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The Amino Terminus of the Fourth Cytoplasmic Loop of Rhodopsin Modulates Rhodopsin-Transducin Interaction*

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Rhodopsin is a seven-transmembrane helix receptor that binds and catalytically activates the heterotrimeric G protein transducin (G_t). This interaction involves the cytoplasmic surface of rhodopsin, which comprises four putative loops and the carboxyl-terminal tail. The fourth loop connects the carboxyl end of transmembrane helix 7 with Cys^{322} and Cys^{323}, which are both modified by membrane-inserted palmitoyl groups. Published data on the roles of the fourth loop in the binding and activation of G_t are contradictory. Here, we attempt to reconcile these conflicts and define a role for the fourth loop in rhodopsin-G_t interactions. Fluorescence experiments demonstrated that a synthetic peptide corresponding to the fourth loop of rhodopsin inhibited the activation of G_t by rhodopsin and interacted directly with the α subunit of G_t. A series of rhodopsin mutants was prepared in which portions of the fourth loop were replaced with analogous sequences from the β_2-adrenergic receptor or the m1 muscarinic receptor. Chimeric receptors in which residues 310–312 were replaced could not efficiently activate G_t. The defect in G_t interaction in the fourth loop mutants was not affected by preventing palmitoylation of Cys^{322} and Cys^{323}. We suggest that the amino terminus of the fourth loop interacts directly with G_t, particularly with $G\alpha_t$, and with other regions of the intracellular surface of rhodopsin to support G_t binding.

Rhodopsin, the dim-light photoreceptor of the rod cell, is a prototypical member of the superfamily of G protein-coupled receptors (GPCRs)[1] (1, 2). Following exposure to light, rhodopsin assumes an active signaling conformation, metarhodopsin II (MII). MII can bind and catalytically activate the retinal heterotrimeric G protein, transducin (G_t). G_t is composed of a guanine-nucleotide binding α subunit ($G\alpha_t$), and a functional heterodimer of β and γ subunits ($G\beta\gamma_t$). Interaction of the trimer with MII promotes the release of GDP from $G\alpha_t$, leading to the formation of a stable MII-$G\alpha_t$(empty pocket) complex. The subsequent binding of GTP activates $G\alpha_t$, leading to its dissociation from the receptor and from $G\beta\gamma_t$. The activated $G\alpha_t$ binds and activates its effector, cyclic GMP phosphodiesterase.

The molecular structure of the complex between rhodopsin and G_t, and the mechanism by which rhodopsin catalyzes nucleotide exchange, are not understood in detail. Numerous studies have localized the G_t-binding site to the cytoplasmic surface of rhodopsin. The cytoplasmic surface is composed of four loops (Fig. 1) and a carboxyl-terminal tail. The first (C1), second (C2), and third (C3) cytoplasmic loops connect adjacent transmembrane (TM) helices. The fourth cytoplasmic loop (C4) is unique in that it is bounded by a helix only at its amino terminus; its carboxyl terminus is formed by the insertion of two palmitoyl groups into the membrane bilayer (3). The palmitoyl groups are attached to Cys^{322} and Cys^{323} via thioester linkages (4, 5). The carboxyl-terminal tail is the region distal to Cys^{322} and Cys^{323}.

Considerable evidence has implicated C-2 and C-3 as participating in the complex with G_t (6–9). However, the literature addressing the role of C4 in interactions with G_t is contradictory. Studies have shown that peptides derived from C4 can disrupt the stabilization of MII by G_t (6), interfere with rhodopsin-stimulated GTPase activity of G_t (10), and bind directly to a fluorescently labeled $G\beta\gamma_t$ and prevent $G\beta\gamma_t$-rhodopsin interactions (11). In contrast, truncation of rhodopsin following Asn^{315}, in the middle of C4, does not impair G_t activation (12). Since truncation at the beginning of C4 precluded proper expression and/or processing of rhodopsin, a follow-up study examined a series of single and double mutations in the amino-terminal half of C4, from Asn^{310} through Asn^{315}. None of the mutations was found to disrupt G_t activation, leading to the conclusion that C4 is not required for productive interactions with G_t (13).

Here we have carefully re-examined and defined the role of the C4 loop in rhodopsin-G_t interactions. We used fluorescence spectroscopy to demonstrate that a synthetic peptide corresponding to C4 of bovine rhodopsin, rho(310–321), binds to G_t and free $G\alpha_t$. Furthermore, we demonstrate the potent inhibition of rhodopsin-catalyzed G_t activation by rho(310–321). We also prepared and characterized a series of site-directed mutants of bovine rhodopsin with alterations of the C4 loop. These data show that when either the entire C4 loop or a tripeptide (Asn^{310}-Lys^{311}-Gln^{312}) at the amino terminus of the loop is replaced with the analogous sequence of the β_2-adrenergic receptor (β_2-AR), the G_t-activating function of rhodopsin is di-

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This article is dedicated by A. G. K. to Prof. D. Balasubramanian on the occasion of his 60th birthday.

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[1] The abbreviations used are: GPCR, G protein-coupled receptor; AR, adrenergic receptor; C1, first cytoplasmic loop of rhodopsin; C2, second cytoplasmic loop of rhodopsin; C3, third cytoplasmic loop of rhodopsin; C4, fourth cytoplasmic loop of rhodopsin; G_t, transducin; $G\alpha_t$, α subunit of transducin; $G\beta\gamma_t$, $\beta\gamma$ heterodimer subunit of transducin; MII, metarhodopsin II; MR, muscarinic receptor; TM, transmembrane; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

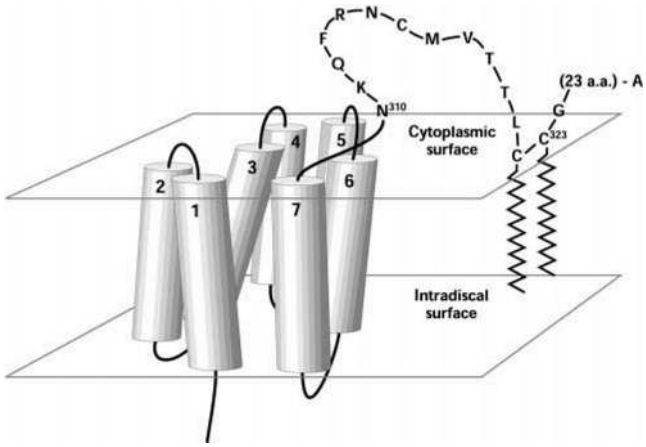

FIG. 1. **Schematic representation of bovine rhodopsin.** Seven putative TM helices are depicted as for previous models of GPCRs. The amino terminus and intradiscal surface are toward the *bottom*, and the carboxyl terminus and cytoplasmic surface is toward the *top* of the figure. The intradiscal and cytoplasmic loops are not drawn to scale. The loop C4 is defined as the 12 amino acids beginning with Asn^{310}, at the membrane border of the TM helix 7, and ending with Cys^{322} and Cys^{323}. Both of these cysteines are palmitoylated (4, 5), and the palmitoyl groups are inserted into the membrane (3).

minished. Neither replacement of the carboxyl-terminal half of the loop, nor removal of the palmitoylation sites disrupt G_t activation. We conclude that the C4 loop is involved in mediating interactions between rhodopsin and G_t.

EXPERIMENTAL PROCEDURES

Preparation of Peptides—Peptides were synthesized at the Rockefeller University Protein/DNA Technology Center and HHMI Biopolymer Facility by solid phase technique using Fmoc (*N*-(9-fluorenyl)methoxycarbonyl) chemistry. All peptides were prepared with free amino termini, and were amidated at the carboxyl termini. The peptides were purified by high performance liquid chromatography and characterized by mass spectrometry. The names and amino acid sequences of the peptides used in this study are the following: rho(132–144), AIERYVVCKPMS; rho(240–252), SATTQKAEKEVTR; rho(310–321), NKQFRNCMVTTL; rho(313–321), FRNCMVTTL; rho(310–321)*scr,* TLTVNMKCQNFR; rho(310–321)*SPD*, SPDFRNCMVTTL.

Preparation of G_t, $G\alpha_t$, and $G\beta\gamma_t$—G_t was prepared from frozen bovine retinas (Lawson, Inc., Lincoln, NE) using standard techniques (14, 15). Specific activities of G_t samples were determined by spectrofluorometric titration, as described previously (16). $G\beta\gamma_t$ and $G\alpha_t$ were isolated from holo-G_t essentially according to published methods (17) using a Hitachi LC-organizer high performance liquid chromatography system with a 1-ml Hi-Trap Blue-Sepharose column (Amersham Pharmacia Biotech). The proteins were eluted from the column by applying a 0–2 M NaCl gradient. Protein concentrations were determined using the Bio-Rad protein assay reagent according to the manufacturer's instructions. The subunits were stored at −20 °C in a 50% glycerol buffer until use.

Measurement of Intrinsic Fluorescence of G_t, $G\alpha_t$, and $G\beta\gamma_t$—Fluorescence measurements were done on a Spex Fluorolog 3–11 τ3 spectrofluorometer equipped with a 450 W Xenon arc lamp. All fluorescence experiments were performed in 10 mM Tris-HCl buffer (pH 7.2) containing 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 5 μM GDP, and 0.01% (w/v) dodecyl maltoside. Spectra were recorded at 10 °C in a 4 × 4-mm quartz cuvette. Protein fluorescence was obtained by exciting at 295 nm and monitoring emission from 315 to 450 nm. The excitation and emission slit bandpass were 1.5 and 5 nm, respectively. Titration experiments were typically performed by adding 10-μl aliquots of the peptide from a stock solution of 250 μM to a protein solution of 200 nM.

Preparation of Rhodopsin Mutants—Site-directed mutagenesis was achieved primarily by using restriction fragment replacement (18) in a synthetic rhodopsin gene (19) cloned into a eukaryotic expression vector (20). Mutants CTr1 and CTr3 were constructed by substituting the *Bsp*EI-*Sal*I restriction fragment with a synthetic duplex containing the desired codon alterations; mutant CTr2 involved a similar substitution of the *Apa*I-*Sal*I fragment. Mutants CTr4 and the Lys^{311} point mutations involved substitution of an *Apa*I-*Bsp*EI fragment. The mutant CysXV (C322S/C323S) was constructed by substituting the *Xho*I-*Bst*EII fragment of the rhodopsin gene into CysXIII (C140S/C316S/C322S/C323S). CysXIII was prepared by substituting the *Bsp*EI-*Sal*I fragment of a C140S mutant (21) with a synthetic fragment that contained the appropriate codon alterations for C316S, C322S, and C323S. The combination mutant CTr4/CysXV was prepared by cloning the *Xho*I-*Bst*EII fragment of CTr4 into a *Xho*I-*Bst*EII digested CysXV vector. Cell culture, transfection, and immunoaffinity purification procedures have been described elsewhere (22–24). Membranes from transfected cells were prepared prior to regeneration with 11-*cis*-retinal using sucrose density gradient centrifugation, as described previously (25).

Fluorescence G_t Activation Assay—The assay was performed essentially as described (16), using 250 nM G_t and 1 nM rhodopsin or mutant pigment. Peptide competition assays were performed with 200 nM G_t and 1 nM purified COS cell-expressed rhodopsin from which 1D4 peptide introduced in the purification procedure was removed by gel filtration on a G-50 Nick column (Amersham Pharmacia Biotech). The appropriate concentration of peptide was added from a 15 mM stock solution and preincubated with G_t for 30 min before the start of the assay.

Measurement of [^{3}H]Palmitic Acid Incorporation—Opsin was metabolically labeled with [^{3}H]palmitic acid (NEN Life Science Products Inc., Boston, MA) essentially as described previously (26). Briefly, 48 h post-transfection, COS cells were grown for 8 h in serum-free media. The cells were then incubated for 30 min in 1% serum, followed by 2 h in 1% serum supplemented with 100 μCi/ml [^{3}H]palmitic acid (43 Ci/mmol). Cells were washed with phosphate-buffered saline, harvested, and solubilized in 0.1% (w/v) dodecyl maltoside solution. The detergent extracts were incubated overnight with resin conjugated with 1D4 monoclonal antibody as used in the standard rhodopsin purification procedure (27). The resin was washed extensively, as monitored by the decreasing tritium counts present in successive washes. Opsin was eluted from the resin by incubation with wash buffer containing the 1D4 peptide. The relative amounts of [^{3}H]palmitic acid incorporated into the eluted samples were analyzed by scintillation counting.

RESULTS

Inhibition of G_t Activation by Synthetic Peptides Corresponding to Cytoplasmic Loops of Rhodopsin—Peptides derived from the C2, C3, and C4 loops of rhodopsin have been shown to disrupt the ability of G_t to stabilize MII as measured by an extra-MII assay (6). We examined whether similar peptides could also disrupt the activation of G_t by catalytic amounts of solubilized rhodopsin in a fluorescence activation assay. In Fig. 2, a dose-dependent decrease in the rate of G_t activation is observed in the presence of synthetic peptides derived from the amino terminus of C2 (rho(132–144)), the carboxyl terminus of C3 (rho(240–252)), and C4 (rho(310–321)). The effective concentration at 50% inhibition (IC_{50}) for all peptides was in the 0.1–0.3 mM range; all peptides inhibited activation completely at concentrations ≤1 mM. A C1-derived peptide, rho(61–75), only modestly inhibited transducin activation at 1 mM (not shown).

A Synthetic Peptide Derived from C4 of Rhodopsin, Rho(310–321), Alters the Fluorescence Emission Wavelength Maximum of $G\alpha_t$ but Not of $G\beta\gamma_t$—In an effort to characterize the interactions of C4 with G_t, the intrinsic fluorescence emission spectra of $G\alpha_t$ and $G\beta\gamma_t$ were collected in the presence of increasing concentrations of rho(310–321). A significant red shift (7.8 ± 0.3 nm, $n = 4$) in the λ_{max} of tryptophan emission of $G\alpha_t$ was observed in the presence of 45 μM peptide (Fig. 3*A*). The shift was accompanied by a modest (~10%) increase in intensity. These spectral changes are indicative of a change in the molecular environment of at least one of the two tryptophans of $G\alpha_t$ caused by the binding of the peptide. The extent of the red shift was dependent on the concentration of peptide (Fig. 3*A, inset*). In contrast, the λ_{max} of tryptophan fluorescence emission of $G\beta\gamma_t$ was only minimally (1.0 ± 0.3 nm, $n = 4$) affected by the peptide (Fig. 3*B*). These data suggest that the peptide does not bind to free $G\beta\gamma_t$, but they do not rule out binding in a manner that does not alter the molecular environment of enough of its 8 intrinsic tryptophans to allow for spectroscopic detection. The emission spectrum of holo-G_t was red shifted by

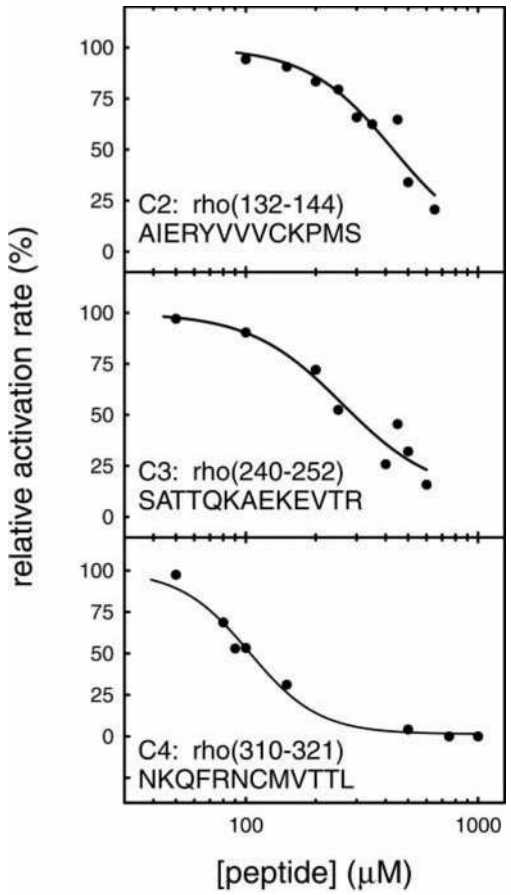

FIG. 2. **Peptides derived from the second, third, and fourth intracellular loops of rhodopsin inhibit activation of G_t by rhodopsin.** Each panel shows the relative initial rate of G_t activation as a function of peptide concentration. Activation rates were determined using a fluorescence assay of G_t activation (16). The peptide used in each experiment is described in the *lower left hand corner* of the corresponding panel. All three peptides completely inhibited activation of G_t, with IC_{50} values in the 0.1–0.3 mM range. A peptide derived from the first intracellular loop only moderately inhibited G_t activation at a concentration of 1 mM (not shown). Each panel represents data from a single set of experiments, which was repeated at least twice with similar results.

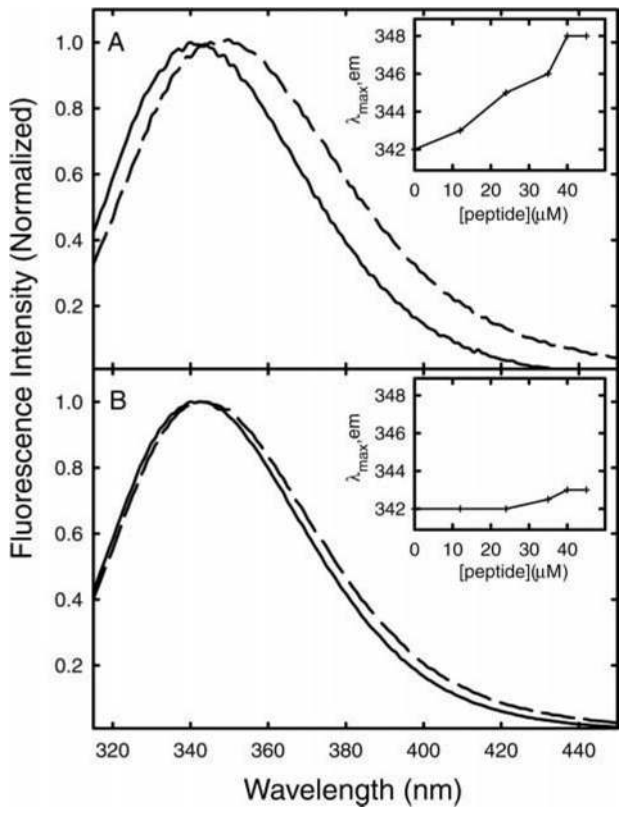

FIG. 3. **Effect of rho(310–321) on the intrinsic fluorescence of $G\alpha_t$ and $G\beta\gamma_t$ subunits.** Fluorescence emission spectra were collected using an excitation wavelength of 295 nm. *A*, fluorescence emission spectra of $G\alpha_t$ (200 nM) before (*solid line*) and after (*dashed line*) incubation with the rhodopsin C4 peptide, rho(310–321) (45 μM). The *inset* shows the fluorescence emission λ_{max} of $G\alpha_t$ as a function of rho(310–321) concentration. The mean maximum $\Delta\lambda_{max} \pm$ S.E. was 7.8 ± 0.3 nm (n = 4). *B*, fluorescence emission spectra of $G\beta\gamma_t$ (200 nM) before (*solid line*) and after (*dashed line*) incubation with rho(310–321) (45 μM). *Inset* shows the fluorescence emission λ_{max} of $G\beta\gamma_t$ as a function of rho(310–321) concentration. The mean maximum $\Delta\lambda_{max} \pm$ S.E. was 1.0 ± 0.3 nm (n = 4). Data shown are representative of at least four independent and reproducible experiments.

approximately 4 nm in the presence of the peptide (data not shown), which is consistent with peptide interaction with $G\alpha_t$ but not $G\beta\gamma_t$ in the context of the heterotrimer. The effects of three additional peptides on the λ_{max} of $G\alpha_t$ emission were examined. The peptides, which were derivatives of rho(310–321), were: (*a*) rho(313–321), in which residues 310, 311, and 312 were not present; (*b*) rho(310–321)*scr* in which the order of the amino acids was scrambled; and (*c*) rho(310–321)*SPD* in which the first three positions of the peptide were changed from NKQ to SPD. The sequence of rho(310–321)*SPD* is derived from the rhodopsin mutant CTr4 (Fig. 4). The peptides rho(313–321) and rho(310–321)*scr* did not affect the λ_{max} of $G\alpha_t$ emission, whereas rho(310–321)*SPD* caused a ~4-nm red shift, with no change in fluorescence intensity (data not shown). As an additional control, the λ_{max} of the emission spectrum of bovine serum albumin was shown to be insensitive to the presence of rho(310–321) (data not shown).

Preparation of Substitution Mutants in C4 of Rhodopsin—Three rhodopsin mutants were prepared in which portions of C4 were replaced with sequences from analogous segments of the β_2-AR or the m1-muscarinic receptor (m1-MR) (Fig. 4). These two receptors were chosen because they have fourth loops of similar lengths to that of rhodopsin and at least one cysteine homologous to Cys^{322} or Cys^{323} of rhodopsin. The β_2-AR has been shown to be palmitoylated (28), while this modification in the m1-MR is inferred to be very likely because of the presence of a Cys residue at the required location. Furthermore, these receptors bind G protein types not related to G_t, which is a member of the $G_{i/o}$ class. The m1-MR couples to G_q, and the β_2 AR couples to G_s.

In mutants CTr1 and CTr2, portions of C4 are replaced with sequence derived from the β_2-AR (Fig. 4). CTr1 involves replacement of the carboxyl-terminal half of the loop, while the entire fourth loop is replaced in CTr2. Only a carboxyl-terminal replacement was constructed with the m1-MR (CTr3), since the amino-terminal halves of the C4 loops of rhodopsin and m1-MR are nearly identical. The chimeric C4 mutant approach offers several advantages. For example, since the replacements are relatively long, and the substituted sequence is derived from receptors that couple to G_s or G_q, the sensitivity of observing a relevant disruption in G protein coupling is high. Additionally, since the fourth loops of the β_2-AR, m1-MR, and rhodopsin are of comparable length, the expression, folding, and palmitoylation of the chimeric fourth loop mutants should not be disrupted. Therefore, the confidence of attributing a loss-of-function phenotype to a specific defect in G_t coupling is high.

The analysis of CTr1, CTr2, and CTr3 described below pointed toward the involvement of the amino-terminal part of C4 in G_t interactions. To further examine this region, mutant CTr4 was constructed in which only those positions that differ

Receptor	Source of mutation	Amino Acid Position (Rhodopsin Numbering) 310	311	312	313	314	315	316	317	318	319	320	321	322	323
Rho		N	K	Q	F	R	N	C	M	V	T	T	L	C	C
β_2 AR		S	P	D	F	R	I	A	F	Q	E	L	L	C	L
m1 MR		N	K	A	F	R	D	T	F	R	L	L	L	L	C
CTr1	β_2 AR	N	K	Q	F	R	I	A	F	Q	E	L	L	C	C
CTr2	β_2 AR	S	P	D	F	R	I	A	F	Q	E	L	L	C	C
CTr3	m1 MR	N	K	Q	F	R	D	T	F	R	L	L	L	C	C
CTr4	β_2 AR	S	P	D	F	R	N	C	M	V	T	T	L	C	C
CysXV		N	K	Q	F	R	N	C	M	V	T	T	L	S	S
CTr4/CysXV	β_2 AR	S	P	D	F	R	N	C	M	V	T	T	L	S	S
K311P		N	P	Q	F	R	N	C	M	V	T	T	L	C	C
K311R		N	R	Q	F	R	N	C	M	V	T	T	L	C	C
K311S		N	S	Q	F	R	N	C	M	V	T	T	L	C	C
K311W		N	W	Q	F	R	N	C	M	V	T	T	L	C	C

FIG. 4. **Amino acid sequences of the fourth loop of bovine rhodopsin, human β_2-AR, human m1-MR, and fourth loop mutants of rhodopsin.** The amino acid sequence of each position in the fourth loop is shown using the standard single letter amino acid code. The numbering of the positions is from bovine rhodopsin. Regions that were replaced or altered in the creation of mutants are highlighted in *gray*.

between CTr1 and CTr2 (*i.e.* 310, 311, and 312) were replaced (Fig. 4). In addition, a series of point mutations in which Lys^{311} was replaced by residues with a variety of physicochemical properties were constructed: K311P, K311S, K311R, and K311W. Position 311 lies in the center of a proposed helical extension of TM7 (29, 30). These mutants were designed specifically to test and control for the possible role of a helix-altering proline in the 311 position in mutant CTr2. Two additional mutants were constructed to assess the role of palmitoylation in the function of C4. In CysXV (C322S/C323S) the sites of palmitoylation were removed, and in Ctr4/CysXV, the CTr4 and CysXV replacements were combined. The mutant CysXV has been previously described and characterized in a detergent-solubilized G_t activation assay (26). The mutant CTr4/CysXV was used to test whether the effects of preventing palmitoylation were different in the background of a mutated C4 as compared with rhodopsin.

The mutants were transiently expressed in COS cells and regenerated with 11-*cis*-retinal to yield pigments. The mutant pigments were either purified in dodecyl maltoside detergent or isolated in cell membrane preparations. UV-visible spectra taken on purified samples in the dark showed that each mutant pigment had a λ_{max} value of 500 nm, identical to that of rhodopsin prepared under the same conditions (Table I). Upon illumination, mutants CTr1, CTr2, and CTr3 formed MII-like pigments with λ_{max} values of 380 nm. Acid denaturation of the photolyzed pigments revealed that the Schiff base bonds of the mutants were at least as stable as that of rhodopsin (data not shown).

Activation of G_t by Solubilized Purified Recombinant Pigments—The ability of the C4 loop substitution mutants to activate purified bovine G_t was measured in a kinetic fluorescence assay. The activation of G_t was observed as an increase in the intrinsic tryptophan fluorescence of $G\alpha_t$ upon binding of GTPγS (16). The initial rate of GTPγS uptake by G_t catalyzed by each mutant was normalized to that of rhodopsin (Fig. 5). Mutants CTr1 and CTr3, in which the carboxyl-terminal half of the loop was replaced, displayed similar initial rates to that of rhodopsin. However, CTr2, in which the entire loop was replaced, displayed a reduced initial rate. The CTr4 mutant, in which only a tripeptide in the amino-terminal part of the loop was replaced with β_2-AR sequence, was also deficient in activating G_t. The level of activity was comparable to that of CTr2. None of the Lys^{311} point mutants was defective in G_t activation. The non-palmitoylated CysXV mutant exhibited similar activity to that of rhodopsin in the detergent assay. When assayed in membranes, CysXV was slightly hyperactive (data not shown). The activity of the combination mutant CTr4/CysXV was similar to that of CTr4.

Characterization of Pigment-catalyzed GTPγS Uptake by $G\alpha_t$ As a Function of $G\beta\gamma_t$ Concentration—Efficient activation of $G\alpha_t$ is known to require the presence of $G\beta\gamma_t$ (31). A previous study of a peptide derived from the C4 loop of rhodopsin suggested that this region binds to $G\beta\gamma_t$ (11). Therefore, the reduced activation of G_t by the mutant CTr2 might be a result of disruption of the $G\beta\gamma_t$-binding site on rhodopsin. To test this hypothesis, the rate of G_t activation by solubilized, COS cell expressed rhodopsin (1 nM) or CTr2 (3 nM) was measured as a function of the concentration of $G\beta\gamma_t$. Higher concentrations of CTr2 were necessary in this experiment due to its reduced activity. Fig. 6 shows the change in fluorescence over time due to rhodopsin- or CTr2-catalyzed GTPγS uptake by $G\alpha_t$ in the presence of different concentrations of $G\beta\gamma_t$. The intrinsic tryptophans of $G\beta\gamma_t$ affected only the background level of fluorescence, which is normalized in Fig. 6. If the defect in CTr2 were solely attributable to decreased binding of $G\beta\gamma_t$, then the concentration of $G\beta\gamma_t$ at which half-maximal activity was observed would likely be significantly higher for CTr2 than for rhodopsin. Additionally, one might expect the relative defect in activation rate of CTr2 to be reduced at high concentrations of $G\beta\gamma_t$. The data do not reveal a significant difference between rhodopsin and CTr2 in the effect of $G\beta\gamma_t$ concentration on $G\alpha_t$ activation, nor does the activity of CTr2 approach that of rhodopsin even at a 2:1 ($G\beta\gamma_t$:$G\alpha_t$) stoichiometric excess (Fig. 6).

TABLE I
Biochemical characterization of rhodopsin fourth loop mutants

Sample	λ_{max}, Abs	Transducin activation rate[a]	Incorporation of [^{3}H]palmitic acid[b]
	nm		
Rho	500	100	1.00
CTr1	500	121 ± 11.2 (5)	1.14 ± 0.20 (4)
CTr2	500	20 ± 5.5 (5)	1.34 ± 0.47 (4)
CTr3	500	82 ± 9.6 (4)	
CTr4	500	25 ± 6.2 (3)	1.10 ± 0.20 (4)
CysXV	500	97 ± 15 (4)	0.24 ± 0.10 (4)
CTr4/CysXV	500	39 ± 5.5 (3)	
K311P	500	85 ± 6.7 (8)	
K311S	500	87 ± 0.9 (3)	
K311R	500	123 ± 13.5 (3)	
K311W	500	74 ± 11.7 (3)	

[a] Activation rates are normalized to that of rhodopsin and are presented as mean ± S.E. (*n*).

[b] The level of incorporation of [^{3}H]palmitic acid for each sample is normalized to that of rhodopsin, after subtraction of nonspecific counts associated with samples prepared from mock transfected cells. Values are presented as mean ± S.E. (*n*).

Palmitoylation of CTr1, Ctr2, and CTr4—We investigated whether replacing portions of the fourth loop with β_2-AR sequence disrupted palmitoylation of mutants CTr1, CTr2, and CTr4. The incorporation of [^{3}H]palmitic acid present in the cell media during transfection into CTr1, CTr2, CTr4, and rhodopsin was comparable (Table I). The levels of incorporation were severalfold higher than the incorporation associated with CysXV, which has been reported not to be palmitoylated (26).

Amino Acid Sequence Analysis of Vertebrate Opsins—The sequence alignments and analyses available in the G Protein-

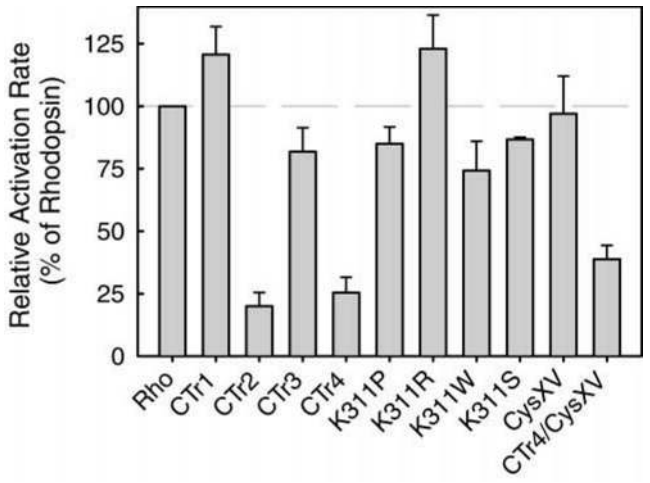

FIG. 5. **Rates of G_t activation catalyzed by solubilized, purified recombinant pigments.** Samples of rhodopsin and C4 rhodopsin mutants were expressed in COS cells, solubilized in dodecyl maltoside, and purified by an immunoaffinity procedure as described under "Experimental Procedures." The rates of G_t activation catalyzed by each sample were determined by linear regression through the first 30–60 s of data collected in a fluorescence activation assay (16). Each assay contained 1 nM rhodopsin or mutant, 250 nM G_t, and 5 μM GTPγS in a volume of 1.5 ml. The *bars* represent the mean rate, normalized to that of rhodopsin. *Error bars* depict the standard error of the mean. The data are presented numerically in Table I. Those mutants in which residues 310, 311, and 312 of rhodopsin are replaced with the analogous sequence of the β_2-AR (*i.e.* CTr2, CTr4, and CTr4/CysXV) are deficient in G_t activation.

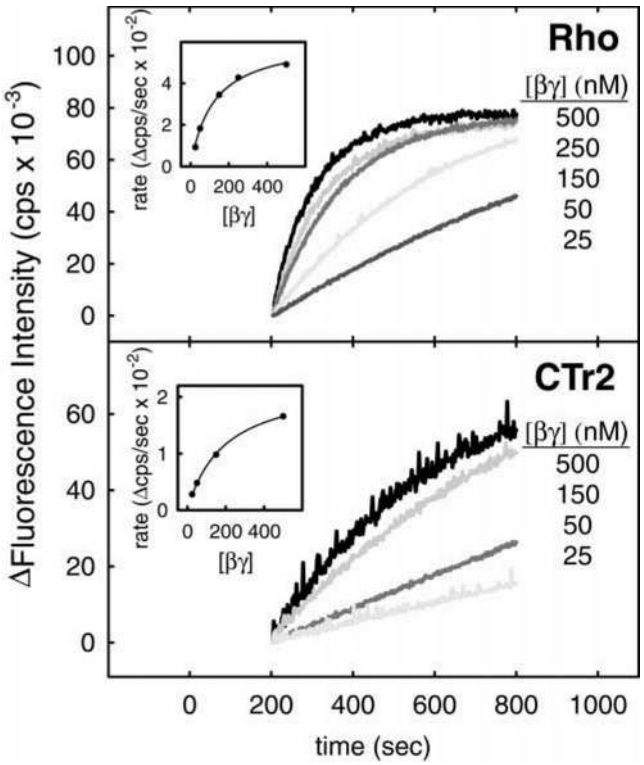

FIG. 6. **The relative rates of pigment-catalyzed GTPγS uptake by Gα_t as a function of G$\beta\gamma_t$ concentration are similar for both rhodopsin and CTr2.** The rate of G_t activation by solubilized, COS cell-expressed pigment was measured as a function of the concentration of G$\beta\gamma_t$. The *top panel* shows fluorescence activation traces of 250 nM Gα_t in the presence of 25–500 nM G$\beta\gamma_t$, and catalyzed by 1 nM rhodopsin. Each trace depicts the change in fluorescence emission intensity following the addition of GTPγS at 200 s. The background fluorescence emission is normalized to zero. The concentration of G$\beta\gamma_t$ in each trace is indicated in the column at *right*, in the same order that the traces are displayed. The *inset* is a plot of activation rate, determined from the initial slopes of the activation traces, *versus* concentration of G$\beta\gamma_t$. The data are fit with a two-parameter hyperbolic function. The *bottom panel* is identical to the top, except that the experiments were conducted with 3 nM CTr2. The data are from a single experiment that was repeated twice with similar results. The similarity of the G$\beta\gamma_t$ concentration dependence for rho and CTr2 argues against the hypothesis that the defect in CTr2 is attributable solely to disruption of the G$\beta\gamma_t$-binding site (see text for further discussion).

coupled Receptor Data Base (GPCRDB) were used to examine the conservation of C4 residues in GPCRs (32). Among the 86 vertebrate opsins in the data base, the residues in the amino-terminal half of C4 were found to be nearly 100% conserved (Fig. 7). In contrast, the carboxyl-terminal half of the loop is only ~65% conserved.

DISCUSSION

Significant efforts have been directed toward elucidating the regions of rhodopsin involved in binding and activating G_t (for reviews, see Ref. 2, 33, and 34). A variety of experiments using peptide competition, mutagenesis, and antibody-based approaches have defined the importance of the intracellular surface, and in particular, loops C2 and C3 in mediating interactions with G_t. Published reports regarding the role of loop C4 are contradictory. Studies based on peptides derived from C4 have suggested the importance of this region (6, 10), but a combination of site-directed mutagenesis and truncation of C4 appeared to rule out an important function for the region in G_t activation (12, 13). Our data demonstrate and characterize the importance of the amino-terminal part of the fourth loop, and suggest the role it plays in rhodopsin-G_t interactions.

Loop C4 of Rhodopsin Is Involved in the Activation of G_t—Several different experimental approaches in this report corroborate the importance of the fourth loop of rhodopsin in interactions with G_t. A peptide derived from C4, rho(310–321), can inhibit the catalysis of G_t activation by rhodopsin. Previously, an identical peptide has been reported to inhibit the binding of G_t to rhodopsin as measured by an extra-MII assay (6). Our results extend and confirm this observation by demonstrating that the peptide is active at similar concentrations in the fluorescence activation assay (Fig. 2). Significantly, the potency of rho(310–321) was comparable to that of peptides derived from very well characterized G protein-interacting regions, including the highly conserved Glu-Arg-Tyr sequence in the C2-derived rho(132–144) peptide. Additionally, rho(310–321) induces a red shift in the fluorescence emission spectrum of Gα_t (Fig. 3), which is evidence that the peptide can bind directly to the Gα_t subunit. The relevance of studies with the isolated C4-derived peptide to the function of C4 in the intact receptor is supported by the report that a rho(306–348) peptide assumed a defined structure in solution (29).

The results of the mutagenesis data strongly support and clarify the involvement of the fourth loop in G_t activation. In particular, the data localize the important region to the amino terminus of C4, as shown by the striking decrease in the rate of G_t activation by the mutants in which residues 310–312 are replaced with β_2-AR sequence (Fig. 5). Osawa and Weiss (13) argued against involvement of this region based on the wild-type phenotype of N310A, K311A, and Q312A point mutants. However, a recent report (35) corroborates our findings by identifying a mutation in the amino terminus of C4 (N310C) that can disrupt G_t activation. The precise role of Asn^{310} in G_t coupling appears complex, and additional work is underway that focuses exclusively on this residue.

Sequence analysis also supports the importance of the amino terminus of the C4 loop in receptor function. The amino terminus of C4 is nearly 100% conserved within the vertebrate opsins, whereas the carboxyl-terminal half of the loop is only ~65% conserved (Fig. 7). Furthermore, the asparagine in posi-

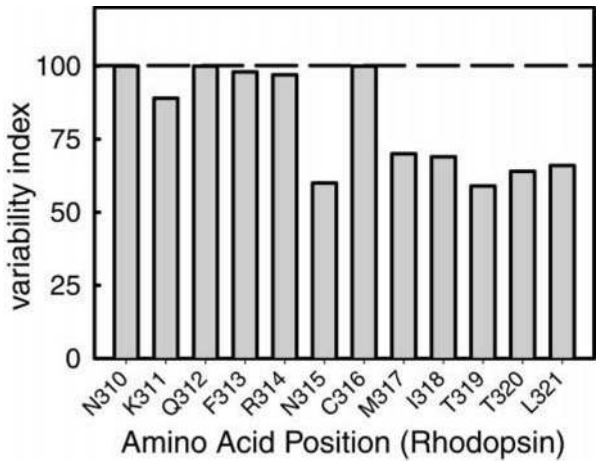

FIG. 7. **The amino-terminal half of the fourth loop of rhodopsin is more conserved than the carboxyl-terminal half within the family of vertebrate opsins.** The conservation of each position within loop C4 of rhodopsin was analyzed in 86 vertebrate opsin sequences. The alignments and the determination of the variability at each position were obtained from the G Protein-coupled Receptor Data Base (32). The *labels* on the *x* axis indicate the consensus amino acid at each position, using bovine rhodopsin numbering. A variability index of 100 means 100% conservation; the lower the number, the greater the variety of residues found at that particular position. The residues in the amino-terminal half of the loop are nearly 100% conserved, whereas those at the carboxyl-terminal half are only ~65% conserved. This pattern of conservation corresponds to the importance of the amino-terminal half of the loop in G_t activation.

tion 310 is very highly conserved within the large biogenic amine family of receptors, with the exception of the non-α_2-ARs, where it nearly always is a serine. This latter observation suggests that the amino-terminal part of the fourth loop may be important in GPCRs other than opsins. Indeed, mutagenesis experiments in this region of the fourth loop identified it as critical for receptor-G protein interactions in the β_2-AR (36, 37).

Several reports have suggested that accessibility to regions in the fourth loop are specifically modulated by photoactivation of rhodopsin, an observation consistent with the regulated involvement of C4 in G_t activation. The cytoplasmic end of TM helix 7 has been shown to become more accessible following light activation of rhodopsin (38), and spin labels attached to positions 316 (39) and 313 (30) in the middle of C4 undergo increased mobility after photolysis. Additionally, a spin label at Cys^{316} was reported to move apart from a label at position 65, at the amino terminus of the TM helix 1, upon MII formation (40).

Factors Affecting the Structure of the C4 Loop Region—We examined several factors that potentially affect the structure of C4, including the palmitoylation of Cys^{322} and Cys^{323}, the integrity of a proposed helical extension of TM7, and the presence of a membrane environment. The palmitoylation of Cys^{322} and Cys^{323} does not appear to affect the function of the amino-terminal part of the loop. Both CTr4 and CTr2 are palmitoylated (Table I) despite alterations in amino acids in the vicinity of the acylation site. However, the non-palmitoylated combination mutant CTr4/CysXV is essentially indistinguishable from CTr4 in detergent assays. In membranes, CysXV is slightly more active than rhodopsin, and CTr4/CysXV is slightly more active than CTr4 (not shown). Both observations corroborate previous reports that a nonpalmitoylated mutant was similar to rhodopsin in detergent solution (26), and that chemical depalmitoylation increased activity of rhodopsin when assayed in rod outer segment membranes (41). Given that residues 310–312 are located adjacent to the membrane border of the TM helix 7, it seems plausible that their structure is unaffected by the membrane anchoring of Cys^{322} and Cys^{323}. The same reasoning might explain why truncation of rhodopsin following Asn^{315} does not diminish G_t activation (12). Furthermore, C4 may form a loop in the absence of palmitoylation, as suggested by the NMR structure of a fourth loop peptide (29). The mechanism of hyperactivity of non-palmitoylated rhodopsin that has been observed in membranes appears to be unrelated to the function of the amino terminus of C4.

The TM helix 7 is likely to extend beyond the membrane border, based on experimental (29, 30) and theoretical (42) grounds. The TM2 (43), TM4, and TM5 (44) helices have all been reported to extend into the aqueous phase. The proline in position 311 of CTr2 and CTr4 might disrupt a helical extension of TM helix 7, leading to the observed phenotype. But the K311P point mutation does not resemble the 310–312 mutation of CTr4 in G_t activation assays, indicating that perturbation of the proposed helical extension does not impair G protein activation.

The Role of the C4 Loop Involves Modulation of Rhodopsin-G_t Interactions—We propose that the role of C4 is to modulate, in conjunction with other structures, the binding site for G_t. We favor this interpretation rather than those in which C4 serves as the sole binding site or participates mechanistically in the catalysis of nucleotide exchange because: (*a*) residual G_t activation was observed with the CTr2 and CTr4 mutants, (*b*) point mutations at the 311 position did not affect activation, (*c*) the amino-terminal C4 sequence of rhodopsin is highly, but not absolutely, conserved in other opsins and in certain other GPCRs (Fig. 7), and (*d*) the substitution of β_2-AR sequence into C4 does not allow rhodopsin to activate G_s (data not shown). The modulatory functions of C4 appear to be mediated both by contacts with G_t and by allosteric interactions with other regions of the receptor.

Specific interaction between C4 and G_t is indicated most directly by the rho(310–321)-$G_t\alpha$ interaction observed by fluorescence emission spectroscopy (Fig. 3). Direct contacts are also suggested by the inhibition of rhodopsin-catalyzed G_t activation in the presence of rho(310–321), an observation plausibly explained by binding of the peptide to the G protein, and occupancy of a receptor contact site (Fig. 2). Which part of the heterotrimer is binding to the C4 loop? A C4-derived peptide, rho(310–324), has been reported to bind $G\beta\gamma_t$ (11). However, the similarity of the activation rate *versus* $G\beta\gamma_t$ concentration profiles for rhodopsin and CTr2, and the failure of high concentrations of $G\beta\gamma_t$ to fully rescue CTr2 activity (Fig. 6), argue against the fourth loop as acting solely as a $G\beta\gamma_t$-binding site. Furthermore, the direct binding of rho(310–321) to $G\alpha_t$ (Fig. 3) demonstrates that C4 is involved with $G\alpha_t$ binding in addition to, or even instead of, $G\beta\gamma_t$ binding. Perhaps C4 binds $G\alpha_t$ directly, and allosterically regulates other regions of the receptor involved in $G\beta\gamma_t$ binding.

The data in this report demonstrate a conclusive role for the amino terminus of C4 of rhodopsin in G_t interactions. We suggest that this region interacts directly with G_t, particularly with $G\alpha_t$, and with other regions of the intracellular surface to support G_t binding. In the following paper, we study a subset of mutant receptors described here using a biophysical assay that detects binding of G_t or peptides derived from G_t subunit sequences (45).

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