducin is a retinal rod cell specific heterotrimeric GTP-binding protein, or G-protein, responsible for transducing visual signals from rhodopsin to the cGMP phosphodiesterase (*1*−*3*). The GTP-bound transducin α subunit, Gtα-GTP, is a molecular switch ultimately responsible for activating the effector enzyme. The initial interaction with the light-activated rhodopsin, R*, and nucleotide exchange on Gtα are dependent on the tight $\beta\gamma$ subunit complex, Gt$\beta\gamma$. Both the GDP-bound transducin α subunit, Gtα-GDP, and Gt$\beta\gamma$ have specialized protein domains involved in R* interactions. The carboxyl-terminal region of Gtγ, which is modified posttranslationally by farnesylation and carboxymethylation, is the primary domain of Gt$\beta\gamma$ involved in R*−Gt$\beta\gamma$ interactions (*4*).

Recent NMR studies of a synthetic peptide, Gtγ(60−71)-farnesyl, bound to R* (*5*) and photoaffinity labeling studies using holotransducin (*6*) show that the C-terminal region of Gtγ is involved in conformational rearrangements that change the position of both the amino acid residues of Gtγ(60−71) and the farnesyl moiety during binding to R*. These results support conclusions made earlier about unmasking of the Gtγ C-terminal domain upon binding of Gt to R*, based on the specific antibody recognition and Gtγ tail accessibility to the carboxypeptidase Y (*7*). The NMR study proposed that Gtγ(60−71) assumes an amphipathic helical conformation upon binding to R*. A molecular model of the R*−Gt complex was on the basis of the computer docking of the peptide structure to the Gt structure in the inactive state. To test this model on the level of holo-Gt, for detailed understanding of the R*−Gt interaction mechanism, and to elucidate the contribution of individual residues of Gtγ in R* binding, we replaced each individual amino acid in the Gtγ(60−71) region with alanine. Recombinant Gt$\beta\gamma$ mutants were expressed in an insect cell/baculovirus expression system, purified, and reconstituted with purified retinal Gtα. The ability of mutant proteins to interact with R* in the native lipid membrane was assessed by a Gt binding and GTP-dependent release assay. The results identify the molecular surface of Gtγ involved in R* interactions, which is important for building a model of the R*−Gt complex.

MATERIALS AND METHODS

Insect Cells and Construction of Baculoviruses Containing Mutant Gtγ**.** *Spodoptera frugiperda* Sf9 cells were obtained from ATCC (ATCC CRL 1711) and maintained in suspension at 27 °C at 120 rpm in Sf-900 II SFM medium (Gibco BRL) or in Grace's complete. Erlenmeyer flasks with the cell suspension were shaken in an INNOVA model 4000 device (New Brunswick Scientific) equipped with a refrigeration unit to maintain a temperature of 27 °C. Cells were kept between 1×10^6 and 3×10^6 cells/mL in the log phase. Gtγ1 (bovine) mutants were constructed using PCR by introducing desired nucleic acid substitutions into the 3′

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primer, which also had an XbaI restriction site introduced immediately after the TAA stop codon for easy subcloning. The 5′ primer contained the engineered PstI site but was otherwise identical to the wild-type nucleic acid sequence in the untranslated region of Gtγ immediately preceding the ATG start codon. The baculoviruses were constructed using the Bac-to-Bac Baculovirus Expression System (Gibco BRL). The mutant cDNA was subcloned into the pFastBac vector (Invitrogen) for transformation of DH5-α competent cells (Gibco). Transformants were grown on LB-ampicillin plates for selection. The DNA from positive colonies was isolated using the Wizard miniprep kit (Promega) and analyzed by digestion with appropriate restriction endonucleases and DNA sequencing.

For transposition into the bacmid, pFastBac/γ1 mutant DNA was added to DH10Bac competent cells (Invitrogen) on ice and incubated for 30 min, heat shocked at 42 °C for 45 s, and placed on ice; 300 μL of SOC medium was added and then the mixture incubated in a 37 °C shaker for 4 h. Serial dilutions of transposition mix in SOC medium were prepared, and 100 μL of undiluted and serially diluted mix was plated onto Luria agar plates containing 50 μg/mL kanamycin, 7 μg/mL gentamicin, 10 μg/mL tetracycline, 100 μg/mL Bluo-gal, and 40 μg/mL IPTG and the mixture incubated for 48 h at 37 °C for color development. White colonies were restreaked on Luria agar complete plates and subcultured. Isolation of the recombinant bacmid and SF9 cell transformation were performed as per the Bac-to-Bac Baculovirus Expression System protocol using cellfectin. The nucleotide sequence of Gtγ in each bacmid was confirmed by DNA sequencing. Each virus was amplified four times to produce a high-titer stock. His-tagged Gtβ1 baculovirus was a gift from T. Kozasa.

Protein Expression and Purification. Purification of the recombinant Gt$\beta\gamma$ subunits was essentially as described previously (*8*). Prior to large-scale protein expression, the β1:γ1 ratio was titrated to that of the native Gt$\beta\gamma$ complex by adjusting the ratios of the β1 and γ1 viruses and monitoring the protein expression by immunoblotting with antibodies against Gtγ1 and Gtβ1 subunits on a Western blot (*9*). Large protein preparations were made in 500 mL cultures at a density of 1×10^6 SF9 cells/mL in Grace's complete. On average, the protein production peaked 36−48 h post-infection. Infected cells were pelleted at 3000 rpm and 4 °C for 15 min, decanted and resuspended in 30 mL of ice-cold membrane wash buffer (MWB) [50 mM sodium phosphate (pH 8), 150 mM NaCl, 10 mM β-mercaptoethanol, 21 μg/mL PMSF, 0.01% TLCK, and 0.01% TPCK], and lysed in Parr bomb on ice at 1000 psi for 30 min. The cell lysate was centrifuged at 310000*g* in a Beckman ultracentrifuge at 4 °C for 30 min, aspirated, and resuspended in 2 mL of MWB.

For membrane binding studies, aliquots of the pellet, which represented the membrane fraction, and aliquots of the supernatant, containing the cytosolic fraction, were saved for immunoblotting analysis. For protein purification, the pellet was homogenized with a Potter-Elvenjem homogenizer and the protein concentration was measured using a Bradford assay (Bio-Rad). The protein was solubilized with 1% octyl glucoside; the protein concentration was adjusted to 5 mg/mL, and the protein was incubated on ice for 1 h with frequent homogenizing and then pelleted in a 70Ti rotor at 310000*g* and 4 °C for 30 min. Proteins were affinity purified on a Ni−NTA agarose column (Qiagen). The purified Gtβ-6-His−Gtγ protein complex was eluted per the Qiagen protocol, dialyzed in 10 mM Tris-HCl (pH 8), 150 NaCl, and 10 mM β-mercaptoethanol, concentrated, and stored in the presence of 50% glycerol at −20 °C. The purified protein was analyzed by silver staining (*10*) and immunoblotting. The concentration of all mutants was normalized to that of a recombinant wild type to ensure accurate comparison of activities in functional assays.

Rod Outer Segment Preparation and Gt Purification. Dark-adapted frozen bovine retinas were obtained from W. L. Lawson, Co. Rod outer segments (ROS) were prepared by the method of Papermaster and Dreyer as we described (*9*). Urea-washed ROS membranes (UM) were prepared using the procedure adapted from Yamazaki et al. (*11*) and Willardson et al. (*12*). The purity of rhodopsin was assessed by SDS−PAGE and protein staining with silver (*10*). The rhodopsin concentration was measured as ΔA_{498} before and after bleaching in the presence of 20 mM hydroxylamine, based on the molar extinction coefficient at 498 nM of 42 700 M^{-1} cm^{-1}. ROS disk membranes were resuspended in ROS-Iso buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF] and stored at −70 °C.

Gt was purified by GTP elution from isotonically washed ROS disks, and Gtα and Gt$\beta\gamma$ were separated by AKTA FPLC on Blue-Sepharose Cl-6B. ROS disks were resuspended under bright light in ROS-Hypo buffer for 10 min. The suspension was centrifuged at 250000*g* for 20 min (Ti-60, Beckman), and the pellet was washed in ROS-Hypo [10 mM Tris-HCl (pH 7.4), 0.5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF] by the same procedure once more. Gt was released by three additional washes with ROS-Hypo buffer and 100 μM GTP. Supernatants of the GTP-containing washes were combined, and Gtα and Gt$\beta\gamma$ were separated by AKTA FPLC on a 25 mL Blue-Sepharose Cl-6B column (Pharmacia) equilibrated in 10 mM HEPES (pH 7.5), 5 mM $MgSO_4$, 1 mM EDTA, 1 mM DTT, and 10 μM PMSF. Gt is applied at a rate of 0.5 mL/min at 4 °C. Unbound fractions were collected and analyzed by SDS−PAGE, and those containing Gt$\beta\gamma$ were combined and concentrated to 1−2 mL on the mini DEAE-Sephacel column. After a short 20 mL wash, Gtα bound to the Blue-Sepharose was eluted with a 75 mL linear gradient of KCl (from 0 to 1 M) in a column equilibration buffer. Fractions containing Gtα were combined and concentrated to 1−2 mL by ultrafiltration using an Amicon PM-10 filter. Finally, Gtα and Gt$\beta\gamma$ were desalted separately on a Superose-12 gel filtration column by AKTA FPLC [flow rate of 1 mL/min; final buffer of 10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 1 mM DTT]. Fractions were analyzed by SDS−PAGE and silver staining. Protein concentrations were determined by the Bio-Rad protein assay, using BSA as a standard. Separated subunits of transducin were aliquoted and stored at −20 °C in the presence of 50% glycerol.

R−Gt Binding and Release Assay*. Various amounts of Gt$\beta\gamma$ (0, 0.1, 0.25, 0.4, 0.55, and 0.7 μg) were reconstituted with 3 μg of Gtα and 30 μg of urea-washed ROS membranes (UM) in 100 μL of buffer [10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF] under dark-red light on ice essentially as we described (*9*).

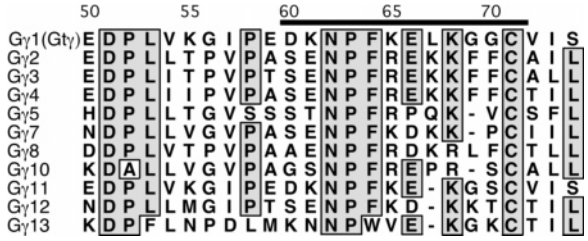

FIGURE 1: Amino acid sequence alignment of the Gtγ(50−71) region with the corresponding amino acid sequences of various subtypes of Gγ subunits. All Gγ subunits are mouse specific, except for Gγ10 and Gγ13 (human) and Gγ12 (bovine). The black bar represents the region targeted by the alanine replacements.

In control reactions, the Gtα was omitted. An aliquot of the reaction mixture was taken at this stage to assess binding of Gt to dark-adapted rhodopsin membranes. This aliquot was centrifuged, and the pellet was washed twice with ROS-Iso buffer in the dark. The reaction was initiated by exposure of the rest of the samples to 480 nm light, followed by a 15 min incubation at 4 °C. UM were centrifuged at 109000*g* and 4 °C for 10 min in a TLA-100.3 rotor on a Beckman TL-100 ultracentrifuge. The pellet was washed twice with ROS-Iso buffer, and another aliquot was taken to measure the amount of Gt bound to R* before the addition of GTPγS. UM with Gt bound were resuspended in 10 mM Tris-HCl (pH 7.4), 0.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and 250 μM GTPγS, incubated on ice for 30 min, and centrifuged as described above. The supernatant and membrane-bound fractions were analyzed for the presence of G-protein subunits by immunoblotting. Quantification was by image analysis of the ECL films. Band intensity calculations were carried out in Image Gauge (FujiFilm).

RESULTS

To study the involvement of individual amino acid side chains of the C-terminal region of Gtγ in interactions with light-activated rhodopisn, R*, we have individually substituted each of 11 amino acid residues of the Gtγ(60−71) domain with alanine. The amino acid sequence of Gtγ and its alignment with other members of Gγ family are shown in Figure 1. Cysteine 71 was preserved in all mutants because it is the site of posttranslational farnesylation and carboxymethylation in native retinal rod cell Gtγ and is critical for Gt functions (*13*). Alanine is one of the most common natural amino acids and is the second-smallest amino acid after glycine. Unlike substitutions with glycine, alanine replacements are less likely to perturb the protein secondary structure, especially in α-helices (*14*). Because of these properties, alanine has been used historically in site-directed alanine scanning mutagenesis to replace the side chains under investigation with methyl group side chains.

Each Gtγ mutant was coexpressed with Gtβ-6-His in insect Sf9 cells using genetically engineered baculoviruses. Previous studies have shown that the baculovirus/insect cell system provides an excellent source of recombinant G-proteins, due to high levels of protein expression and the presence of intracellular machinery for effective posttranslational isoprenylation of G-protein γ subunits (*5*, *15*−*20*).

To determine whether alanine replacements affected the membrane binding properties of the Gt$\beta\gamma$ dimers, the aliquots of infected cells were lysed and the distribution of recombinant G$\beta\gamma$ between the membrane and cytosolic fraction was compared to that of a wild-type recombinant Gt$\beta\gamma$. All mutants exhibited a distribution in the membrane and cytosol similar to that of wild-type G$\beta\gamma$ (Figure 2). Only the membrane fraction, containing farnesylated Gt$\beta\gamma$, was used for further detergent extraction and purification on Ni−NTA affinity resin.

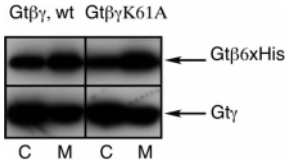

FIGURE 2: Interactions of Gt$\beta\gamma$ with lipid membranes. Immunoblot of Gtβ (top) and Gtγ (bottom) in the cytosolic fraction (C) and the membrane fraction (M) of Sf9 cells after expression of the wild type (wt) and mutant Gt$\beta\gamma$. Identical results were obtained for all mutants. The data are representative of at least two independent experiments.

Affinity-purified recombinant Gt$\beta\gamma$ dimers contained both Gtβ-6-His and mutant Gtγ in ratios identical to those of the wild-type Gt$\beta\gamma$ dimers, which shows that none of the mutations affected the formation of the $\beta\gamma$ complex. Protein concentrations were normalized between the various Gt$\beta\gamma$ mutants by quantitative immunoblotting to ensure functional results can be compared directly. Purified recombinant Gtβ-6-His showed the expected increase in molecular mass of ∼840 Da compared to the Gtβ purified from bovine retina rods (mass of 37 377 Da), due to the presence of a six-histidine tag at the N-terminus of the β subunit. This difference in molecular mass was helpful in distinguishing between binding of recombinant Gt$\beta\gamma$-6-His to rhodopsin membranes and the background level of native retinal Gt$\beta\gamma$ always present in small amounts, even in urea-washed ROS membranes. Recombinant Gtγ mutants had a molecular mass similar to that of native Gtγ as determined from immunoblots of 12% Laemmli gels.

Transducin binds to light-activated rhodopsin in the absence of guanine nucleotide GTP or GDP to form a nucleotide-empty ternary R*−Gt complex in the membrane. Addition of GTP, or nonhydrolyzable analogue GTPγS, activates transducin and dissociates the R*−Gt complex. The amount of Gt bound and released by GTP can be visualized by gel staining or immunoblots. Originally developed by Kühn (*21*), the assay is based on the R*−Gt binding and release which allows quantitative measurement of interactions of R* with individual subunits of Gt (*5*, *7*, *9*, *16*). Binding and GTP-induced release of Gt$\beta\gamma$ from R* are dependent on the presence of Gtα. No interactions of recombinant Gt$\beta\gamma$ with R* were observed when Gtα was omitted from the reaction mixture, which is evident from the lack of the higher-molecular mass band of Gtβ-6-His in GTPγS eluates even at high concentrations of the recombinant protein (Figure 3A).

To investigate the rhodopsin-interacting surface of the transducin γ subunit, and to evaluate how site-directed alanine replacements in the Gtγ(60−71) domain affect interactions with rhodopsin, equal amounts of recombinant Gt$\beta\gamma$ mutants in identical dilution series were reconstituted with Gtα purified from bovine retinas and analyzed for interactions with R*. GTPγS eluates were analyzed by quantitative immunoblotting for the presence of Gt, using a

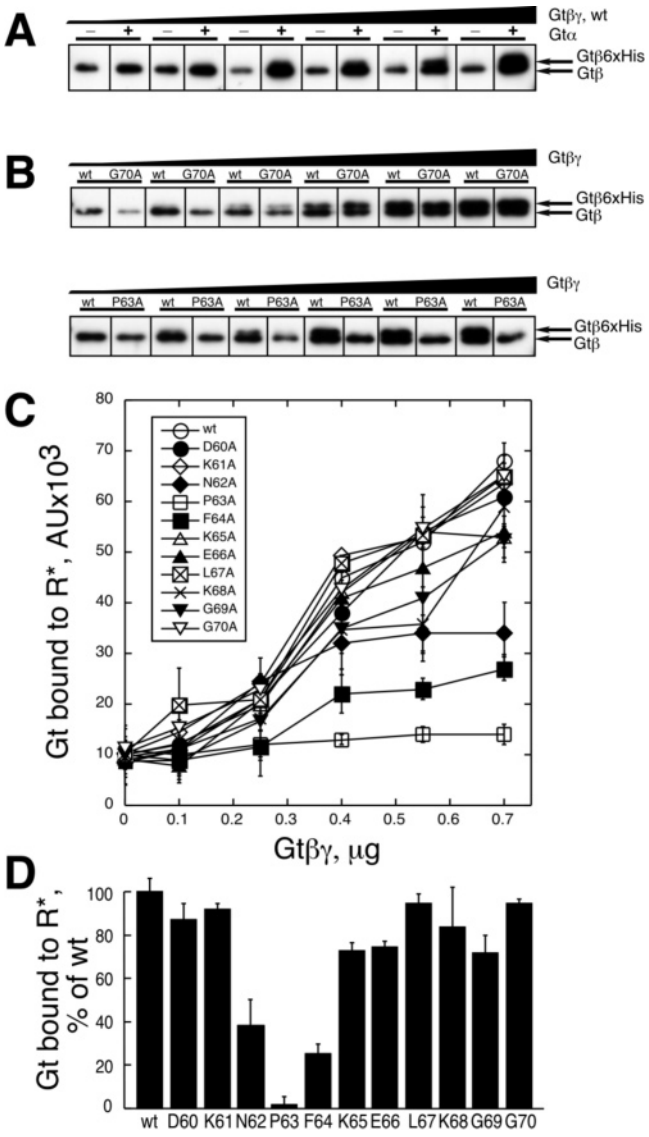

FIGURE 3: Interaction of Gt$\beta\gamma$ mutants with light-activated rhodopsin in native membranes measured by binding and subsequent GTPγS-induced dissociation of the R*−Gt complex. (A) The interaction of Gt$\beta\gamma$ with R* is Gtα subunit-dependent. Increasing amounts of Gt$\beta\gamma$ (0, 0.1, 0.25, 0.4, 0.55, and 0.7 μg) from left to right are shown by the top bar. The absence and presence of 3 μg of Gtα for each dose of Gt$\beta\gamma$ are indicated with minus and plus signs, respectively. The immunoblot of GTPγS eluates represents three independent experiments. (B) Western blot analysis of Gt$\beta\gamma$, wild type and mutants in pairs, in GTPγS eluates after interaction with the rhodopsin membranes in the presence of purified Gtα. The bottom band (Gtβ) represents the background endogenous Gt$\beta\gamma$ complex. The top band (Gt$\beta\gamma$-6-His) represents interactions of the recombinant Gt$\beta\gamma$ (G70A or P63A) with rhodopsin under identical conditions. Starting amounts of the Gt$\beta\gamma$ subunits were normalized in preliminary experiments. (C) Dose-dependent interaction of mutant Gt$\beta\gamma$ with rhodopsin membranes, based on the quantitative densitometry of Western blots. The data represent two to five independent experiments for each mutant. (D) Maximal binding for individual mutants expressed in percentage points compared to the control, wild-type (wt) Gt$\beta\gamma$.

highly specific anti-Gtβ antibody (*9*), as shown for Gt$\beta\gamma$ mutants G70A and P63A as examples (Figure 4B). For all mutants, the intensity of the recombinant Gtβ-6-His band was quantified as shown in Figure 4C, and the maximal values were expressed in percentage points using interactions of wild-type (wt) Gt with R* as 100% (Figure 4D). Four

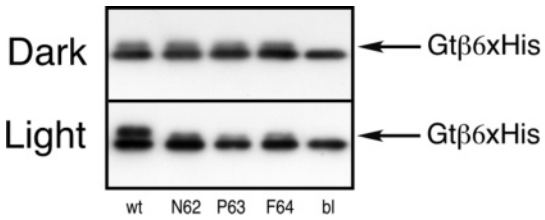

FIGURE 4: Gt$\beta\gamma$ mutations affect formation of the R*−Gt complex. Wild-type and mutant Gt$\beta\gamma$ bound to dark-adapted (Dark) and light-activated (Light) rhodopsin membranes in the absence of nucleotides. The experimental conditions are as described in the legend of Figure 3 at 0.7 μg of Gt$\beta\gamma$, 3 μg of Gtα, and 30 μg of rhodopsin membranes. Blank reaction (bl) contained only rhodopsin membranes. The immunoblot represents two independent experiments.

mutants, Gt$\beta\gamma$D60A, Gt$\beta\gamma$K61A, Gt$\beta\gamma$L67A, and Gt$\beta\gamma$G70A, had activity almost identical to that of wild-type Gt$\beta\gamma$. Gt$\beta\gamma$K65A, Gt$\beta\gamma$E66A, Gt$\beta\gamma$K68A, and Gt$\beta\gamma$G69A mutations affected R* interactions mildly, showing an activity that was ~70% of that of the wild type. GtγP63A exhibited only residual activity compared to wild-type Gt$\beta\gamma$, while adjacent mutations GtγN62A and GtγF64A were at 38 and 25%, respectively. These functional defects can result from abnormalities at three different steps of the activation process: (1) binding of Gt to dark-adapted rhodopsin membranes, (2) formation of a nucleotide-free R*−Gt complex, and (3) GTP-induced activation of Gtα and dissociation of the complex. To discriminate between these possibilities, we reconstituted the three most affected Gt$\beta\gamma$ mutants (GtγN62A, -P63A, and -F64A) with Gtα and analyzed the amount of Gt bound to rhodopsin membranes in the dark, and after light activation in the absence of nucleotides. Binding to the dark rhodopsin membranes was identical for all mutants and the wild type (Figure 4). The amount of mutant Gt bound to light-activated rhodopsin membranes was affected significantly compared to that of the wild type (Figure 4). The results of light binding parallel well the results of GTPγS activation and complex dissociation (Figure 3D), strongly suggesting that the initial step of formation of the R*−Gt complex is affected in all three mutants.

DISCUSSION

Phototransduction in retinal rod cells is initiated by the interactions between photoactivated rhodopsin and the heterotrimeric transducin. In addition to the interactions between R* and Gtα leading to the nucleotide exchange, Gt$\beta\gamma$ has been shown to play a pivotal allosteric role in supporting docking of the heterotrimer to R* and facilitating Gtα activation (*22*, *23*). Early reports of direct interactions of Gt$\beta\gamma$ with R* (*24*−*26*) and the demonstration of an indispensable role of the farnesyl group in coupling with the activated receptor (*13*, *27*) have been substantiated by the identification of the C-terminal domain on Gtγ involved in direct contacts with R* (*28*).

The site of interactions with R* on Gt$\beta\gamma$ has been mapped to the Gtγ(60−71) region by peptide competition, peptide-induced extra Meta II, amino acid reversal mutagenesis, and NMR studies (*5*, *7*, *28*, *29*). Experiments in two independent laboratories have shown recently that the C-terminal region of Gtγ, including the farnesyl group, undergoes significant conformational change upon interactions with R* in native

lipid membranes (*5*−*7*). The changes include the movement of the farnesyl moiety and the reorganization of the Gtγ C-terminal domain into an amphipathic helix. These results suggest strongly that the molecular surface on Gt$\beta\gamma$ presented for interactions with R* is substantially different from the conformation observed in the X-ray structures of Gt$\beta\gamma$ (*30*).

It has specifically been proposed that the F64 side chain in Gtγ changes orientation dramatically from facing Gtβ to facing R* and that P63 immediately preceding the helix may serve as an anisotropic molecular hinge (*5*). This model, based on the NMR of a synthetic peptide, requires testing on the level of a holo-G-protein. We introduced alanine substitutions in 11 positions along the Gtγ(60−70) stretch, coexpressed the recombinant proteins with Gtβ using the Sf9/baculovirus system, and purified the Gt$\beta\gamma$ dimers containing corresponding C-terminal mutations in Gtγ. After reconstitution with pure retinal Gtα, holo-Gts with mutations N62A, P63A, and F64A were unable to interact with R* effectively (Figure 3). Additional analysis revealed that while binding to the dark-adapted rhodopsin membranes was normal for these mutants, binding to R* was significantly impaired (Figure 4). These results argue that N62A, P63A, and F64A mutations affect formation of the nucleotide-empty R*−Gt complex, rather than the subsequent step of GTP-induced activation of Gtα. The location of functionally important N62, P63, and F64 at the N-terminus of Gtγ(60−71) is consistent with metarhodopsin II stabilization by farnesylated peptides of varying sizes. The peptide data showed that inclusion of Gtγ(62−64) is obligatory for interactions with Meta II, while the Gtγ(65−71) region was necessary but insufficient for activity (*31*).

Four positions in Gtγ, D60, K61, L67, and G70, tolerated replacements with alanine well, which suggests that these amino acids are not involved in R* interactions. Previous mutational studies using synthetic peptides identified the F64−L67 region as being generally important for stabilization of Meta II (*7*). The data presented here narrow this region to the aromatic side chain of F64. The mechanism of inactivating mutations in the Gt(60−71) region by multiple mutations reported earlier can be more complex, however, through disruption of specific involvement of the F64 side chain in R* interactions and also through structural interference in helix formation as has been shown for the K65E66 reversal mutant (*5*).

Several mutants, K65A, E66A, K68A, and G69A, exhibited only mild reduction in activity. Because the side chains of these amino acids do not appear to form a unified interface, the mechanism of these mutations is likely due to the partial negative effect on helix formation in this region during binding to R*. Mutation of two adjacent glycine residues at the very C-terminus immediately before farnesylated C71 had a mixed effect on R* coupling: the G69A mutant was partially active at ~70% of the level of the wild type, while G70A was fully active. The fact that G69A and G70A individually did not have a major effect on R* interactions suggests that either the high mobility of the polypeptide chain in this region is not necessary for the conformational changes involving farnesyl group and helix formation or only one glycine is sufficient for ensuring high conformational mobility. Double mutant G69A/G70A may answer this question fully. Overall, it appears that the N-terminal portion of the C-terminal helical domain of Gtγ in the R*-bound form is critically important for R* interactions (Figure 5).

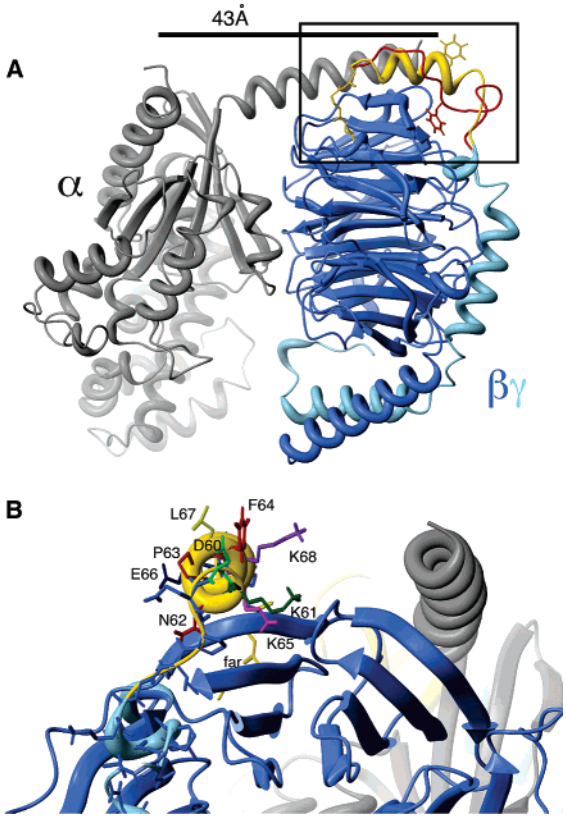

FIGURE 5: Ribbon diagram of Gt based on the X-ray crystallographic studies (PDB entry 1GOT). The conformations of Gtα(340−350) [PDB entry 1AQG (*44*)] and Gtγ(60−71) [PDB entry 1MF6 (*5*)] are shown in the R*-bound state. (A) The ground conformation of Gtγ(60−71)farnesyl is colored red and the R*-bound form yellow. The change in the position of F64 from facing the Gtβ to facing rhodopsin is highlighted. The distance between Gtα L349 and Gtγ F64 is 43 Å. (B) Close-up of the inset in panel A, rotated horizontally by 90°. Residues affected most by alanine replacements, N62, P63, and F64, are colored red.

Amino acid residues N62, P63, and F64 are highly conserved among all members of the Gγ subunit family (Figure 1). Our results explain this conservation, as mutations at these positions lead to the reduced level of interaction with rhodopsin. The pattern of P63 conservation reveals an interesting observation.

P63 is highly conserved among all classes of mammalian Gγ subunits, from subtype 1 to 13 (Figure 1), and Gγs found in *Caenorhabditis elegans* and *Drosophila melanogaster*. Interestingly, Gγ subunits involved in pheromone responses in budding yeast are exceptions to this rule. This unique feature of yeast Gγ may explain a report that the Gγ tail is dispensable in pheromone signaling (*32*), although the contribution of other factors, such as the much larger length of the yeast Gγ C-terminal domain, cannot be ruled out (mammalian Gγ domains have seven amino acids between P63 and C71 of the CAAX box, while the corresponding domain in yeast is 21 amino acids long). It is also possible that pheromone signaling in budding yeast relies on the evolutionarily earlier and simpler GPCR/G-protein system.

Of special interest for the mechanism of Gt activation is the observation that mutations that affect R* binding the most are located on the outer edge of Gtγ that is farther from the Gtα subunit. The distance between amino acid side chains

that have been shown to be critical for R* binding, Gtα L349 and Gtγ F64, is 43 Å (Figure 5B). The rhodopsin cytoplasmic surface in the inactive dark state is elliptical with physical side-to-side dimensions of 45 Å for a major axis and 27 Å for a minor axis. The more realistic functionally important area is only ∼30 Å in diameter, which encompasses loop 2, loop 3, and the N-terminus of helix 8. Numerous studies have shown that upon light activation the cytoplasmic surface increases in size by up to 8 Å, in response to the outward movements of transmembrane helices, most notably helix 6 (TM6) (*33*−*35*). The model of the Meta II state built on the basis of these experimental data has taken into account energetically favorable modes of conformational changes and inherent flexibility of the cytoplasmic loops (*36*, *37*). It reveals that light activation increases the size of the minor axis to approximately 31−33 Å. It is clear that the distance of 43 Å between the two Gt domains is too large for simultaneous interaction with the R* monomer, as predicted in a 1:1 model of the R*−Gt complex (*38*).

Three potential scenarios may help to reconcile this discrepancy. (1) A significant change in tilt between subunits, bringing the C-termini of Gtα and Gtγ closer together (*39*), has been proposed. The lever hypothesis provides a compelling way for R* to exert an allosteric effect on the nucleotide-binding site via Gtβγ contacts with switch regions II and III on Gtα (*39*). (2) Interaction of a single Gt with a rhodopsin dimer has strong support from the atomic microscopy experiments (*40*). This hypothesis does not call for a large reorganization of the Gt subunit interface, thus satisfying the current paradox between the small diameter of the rhodopsin monomer and the large footprint from R* on Gt. The dimer hypothesis does not explain at present, however, the fact that both C-terminal domains of Gt can interact only with active Meta II and not inactive dark-adapted rhodopsin. Both rhodopsin units in a dimer must be light-activated to interact with the C-termini of Gtα and Gtγ. This requirement clashes with the known property of the phototransduction pathway which is to be activated by a single photon of light (*41*). (3) The third mechanism, R*−Gt coupling via two-site sequential fit interactions, has also been proposed (*29*, *42*, *43*). This mechanism suggests temporal separation of interactions between Gtα−R* and Gtγ−R* complexes, with each step having a discrete structural impact on Gt, and possibly on R* conformations, ultimately leading to nucleotide release. Further experiments are required to discriminate among the three mechanisms.

In conclusion, using site-directed alanine scanning mutagenesis of the C-terminal region of Gtγ, we mapped the location of a conserved regulatory switch involved in R*−Gt interactions. Disabling of this switch, which includes amino acids N62, P63, and F64, significantly impairs Gt activation by weakening the ability of mutant Gt to form a complex with light-activated rhodopsin. The location of N62, P63, and F64 at the N-terminal part of an amphipathic helix formed during binding to R* places important distance constraints on the R*−Gt complex.

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