Rhodopsin Mutants Discriminate Sites Important for the Activation of Rhodopsin Kinase and Gt

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The cytoplasmic loops of rhodopsin, the rod cell photoreceptor, play important regulatory roles in the activation of both rhodopsin kinase and the rod cell G protein, Gt. A number of studies have identified domains in rhodopsin that are important for the activation of Gt. However, less is known concerning the cytoplasmic regions that regulate phosphorylation of the photoreceptor by rhodopsin kinase. To identify regions that participate in these processes, a series of alanine mutations were generated in the three cytoplasmic loops of rhodopsin and transiently expressed in HEK-293 cells. Membranes prepared from these cells were reconstituted with the opsin chromophore, 11-cis-retinal, and characterized for their ability to undergo light-dependent phosphorylation by rhodopsin kinase and to catalyze GTPγS (guanosine 5′-O-(3-thiotriphosphate)) binding to Gt. We have identified mutants that fall into three distinct categories: 1) those that show altered phosphorylation but normal Gt activation, such as T62A/V63A/Q64A and R147A/F148A/G149A in Loops I and II, respectively; 2) mutants that have reduced ability to activate Gt but are phosphorylated normally, including T242A/T243A and V250A/T251A/R252A in Loop III; and 3) mutants that affect both phosphorylation and Gt activation, including A233C/A234G/A235G and A233N/A234N/A235N in Loop III. The use of these two assays in parallel have allowed us to distinguish the presence of distinct functional domains within the cytoplasmic loops which are specific for interaction with rhodopsin kinase or Gt.

G protein-coupled receptors are members of a large multigene family having seven transmembrane-spanning domains that regulate intracellular signaling pathways in response to external stimuli (Fig. 1). Their function is the activation of heterotrimeric G proteins, which regulate a growing list of effector enzymes important in cell growth control and metabolism. One mechanism to limit continuous G protein activation is homologous desensitization, which is mediated by phosphorylation of the receptors followed by the binding of a member of the arrestin family of proteins. These events result in the generation of receptors unable to interact with their G proteins. The phosphorylation is mediated by a class of serine/threonine kinases, known as G protein-coupled receptor kinases (GRKs),1 which bind only the ligand-activated form of the receptors (1, 2). Rhodopsin, the photoreceptor of the mammalian rod cell, has been used extensively as a model for defining the molecular interactions between receptors, their G proteins, and the GRKs that mediate desensitization.

Studies of several different G protein-coupled receptors, including rhodopsin, have suggested that the cytoplasmic loops (Fig. 1) play important roles in their interactions with G proteins (3–11). In contrast, far less is known concerning the critical sites of interaction between these receptors and GRKs. A series of 7 serines and threonines on the C terminus of rhodopsin serve as phosphoacceptor sites for rhodopsin kinase, the rod cell GRK, although only one or two are thought to be physiologically relevant (4, 12, 13). Peptide competition studies have suggested that the cytoplasmic loops of the photoreceptor protein are involved in phosphorylation of rhodopsin by rhodopsin kinase (14, 15). In addition, the efficient phosphorylation of a synthetic peptide corresponding to the C terminus requires activation of the kinase by the cytoplasmic loops of rhodopsin (16–18). Mastoparan, which can activate several G proteins in place of their receptors, also potentiates the phosphorylation of the synthetic peptide substrate by rhodopsin kinase (18). These data suggest that the cytoplasmic loops of rhodopsin participate in the light-dependent activation of the kinase, allowing it to phosphorylate its substrate sites on the C terminus.

Using a series of alanine mutations in the three cytoplasmic loops of rhodopsin, we have examined the phosphorylation of rhodopsin by rhodopsin kinase. To control for mutations that may cause extensive conformational changes in the cytoplasmic loops and therefore disrupt the ability of light to activate rhodopsin, we have also measured the activation of the rod cell G protein, Gt. We have identified mutants that fall into three distinct categories: 1) those that affect phosphorylation by rhodopsin kinase, 2) those that affect Gt activation, and 3) mutations that affect both of these processes. The parallel use of these two assays has allowed us to begin to discriminate domains on the cytoplasmic surface of rhodopsin that participate in the phosphorylation of rhodopsin by rhodopsin kinase from those that are involved in the activation of Gt.

EXPERIMENTAL PROCEDURES

Materials—All reagents for mutagenesis, including the Altered Sites mutagenesis kit, were obtained from Promega. The expression vector pCDNA1/Amp was purchased from Invitrogen. Bovine retina were purchased from J. A. Lawson Inc. (Lincoln, NB). Human embryonic kidney-293 (HEK-293) cells were from American Type Culture Collection. The

1 The abbreviations used are: GRK, G protein-coupled receptor kinase; Gt, transducin; HEK-293, human embryonic kidney-293; ROS, rod outer segment; GTPγS, guanosine 5′-O-(3-thiotriphosphate); PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]-dimethylammonio)propanesulfonic acid; TBS, Tris-buffered saline.
cDNA for bovine opsin and pRSV-TAG, a vector expressing T antigen, were gifts from Dr. Jeremy Nathans. The monoclonal antibody R2-15N was a gift from Dr. Paul Hargrave. The opsin chromophore, 11-cis-retinal, was a gift from Hoffman-La Roche. Radiolabeled nucleotides were purchased from Amersham Corp. or DuPont NEN. Oligonucleotides were synthesized at a facility of the University of North Carolina Lineberger Comprehensive Cancer Center using an automated synthesizer from Applied Biosystems.

**Cloning and Mutagenesis**—The insertion of the cDNA for bovine opsin into pSelect and the methods for generating point mutants using the Altered Sites mutagenesis kit (Promega) have been described previously (19). All mutations were confirmed by dideoxy chain termination sequencing methods using Sequenase (U. S. Biochemical Corp.). The cDNAs for mutants showing differences in phosphorylation or G, activation were fully sequenced at the Automated DNA Sequencing Facility at the University of North Carolina at Chapel Hill on a model 373A DNA Sequencer (Applied Biosystems) using the Taq DyeDeoxy® terminator cycle sequencing kit (Applied Biosystems).

**Expression of Bovine Rhodopsin in HEK-293 Cells**—The cDNAs for wild-type opsin and for the mutants were ligated into pcDNAI/Amp and cotransfected with pRSV-TAG into HEK-293 cells. All procedures for transfection and expression of bovine rhodopsin in HEK-293 cells using DEAE-dextran have been described previously (19).**Preparation of Membranes**—Membranes were prepared from transfected cells approximately 65–70 h after transfection according to methods described previously (19). Protein concentrations were determined using procedures developed by Bradford (20). The expression of rhodopsin in HEK-293 cells was determined by Western analysis using the monoclonal antibody R2-15N, which recognizes the N terminus of the photoreceptor protein (21). Methods for electrophoresis and immunoblotting have been described previously (19). The levels of expression for wild-type and mutant rhodopsins were estimated using a Molecular Dynamics PhosphorImager. Because the expressed rhodopsin migrates as multiple bands on polyacrylamide gels, the entire lane was measured for each sample. After subtraction of a background estimated from a lane of nontransfected cell membranes, the amount of rhodopsin was calculated using the concentration of rhodopsin in rod outer segment (ROS) membranes as a standard. The purification of ROS membranes has been described previously (22).

**Phosphorylation of Rhodopsin Mutants By Rhodopsin Kinase**—Light-exposed ROS, isolated from bovine retina on sucrose gradients, were incubated in 200 mM NaHepes, 20 mM EDTA, 2 mM dithiothreitol, pH 8.0, and centrifuged for 30 min at 200,000 × g to extract rhodopsin kinase as described (14). To standardize the level of kinase activity in the phosphorylation reaction, each rhodopsin kinase preparation was used to phosphorylate 2 μg of ROS membranes in the kinase assay buffer (described below) for 60 min and quantified by SDS-PAGE and phosphorimage analysis. For the expressed wild-type and mutant opsins, HEK-293 cell membranes containing 1 μg of rhodopsin were used in the phosphorylation assay. The amount of total protein in the reaction mixture was held constant at approximately 75–80 μg by supplementing the samples with membranes from nontransfected cells. The membranes were reconstituted with 25 μM 11-cis-retinal (14 nmol/μg membrane protein) for 1 h at room temperature in the dark. After centrifugation at 12,000 × g for 15 min at room temperature to pellet membranes and remove unbound material, the membranes were resuspended in 10 mM Tris-HCl, pH 7.4, 260 mM NaCl, 5 mM MgCl₂, 0.125 mM EDTA, 0.125 mM EGTA, 2 mM dithiothreitol, 50 mM

**Fig. 1. Amino acid sequence of the cytoplasmic domains of bovine rhodopsin.** I, II, and III designate the three intracellular loops. IV represents the fourth cytoplasmic loop formed by the palmitoylation of cysteines 322 and 323 and their attachment to the plasma membrane. K296 is the lysine that forms the attachment site for 11-cis-retinal. The asterisks (*) represent the sites of palmitoylation.

**Table 1.** Mutations in the cytoplasmic loops of bovine rhodopsin

| Loop I | 62 | 73 |
|--------|----|----|
| T62A/V63A/Q64A | TVQHKKLRTPLN | AAAAAAA----|
| H65Y | - | Y----|
| K66A/K67A | - | - - A A |
| L68A/R69A/T70A | - | - - A A A - |
| P71A | - | --- A - |
| L72A/N73A | - | - A - |

| Loop II | 141 | 152 |
|--------|-----|-----|
| K141A/P142A/N143A | KPHSNFPRFGENH | AAAAA - - - - - - |
| S144A/N145A/F146A | - - - A AA - - - - |
| R147A/F148A/Q149A | - - - A AA - - - - |
| E150A/N151A/H152A | - - - - - - A A A |

| Loop III | 231 | 252 |
|---------|-----|-----|
| K231A/E232A | KEAAQQQESATTQKAEXVTR | A---- - - - - - - - - |
| A233G/A234G/A235G | GGGG - - - - - - - - |
| A233N/A234N/A235N | NNNN - - - - - - - - |
| Q236A/Q237A/Q238A | - - - A AA - - - - |
| E239A/S240A | - A - - - - - - - - |
| T242A/T243A | - - - - - - - - - - |
| Q244A/K245A | - - - - A A - - - - |
| E247A/K248A/E249A | - - - - A AA - - - - |
| V250A/T251A/R252A | - - - - - A A A |
NaF, 1 μM okadaic acid, and 150 μM [32P]ATP (50 μCi/ml) in a reaction volume of 250 μl. The reaction was initiated by the addition of the appropriate amount of rhodopsin kinase extract (approximately 60 μl). The samples were incubated for 8 min at 30 °C under fluorescent room lights or in the dark under Kodak No. 2 safelight filters. The reaction was terminated by incubation on ice for 2 min followed by the addition of 500 μl of ice-cold 10 mM Tris-HCl, pH 7.5, and centrifuged for 15 min at 12,000 × g at room temperature. The pellets were extracted for 1 h with 1.5% octylglucoside in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, pH 7.4 (TBS), containing 50 mM NaF and centrifuged for 15 min at 12,000 × g to remove insoluble material. The supernatants were incubated with the R2-15N monoclonal antibody for 1 h at room temperature, followed by incubation with Protein A-Sepharose beads (30 μl of a 50% slurry in TBS containing 50 mM NaF) for 1 h. The mixtures were centrifuged for 4 min at 50 × g to pellet the immune complexes. The immunoprecipitates were washed three times in TBS containing 0.1% sodium deoxycholate and 50 mM NaF. The antigen/antibody complexes were released from the beads by incubation in SDS-Laemmli sample buffer (23) and analyzed by SDS-PAGE on 10% polyacrylamide gels. The radioactivity was quantified by phosphorimage analysis as described above. The level of phosphorylation in the

Fig. 2. Time course of phosphorylation of wild-type rhodopsin expressed in HEK-293 cells. A, autoradiogram of time course of phosphorylation. Membranes reconstituted with 11-cis-retinal were phosphorylated in the light or in the dark for the indicated times, as described under “Experimental Procedures,” and chromatographed on a 10% polyacrylamide gel, followed by autoradiography. B, quantification by phosphorimage analysis. The dried gel from A containing samples phosphorylated in the light (○) and in the dark (●) was exposed to a Molecular Dynamics phosphorimage screen and subjected to phosphorimage analysis using a Molecular Dynamics PhosphorImager.

Fig. 3. Phosphorylation and Gα activation of Loop I mutants. A, phosphorylation by rhodopsin kinase. Membranes from transfected HEK-293 cells were harvested, reconstituted with 11-cis-retinal, and assayed for phosphorylation in the light and in the dark according to procedures described under “Experimental Procedures.” After subtracting the dark values from the phosphorylation in the light, the results were expressed as a fraction of the level of light-dependent phosphorylation for wild-type rhodopsin normalized to a value of 1.0. The results represent the averages of duplicates from at least two transfections. The error bars represent S.E. B, time course of phosphorylation of T62AV63AQ64A compared to wild-type rhodopsin. Membranes were phosphorylated and quantified as described under “Experimental Procedures” and in the legend to Fig. 2. Phosphorylation was performed in the light (open symbols) or in the dark (closed symbols). □ and △.
dark was subtracted from the amount in the light. The data were normalized to the level of light-dependent phosphorylation of wild-type rhodopsin expressed in HEK-293 cells. The absolute amount of phosphate incorporated per mole of rhodopsin was estimated from the immunoprecipitates by cutting out the lanes from the gel and quantifying the level of radioactivity by liquid scintillation spectrometry.

**Activation of G<sub>τ</sub>**—Light-dependent activation of G<sub>τ</sub> was assayed using [35S]GTP·S binding as described previously (19). The assay mixture contained 2.5 µg of membrane protein and 0.5 µg G<sub>τ</sub> purified from ROS membranes. The reaction was initiated by the addition of 1 µM [35S]GTP·S (6.25 Ci/mmol). At 30, 60, 90, 120, and 150 s, aliquots were removed from the reaction mixture, applied to nitrocellulose filter discs (Millipore) and washed with assay buffer to remove unbound GTP·S. The level of GTP·S binding to G<sub>τ</sub> was quantified by liquid scintillation spectrometry. Under these conditions, the time courses were linear. Therefore, initial rates of activation could be determined from the slopes of the curves generated by linear regression analysis using the computer program Deltagraph Professional. The rates of activation were normalized to the levels of expression of the rhodopsin mutants quantified by Western analysis and normalized to the rate of activation in wild-type expressed rhodopsin.

**Results**

**Expression of Bovine Rhodopsin Mutants in HEK-293 Cells**—The cytoplasmic loops of rhodopsin were subjected primarily to alanine-scanning mutagenesis (Table I). Alanine was chosen because it is a neutral amino acid found at both exposed and buried positions that does not cause severe changes in secondary structure (24–26). These mutants were expressed in HEK-293 cells, and membranes were prepared for phosphorylation and G<sub>τ</sub> activation experiments. Previously, truncation mutants expressed in these cells showed changes in glycosylation pattern that appeared to result from altered transmembrane folding (19, 27). Western analysis showed no obvious difference in glycosylation pattern between the wild-type protein and the mutants, suggesting that the transmembrane folding of these proteins is not affected (data not shown). All of the mutants showing differences, compared to wild-type expressed rhodopsin, in phosphorylation by rhodopsin kinase or in the activation of G<sub>τ</sub> were also assayed for their ability to bind 11-cis-retinal. None of these mutants displayed altered retinal binding, also suggesting that the overall tertiary structure of these proteins was normal (data not shown).

**Phosphorylation of Bovine Rhodopsin Expressed in HEK-293 Cells**—A method for measuring the phosphorylation of rhodopsin expressed in HEK-293 cells was developed using an antibody directed against the N terminus of rhodopsin (21) to immunoprecipitate phosphorylated rhodopsin from the membranes. A time course in the light, shown in Fig. 2, demonstrates that the reaction reaches a plateau in approximately 5 min, similar to results obtained by Bhattacharya et al. (28) using COS-1 cell-expressed bovine rhodopsin and to results in our laboratory using ROS membranes (data not shown). In contrast, membranes incubated in the dark demonstrate no significant increase in phosphorylation even after 60 min. On the basis of this time course, membranes from cells expressing mutant rhodopsins were incubated for 8 min in the presence of rhodopsin kinase. This time represents a point in the curve that gives a detectable signal and is close to linearity. Approximately 0.2 mol of phosphate/mol of rhodopsin is incorporated under these conditions.

**Mutations in Loop I**—Cytoplasmic Loop I is a relatively short, highly basic loop that is well conserved between invertebrate and vertebrate rhodopsin and between the mammalian cone and rod opsins (4). Table I shows the sequences of the mutations generated in this loop. Each amino acid was changed to alanine with the exception of Hist<sup>26</sup>, which was mutated to a tyrosine. Fig. 3A demonstrates that most of the mutants are similar to the wild-type protein in their ability to be phosphorylated by rhodopsin kinase except for T62A/V63A/Q64A, which displays significantly enhanced phosphorylation (approximately 48% higher). A time course of the reaction (Fig. 3B) demonstrates that the increase in phosphorylation observed for T62A/V63A/Q64A is due to an increase in the rate of phosphorylation compared to the wild-type protein.

The Loop I mutants were also assayed for their ability to activate G<sub>τ</sub> (Fig. 3C). Initial rates of activation comparing the mutants with wild-type rhodopsin expressed in HEK-293 cells suggest that T62A/V63A/Q64A is normal in its ability to catalyze the binding of GTP·S to G<sub>τ</sub>, despite its enhanced rate of phosphorylation described above. L72A/N73A also showed a small (approximately 27%) decrease in G<sub>τ</sub> activation although it was phosphorylated normally by rhodopsin kinase. The other four mutants demonstrated no significant differences in either G<sub>τ</sub> activation or phosphorylation compared to the wild-type protein.

**Mutations in Loop II**—Loop II has been implicated in the activation of G<sub>τ</sub> by peptide competition experiments as well as deletion and substitution mutagenesis (6, 8). The triple alanine mutants generated in Loop II are shown in Table I. R147A/F148A/G149A demonstrated significantly reduced levels of phosphorylation, approximately 43% lower than wild-type rhodopsin (Fig. 4A). S144A/N145A/F146A also demonstrated a small reduction (26%) in phosphorylation. In Fig. 4B, a time course for R147A/F148A/G149A indicates that the reduced phosphorylation is due to a decreased rate of phosphorylation by rhodopsin kinase. The Loop II mutants all displayed normal activation of G<sub>τ</sub> (Fig. 4C). These data suggest that sites in Loop II participate specifically in regulating the phosphorylation of rhodopsin by rhodopsin kinase.

**Mutations in Loop III**—For many G protein–coupled receptors, including rhodopsin, the third cytoplasmic loop has been shown to be important for both G protein activation and G protein selectivity (6, 8, 29–32). Peptide competition studies also suggested that rhodopsin’s Loop III plays a role in regulating the phosphorylation of rhodopsin by rhodopsin kinase and the binding of arrestin (14, 15, 18, 33). Mutations were generated in this loop by alanine-scanning mutagenesis with the exception<sup>25</sup> A81<sup>A</sup>A82<sup>A</sup>A83<sup>Ala</sup>, which was mutated first to glycines (Table I). Because the introduction of glycine is known to disrupt the secondary structure of α helices (34), asparagines were also substituted for these three alanines. All of the mutants appeared to be normally phosphorylated compared to wild-type rhodopsin except for A233G/A234G/A235G (Fig. 5A), reduced by approximately 80%, and A233N/A234N/A235N (Fig. 6A), reduced by approximately 78%. A233G/A234G/A235G also showed a dramatic loss in ability
FIG. 4. Phosphorylation and G, activation of Loop II mutants.
A, phosphorylation by rhodopsin kinase. Membranes from transfected HEK-293 cells were harvested and assayed for phosphorylation as described under “Experimental Procedures” and the legend to Fig. 3. The results are expressed as a fraction of the level of light-dependent phosphorylation for wild-type rhodopsin normalized to a value of 1.0 and represent the averages of duplicates from at least two transfections. The error bars represent S.E. B, time course of phosphorylation of K141A/F146A/G149A compared to wild-type rhodopsin. Membranes were phosphorylated and quantified as described under “Experimental Procedures” and in the legend to Fig. 2. Phosphorylation was performed in the light (open symbols) and in the dark (closed symbols). □ and ■, wild-type rhodopsin; △ and ▲, K141A/F146A/G149A. C, activation of G, Membranes expressing mutant rhodopsins were reconstituted with 11-cis-retinal and assayed for the light-dependent binding of GTP-γ-S to G, to activate G, (Fig. 5B). Therefore, this mutant displays a reduced coupling to its G protein as well as reduced phosphorylation. Interestingly, A235N/A234N/A235N seems to be less severely affected for G, activation (Fig. 5B) than the glycine mutant of this sequence. Measurements of absorbance for A235G/A234G/A235G and A233N/A234N/A233N indicated that retinal binding is equivalent to wild-type (Fig. 7). Therefore, the integrity of the transmembrane domains in these

FIG. 5. Phosphorylation and G, activation of Loop III mutants.
A, phosphorylation by rhodopsin kinase. Membranes from transfected HEK-293 cells were harvested and assayed for phosphorylation as described under “Experimental Procedures” and the legend to Fig. 3. The results are expressed as a fraction of the level of light-dependent phosphorylation for wild-type rhodopsin normalized to a value of 1.0 and represent the averages of duplicates from at least two transfections. The error bars represent S.E. B, activation of G, Membranes expressing mutant rhodopsins were reconstituted with 11-cis-retinal and assayed for the light-dependent binding of GTP-γ-S to G, as described under “Experimental Procedures” and the legend to Fig. 3. Rates of activation calculated from the slopes of the lines are expressed as a fraction of wild-type rhodopsin normalized to a value of 1.0. The results represent the average of at least two independent transfections performed in duplicate. The error bars represent S.E. The average rate of GTP-γ-S binding for wild-type rhodopsin was 0.1 mol/s-mol.
FIG. 6. Phosphorylation and Gt activation of the Loop III mutants A233N/A234N/A235N and A233G/A234G/A235G. A, phosphorylation by rhodopsin kinase. Membranes from transfected HEK-293 cells were harvested and assayed for phosphorylation as described under "Experimental Procedures" and the legend to Fig. 3. The results are expressed as a fraction of the level of light-dependent phosphorylation for wild-type rhodopsin normalized to a value of 1.0 and represent the averages of duplicates from at least two transfections. The error bars represent S.E. B, activation of Gt. Membranes expressing mutant rhodopsins were reconstituted with 11-cis-retinal and assayed for the light-dependent binding of GTP·γS to Gt as described under "Experimental Procedures" and the legend to Fig. 3. Rates of activation calculated from the slopes of the lines are expressed as a fraction of wild-type rhodopsin normalized to a value of 1.0. a, the results represent the average of two independent transfections performed in duplicate. Error bars represent S.E. b, error bars represent the range of duplicates from a single transfection. The average rate of GTP·γS binding for wild-type rhodopsin was 0.05 mol/s/mol.

mutants is preserved.

The other Loop III mutants were also assayed for their ability to catalyze GTP·γS binding to Gt (Fig. 5B). Two of these mutants, T242A/T243A and V250A/T251A/R252A, demonstrated 57% and 69% reduced ability to activate Gt, respectively, despite normal levels of phosphorylation. These data suggest that multiple domains within Loop III are important specifically for the regulation of Gt activation.

FIG. 7. Subtraction spectra of the Loop III mutants A233G/A234G/A235G and A233N/A234N/A235N. Membranes were reconstituted with 11-cis-retinal and assayed for retinal binding in the dark and in the light as described under "Experimental Procedures." The spectra generated in the light were subtracted from the spectra generated in the dark to define the absorption peak of membranes from nontransfected, wild-type, and mutant-transfected cells.

DISCUSSION

This report describes the use of alanine-scanning mutagenesis to define the participation of the cytoplasmic loops in the interaction of rhodopsin with rhodopsin kinase and with its G protein, Gt. Previously, this technique has been used to uncover the functions of specific domains for a variety of proteins, including the sites of interaction between human growth hormone and its receptor (35), the effector binding region of Gt (36), and muscarinic acetylcholine receptor sequence required for G protein activation (37). Although the role of amino acids similar to alanines may not be successfully evaluated by this technique, it has the advantage of being less disruptive than other methods of analysis. Palczewski et al. (18) demonstrated that a proteolytic fragment of rhodopsin missing the C-terminal phosphorylation sites is required for maximal phosphorylation of a synthetic peptide substrate by rhodopsin kinase. Therefore, cytoplasmic domains distinct from the phosphorylation sites participate in the activation of the kinase. Rhodopsin kinase and Gt have been shown to compete for interaction with rhodopsin, suggesting the possibility of overlapping binding sites (38, 39). We have examined the phosphorylation of rhodopsin by rhodopsin kinase and the activation of Gt in parallel in order to identify regions of the cytoplasmic surface that participate specifically in the activation of these two proteins.

In Loops I and II we were able to define regions that affect phosphorylation when they are mutated to alanines. We detected a significant increase in phosphorylation in the Loop I mutant T62A/V63A/Q64A, although Gt activation was similar to wild-type rhodopsin. Several potential mechanisms may account for this observation, including a release of steric restraint or an increase in affinity for the kinase. In contrast, R147A/F148A/G149A in Loop II showed reduced ability to be phosphorylated by rhodopsin kinase but normal activation of Gt. These
...data suggest for the first time that domains that participate in the activation of rhodopsin kinase reside in these two loops. None of the mutations in Loop II described in this study appeared to significantly affect G activation. Mutation of the highly conserved Arg132 (found at the border between the third transmembrane domain and Loop II of most G protein-coupled receptors) to uncharged amino acids abolishes the binding and activation of G (8, 10). Therefore this amino acid, which was not included in our present studies, is critical for interaction with G. It would be interesting to determine the effects of mutations in Arg132 on phosphorylation by rhodopsin kinase in order to fully understand the role of this important amino acid.

In Loop III we have detected a significant loss of both phosphorylation and G interaction by mutation of Ala233-Ala234. Ala235 to either glycines or asparagines. Interestingly, A233N/A234N/A235N was less severely affected for G activation than the glycine mutant of this sequence. These observations suggest that the conformational requirements may be more severely different for phosphorylation and G protein activation, as demonstrated by Robinson et al. (40). It is not clear from our data whether these mutations have affected just the Ala233-Ala234, Ala235 region or whether they have affected the secondary structure of the entire loop. Loop III is predicted by Fourier transform infrared spectroscopy to be an a helix (41). Computer analysis, based on Chou-Fasman algorithms (42), predicts that either glycines or asparagines substituted for the alamines will introduce turns in the Loop III sequence (data not shown). Previously, substitution of glycines, which are known to disrupt a helices, at Thr242-Thr243 (located in the center of Loop III) resulted in a 90% loss in GTPase activity compared to a 66% loss when these amino acids were substituted with valines (9). In addition, proteolytic cleavage of Loop III between Ghe235 and Ser240 destroys both the ability of the photoreceptor to activate rhodopsin kinase (18) and disrupts activation of G (43). These data, taken together with the present report, strongly suggest that the secondary structure of a large segment of this loop is critical for the ability of rhodopsin to couple to G, as well as to promote phosphorylation of the C terminus by rhodopsin kinase. Loop III may be important for the conformational changes that must occur in response to light to convert rhodopsin to an active protein. Nevertheless, we cannot rule out through our present studies that Ala233-Ala234-Ala235 alone may be the required sequence for these light-dependent conformational changes. Nor can we rule out the possibility that G and rhodopsin kinase have partially overlapping sites of interaction. Future studies will focus on additional mutations using amino acids that are less disruptive to secondary structure to distinguish between these possibilities.

Previously, the use of peptides as competitive inhibitors suggested that the C-terminal region of Loop III is important for phosphorylation of the C terminus by rhodopsin kinase (14, 15). However, none of the mutants in that region affected phosphorylation in our assay. In contrast, we did observe reduced activation of G in the Loop III mutants T242A/T243A and V250T251R252. T242A/T243A was reduced by approximately 55%, similar to the results by Franke et al. (9) where these amino acids were substituted with various charged alamines, as described above. Val250-Thr251Arg252, adjacent to the transmembrane domain, has been identified for the first time by our studies as a critical site for G activation. Since both mutants showed normal phosphorylation, they appear to be important specifically for G activation. Franke et al. also demonstrated that a mutant containing a deletion of the central region of Loop III that includes Thr242-Thr243 (amino acids #237-249) can bind G, but is unable to catalyze guanine nucleotide exchange (8), suggesting that this region may be needed not for binding, but for the catalysis of guanine nucleotide exchange. Whether Val250-Thr251Arg252 is involved in the binding or the activation of G remains to be determined in future experiments. Two amino acid mutations in Loop III (S240A and K348L) reported previously to be important in the activation of G (9) were not detected in our studies. The reasons for these differences between the two laboratories are unclear. Perhaps they are due to differences in the assay conditions, such as the use of membranes in our case compared to detergent-extracted proteins in the earlier report, and/or the use of different mutations at these sites.

Little is known concerning the involvement of the cytoplasmic loops in the regulation of phosphorylation of other G protein-coupled receptors by G protein-coupled receptors. In contrast, there have been a number of studies implicating Loops II and III in the activation of various G proteins. For example, a hydrophobic amino acid located in Loop II, leucine in the M1 muscarinic acetylcholine receptor and phenylalanine in the b-adrenergic receptor, is required for G protein-mediated signal transduction in these two receptors (37). Replacement of either amino acid with alanine led to 85% greater than 85% loss in coupling. In the present study, replacement of the equivalent amino acid in rhodopsin, Met143, had no effect on either G activation or phosphorylation by rhodopsin kinase. Therefore, the critical domains in rhodopsin may differ from those in some closely-related receptors. Both Loops II and III, as well as the "fourth cytoplasmic loop" (Loop IV, Fig. 1), have been implicated in the selectivity of coupling of specific G proteins to receptors such as the metabotropic glutamate, muscarinic acetylcholine, and b-adrenergic receptors (31, 44). The participation of the rhodopsin cytoplasmic domains in the selectivity of coupling to G proteins remains to be determined.

In summary, we have, for the first time, defined domains in Loops I and II of rhodopsin that specifically affect the activation of rhodopsin kinase and that are distinct from regions involved in G activation represented by mutations in Loop III. In addition, mutation of Ala233-Ala234-Ala235 in the N-terminal domain of Loop III disrupted both G protein activation and phosphorylation. Additional experiments will be needed to separate and define the specific amino acids that play a role in these two pathways and to understand their significance in signaling events mediated by rhodopsin.

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