Transducin-α C-terminal Peptide Binding Site Consists of C-D and E-F Loops of Rhodopsin*

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The binding of heterotrimeric GTP-binding proteins (G-proteins) to seropentine receptors involves several independent contacts. We have deduced the points of interaction between mutant bovine rhodopsins and αt-(340–350), a peptide corresponding to the C terminus of the α subunit (αt) of bovine retinal G-protein, transducin. Direct binding of αt-(340–350) to rhodopsin stabilizes the activated metarhodopsin II state (M II), consequently uncoupling the rhodopsin-transducin interaction. This peptide action requires two segments on the cytoplasmic domain of rhodopsin: the Tyr136-Val137, Val139-Val138 sequence on the C-D loop and the Glu247-Val250, Lys246-Glu245-Val250-Thr251 sequence on the E-F loop. We propose that a tertiary interaction of these two loop regions forms a pocket for binding the αt C terminus of the transducin during light transduction in vivo. In most G-proteins, the C termini of α subunits are important for interaction with receptors, and, in several serpentine receptors, regions similar to those in rhodopsin are essential for G-protein activation, indicating that the interaction described here may be a generally applicable mode of G-protein binding in signal transduction.

Activation of heterotrimeric guanine nucleotide-binding proteins (G-proteins)1 by transmembrane receptors is a general paradigm for signal transduction by a large variety of hormones, neurotransmitters, and physical stimuli. The G-protein coupled receptors (GPCRs) contain an extracellular N-terminal tail, seven transmembrane helices, three interhelical loops on either side of the membrane, and a cytoplasmic C-terminal tail. The cytoplasmic domain of the receptors binds and activates the G-protein (1–5). Visual transduction in rod cells is a prototypical example of a G-protein-coupled signaling system. In rod cells, signal transduction is initiated by photon-induced isomerization of the 11-cis-retinal chromophore, to all-trans-retinal. As shown in Fig. 1, this generates an inactive intermediate, metarhodopsin I (M I), and structural changes in the apoprotein leads to an active intermediate, metarhodopsin II (M II). The M II then binds and activates the retinal G-protein, transducin (Gt). Evidence from peptide competition (6), mutational (7–9), and biochemical (10) studies have implicated three cytoplasmic regions of M II as being critical for Gt interaction. Likewise, in transducin, the α subunit residues 340–350 at the C terminus, 311–323 at ø4/ø6/ø5 regions, 8–23 at the N terminus, and the farnesylated at the C-terminal tail of the γt subunit have been shown to be specific contact sites for rhodopsin (11, 12). Additional contact sites involving the β subunit are anticipated but have not been mapped. Thus, several distinct contacts are involved in the signal transfer from rhodopsin to Gt, but which segment of Gt interacts specifically with a particular region of rhodopsin is not known.

This paper focuses on the identification of the residues of bovine rhodopsin that interact with the transducin α subunit C-terminal residues Glu247-Lys246-Glu245-Val250-Thr251, a region that is important in rhodopsin-transducin coupling (11, 13–16). The ability of an 11-amino acid αt-(340–350) peptide to directly stabilize the M II state of rhodopsin mutants was employed. We report that the binding site consists of the residues Tyr136 through Val138 in the C-D loop and the residues Glu247 through Thr251 in the C-terminal portion of the E-F loop of bovine rhodopsin.

EXPERIMENTAL PROCEDURES

Expression, Purification, and Characterization of Mutant Rhodopsins—Procedures for the construction of mutants and expression of opsin genes have been described earlier (17, 18). Wild-type and mutant opsin genes (Table I) were expressed in COS1 cells by transient transfection of corresponding gene. The rhodopsin chromophore was generated by adding 11-cis-retinal (40 μM) to a cell suspension, the cells were solubilized in 1% dodecyl maltoside, and rhodopsin was purified by immunoaffinity chromatography (17, 18). The pigment concentration was calculated from its absorbance at 500 nm based on ε500 = 42,700 M−1 cm−1. For rhodopsin samples prepared for M I ↔ M II equilibrium studies, the dodecyl maltoside was replaced with 1% digitonin in all washes and elution.

Transducin Activation—Transducin was isolated from the bovine rod outer segment as described by Fung et al. (19). Catalytic activation of transducin by wild-type and mutant rhodopsins was assayed by a (GTPγS)-binding assay as described by Wesselingh,Resnick and Johnson (20). The assay mixtures consisted of 1–5 mM purified rhodopsin, 2 μM transducin, 20 μM [35S]GTPγS (1130 Ci/mmol) in 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 5 mM dithiothreitol, and 0.012% dodecyl maltoside. The assay was initiated by illumination for 2 min at a wavelength greater than 495 nm. The reaction mixture then remained in the dark at 23 °C for 60 min. The number of moles of [35S]GTPγS bound per mol of rhodopsin in 60 min was estimated from the [35S]GTPγS retained on the filter after filtration and elution.

Synthesis and Characterization of Peptides—The αt-(340–350) peptide, Ac-IKENLKDCGLF, and seven analogues (Fig. 2A) were synthesized, purified, and characterized by the protein chemistry core services of the Research Institute of the Cleveland Clinic as described earlier (18). These peptides will be referred to as peptides 1 (the parent peptide) through 8.

The αt-(340–350)-induced M I ↔ M II Equilibrium Assay—The principles and procedure for the M I ↔ M II equilibrium assay have been described previously (16, 18). In a typical experiment, ~5 to 8 × 10−8 M...
FIG. 1. Schematic representation of the steps involved in the stabilization of the M II state, GDP-GTP exchange, and the $\alpha_t$-(340–350)-mediated inhibition of Gt activation.

rhodopsin was evaluated with $1 \times 10^{-4}, 5 \times 10^{-4},$ and $1 \times 10^{-3}$ M concentrations of each peptide. The $\alpha_t$-(340–350) peptide or its analogues were mixed with wild-type and mutant rhodopsins in 1% digitonin in the dark and kept on ice for 20 min. Dark spectra were recorded at 5°C. The samples were then exposed to light for 20 s using a 150-watt Fiber-Lite fitted with 490 nm cut-off filter; the sample was at 5°C. The samples were then exposed to light for 20 s using a 150-watt Fiber-Lite fitted with 490 nm cut-off filter; the sample was allowed to equilibrate in the dark at 5°C for 20 min, and then light spectra were recorded (see Figs. 2B and 3).

RESULTS AND DISCUSSION

The conclusions in this study are based on the analysis of the ability of the $\alpha_t$-(340–350) peptide to directly bind and stabilize the M II intermediate. Two different assays were employed: (i) $\alpha_t$-(340–350) inhibition of rhodopsin-stimulated transducin activation and (ii) formation of M II from the M I intermediate by $\alpha_t$-(340–350)-dependent stabilization of M II. The relationship between these two assays is schematically shown in Fig. 1. Previous studies have found that bleaching rhodopsin in dodecyl maltoside in the absence of Gt yields the active M II state with a $\lambda_{max}$ ~380 nm, an intermediate that stimulates t-(340–350) peptide (11, 18). Thus, $\alpha_t$-(340–350) peptide or its analogues was used. The C-terminal 347 CGLF350 sequence was used to form a $\alpha$-turn structure, and the subtype of the $\alpha$-turn is speculated to be important for receptor selectivity (16, 22). Conservative single amino acid substitution of the remaining seven residues led to varying phenotypes (Fig. 2). Peptides 2 and 8 competed as effectively against Gt as the parent peptide 1 in both assays. Peptide 5 was a slightly (~2-fold) more effective competitor of Gt, and also better shifted the M I M II equilibrium in favor of M II (Fig. 2, A and B). Peptide 3 had a slightly lower potency (~2.5-fold less) than peptide 1. Peptides 4, 6, and 7 were very poor competitors in the Gt activation assay and were completely ineffective in shifting the M I M II equilibrium (Fig. 2B).

Studies using transferred nuclear Overhauser effect spectroscopy suggested that a salt bridge between Glu342 and Lys345 exists in $\alpha_t$-(340–350) bound to rhodopsin in the dark, that is broken during M II stabilization and replaced by a new salt bridge between Lys345 and the free $\alpha$-COO$^-$ group of the peptide (16). The Gln substitution can provide hydrogen bonding interactions in the place of a salt bridge. However, the Lys345 $\rightarrow$ Gln change (peptide 7) is likely to affect both conformations required for binding to rhodopsin, in the dark, as well as M II. But, the Glu342 $\rightarrow$ Gln change in peptide 4 is expected to favor the conformation that enables M II stabilization. The lack of peptide 4 binding suggests that Glu342 is essential for stabilizing M II state. Lys345 made a negligible contribution. Leu344 is a critical residue. Substitution with shorter Ala (peptide 6) produced an inactive peptide indicating that hydrophobicity and the side chain size of Leu344 side chain are stringent requirements for interaction with rhodopsin. Consistent with this observation, Martin et al. (23) discovered that combinatorial analogues of $\alpha_t$-(340–350) preserve the shape of the hydrophobic “face” with little variation, whereas larger changes in the hydrophilic face are tolerated. Mutagenesis studies suggest to a native state. All mutant rhodopsins purified in dodecyl maltoside and digitonin yielded a chromophore with a $\lambda_{max}$ ~500 nm (data not shown). Light-activated rhodopsin samples in dodecyl maltoside were used for measuring $^{[35S]}$GTP$\gamma$S binding to Gt (Fig. 2A and Table I). The wild-type rhodopsin activated nearly 267 ± 29 mol of Gt/mol of rhodopsin. Unreconstituted opsin and rhodopsin not exposed to light activated 15 ± 2 mol of Gt/mol of rhodopsin.

The synthetic 11-amino acid $\alpha_t$-(340–350) peptide 1 inhibited (50 ± 10% of maximal) Gt activation by bleached rhodopsin. The apparent $K_i$ for inhibition by peptide 1 was 80 $\mu$M. In agreement with earlier studies using urea-washed rod outer segment disc membranes, an inhibition greater than 50% could not be achieved at a higher peptide 1 concentration (11). As shown in Figs. 2B and 3, bleaching wild-type rhodopsin in 1% digitonin yielded predominantly the M I intermediate ($\lambda_{max}$ ~480 nm) and a small amount of the M II intermediate. The peptide did not affect the spectrum of rhodopsin in the dark. The presence of 500 $\mu$M $\alpha_t$-(340–350) yielded ~50% M II at the expense of M I. Comparing the potency of the peptide in the two assays indicated that an approximately 200-fold molar excess of peptide 1 was required for half-maximal inhibition of $^{[35S]}$GTP$\gamma$S binding to Gt ($K_i$ = 80 $\mu$M), and an approximately 1000-fold molar excess of peptide was needed for a half-maximal shift of M I M II. Thus, the interaction of $\alpha_t$-(340–350) and Gt with the detergent-solubilized wild-type rhodopsin obtained from COS1 cells had properties identical to those reported for interaction with bovine retinal rhodopsin (11).
Points of Rhodopsin-Transducin Contact

Transducin activation by mutant and wild-type rhodopsin were carried out as described under “Experimental Procedures.” The relative mean GTP-γS binding values (S.E. < 5%) are equilibrium values obtained without any correction for the decay of metarhodopsin II that might have occurred during the 60-min assay period. The results presented are a mean of 3 to 5 separate experiments in which mutant and wild-type rhodopsin expressed in COS cells were assayed in parallel and the mutant activities were normalized to the wild-type activity. The concentration of opsin was estimated from the \( \lambda_{\text{max}} \) assuming a molar extinction coefficient of 42,000 mol\(^{-1}\) cm\(^{-1}\).

| Mutant | Sequence \( ^a \) | Transducin activation (fraction of wild-type) | Peptide 1 binding \( ^b \) |
|--------|------------------|------------------------------------------|----------------------|
| C-D loop | | | |
| Wild-type | ERYVVVCKPMSNRFGE | 1.00 | + |
| CD1 | ---AAAA----------- | 0.69 | +/- |
| CD2 | ---LAAAA--------- | 0.22 | - |
| CD3 | -------EPNHMFN--- | 0.55 | + |
| CD4 | -----------NIMFN--- | 0.45 | + |
| CD5 | ------EPNHM----- | 0.44 | + |
| E-F loop | | | |
| Wild-type | KEAAAAQQPESATTQKEVTR | 1.00 | + |
| EF1 | ----TSLHGSVTGPTGSNL-- | 0.09 | - |
| EF2 | ----TSLHGSV---------- | 0.59 | + |
| EF3 | -----------GG--------- | 0.45 | + |
| EF4 | ------------GLTGSNL-- | 0.09 | + |
| EF5 | ------------GLT---L-- | 0.29 | + |
| EF6 | -------------------GG- | 0.58 | +/− |

\( ^a \) Wild-type bovine rhodopsin amino acid sequence corresponds to arbitrarily assigned interhelical regions. The solid lines indicate the amino acid sequence not altered in the specific mutant.

\( ^b \) Significant shift of M II/M I equilibrium toward formation of M II in the presence of 500 \( \mu M \) \( \alpha\text{-}(340–350) \) peptide 1.

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**TABLE I**

**Influence of rhodopsin cytoplasmic domain mutants**

Transducin activation by mutant and wild-type rhodopsin were carried out as described under “Experimental Procedures.” The relative mean GTP-γS binding values (S.E. < 5%) are equilibrium values obtained without any correction for the decay of metarhodopsin II that might have occurred during the 60-min assay period. The results presented are a mean of 3 to 5 separate experiments in which mutant and wild-type rhodopsin expressed in COS cells were assayed in parallel and the mutant activities were normalized to the wild-type activity. The concentration of opsin was estimated from the \( \lambda_{\text{max}} \) assuming a molar extinction coefficient of 42,000 mol\(^{-1}\) cm\(^{-1}\).

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**Points of Rhodopsin-Transducin Contact**

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**Fig. 2.** Effect of \( \alpha\text{-}(340–350) \) analogues on the \([35S]GTP\text{-γS} \) binding to G\( \text{a} \), stimulated by light-activated rhodopsin (A) and the formation of M II state from bleached rhodopsin (B). The \([35S]GTP\text{-γS} \) binding assay mixtures consisted of 1 nM purified rhodopsin, 2 \( \mu M \) transducin, 20 \( \mu M \) \([35S]GTP\text{-γS} \), and 0.012% dodecyl maltoside. The bars represent maximal binding in the presence of 500 \( \mu M \) \( \alpha\text{-}(340–350) \) analogues at 23 °C for 60 min. In separate experiments 1–1000 \( \mu M \) concentrations of each peptide analogue were used to obtain inhibition curves, and apparent the \( K_i \) values shown were estimated by linear regression analysis of the inhibition curves. The values represent the mean of three measurements on the same batch of purified rhodopsin. The \( K_i \) values have a \( \pm 15\% \) error. The M II stabilization assay used rhodopsin samples in 1% digitonin at rhodopsin: \( \alpha\text{-}(340–350) \) ratios of 1:700. Representative spectra yielded by four \( \alpha\text{-}(340–350) \) analogues with a significant affinity change are shown. Please refer to Fig. 3 for the wild-type spectrum with peptide 1. The samples in each case were: dark rhodopsin + 500 \( \mu M \) peptide (1), light-activated rhodopsin without the peptide (2), and light-activated rhodopsin + 500 \( \mu M \) peptide (3).

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**Localization of \( \alpha\text{-}(340–350) \) Binding Residues of Rhodopsin—**To identify the rhodopsin \( \alpha\text{-}(340–350) \) binding site, we created rhodopsin mutants in three distinct cytoplasmic regions involved in the G\( \text{a} \) interaction. Amidst these, it should be possible to identify mutants in which the \( \alpha\text{-}(340–350) \) binding is abolished even though the interaction with G\( \text{a} \) is not completely abolished. The abolished \( \alpha\text{-}(340–350) \) binding should be restored upon re-introduction of the wild-type amino acid sequence. Five C-D loop mutants and six E-F loop mutants were chosen for analysis. In these mutants, formation of rhodopsin—
rhodopsin mutants in 1% digitonin. Hence, they were not examined further. The mutant CD1, in which the residues 136–140 (YVVVC of the wild-type) were replaced with LAAAA (mutant CD2). Transducin activation was reduced ~80%. A nearly 6000-fold excess of $\alpha_t$-(340–350) did not shift the M II ↔ M I equilibrium (Fig. 3). Thus, Tyr$^{136}$ is an important determinant for $\alpha_t$-(340–350) stabilization of the M II state. Previously, Ridge et al. (24) used cysteine scanning mutagenesis to demonstrate that individual replacement of Tyr$^{136}$, Val$^{137}$, Val$^{138}$, and Val$^{139}$ led to partial loss of Gt activation. The Cys$^{140}$ residue was found to be not essential (17, 24). Therefore, the VVV sequence following Tyr$^{136}$ may contribute to stabilizing interactions. In our study, the remaining substitution mutants (CD3, CD4, and CD5) caused partial loss of Gt activation but showed normal affinity for the $\alpha_t$-(340–350) (data not shown). Thus, the residues 141–150 of the C-D loop do not participate in $\alpha_t$-(340–350) binding.

Franke et al. (7) found that replacing the E-F loop region between residues 231 and 252 with an amino acid sequence from the extracellular loop B-C produced a rhodopsin molecule that was normally activated by light but stimulated transducin very poorly. We constructed the same mutant (EF1 in Table I). The mutant exhibited normal photocycle properties as reported earlier and also activated transducin at only ~9% of the wild-type control. This mutant produced an M I-like state when bleached in digitonin. The M II ↔ M I equilibrium of the mutant was not shifted by $\alpha_t$-(340–350), suggesting that this mutant lacks the binding site for the peptide (Table I and Fig. 3).

We constructed mutants EF2 through EF5 by restoring the wild-type amino acid sequence in different parts of the E-F loop region. The mutant EF6 was constructed to examine the remaining two residues predicted to be part of the E-F loop. As indicated in Fig. 3 and Table I, mutants EF2, EF3, and EF6 activated transducin at ~40–60% of the wild-type. The mutants EF2 and EF3 bound $\alpha_t$-(340–350) almost as well as the wild-type. The mutant EF6 exhibited an interesting phenotype. The $\alpha_t$-(340–350) binding was evident because the M I peak decreased. However, this decrease was not accompanied by a transition to a distinct M II peak but rather by an increase in light scattering at the spectral region below 380 nm. The EF6 mutation must either alter the affinity for $\alpha_t$-(340–350) or decrease the stability of the M II-$\alpha_t$-(340–350) complex. Therefore, removing the Val$^{250}$-Thr$^{251}$ side chains likely indirectly influences the $\alpha_t$-(340–350) interaction. The mutant EF4 was essentially inactive in both the peptide binding and Gt stimulation assays. Examination of the residues in this mutant indicates that hydrophilic and charged residues present in the wild-type rhodopsin E-F loop are replaced by hydrophobic (Leu), shorter (2 Gly residues), and hydrogen-bonding (Asn, Ser, and Thr) residues.

On the basis of earlier mutagenesis studies, the Glu$^{247}$-Lys$^{248}$-Glu$^{249}$ sequence is thought to be essential for efficient activation of transducin and that the other residues play a relatively minor role (7, 9). The EKE triad sequence was kept in the mutant EF5. The M II ↔ M I mixture generated by bleaching this mutant was shifted toward M II formation by $\alpha_t$-(340–350), suggesting that the peptide was now able to bind and stabilize the M II intermediate. Transducin activation was partially restored (~30%). Therefore, it seems reasonable that the charged triad Glu$^{247}$-Lys$^{248}$-Glu$^{249}$ is necessary for the M II ↔ M I equilibrium shift promoted by $\alpha_t$-(340–350) binding. We conclude that $\alpha_t$-(340–350)-mediated stabilization requires the hydrophobic residues Tyr$^{136}$-Val$^{137}$-Val$^{138}$ on the C-D loop and the hydrophilic charged residues Glu$^{247}$-Lys-Glu-Val-Thr$^{251}$ on the E-F loop of rhodopsin. The type of analysis used here is not sensitive enough to determine which side chains of the $\alpha_t$-(340–350) interact with each of the regions.
Fig. 4 depicts the location of the two sites required for αt-(340–350) binding in the cytoplasmic extensions of the C and F helices. In conventional models, the transmembrane helices of rhodopsin are terminated at the membrane-aqueous interface. However, recent site-directed spin-labeling studies suggest that 1 to 3 turns of the helices extend into the cytoplasm, with helix C having a close tertiary interaction with helices B, D, E, and F. In a revised model, the Tyr136 and Val139 of helix C faces helix F, and Lys247 of helix F faces helix C (24, 25). This observation supports our hypothesis that the tertiary interaction of Tyr136-Cys140 and Glu247-Thr251 regions forms a subsite that is stabilized by αt-(340–350). The spin-labeling studies suggest that both these segments are rigid relative to the helix E extension, which is more dynamic and not essential for binding the αt-(340–350). Based on these observations, some qualitative conclusions can be drawn regarding M II stabilization by αt-(340–350) and holotransducin in vivo. Perhaps the rigidity of the cytoplasmic helix C and helix F extensions is required to provide an optimal surface for binding. The M II stabilization may occur because entropy is lost after αt-(340–350) has bound to the rigid cytoplasmic extensions of helices C and F. This loss explains M II-Gt complex stabilization by the Gt-α residues 340–350, which are currently believed to be disordered in the heterotrimer (26). It is noteworthy that these two rhodopsin helices contact the ionone ring of the 11-cis-retinal chromophore (27, 28).

It is now generally assumed that the G-protein binding site of all GPCRs comprises regions from the C-D loop, E-F loop, and the membrane-proximal segment of the cytoplasmic tail (1–9). Various types of studies indicate that the E-F loop is preeminent in the G-protein activation process in GPCRs (3–5). A hydrophobic site near the N-terminal region of the E-F loop that is important for G-protein coupling in several GPCRs (4, 29) appears not to be crucial for αt-(340–350) interaction with rhodopsin. Instead, our results indicate that the αt-(340–350) binding involves a hydrophobic region of the C-D loop. The hydrophilic and charged portion of this pocket near the C terminus of the E-F loop corresponds to a site that is important for G-protein activation in several GPCRs (4). E-F loop regions of different GPCRs were found to cross-link to specific α-subunits (30), as well as β-subunits of G-protein heterotrimers (31). All these evidences suggest that the E-F loop may wrap around the G-protein heterotrimer, establishing contacts with critical regions of the α-subunit, as well as with the βγ complex. Our results are the first description of an interaction between a defined region on transducin and a specific site on the receptor. Our approach could be used to explore the three other subsites on rhodopsin for Gt regions, αt-(311–323), αt-(8–23), and the farnesylated γt-(60–71) residues.

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Note Added in Proof—During the review of this manuscript two papers were published (Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., and Khorana, H. G. (1996) Science 274, 768–770; Sheikh, S. P., Zvyaga, T. A., Lichtarge, O., Sakmar, T. P., and Bourne, H. R. (1996) Nature 383, 347–350). Using two different experimental systems the authors have reached the same conclusion which indicates that movement of transmembrane helices C and F is required for light activation of rhodopsin. Furthermore these studies demonstrated the proximity of cytoplasmic extensions of transmembrane helices C and F which are identical to the segments identified in our study.

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