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The Carboxyl Terminus of Bovine Rhodopsin Is Not Required for G Protein Activation

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SUMMARY

Rhodopsin, the photoreceptor of mammalian rod cells, shares regions of structural homology with many G protein-coupled receptors. One of these domains is the "fourth cytoplasmic loop" formed by palmitoylation of two cysteines (Cys-322 and Cys-323) in the carboxyl terminus. Evidence from several laboratories suggests that this domain is important for the activation of the G protein for rhodopsin, G_t, and that it undergoes conformational changes upon exposure to light. Previously we reported that a truncation mutant with only six amino acids remaining at the proximal end of the carboxyl terminus was able to activate G_t, whereas a mutant lacking an additional five amino acids was misfolded and unable to bind retinal. In the present report, these six amino acids were mutated, to define their roles in the formation of a functional photoreceptor and in the activation of G_t. All of the point mutants displayed normal expression, post-translational processing, and G_t activation, suggesting that the fourth cytoplasmic loop in the carboxyl terminus does not play a major role in the activation of G proteins and that the specific amino acid sequence in this domain is not required for the production of a properly folded, functional photoreceptor.

Rhodopsin, the rod cell photoreceptor, is a member of the family of G protein-coupled receptors. It has been used extensively as a model system for studying the functions of specific receptor domains. A number of peptide competition studies (1–5) have suggested that the carboxyl terminus, including the highly conserved fourth cytoplasmic loop, is involved in the interaction with its G protein, G_t. However, the role of specific amino acid residues in this region has not been determined. Previously, we reported that a truncation mutant of bovine rhodopsin that retains only six amino acids of the proximal region of the carboxyl terminus is a functional photoreceptor that demonstrates normal binding to 11-*cis*-retinal, the opsin chromophore, and enhanced activation of G_t (6). Removal of five additional amino acids resulted in the production of a misglycosylated nonfunctional mutant unable to bind retinal. These data suggested that the proximal region of the carboxyl terminus is important for the proper folding and integration of the rhodopsin polypeptide into the membrane, but they did not resolve the question of participation of these six individual amino acids in G protein activation.

In the present report we have made a series of point mutations in the bovine rhodopsin cDNA corresponding to the proximal six amino acids of the carboxyl terminus. These mutants have been expressed transiently in HEK-293 cells and their level of expression, ability to bind 11-*cis*-retinal, and ability to activate G_t have been tested. Surprisingly, none of the mutants shows significant differences in these properties, compared with wild-type rhodopsin. These data suggest that the specific amino acid sequence of the fourth cytoplasmic loop is not required for G protein activation or for the proper folding and expression of a functional photoreceptor. The implications of our results for generally accepted views of receptor-G protein interaction are discussed.

Experimental Procedures

Mutagenesis. Point mutations were made in the cDNA for bovine rhodopsin (7) using oligonucleotide mutagenesis, as described previously (6). A sequence within the opsin cDNA encoding the proximal region of the carboxyl terminus contains a palindrome that disrupted efforts to perform mutagenesis. Therefore, this sequence was altered, without changing the amino acid sequence of opsin, using an oligonucleotide with the following 56-base composition: TACATCATGATGAACAAACAATTTAGGAACTGCATGGTGACCACTCTCTGCTTGTGG. The underlined bases represent changes from the original sequence. The mutant DNA sequence was confirmed by dideoxy sequencing using the enzyme Sequenase (United States Biochemicals), according to the manufacturer's directions. Expression of this altered cDNA and reconstitution with 11-*cis*-retinal indicated no change in the biochemical properties of this construct (data not shown). This cDNA

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ABBREVIATIONS: HEK, human embryonic kidney; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate.

was used for making the point mutations and the truncation mutant C322stop using the Altered Sites mutagenesis kit (Promega), according to the manufacturer's directions. The construction of C316stop and K325stop has been described previously (6).

Transfection of HEK-293 cells and preparation of membranes. Details of the transfection have been described previously (6). Briefly, HEK-293 cells were co-transfected with pcDNA/Amp (Invitrogen), containing the rhodopsin cDNA, and pRSV-TAg (8), containing the gene for simian virus 40 T antigen (a gift from J. Nathans, The Johns Hopkins University School of Medicine, Baltimore, Maryland), using DEAE-dextran. Approximately 70 hr after transfection, the cells were harvested and membranes were prepared by sucrose gradient centrifugation. Protein determinations were performed as described by Bradford (9).

Quantitation of expressed rhodopsin. The level of rhodopsin expression was estimated using aliquots from membranes and CHAPS extracts of membrane preparations. These samples were examined by Western analysis using the antirhodopsin monoclonal antibody R2–15N (a gift from Dr. Paul Hargrave, University of Florida, Gainesville, Florida) (10), followed by incubation with ^{125}I-Protein A. A Molecular Dynamics PhosphorImager was used to quantify the level of rhodopsin in the Western blots.

Absorption measurements of retinal binding to rhodopsin. Approximately 200 μg of membrane protein were incubated for 1 hr in buffer A (50 mM HEPES, pH 6.5, 140 mM NaCl, 3 mM $MgCl_2$, 2 mM EDTA, 1 mM DTT) containing 14 μM 11-*cis*-retinal, at room temperature in the dark. Forty micrograms of the mixture were removed for the GTPγS assay (see below) and the remaining solution was centrifuged for 5 min at room temperature. The pellet containing the membranes (approximately 160 μg) was extracted with buffer A containing 2% CHAPS. After centrifugation at 12,000 × *g* for 10 min, the supernatant was used for spectrophotometric assays before and after exposure to fluorescent room light, as described previously (6). A difference spectrum was calculated for each sample by subtracting the absorbance measured in the light from that for the same sample measured previously in the dark. A molar extinction coefficient of 42,700 M^{-1} cm^{-1} at 498 nm was used to estimate the concentration of rhodopsin in these preparations (11). The mutant rhodopsins were assumed to have the same extinction coefficient as the wild-type protein. The measurements, performed in duplicate, varied by approximately 9%.

Activation of G_t. The ability of rhodopsin to activate its G protein was determined by assaying the initial rates of [^{35}S]GTPγS binding to G_t using a nitrocellulose filter binding assay. The 40 μg of HEK-293 cell membranes removed from the retinal incubation mixture (see above) were centrifuged at 12,000 × *g* for 10 min at room temperature in the dark, to remove excess 11-*cis*-retinal. The pellets were resuspended in 20 mM Tris·HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT. For each sample, 2.5 μg of membranes expressing either wild-type or mutant rhodopsin were assayed in the light and in the dark, as described previously (6, 12). The initial rates of GTPγS binding were normalized to the level of expression of rhodopsin estimated by PhosphorImager analysis and are presented as a percentage of the rate measured for expressed wild-type rhodopsin.

Palmitoylation of rhodopsin. HEK-293 cells were plated in 5.5-cm plates 1 day before transfection. Transfections with the mutant cDNAs were carried out as described above. Approximately 60 hr later, the medium was changed to Dulbecco's modified Eagle's medium/F-12 medium (without fetal calf serum). After 3 hr, the medium was replaced with Dulbecco's modified Eagle's medium/F-12 medium containing [^{3}H]palmitic acid (100 μCi/ml) and 1% fetal calf serum, and the cells were incubated for 2 hr. At the end of the incubation, the cells were washed three times with phosphate-buffered saline, scraped, and collected in centrifuge tubes. After centrifugation at 600 × *g*, the cell pellets were solubilized in Tris-buffered saline (20 mM Tris-HCL, pH 7.5, 150 mM NaCl) containing 1.5% octylglucoside, 2 mM $MgCl_2$, 2 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM DTT, and 0.1 mM EDTA. The solution was centrifuged at 1100 × *g* for 5 min, and the supernatant was centrifuged again at 12,000 × *g* for 5 min to remove insoluble material. The antirhodopsin monoclonal antibody R2–15N was added to this supernatant and incubated for 1 hr at room temperature, followed by the addition of Protein A-Sepharose 4B (50% slurry) and incubation at room temperature for 30 min. The mixture was centrifuged at 600 × *g* for 2 min at 4°. The pellet was washed three times with Tris-buffered saline containing 0.1% sodium deoxycholate, then dissolved in Laemmli buffer without β-mercaptoethanol (20 mM Tris HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue), and analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After treatment with Amplify (Amersham), according to the manufacturer's instructions, the gel was exposed to Kodak XAR film for autoradiography.

Results

Mutagensis of the carboxyl terminus. The potential functions for the six proximal amino acids of the carboxyl terminus include 1) acting as sites of interaction between rhodopsin and G_t, 2) regulating the stability or folding of the photoreceptor, and 3) controlling retinal binding. Individual point mutations were made to convert each of these six amino acids to alanines within the full length photoreceptor sequence (Fig. 1). Alanine was chosen because it has neutral properties, it is prevalent at both exposed and buried surfaces, and it does not cause severe changes in secondary structure (13–15). Because mutation of Gln-312 and Asn-315 to alanines might be considered conservative replacements, the more hydrophobic amino acids leucine and isoleucine were also substituted for these two residues, respectively. Lys-311 and Arg-314 were also mutated simultaneously to alanines to test for the presence of a stop/transfer sequence (16).

Expression and post-translational modification. Rhodopsin expressed in cultured cell lines is typically modified with a heterogeneous complex of carbohydrates, compared with natural rhodopsin from bovine rod outer segments. This glycosylation pattern is dependent on the specific cell type expressing rhodopsin (8, 17, 18). Previously, a truncation mutant that terminates at Asn-310 (K311stop) and proteins with mutations in the amino terminus were shown to have abnormal glycosyl-

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              310       315           322                 348
Op(wt)        N K Q F R N C M V T T L C C G . . . . . A*
C322stop      N K Q F R N C M V T T L *
C316stop      N K Q F R N *
N310A         A . . . . . . . . . . . . . . . . . . . A*
K311A         . A . . . . . . . . . . . . . . . . . . A*
Q312A         . . A . . . . . . . . . . . . . . . . . A*
F313A         . . . A . . . . . . . . . . . . . . . . A*
R314A         . . . . A . . . . . . . . . . . . . . . A*
N315A         . . . . . A . . . . . . . . . . . . . . A*
K311A/R314A   . A . . A . . . . . . . . . . . . . . . A*
Q312L         . . L . . . . . . . . . . . . . . . . . A*
N315I         . . . . . I . . . . . . . . . . . . . . A*
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Fig. 1. Amino acid sequence of the carboxyl terminus of wild-type rhodopsin, the truncation mutants C316stop and C322stop, and the point mutants. *Op(wt)*, amino acid sequence of the rhodopsin carboxyl terminus. The two *underlined* cysteines are palmitoylated and form the fourth cytoplasmic loop of rhodopsin. *, Termination sites for each polypeptide.

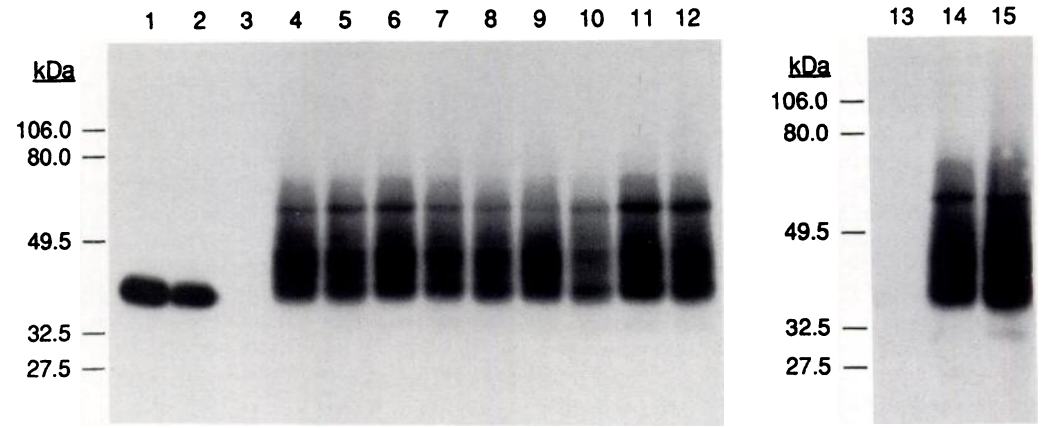

Fig. 2. Western analysis of the point mutants of rhodopsin expressed in HEK-293 cells. Membrane preparation and Western analysis were performed as described in Experimental Procedures. *Lane 1*, rod outer segments, 200 ng; *lane 2*, rod outer segments, 100 ng; *lane 3*, nontransfected cells; *lane 4*, wild-type rhodopsin; *lane 5*, K311A; *lane 6*, Q312A; *lane 7*, F313A; *lane 8*, R314A; *lane 9*, N315A; *lane 10*, K311A/R314A; *lane 11*, Q312A; *lane 12*, N315I; *lane 13*, nontransfected cells; *lane 14*, wild-type rhodopsin; *lane 15*, N310A. *Lanes 3-15* contain 15 μg of membrane protein.

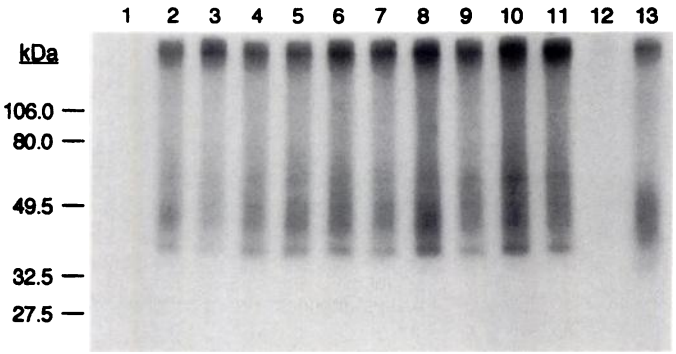

Fig. 3. Palmitoylation of the point mutants of rhodopsin. Rhodopsin point mutants and two truncation mutants (C322stop and K325stop) were expressed in HEK-293 cells using DEAE-dextran. As described in detail in Experimental Procedures, 3 days after the transfection the cells were incubated with [^{3}H]palmitic acid, extracted with octylglucoside, immunoprecipitated, and analyzed on nonreducing gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. *Lane 1*, nontransfected cells; *lane 2*, wild-type rhodopsin; *lane 3*, N310A; *lane 4*, K311A; *lane 5*, Q312A; *lane 6*, F313A; *lane 7*, R314A; *lane 8*, N315A; *lane 9*, K311A/K314A; *lane 10*, Q312L; *lane 11*, N315I; *lane 12*, C322stop; *lane 13*, K325stop.

TABLE 1

Retinal binding to rhodopsin expressed in HEK-293 cells

The percentage of CHAPS-extracted rhodopsin that bound to 11-*cis*-retinal was determined according to the difference spectra measured in the dark and in the light at 498 nm, as described in Experimental Procedures. For each sample, the amount of functional rhodopsin, estimated by using the extinction coefficient at 498 nm, is reported as a percentage of the amount of CHAPS-extracted rhodopsin measured on Western blots. Values represent the averages of duplicate experiments for wild-type rhodopsin, K311A, Q312A, F313A, R314A, N315A, and K311A/R314A. Experiments using the point mutants N310A, Q312L, and N315I were performed once.

	Retinal binding
	%
Wild-type rhodopsin	98.0
N310A	72.0
K311A	88.6
Q312A	88.7
F313A	79.0
R314A	76.0
N315A	96.1
K311A/R314A	65.7
Q312L	62.2
N315I	81.4

ation patterns, compared with the expressed wild-type protein, and lacked the ability to bind 11-*cis*-retinal, suggesting that they were misfolded (6, 19). Fig. 2 shows a Western blot analysis of the wild-type and mutant rhodopsins expressed in HEK-293 cells, with rhodopsin from bovine rod outer segments as a control. All nine rhodopsin mutants demonstrate a pattern of glycosylation similar to that of wild-type rhodopsin expressed in HEK-293 cells, suggesting the absence of severe structural defects that lead to altered glycosylation.

A second post-translational modification found in native rhodopsin is the palmitoylation of two cysteines, Cys-322 and Cys-323, which is reported to anchor the "fourth cytoplasmic loop" to the membrane bilayer (20, 21). This domain in rhodopsin is one of the most structurally conserved regions on the cytoplasmic surface within this multigene family of cell surface receptors, and palmitoylation of at least one cysteine in the carboxyl terminus is a common feature. The results of metabolic labeling of rhodopsin with [^{3}H]palmitic acid (Fig. 3) demonstrate that all of the mutants are able to incorporate the labeled fatty acid except for the truncation mutant C322stop, which lacks the two cysteine residues. Therefore, this sequence of amino acids is not necessary for palmitoylation of the carboxyl terminus. K325stop, a truncation mutant with only one amino acid beyond the sites of acylation, is also palmitoylated, suggesting that the amino acids downstream from Asn-324 are also not required for palmitoylation of the carboxyl terminus.

Retinal binding. Retinal binding is also a measure of the structural integrity of the photoreceptor, because it requires the proper alignment of the seventh transmembrane domain containing Lys-296, the site of covalent attachment to the chromophore (22), and the third transmembrane domain where Glu-113, the protonated Schiff base counterion, is located (23, 24). The mutants were examined for their ability to produce the characteristic spectrum of rhodopsin, with an absorbance peak at approximately 498 nm. Table 1 shows the extent of chromophore formation for the mutants and wild-type rhodopsin. All of the mutants, including the double-mutant K311A/R314A, are able to bind 11-*cis*-retinal at levels similar to that of the wild-type protein.

G protein activation. The rates of G_t activation for wild-type and mutant rhodopsins expressed in HEK-293 cell membranes were measured. The three truncation mutants,

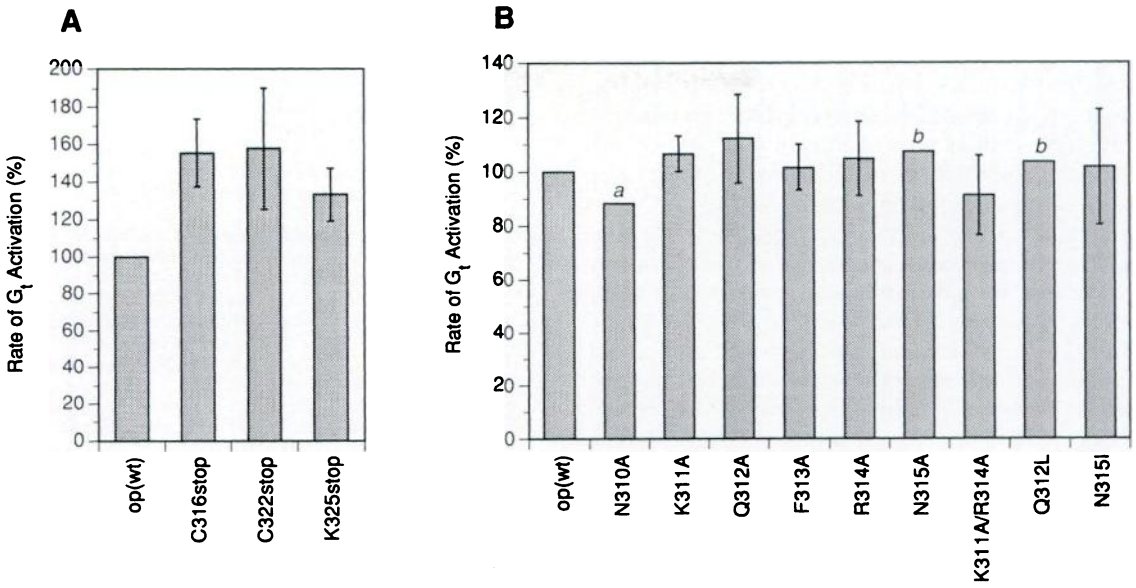

Fig. 4. Rate of G_t activation by mutants of rhodopsin. The initial rates of GTPγS binding to G_t were measured for wild-type rhodopsin [*op(wt)*] and the mutants and then normalized to the level of expression for each construct, as described in Experimental Procedures. A, Truncation mutants. The data represent the average of duplicate determinations from a representative of three independent transfections. *Error bars*, ranges for the duplicate samples. The rate of activation for wild-type rhodopsin was 0.110 mol/sec·mol. B, Point mutants. Unless otherwise noted, the data represent averages calculated from three independent transfections, each performed in duplicate. *Error bars*, standard errors calculated for these three experiments. *a*, Single experiment performed in duplicate; *b*, average of duplicate determinations from two transfections. The rates of activation for wild-type rhodopsin for each of the transfections were 0.079, 0.109, and 0.119 mol/sec·mol. The rate of GTPγS binding activity was 0.007 pmol/sec for nontransfected membranes (data not shown).

C316stop, C322stop, and K325stop, demonstrate rates of G_t activation approximately 30–50% higher than that of wild-type rhodopsin (Fig. 4A). In contrast, rates for the point mutants, which retain the carboxyl terminus, are similar to that for the wild-type protein (Fig. 4B). These data suggest that the amino acid sequence of the carboxyl terminus is not required for receptor-mediated activation of G_t.

Discussion

The present studies were undertaken to examine the participation of specific amino acids within the fourth cytoplasmic loop in the activation of G_t. Using synthetic peptides, other laboratories have proposed that sequences within the carboxyl terminus may bind to G_t. For example, synthetic peptides corresponding to amino acids 317–339 (1, 2) or 325–338 (5) serve as competitive inhibitors of receptor-stimulated GTPase activity of G_t. In another report, a peptide corresponding to amino acids 310–321 (the fourth cytoplasmic loop) disrupted the stabilization of metarhodopsin II by G_t (3). Finally, it has also been suggested that $G_{t\beta\gamma}$ binds within this region of rhodopsin (4), based on the ability of a peptide corresponding to amino acids 310–324 to interact directly with the βγ complex and to prevent its interaction with rhodopsin. Interestingly, our results demonstrate an increase in G_t activation by C316stop, which is missing all but six amino acids of the carboxyl terminus (6). As described in the present study, individual mutation of the six remaining amino acids, Asn-310 to Asn-315, to alanines had no effect on the ability of the photoreceptors to catalyze guanine nucleotide exchange on its G protein. Receptors with the less conservative mutations of Gln-312 and Asn-315 to the hydrophobic amino acids leucine and isoleucine also were normal in their ability to activate G_t. Therefore, the carboxyl terminus cannot be shown to have a critical function in G_t protein activation, forcing us to reevaluate what has been the accepted model for receptor-G protein coupling, partcularly for rhodopsin (25, 26).

Although our data suggest that the carboxyl terminus is not required for G protein activation, a regulatory function for this domain cannot be ruled out. Evidence from our laboratory using the truncation mutants (Ref. 6 and the present report), as well as reports by others that enhanced activation of G_t occurs when rhodopsin is proteolytically cleaved to remove the last 12 amino acids (27, 28) or when the protein is depalmitoylated (29), suggests that the carboxyl terminus may operate as a negative regulator of guanine nucleotide exchange. This domain may interact with other domains of the photoreceptor that are more directly involved in G protein activation, or it may bind to the G protein itself, as suggested by the peptide competition experiments. The loss of this sequence by truncation, proteolysis, or depalmitoylation could release the receptor-G protein complex from such negative regulation and allow increased guanine nucleotide exchange. The synthetic peptides that are reported to disrupt the activation of G_t by rhodopsin (1–4) may be mimicking the activity of the carboxyl-terminal domain in the intact protein.

Our studies also addressed the potential role of this domain in the proper folding and post-translational modification of the photoreceptor. Previously, we demonstrated that a truncation at Lys-311 resulted in a misfolded misglycosylated protein that was unable to bind retinal and unable to activate G_t (6). Therefore, we speculated that the region between Lys-311 and Cys-316 was involved in regulating the folding of the opsin protein. The presence of a stop/transfer sequence in the seventh membrane-spanning domain has been proposed previously (30), and cytoplasmic sequences following hydrophobic stop/transfer domains frequently contain positively charged amino acids (16). Deletion or alteration of such a sequence might cause the transfer of the seventh transmembrane domain (which contains the retinal binding site) across the bilayer, resulting in the formation of an incorrectly folded protein. Therefore, the influence of positively charged amino acids on receptor folding was tested by constructing a double mutation, converting both Lys-311 and Arg-314 to alanines (Fig. 1). However, no defects in the synthesis of or signaling by this mutant could be detected, suggesting that the length of this sequence may be more critical than the presence of specific amino acids.

In summary, we could not detect any loss of the ability of rhodopsin to activate its G protein either with truncation mutants where the carboxyl terminus was shortened to six amino acids or with mutants where those six amino acids were converted individually to alanines, despite evidence from other laboratories, using synthetic peptides, for a role of these amino acids in G_t activation. Therefore, the carboxyl terminus does not appear to be essential for G protein activation but may play a regulatory role in this process. In contrast, mutations in loops 1, 2, and 3 (31, 32) do result in disruption of G_t activation and are, therefore, more likely to be critical for G protein activation.[1] Interestingly, the proximal end of the fourth cytoplasmic loop of rhodopsin, which contains both positively charged and hydrophobic amino acids, is conserved in most G protein-coupled receptors (25). The possibility that these amino acid residues are important for the binding of other proteins, such as rhodopsin kinase and arrestin, that participate in desensitization will be the focus of future investigations.

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