Mutation of the Fourth Cytoplasmic Loop of Rhodopsin Affects Binding of Transducin and Peptides Derived from the Carboxyl-terminal Sequences of Transducin α and γ Subunits*

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The role of the putative fourth cytoplasmic loop of rhodopsin in the binding and catalytic activation of the heterotrimeric G protein, transducin (Gt), is not well defined. We developed a novel assay to measure the ability of Gt, or Gt-derived peptides, to inhibit the photoregeneration of rhodopsin from its active metarhodopsin II state. We show that a peptide corresponding to residues 50–71 of the α subunit of Gt, or a cysteinyl-thioetherfarnesyl peptide corresponding to residues 50–71 of the γ subunit of Gt, are able to interact with metarhodopsin II and inhibit its photoconversion to rhodopsin. Alteration of the amino acid sequence of either peptide, or removal of the farnesyl group from the γ-derived peptide, prevents inhibition. Mutation of the amino-terminal region of the fourth cytoplasmic loop of rhodopsin affects interaction with Gt, (Marin, E. P., Krishna, A. G., Zvyaga T. A., Isele, J., Siebert, F., and Sakmar, T. P. (2000) J. Biol. Chem. 275, 1930–1936). Here, we provide evidence that this segment of rhodopsin interacts with the carboxyl-terminal peptide of the α subunit of Gt. We propose that the amino-terminal region of the fourth cytoplasmic loop of rhodopsin is part of the binding site for the carboxyl terminus of the α subunit of Gt and plays a role in the regulation of βγ subunit binding.

G protein-coupled receptors transmit extracellular signals to the cell's interior via heterotrimeric G proteins and effector enzymes or ion channels (1, 2). Rhodopsin is one of the archetypes of the G protein-coupled receptor superfamily. It triggers the biochemical amplification machinery of the visual cascade in the rod photoreceptor cell, which comprises the G protein transducin (Gt), and the effector, a cyclic GMP-specific phosphodiesterase (3, 4). The transduction of a light signal begins with the photochemical cis-trans isomerization of the chromophore, 11-cis-retinal. Protein conformational changes are transmitted from the ligand-binding site to the cytoplasmic surface of the receptor where catalytic activation of Gt occurs. This intramolecular conversion from inactive (rhodopsin) to active (R*) states mediated by chromophore isomerization has been termed “signal transmission” (5). Key structural correlates of the transition to the active state include deprotonation of the retinylidene Schiff base (6) with concomitant protonation of the Glu117 counterion (7, 8) and the protonation of the cytoplasmic surface of rhodopsin (9, 10) mediated by the highly conserved Glu334 residue at the cytoplasmic border of transmembrane (TM) helix 3. Movements of TM helices have been proposed to accompany the signal transmission process, with a change in the orientation of TM helices 3 and 6 relative to each other as the most prominent feature (11–13).

The cytoplasmic surface of rhodopsin comprises four loops and a carboxyl-terminal tail. The first (C1), second (C2), and third (C3) cytoplasmic loops connect adjacent TM helices. The fourth cytoplasmic loop (C4) is bounded by TM helix 7 at its amino terminus and two palmitoyl groups inserted into the membrane bilayer at its carboxyl terminus. The palmitoyl groups are attached to Cys322 and Cys325 via thioester linkages. A schematic of the structure of rhodopsin is presented in the preceding paper (14). A variety of experimental approaches, including proteolysis, chemical modification, peptide competition, and site-directed mutagenesis in combination with biochemical and biophysical assays, have been employed to map the sites of rhodopsin responsible for the binding and activation of Gt. The salient results have indicated that loops C2 and C3 are involved in Gt binding and activation (15, 16). In addition, recent studies indicate a role for the loop C4 in Gt activation (14, 17, 18). Despite these studies and the availability of a high-resolution crystal structure for the Gt holoprotein (19), there is little information concerning: 1) the key functional intramolecular interactions on the cytoplasmic surface of rhodopsin that form and regulate the catalytic site for Gt, 2) Gt subunit specificity for binding to particular cytoplasmic regions of rhodopsin, 3) the molecular mechanism of rhodopsin-catalyzed nucleotide release by Gt.

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0.1 mmol small-scale cycles) on an ABI Model 433A peptide synthesizer. Farnesylation of Gαγ-derived peptides was carried out by dissolving pure peptide (60 mg) in 5 ml of a solution of 50% (v/v) 1-propanol containing 35 mmol of sodium carbonate. The resulting solution was saturated with nitrogen and 0.6 ml of a freshly prepared 10% (v/v) formaldehyde solution in 1-propanol was slowly added under nitrogen stirring while pH was adjusted to >9. The solution was flushed with nitrogen again and incubated for 24–48 h with shaking. The farnesyl peptides were purified by reverse phase high performance liquid chromatography.

Instrumentation—Time-resolved absorption traces were recorded on a home-built single-wavelength absorption photometer. Light from a 150-W halogen light source passes through a John-Yvon HR460 monochromator (focal length 460 mm, 1200 lines/mm, slit width set to 1 mm) tuned to 543 nm, and from there through the cuvette (4-mm optical path length) and a band-pass interference filter onto a large surface PIN photodetector. The output is nulled and amplified twice, filtered with a 500-µs electronic low-pass filter and recorded using a modified Nicolet 2096-III A digital oscilloscope.

Photoregeneration Experiments—The rhodopsin photoregeneration assay was performed as reported (20) with adaptation for recombinant pigments as follows. All samples contained 2 µl pigment in a volume of 0.26 ml 200 mM Na2HPO4 (pH 8.0), 10 mM NaCl, and approximately 0.03% (w/v) dodecyl maltoside (DM). Due to the concentration procedure required for recombinant samples, the final DM concentration was not precisely known, but was estimated not to exceed 0.035% (w/v). After equilibrating the sample cuvette to 13 °C, the sample was illuminated for 30 s with a green HeNe laser (543.5 nm, 5 mW, Melles Griot) to cause quantitative formation of MII. Absorption at 543 nm was recorded continuously. After 50 ms, a flash of blue light (412 ± 7 nm, about 20-µs duration) was applied to the sample and formation of photo-regenerated pigment was measured at 543 nm for an additional 200 ms. Discharge of the flashlamp affected the sensitive electronics of the detector, causing a brief artificial negative deflection. Four records induced by four separate flashes were collected from each sample with 30-s intervals between the recordings. Starting with the initial illumination, each experiment took approximately 140 s. The four records were averaged to produce experimental data traces as presented in Fig. 2. The experimental photoregeneration signal traces are depicted as absorbance changes at 543 nm versus time (i.e. a rising signal indicates a proportional increase of absorbance due to reproto- nation of the Schiff base).

Numerical Fitting Procedures and Determination of Initial Slope Values—The photoregeneration signal comprises a fast phase, which is not resolved, and a slow phase, which is monitored for 200 ms (see Fig. 4). The rate of discharge of the flashlamp affected the sensitive electronics of the detector, causing a brief artificial negative deflection. Four records induced by four separate flashes were collected from each sample with 30-s intervals between the recordings. Starting with the initial illumination, each experiment took approximately 140 s. The four records were averaged to produce experimental data traces as presented in Fig. 2. The experimental photoregeneration signal traces are depicted as absorbance changes at 543 nm versus time (i.e. a rising signal indicates a proportional increase of absorbance due to reproto- nation of the Schiff base).

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RESULTS

The Effect of Gα and Gα-derived Peptides on the Photoregen- eration of Rhodopsin—A photoregeneration assay was employed that measures the kinetics of photoreconversion of MII to rhodopsin (20). The assay takes advantage of the conformational coupling of the cytoplasmic surface of the active state of rhodopsin, R*, to the chromophore-binding pocket in the membrane-embedded domain of the receptor. The slow phase of the photoconversion kinetics essentially monitors the reproto- nation of the retinylidene Schiff base as a function of time after a sample of R* is subjected to a blue flash. Photoconversion of R* (λmax = 380 nm) to rhodopsin (λmax = 500 nm) is inhibited if R* is bound to Gα or certain Gα-derived peptides. R* that is not bound in a stabilizing complex with Gα or Gα-derived peptides is

Experimental Procedures

Preparation of Rhodopsin and Gα—Purified bovine rhodopsin was prepared from hypotonically washed rod outer segment membranes essentially as described (23). The recombinant mutant pigments used in this study are shown in Fig. 1A. The construction, expression, and purification of these samples were reported (14). Following purification, the samples were concentrated to 10-fold using Centricon-30 filtration devices (Amicon). Gα holoprotein was purified from rod outer segment preparations essentially as described previously (24).

Peptide Synthesis—The synthetic peptides used in this study are listed in Fig. 1B. Peptides were synthesized using the Fmoc (N-[9- fluorenylmethoxycarbonyl] strategy with HBTU activation (Fastmoc,
The initial slopes of the photoregeneration traces are plotted as a function of $G_t$ concentration in the inset. These plots were fit using a hyperbolic function with additive offset. $IC_{50}$ values and errors are derived from the fits to the dose-response data and presented in the insets. A, photoregeneration of rhodopsin in the presence of increasing concentrations of $G_t$, $IC_{50} = 2.56 \pm 2.0 \mu M$. B, photoregeneration of rhodopsin in the presence of increasing concentrations of $a(340–350)$ peptide, $IC_{50} = 49.5 \pm 6 \mu M$. C, photoregeneration of rhodopsin in the presence of increasing concentrations of $\gamma(50–71)$-far peptide, $IC_{50} = 285 \pm 74 \mu M$.

more readily photoconverted.

The effect of $G_t$ on the photoconversion of R* was studied first (Fig. 2A). The change in absorbance at 543 nm is plotted as a function of time. The blue flash is applied to the sample at 50 ms. Superimposed upon an initial rapid change in amplitude, the slow phase of the trace represents the back conversion of R* to rhodopsin. The experiment was repeated with identical rhodopsin samples in the presence of increasing concentrations of $G_t$, $IC_{50} = 0.5, 1.0, 2.0, 3.0, and 5.0 \mu M$. The traces are superimposed to show a clear dose-dependent inhibition of the photoregeneration by $G_t$. The experimental traces were fit to a hyperbolic function in order to calculate values for initial slopes. The calculated fits are shown as dotted lines in Fig. 2. The initial slopes of the photoregeneration traces are plotted as a function of $G_t$ concentration in the inset. A satisfactory hyperbolic fit yielded an effective concentration at 50% inhibition ($IC_{50}$) value of 2.56 ± 2.0 $\mu M$. This value effectively represents a binding constant for the interaction between R* and $G_t$ under the conditions of the assay.

The effects of two peptides corresponding to the carboxyl-terminal regions of $G_t$ and $G_{\gamma}$ on the photoconversion of R* were studied next (Fig. 2, B and C). The amino acid sequences of $a(340–350)$ and $\gamma(50–71)$-far are presented in Fig. 1B. The $\gamma(50–71)$-far peptide shows the post-translational isoprenylation that is characteristic of $G_{\gamma}$. Fig. 2B shows six superimposed photoregeneration traces obtained from identical rhodopsin samples in the presence of increasing concentrations of $a(340–350)$ (0 to 1000 $\mu M$). The traces show a clear dose-dependent inhibition of the photoregeneration of R* by $a(340–350)$. The initial slopes of the photoregeneration traces are plotted as a function of $a(340–350)$ concentration in the inset to Fig. 2B. A satisfactory hyperbolic fit yielded an $IC_{50}$ value of 49.5 ± 6 $\mu M$ for the interaction between R* and $a(340–350)$.

Fig. 2C shows six superimposed photoregeneration traces obtained from identical rhodopsin samples in the presence of increasing concentrations of $\gamma(50–71)$-far (0 to 1000 $\mu M$). The traces show a clear dose-dependent inhibition of the photoregeneration of R* by $\gamma(50–71)$-far. Plotting the initial slopes of the photoregeneration traces as a function of $\gamma(50–71)$-far concentration (inset to Fig. 2C) permitted a satisfactory hyperbolic fit that yielded an $IC_{50}$ value of 285 ± 74 $\mu M$ for the interaction between R* and $\gamma(50–71)$-far.

Specificity of the Effects of $a(340–350)$ and $\gamma(50–71)$-far Peptides—The specificity of the effect of the $G_t$-derived peptides was studied by performing control experiments with altered peptides. The peptide sequences are shown in Fig. 1B. One control peptide, $a(340–350)$ K341I/L349A, was studied to evaluate the specificity of the carboxyl-terminal sequence of $G_t$ in R* interaction. This peptide failed to show peptide-R* interaction (25, 26) and the substitution of Leu$^{341}$ by alanine in $G_t$ was reported to abolish coupling to active rhodopsin (27, 28). As shown in Fig. 3, the $a(340–350)$ K341I/L349A peptide failed to inhibit the photoregeneration of R*. Similarly, the requirements for length, primary structure, and farnesylation of the $G_{\gamma}$-derived peptide were tested. Peptides $\gamma(60–71)$-far and $\gamma(50–71)$-far both inhibited photoregeneration similarly (Fig. 3). Positions 64 and 67 in $G_{\gamma}$ have been reported to be critical for interaction with MII, as observed with the altered peptide $\gamma(60–71)$-far F64T/L67S (29). The $\gamma(60–71)$-far F64A/L67A peptide did not inhibit photoregeneration (Fig. 3). In addition, the $\gamma(60–71)$ peptide, which lacked cysteinyl farnesylation, did not inhibit photoregeneration (Fig. 3). This finding is consistent with earlier results showing that lack of farnesylation prevented MII stabilization by $G_{\gamma}$-derived peptides (30, 31). In
other control experiments, the 1D4 peptide, corresponding to the carboxyl-terminal 18 amino acids of rhodopsin, did not affect photoregeneration, nor did it affect the inhibition of photoregeneration by Gt and the Gt-derived peptides (data not shown).

It has been reported that detergent concentration has an influence on the activation rate of Gt by R* (16, 32). The effect of varying concentrations of DM on the photoregeneration kinetics of rhodopsin and on the inhibition of photoregeneration by Gt (3 μM), α(340–350) (200 μM), or γ(50–71)-far (500 μM) was studied. Varying DM concentrations from 0.01 to 0.10% (w/v) had no effect on the photoregeneration kinetics of rhodopsin, and no effect on the inhibition of photoregeneration by α(340–350) (data not shown). However, the inhibition of photoregeneration by Gt and γ(50–71)-far was reduced by increasing DM concentrations from 0.01% to 0.10% (data not shown). This effect mirrors the reduction in the rate of Gt activation by R* in the presence of increasing DMI, as previously reported (16, 32). The final DM concentration under the standard conditions of the photoregeneration assay using heterologously expressed and purified mutant pigments is estimated to be 0.01–0.035%, a range in which detergent effects were found to be modest. Furthermore, the final DM concentration in assays of each of the recombinant samples is virtually identical.

Photoregeneration Assay of Recombinant Rhodopsin and Loop C4 Mutants—Photoregeneration assays were carried out on wild-type recombinant rhodopsin and four mutant rhodopsins (Fig. 1A). Representative photoregeneration traces are presented in Fig. 4. In each panel, the change in absorbance at 543 nm is plotted as a function of time. The blue flash is applied to the sample at 50 ms. Superimposed upon an initial rapid change in amplitude, the slow phase of the trace represents the back conversion of R* to rhodopsin. The black trace shows the result with pigment alone. The red trace shows the result with pigment in the presence of Gt (3.0 μM), α(340–350) (200 μM), or γ(50–71)-far (500 μM) as indicated. The behavior of COS cell-expressed rhodopsin was similar to that of bovine rhodopsin in the photoregeneration assay. Fig. 4 shows typical experimental traces obtained with purified recombinant rhodopsin in the presence of Gt, α(340–350), and γ(50–71)-far. Typical traces obtained with bovine rhodopsin are presented in Fig. 2. These results confirm that the photoregeneration assay can be employed to study recombinant pigments prepared in relatively small quantities in a heterologous expression system.

Four mutant pigments with alterations of the amino acid sequence of the C4 loop were prepared (Fig. 1A). Mutants CTr1, CTr2, and CTr4 are essentially chimeric receptors in which parts of the C4 loop of rhodopsin are replaced by sequences from the β2-adrenergic receptor (β2-AR). Mutant CysXV (C322S/C323S) was designed to evaluate the effect of receptor palmitoylation on the photoregeneration kinetics. The mutant opsin genes were expressed in COS cells, treated with 11-cis-retinal and purified in DM detergent solution. The levels of palmitoylation of the expressed mutant pigments CTr2 and CTr4 were similar to that of the wild-type receptor expressed in parallel (14); CysXV was not palmitoylated (14, 33). The ability of each of the mutant pigments to activate Gt was evaluated using a fluorescence Gt activation assay (Table I). Mutant pigments CTr2 and CTr4 were significantly defective in their

![Transducin](image-url)

**Fig. 4.** The effect of Gt, α(340–350), and γ(50–71)-far on photoregeneration of heterologously expressed rhodopsin and C4 loop mutants. Experiments were carried out as described in the legend to Fig. 2. Concentrations of Gt, α(340–350), and γ(50–71)-far were 3, 200, and 500 μM, respectively. The vertical scale bar, which depicts 0.075 mOD at 543 nm, applies to all traces. Black traces show photoregeneration of pigment alone. Red traces show photoregeneration of each pigment in the presence of Gt (Transducin), α(340–350), or γ(50–71)-far as indicated at the top of each column of panels. The sample used in each row is indicated in the labels at left. Each pair of traces is representative of at least two sets of experiments performed on different samples. WT, CTr1, and CysXV show distinct effects of Gt or Gt-derived peptides. Photoregeneration of CTr4 is not influenced by Gt, or Gt-derived peptides, and CTr2 shows an effect with γ(50–71)-far, a minor effect with Gt holoprotein and no effect with α(340–350).

| Table 1 | Gt activation and photoregeneration data |
|---------|----------------------------------------|
|         | 3 μM Gt | 200 μM α(340–350) | 500 μM γ(50–71)-far |
| %       | Gt      | Relative slope | Normalized inhibition | Gt      | Relative slope | Normalized inhibition | Gt      | Relative slope | Normalized inhibition |
|         | α(340–350) |                  |                     | α(340–350) |                  |                     | α(340–350) |                  |                     |
| WT-Rho  | 100     | 2                | 0.67 ± 0.05         | 1.00 ± 0.15 | 2                | 0.26 ± 0.01        | 1.00 ± 0.01 | 2                | 0.59 ± 0.03         | 1.00 ± 0.08 |
| CTr1    | 121 ± 11 | 2                | 0.73 ± 0.06         | 0.82 ± 0.16 | 3                | 0.89 ± 0.09        | 0.15 ± 0.13 | 3                | 0.62 ± 0.01         | 0.93 ± 0.05 |
| CTr2    | 20 ± 5.5 | 2                | 0.98 ± 0.02         | 0.06 ± 0.04 | 2                | 0.96 ± 0.13        | 0.06 ± 0.12 | 2                | 0.95 ± 0.07         | 0.13 ± 0.11 |
| CTr4    | 25 ± 6.2 | 2                | 1.03 ± 0.04         | −0.08 ± 0.09 | 2                | 0.24 ± 0.06        | 1.03 ± 0.06 | 4                | 0.41 ± 0.06         | 1.42 ± 0.10 |
| CysXV   | 97 ± 15  | 2                | 0.61 ± 0.12         | 1.19 ± 0.30 | 2                | 0.26 ± 0.01        | 1.00 ± 0.01 | 2                | 0.59 ± 0.03         | 1.00 ± 0.08 |

*Previously reported (14).

*The number given for n refers to the number of independent samples studied. Each kinetic trace (Fig. 4) resulted from four separate photoregeneration experiments per sample.

*The relative slope is defined as the initial slope of the slow phase of the photoregeneration trace in the presence of Gt, α(340–350), or γ(50–71)-far divided by the initial slope of the trace in the absence of Gt or Gt-derived peptide. The initial slope of the slow phase of the photoregeneration trace was determined from the numerical fit of a simple exponential-rise function offset by the amplitude of the fast phase. The data are presented as mean ± S.E.

*The normalized inhibition is defined as: (1 − mean relative slope)mutant/(1 − mean relative slope)rhodopsin. The propagated S.E. values are also presented.
Photoregeneration of mutant CTr1 was inhibited by Gα and the Gα-derived peptides. The degrees of inhibition were identical to those seen with wild-type rhodopsin. The behavior of mutants CTr2 and CTr4 was different from that of rhodopsin. Gα and the peptide α(340–350) did not inhibit photoregeneration of CTr2. This result is best appreciated in Fig. 5A, where the relative slopes for CTr2 in the presence of Gα and α(340–350) are −1.0. However, the γ(50–71)-far peptide was able to inhibit photoregeneration of CTr2 to the same extent observed with rhodopsin. The photoregeneration of mutant CTr4 was not affected by Gα, α(340–350), or γ(50–71)-far. This result is best seen in Fig. 5A, where the relative slopes for CTr4 in the presence of Gα and the Gα-derived peptides are −1.0. Photoregeneration of mutant CysXV was inhibited by Gα and the Gα-derived peptides. However, the inhibition by peptide γ(50–71)-far was relatively more pronounced for CysXV than for rhodopsin.

Each of the peptides and Gα inhibit the photoregeneration of rhodopsin to different degrees. Therefore, the ability of each peptide and Gα to affect a particular mutant cannot be compared directly using relative slopes. However, by normalizing the relative slope of a mutant to that of rhodopsin, such a comparison can be made. This expression, termed the normalized inhibition, is obtained from the following equation: (1 – mean relative slope)mutant/(1 – mean relative slope)rhodopsin. The normalized inhibition for rhodopsin is defined to be 1.0. A value of zero indicates no inhibition of the photoregeneration of the mutant pigment by a particular ligand. Data are plotted in Fig. 5B and listed in Table I. Mutant CTr1 is similar to rhodopsin with respect to inhibition of photoregeneration by Gα, α(340–350), and γ(50–71)-far. Mutant CTr2 shows essentially normal interaction with γ(50–71)-far, but fails to be affected by Gα and α(340–350). Mutant CTr4 is unaffected by Gα and both Gα-derived peptides. The photoregeneration of mutant CysXV is inhibited by α(340–350) and Gα normally, but displays an enhanced sensitivity to γ(50–71)-far.

DISCUSSION

Several lines of evidence suggest that the conformation of the cytoplasmic surface of the active state of rhodopsin, R*, is coupled to the conformation of the chromophore-binding pocket in the membrane-embedded domain of the receptor (3, 4). In analogy to G protein-coupled receptors with diffusible ligands in which G protein binding stabilizes a receptor conformation with a high affinity ligand-binding site, MII is stabilized at the expense of its tautomeric forms by the binding of Gα or Gα-derived peptides. This stabilization of MII is the basis of the "extra-MII" assay (34, 35). This assay, however, can only be applied under conditions of a dynamic equilibrium between metarhodopsin I and MII, which is exquisitely sensitive to membrane environment, pH, temperature, ionic strength, etc. Therefore, an assay was developed that measures the kinetics of photoconversion of MII to rhodopsin in detergent solution (20–22). The assay uses the fact that photoconversion of MII to rhodopsin in detergent solution (340–350 nm) following a blue actinic flash is inhibited if the MII molecule is bound to Gα (20), or certain Gα-derived peptides (21, 22) as a result of the coupling between the conformation of the cytoplasmic surface and that of the chromophore-binding pocket.

The initial step in photoregeneration, the photoisomerization of the retinal to its cis conformation, may be compared with loading a spring that subsequently drives the protein, including its cytoplasmic domain, back to the ground state conformation (20). The product formed in this initial step, termed "reverted meta (RM)," is characterized by a MII-like protein conformation and a cis-retinal with a deprotonated Schiff base; it is spectrally indistinguishable from MII. RM rapidly converts...
to a rhodopsin-like species characterized by a rhodopsin-like protein conformation and a cis-retinal with a protonated Schiff base. The presence of Gt does not affect RM formation, indicating that the isomerization of the retinal itself is unaffected. However, bound Gt prevents RM from converting to rhodopsin, by stabilizing the MII-like conformation of RM. Dissociation of Gt, from RM by GTPyS treatment allows RM to revert to rhodopsin quantitatively (20). The effects, and presumably the mechanism of action, of certain Gt-derived peptides on photoregeneration are similar to those of Gt itself (22).

**Photoregeneration Is Sensitive to Interactions with Gt and Certain Gt-Derived Peptides—**Gt interacts with R* to stabilize the active signaling state such that photoregeneration to rhodopsin is effectively blocked (20). Recently, a peptide corresponding to the carboxyl terminus of Gt and a peptide analogue related to the carboxyl terminus of Gaq were demonstrated to mimic the effect of Gt, by inhibiting photoregeneration of R* (22). Here we showed that synthetic peptide α(340–350) could cause the same effect as Gt (Fig. 2). In addition, the effect of α(340–350) was specific to its primary structure since a mutant peptide had no effect (Fig. 3). Synthetic peptide γ(50–71)-far also inhibited photoregeneration of R* (Fig. 2). The effect was specific to its primary structure and to the presence of cysteinyl thioether farnesylation (Fig. 3). Using single peptides that represent small regions of Gt provides a powerful probe of subunit- and domain-specific interactions.

The potencies of the α(340–350) and γ(50–71)-far peptides are about 20- and 100-fold less than that of Gt, respectively (Fig. 2). This finding is reasonable considering that the tertiary structure of a short peptide is less defined, so that a higher binding energy, and thus concentration, is needed for the “induced fit.” Also, the cytoplasmic surface domain of R* comprises multiple interaction sites for Gt, binding, including the loops C2 and C3 (15, 16, 36). Gt also has at least two, and probably more, sites that interact with the receptor during binding and activation. Peptide α(340–350) showed a clear inhibition of photoregeneration with an almost complete suppression at saturating concentrations (Fig. 2B). The peptide γ(50–71)-far showed a lower efficacy to inhibit photoregeneration. Although the inhibitory effect saturated at high concentrations with a normal first-order binding isotherm, there was not a complete suppression of the photoregeneration effect (Fig. 2C). The binding of γ(50–71)-far to R* is likely to be quite complex due to specificity which arises from both the farnesyl moiety and the peptide sequence (Fig. 3). The carboxyl-terminal region of GYt was also studied using a MII difference spectroscopy assay with similar findings (30, 31).

**The Role of a Conserved Region at the Amino Terminus of Loop C4 of Rhodopsin in Gt Binding—**We used the photoregeneration assay to probe the effects of Gt and Gt-derived peptides on expressed rhodopsin and rhodopsin mutants. The rhodopsin loop C4 mutations did not significantly affect the signal transmission path itself, as is seen from the similar kinetics of the photoregeneration signals in the absence of Gt and Gt-derived peptides (Fig. 4). The results in Figs. 2 and 3 show that Gt, and peptides α(340–350) and γ(50–71)-far are specific probes of rhodopsin signaling. Therefore, inhibition of photoregeneration by Gt or Gt-derived peptides is interpreted as the specific interaction of these reagents with the intracellular surface. A lack of inhibition due to alteration of either the peptide or the C4 loop is interpreted as a disruption of interaction. An advantage of the photoregeneration assay is that only productive binding interactions that stabilize specific conformations of the protein are reported.

In theory, a particular mutation might have the effect of uncoupling the conformation of the cytoplasmic surface from that of the chromophore binding pocket. For example, an E134Q mutant has been shown to assume a partially activated conformation at the cytoplasmic surface while the chromophore and surrounding structures remain in the dark, inactive conformation (12). This type of mutant might give misleading results, as photoregeneration (monitored by structural rearrangements surrounding the chromophore) could proceed unhindered, even as Gt, or peptides bound normally to the cytoplasmic surface. The rhodopsin loop C4 mutants showed no evidence of any uncoupling between the chromophore-binding pocket and cytoplasmic surface conformations. All of the mutants showed similar photoregeneration kinetics in the absence of peptide (Fig. 4, black traces). This suggests that the effects of the mutations are localized to the cytoplasmic surface, and do not affect the photoregeneration process itself. However, the mutant E134R/R135E photoregenerated with kinetics that were distinctly different from that of rhodopsin (data not shown), and therefore was not considered further in this study. Of course, mutants could exist that would foil all assays that rely on the detection of binding events at the intracellular surface resulting from conformational changes that are induced elsewhere in the protein. The extra-MII assay commonly used for rhodopsin, and the GTP-induced agonist affinity shift assay extensively used for other G protein-coupled receptors have the same potential limitations and are much less sensitive and specific.

Taken together, the results in the preceding paper (14) and the biophysical analysis of selected rhodopsin mutants herein strongly suggest that the amino terminus of C4 plays an important role in Gt binding and activation. Both Gt, and α(340–350) binding are disrupted when a tripeptide in this region is replaced by a sequence from the β2-AR (Fig. 5). The most straightforward interpretation of the data is that the amino-terminal region of loop C4 directly influences or is part of a direct binding site for the carboxyl-terminal tail of Gaq.

It is surprising that CTR4, in which residues 310–312 are replaced with analogous sequence from the β2-AR, binds neither α(340–350) nor γ(50–71)-far. Can the two peptides bind to overlapping sites on the receptor, each of which includes the 310–312 region? This seems unlikely, because the carboxyl termini of Gaq and GYt are located at a significant distance apart from each other in the structure of the heterotrimer (19).

Two potential explanations, which are not mutually exclusive, arise: 1) the peptides bind to different sites on the receptor and the sites are allosterically coupled; 2) Gt undergoes a large conformational change on contact with R* to bring the carboxyl termini of Gaq and GYt into close proximity with the interaction domain near residues 310–312. A conformational switch in Gt, induced by the contact with R*, is an element of the “sequential fit” model (22), and it may be identical to the switch in GβYt that was suggested earlier (31).

**Possible Role of GYt-farnesyl in Docking of Gt to the Active Receptor—**The relevance of the data to the binding site of GβYt is more subtle. The observation that CTR2, but not CTR4, binds γ(50–71)-far highlights the complexity of the binding interaction between γ(50–71)-far and rhodopsin. Further evidence of this complexity, as noted above, is that γ(50–71)-far fails to fully inhibit the photoregeneration reaction, even at saturating concentrations. This behavior contrasts with that of α(340–350) (Fig. 2). In addition, both the farnesyl moiety and the peptide itself are required for binding to R* (Fig. 3). Each is likely to have a distinct binding site that may be differentially altered in the mutants studied. The binding of γ(50–71)-far to CTR2, but not CTR4, suggests that the structural integrity of the fourth loop is disrupted by substitution of 310–312 with β2-AR sequence, but that the substitution of the entire loop
restores the structural determinants required for γ(50–71)-far binding. In this scenario, the tertiary, but not necessarily the primary structure of C4 would be critical for γ(50–71)-far binding. We have recently provided direct evidence, based on monolayer expansion measurements, that both the farnesylated carboxyl terminus of Gγ and the myristoylated amino terminus of Gα are involved in the membrane interaction of Gt (37). Gγ-far plays a role in both membrane and receptor interactions of Gt. We hypothesize that opening of the fourth loop structure could guide the farnesylated carboxyl terminus of Gγ toward the receptor, thus ensuring the docking of Gt. By definition, such a process proceeds through predominantly hydrophobic interactions and avoids the need for Gt to “jump” onto the receptor via a transiently soluble intermediate state, which would slow the catalytic interaction (38, 39). Assuming a fundamentally similar G protein activation mechanism for rhodopsin and the βγ-AR, it is conceivable that the intact tertiary fourth loop structure in the CTr2 mutant has the capability to form a docking site for the γ-peptide from Gt.

In summary, we developed a novel biophysical assay to probe the Gt-binding domain of rhodopsin and expressed rhodopsin. Gt and peptides corresponding to the carboxyl-terminal regions of Gα and Gγ specifically bind to Rγ and stabilize the active state of the receptor. We conclude that the amino-terminal region of loop C4 acts as part of the binding site for Gα and modulates the Gt-binding domain of Rγ. Future work is underway to reconcile the various models for allosteric regulation of the Gt-binding surface of Rγ, especially concerning the binding of Gβγ. This work will require the use of additional rhodopsin mutants and expressed G protein subunits.

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