Effects of Carboxyl-Terminal Truncation on the Stability and G Protein-Coupling Activity of Bovine Rhodopsin†

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ABSTRACT: A number of studies have suggested that G protein-coupled receptors possess domains within the carboxyl terminus that are important for the catalytic activation of G proteins. To define these regions, truncation mutants were generated in the cDNA of bovine rhodopsin, the receptor responsible for visual signal transduction in the retinal rod cell. The mutants were expressed in HEK-293 cells and analyzed for their ability to bind the chromophore, 11-*cis*-retinal, and for activating G_t, the G protein of the rod cell regulated by rhodopsin. Removal of 38 carboxyl-terminal amino acids resulted in the production of a mutant (K311stop) that does not bind 11-*cis*-retinal, has an abnormal pattern of glycosylation, and does not catalyze light-dependent binding of GTPγS to G_t, suggesting that it is unable to fold properly during biogenesis. However, a truncation mutant with only five additional amino acids (C316stop) coupled normally to G_t, using membranes from transfected cells, despite the fact that it lacked the "fourth cytoplasmic loop" formed by palmitoylation of cysteines-322 and -323. When C316stop is extracted from the membrane with detergent, only a fraction is able to bind 11-*cis*-retinal, but the fraction that binds retinal activates G_t normally. In contrast, detergent-solubilized wild-type rhodopsin and K325stop (a truncation mutant with the longest carboxyl terminus) both bind retinal and activate G_t normally. These data suggest that the proximal region of the carboxyl terminus is critical for the proper folding and stability of the rhodopsin molecule and that amino acids Cys316 to Ala348 are not necessary for the activation of G_t.

G proteins are heterotrimeric, guanine nucleotide-binding proteins that regulate multiple cellular pathways controlling protein phosphorylation, ion channel activity, and the generation of additional second-messenger cascades. These intracellular signal transduction proteins are activated by structurally-related members of a multigene family of cell-surface receptors. The sequencing and cloning of many of these receptors, including the α- and β-adrenergic, muscarinic acetylcholine receptors and rhodopsin, have provided a foundation for structural and functional comparisons between these receptors and for a detailed analysis of domains thought to be involved in ligand binding and in their major catalytic function, the activation of G proteins (Weiss et al., 1988b). These G protein-coupled receptors share a characteristic structure consisting of seven transmembrane domains oriented with three loops on either side of the membrane, the amino terminus on the extracellular side and the carboxyl terminus facing the cytoplasmic surface. The cytoplasmic loops and the carboxyl terminus are reported to participate in the activation of G proteins (Franke et al., 1992; Hargrave & McDowell, 1992; Khorana, 1992).

Rhodopsin, the light receptor of the mammalian rod cell, is one of the best characterized of the G protein-coupled receptors. In response to light, rhodopsin catalyzes the activation of its G protein, G_t, which in turn activates a cGMP phosphodiesterase, causing a reduction in rod cell cGMP levels and closure of cGMP-sensitive cation channels (Stryer & Bourne, 1986). This pathway of G protein activation is directly analogous to that of other G protein-coupled receptors involved in the regulation of effector enzymes such as adenylyl cyclase, phospholipase C-β, and a number of ion channels. Several studies have suggested that the cytoplasmic (extradiscal) domains of rhodopsin share structural and functional homology with other members of this multigene family. First, antipeptide antibodies directed against the first cytoplasmic loop and the carboxyl terminus of rhodopsin recognize both the β-adrenergic receptor and a putative μ-opioid receptor (Weiss et al., 1987; Gioannini et al., 1991). Second, rhodopsin can couple to G_i-like proteins *in vitro* (Tsai et al., 1987) and has been shown to inhibit adenylyl cyclase activity in a pertussis toxin-sensitive manner in response to light when expressed in Chinese hamster ovary cells (Weiss et al., 1990). Despite this evidence for structural and functional homology, very little amino acid homology exists that has been shown to be related to the activation of G proteins. One such region is the conserved Glu (or Asp)/Arg pair in cytoplasmic loop 2 (Franke et al., 1990), found in many receptors coupled to different G proteins. Another domain which is structurally conserved among many G protein-coupled receptors (Hargrave & McDowell, 1992) is the proximal region of the carboxyl terminus. This sequence forms an additional cytoplasmic loop by palmitoylation of a cysteine residue (two cysteines in rhodopsin; Ovchinnikov et al., 1988). Several studies have suggested the presence of a domain important for G protein activation within this sequence (Konig et al., 1989; Phillips & Cerione, 1992), but a detailed analysis of this domain has not been performed.

Site-directed mutagenesis has been used by many laboratories as an approach to define the functional significance of individual receptor domains with the intent of establishing potential consensus sequences for receptor interaction with G proteins and with other proteins involved in signal transduction. The present report describes the consequences of progressive truncation of the carboxyl terminus of bovine rhodopsin. We have determined that five amino acids (K311–N315) located at the proximal end of the rhodopsin carboxyl terminus are

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critical for the expression of a functional photoreceptor capable of the activation of G_t. These amino acids appear to be important for the proper folding of the receptor within the membrane. In addition, the presence of amino acids C316–G324 aids in maintaining the properly folded, detergent-solubilized receptor.

MATERIALS AND METHODS

Purification of Rhodopsin and G_t from Rod Outer Segments. The purification of rhodopsin and G_t was performed as described previously (Weiss et al., 1988a). Briefly, rod outer segment (ROS)[1] membranes were isolated under dim red light (Kodak no. 2 filters) by sucrose density gradient centrifugation after mechanical agitation of dark-adapted retina to detach them from the rod inner segments. The membranes were stripped of extrinsic proteins by a series of hypertonic and hypotonic washes followed by incubation with urea. After washing and centrifugation to remove the urea, these membranes contain rhodopsin at approximately 90% purity estimated from Coomassie Blue-stained SDS–polyacrylamide gels. The amount of rhodopsin in ROS was estimated by spectrophotometric measurements as described below. This value was the same whether or not the ROS membranes were preincubated with excess 11-*cis*-retinal (data not shown).

G_t was purified from ROS membranes isolated on sucrose density gradients as described above except that all manipulations were performed in the light in order to promote the binding of the G protein to its receptor. After removal of light-insensitive extrinsic membrane proteins with hypertonic and hypotonic washes (Weiss et al., 1988a), G_t was specifically extracted from the ROS by incubation with 40 μM GTP. These membranes were centrifuged to isolate the G protein in the supernatant. The preparation was concentrated and dialyzed against 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1 mM EDTA, 5 mM $MgCl_2$, and 1 mM DTT (buffer A).

Mutagenesis. Point mutations were generated in bovine rhodopsin by site-directed mutagenesis. The *Sma*I fragment of the cDNA for bovine opsin (Nathans & Hogness, 1983) was inserted into the plasmid M13 at the *Hin*dIII restriction site using *Hin*dIII linkers (New England Biolabs Inc.). Oligonucleotides containing a single-base mismatch were synthesized and annealed to single-stranded DNA generated from these plasmids. Mutants for K311stop and K325stop were selected and identified using procedures and reagents supplied by Amersham (In Vitro Mutagenesis Kit). For C316stop, mutagenesis was difficult because of a palindromic structure in this region of the cDNA. Therefore, Taq polymerase (Cetus) was used according to the manufacturer's directions to generate this mutant by polymerase chain reaction. All mutations were confirmed by dideoxy sequencing of the entire cDNA for each construct using the enzyme Sequenase (United States Biochemicals) according to the manufacturer's directions.

Transfection of HEK-293 Cells. The cDNA constructs described above were inserted into the plasmid pcDNAI/Amp (Invitrogen) at a *Hin*dIII site within the polylinker. HEK-293 cells were cultured in DMEM/F12 with 10% fetal calf serum (Nathans, 1990). Cells were cotransfected, using methods adapted from Ausubel et al. (1987), with pcDNAI/Amp containing the rhodopsin cDNA inserts and pRSV-TAg kindly supplied by Dr. Jeremy Nathans. One day prior to transfection, cells were plated at approximately 70% confluence in 10-cm dishes. Typically for each dish, 4 μg of pcDNAI/Amp-opsin (or opsin mutants) and 2 μg of pRSV-TAg were mixed with 0.2 mL of 10 mg/mL DEAE-dextran dissolved in TBS and 40 μL of 10 mM chloroquine. This material was added to a dish containing 4 mL of DME/F12 and 10% Nu-serum (Collaborative Biomedical Products) and incubated for 4 h. At the end of this period, the transfection mixture was removed, and the cells were incubated for 2.5 min in 10% DMSO in PBS followed by a wash with complete medium. After this wash, the cells were returned to complete medium. Cell membranes were prepared approximately 70 h after transfection.

Preparation of Membranes from HEK-293 Cells. Membranes were isolated using methods described previously (Nathans, 1990) with some modification. Briefly, cells were scraped from their dishes in PBS and centrifuged at 1500 rpm for 5 min at 4 °C. The cell pellets were frozen at −80 °C, thawed on ice, and resuspended in 18 mL of 0.1 M sodium phosphate, pH 6.5, 1 mM EDTA, and 1 mM DTT (buffer B) containing 250 mM sucrose and the protease inhibitors aprotinin (2 μg/mL) and leupeptin (1 μg/mL). The cells were disrupted by dounce homogenization, layered over a 20-mL cushion of 1.15 M sucrose in buffer B, and centrifuged at 24 000 rpm for 30 min in an SW28 rotor. Membranes were collected at the interface, diluted 8-fold with buffer B, and centrifuged at 20 000 rpm for 20 min in a Ti70 rotor to remove the sucrose. The pellet was resuspended in 50 mM Hepes, pH 6.5, 140 mM NaCl, 3 mM $MgCl_2$, 2 mM EDTA, and 1 mM DTT (buffer C) and frozen in aliquots at −80 °C until used for experiments. Protein determinations were performed as described by Bradford (1976).

Retinal Binding. Sucrose gradient-purified membranes isolated from HEK-293 cells were incubated with 14 μM (7 nmol/200 μg of membrane protein) 11-*cis*-retinal (a gift from Hoffmann-La Roche) for 1 h at room temperature followed by centrifugation at 12000*g* for 10 min to remove the unbound chromophore. All incubations were performed under dim red light or wrapped in aluminum foil to prevent exposure of the rhodopsin. The pellets, containing regenerated rhodopsin, were solubilized with 160 μL of buffer C containing 2% CHAPS and incubated for 15 min at room temperature, followed by the addition of hydroxylamine at a final concentration of 50 mM. After a further 15 min of incubation, the membranes were centrifuged at 12000*g* for 10 min at room temperature to remove insoluble material. The supernatant, containing the extracted membrane proteins, was used for spectrophotometric measurements before and after exposure to fluorescent room light for 10 min. The data were collected using a Beckman microcell and a Beckman DU-64 spectrophotometer. Typically, 50 μL of sample was used for these measurements. Difference curves were calculated using the Macintosh computer program Deltagraph Professional from Deltapoint by subtracting the spectra of the light-exposed samples from spectra of the same samples measured before light exposure. These difference curves were corrected to an absorbance = 0 Arbitrary Units (AU) at 600 nm for direct comparison between samples. 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 (Hong & Hubbell, 1973). The mutant rhodopsins were assumed to have the same extinction coefficient as the wild-type protein.

[1] Abbreviations: ROS, rod outer segment; HEK-293, human embryonic kidney-293; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CNBr, cyanogen bromide; TSH, thyroid stimulating hormone.

Electrophoresis and Immunoblotting. In order to determine the level of expressed protein, membranes prepared as described above were dissolved in Laemmli sample buffer (Laemmli, 1970), chromatographed on 10% SDS–polyacrylamide gels, transferred to nitrocellulose, and immunoblotted as described previously (Weiss et al., 1987) with the monoclonal antibody R2-15N (a gift from Dr. Paul Hargrave) against the amino terminus of rhodopsin (Hicks & Molday, 1986). The levels of the expressed rhodopsin constructs were estimated using a Molecular Dynamics PhosphorImager. Because 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 ROS as a standard.

Activation of G_t. The ability of expressed rhodopsin to activate G_t was measured by assaying the light-dependent binding of [^{35}S]GTPγS to G_t using a nitrocellulose filter binding assay as described previously (Weiss et al., 1987). Briefly, HEK-293 cell membranes from cells transfected with rhodopsin or rhodopsin mutant cDNAs were preincubated with 11-*cis*-retinal as described above to regenerate the functional photoreceptor. After centrifugation, the membranes were resuspended in buffer A. For some experiments, membranes were solubilized in buffer C containing 2% CHAPS and prepared as described in the figure legends. G_t was added at 0.5 μM (250 pmol per reaction) and the mixture preincubated at room temperature under fluorescent room light for 1 min. To initiate the reaction, 1 μM [^{35}S]GTPγS (6.25 μCi/nmol) was added. At specified time points, aliquots were applied to nitrocellulose filters and washed to remove unbound GTPγS. The filters, containing [^{35}S]GTPγS bound to G_t, were quantified by liquid scintillation spectrometry. Rates of activation were determined from the slopes of the lines fitted by linear regression analysis using the Macintosh computer program Deltagraph Professional.

Phosphorylation of Rhodopsin Kinase. Extraction of rhodopsin kinase from bovine rod outer segments and the kinase reaction were performed essentially as described by Kelleher and Johnson (1990). HEK-293 cell membranes prepared as described above were incubated with 70 μM 11-*cis*-retinal (35 nmol/500 μg of membrane protein) for 3 h at 4 °C under dim red light, followed by centrifugation at 150000*g* for 30 min to pellet the membranes and remove unbound retinal. The reaction mixture contained 15 μM [^{32}P]ATP (50 μCi/mL), 10 mM Tris-HCl, pH 7.4, 260 mM NaCl, 5 mM $MgCl_2$, 0.125 mM EDTA, 0.125 mM EGTA, 2 mM DTT, 50 mM NaF, 1 μM okadaic acid, and 250 μg of membrane protein. The reaction was initiated by the addition of 60 μL of kinase extract and incubated for 60 min at 30 °C under dim red light (for "dark" samples) or under fluorescent room light (for light-exposed samples). To terminate the reaction, the mixture was centrifuged for 15 min at 12000*g* at room temperature. The pellets were resuspended in TBS (15 mM Tris-HCl, pH 7.4, and 150 mM NaCl) containing 1.5% octyl glucoside and incubated with the R2-15N monoclonal antibody for 1 h at room temperature. Protein A–Sepharose beads (30 μL of a 50% slurry in TBS) were then added for 1 h, and the mixture was centrifuged to pellet the antigen/antibody complexes. The immunoprecipitates were washed 3 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 and analyzed by SDS–PAGE and autoradiography.

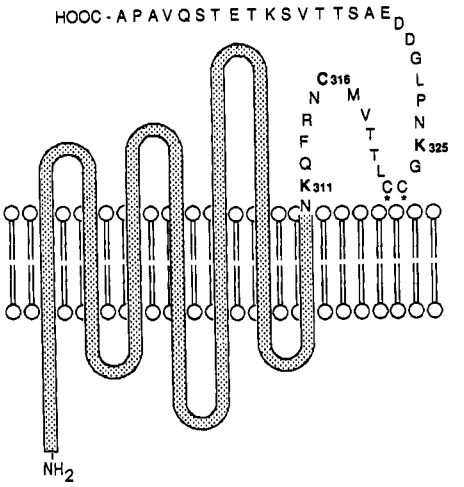

FIGURE 1: Mutations in bovine rhodopsin. The three mutations K311stop, C316stop, and K325stop are located within the carboxyl terminus of the photoreceptor which faces the cytoplasmic (extradiscal) surface. The amino terminus faces the inside surface (intradiscal) of the rod disk membrane. Asterisks (*) represent sites of palmitoylation.

RESULTS

Expression. To examine the participation of rhodopsin's carboxyl terminus in the activation of its G protein, oligonucleotide-directed mutagenesis was used to generate stop codons at three positions within the cDNA for bovine rhodopsin corresponding to the amino acids K311 (K311stop), C316 (C316stop), and K325 (K325stop). This resulted in the synthesis of 310, 315, and 324 amino acid polypeptides, respectively (Figure 1). The mutants and the wild-type protein were expressed in HEK-293 cells to examine their structural and functional properties. Western analysis of membranes isolated from nontransfected cells (Figure 2A, lane 4) and cells transfected with the wild-type and mutant cDNAs (Figure 2A, lanes 5–8) was performed using an antibody directed against the amino terminus of bovine rhodopsin (Hicks & Molday, 1986). A heterogeneous series of bands of differing mobilities reacted with this anti-rhodopsin antibody in the transfected but not in the nontransfected cell membranes. This heterogeneity has also been reported by others in HEK-293 cells (Nathans, 1990) and is similar but not identical to the pattern observed when bovine rhodopsin is expressed in other cell types (Oprian et al., 1987; Weiss et al., 1990). These differences suggest that protein glycosylation patterns are unique to individual cell types and are not determined by the primary sequence of the rhodopsin polypeptide. The K311stop mutant does not possess the extensive carbohydrate heterogeneity seen for the wild-type protein. In contrast, K325stop has a pattern very similar to the wild-type protein. C316stop appears to have a glycosylation pattern that is intermediate in heterogeneity between K311stop and the wild-type protein. After treatment with endoglycosidase F to remove the carbohydrate, the mutants and wild-type rhodopsin expressed in HEK-293 cells all migrate with greater mobility, suggesting that each of them, including K311stop, undergoes some degree of glycosylation (data not shown).

To distinguish the major functional from nonfunctional forms of the photoreceptor, membranes from cells transfected with the wild-type rhodopsin cDNA were phosphorylated by rhodopsin kinase and immunoprecipitated with the anti-rhodopsin antibody (Figure 2B). The phosphorylation of rhodopsin was light-dependent, and the pattern of bands detected by autoradiography was similar to that shown by Western analysis except for a single 57-kDa band in the

Table 1: Extraction of Rhodopsin from HEK-293 Cell Membranes

	membrane protein (μg)[a]	rhodopsin in membranes (μg)[b]	CHAPS-extracted protein (μg)[a]	CHAPS-extracted rhodopsin (μg)[b]	CHAPS-extracted rhodopsin (%)[c]
op(wt)	224.2	7.23	106.9	4.20	58.1
K311stop	227.6	0.89	103.0	0.16	18.0
C316stop	229.7	1.58	93.6	0.60	38.0
K325stop	224.8	3.29	102.6	1.71	52.0

[a] Protein quantitated by Bradford analysis. [b] Amount of rhodopsin quantitated by phosphorimage analysis. [c] Represents the percent of rhodopsin that can be extracted from the membranes as a percentage of the total rhodopsin.

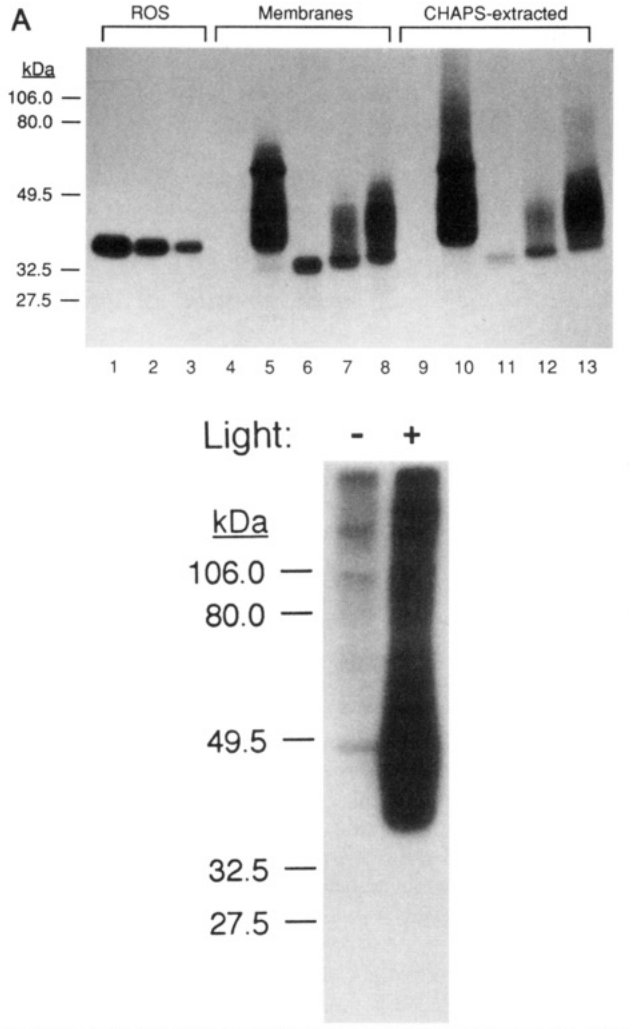

FIGURE 2: Analysis of expressed rhodopsin in HEK-293 cells. (A) Western analysis was performed on membranes expressing wild-type and mutant rhodopsin and on protein extracted with buffer C containing 2% CHAPS from membranes as described under Materials and Methods. The level of rhodopsin in each lane was quantitated by phosphorimage analysis using ROS membranes as a standard. Lanes: 1, ROS, 200 ng; 2, ROS, 100 ng; 3, ROS, 50 ng; 4 and 9, nontransfected; 5 and 10, op(wt); 6 and 11, K311stop; 7 and 12, C316stop; 8 and 13, K325stop. Lanes 4–8 each contain 25 μg of membrane protein; lanes 9–13 contain 25 μg of CHAPS-extracted protein. (B) Phosphorylation of wild-type rhodopsin from HEK-293 cells by rhodopsin kinase. Isolated membranes were phosphorylated in the dark (–) and in the light (+) and immunoprecipitated using an antibody directed against the amino terminus of rhodopsin according to procedures described under Materials and Methods.

Western blot of the wild-type protein that did not appear to be phosphorylated. This band was estimated by phosphorimage analysis to contain approximately 9% of the total immunoreactivity. Interestingly, this 57-kDa band was not present in any of the mutants. These observations indicate that the majority of the bands detected by the antibody represent properly folded, functional rhodopsin.

Detergent Extraction and Retinal Binding. A critical aspect of the function of G protein-coupled receptors is the proper folding and integration of the transmembrane domains into the membrane. The binding of 11-*cis*-retinal to opsin serves as a measure of the overall structural integrity of the photoreceptor protein; it requires the proper alignment of the seventh transmembrane domain, where Lys296 that forms the Schiff base with the retinal is located (Bownds, 1967), and the third transmembrane domain containing Glu113, the protonated Schiff base counterion (Sakmar et al., 1989; Zhukovsky & Oprian, 1989). Therefore, the ability of the mutants to bind 11-*cis*-retinal was examined to identify major structural changes caused by removal of the carboxyl terminus. For these experiments, membranes from transfected HEK-293 cells were reconstituted with 11-*cis*-retinal and extracted with CHAPS, as described under Materials and Methods. Western analysis (Figure 2A, lanes 9–12) and quantitation of the CHAPS-extracted protein (Table 1) suggest that the mutants differ in the level at which they can be extracted from the membrane. The amounts of wild-type- and K325stop-expressed rhodopsin extracted by CHAPS were similar, 58.1% and 52.0%, respectively. For the other two mutants, only 38.0% of C316stop and 18.0% of K311stop were extracted. In contrast, the amount of total protein extracted varied less than 13% for all samples. Therefore, it appears that the truncations at C316 and K311 generate structural changes which are specific to the protein itself, leading to a reduction in the extractability of the photoreceptor.

Measurement of the difference spectrum for retinal binding by wild-type rhodopsin expressed in HEK-293 cells (Figure 3A) demonstrated the presence of a light-dependent peak at approximately 500 nm that is characteristic of the native photoreceptor (Crescitelli & Dartnall, 1953; Wald & Brown, 1958). Protein extracted from nontransfected membranes showed no absorbance at that wavelength. Of the three mutants, only K325stop appeared to have significant retinal-binding activity. C316stop did not demonstrate significant ability to bind retinal unless approximately 3.5 times more concentrated membrane protein was used (Figure 3B). When the absorbance at 500 nm is normalized to the amount of CHAPS-extracted rhodopsin by Western analysis, approximately 43% of the CHAPS-extracted C316stop mutant is able to bind 11-*cis*-retinal compared with approximately 100% of the wild-type protein. An extended incubation period of 2 h in CHAPS did not alter the level of retinal-bound photoreceptor (data not shown), suggesting that two distinct, stable populations of C316stop are present in the detergent extract. In experiments using similar amounts of highly concentrated protein (data not shown), the spectrum for K311stop appears identical to that in Figure 3A. Therefore, this mutant is unable to bind 11-*cis*-retinal.

G Protein Activation by Membrane-Associated Rhodopsin. The ability of these mutants to function as photoreceptors was determined by measuring the rate of light-dependent

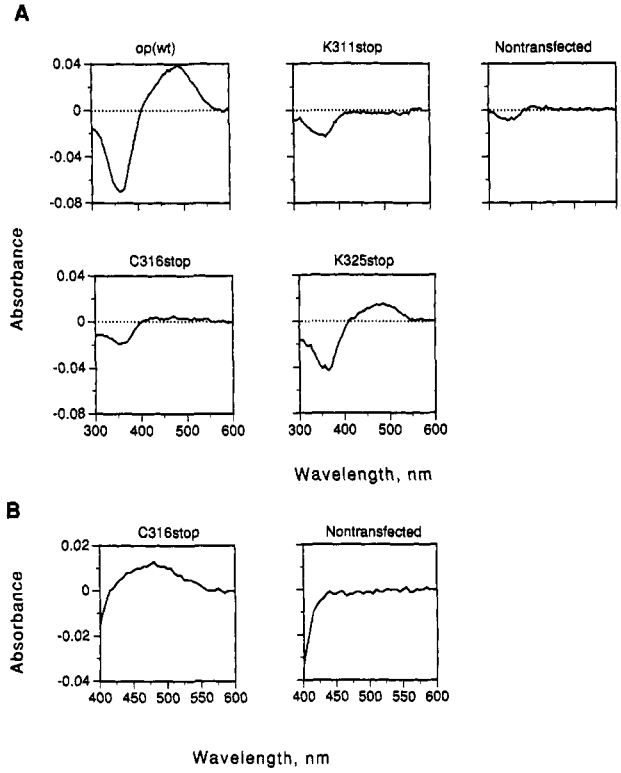

FIGURE 3: Absorbance spectra of wild-type and mutant rhodopsins. (A) Approximately 225 μg of membrane protein isolated from transfected HEK-293 cells was incubated with 11-*cis*-retinal, extracted with buffer C containing 2% CHAPS, and analyzed for retinal binding. For each sample, the following amounts of CHAPS-extracted protein were used: nontransfected, approximately 30 μg; op(wt), 33.4 μg; K311stop, 32.1 μg; C316stop, 29.3 μg; K325stop, 32.1 μg. Spectra are representative of three independent experiments. (B) Approximately 920 μg of membrane protein was prepared and analyzed as described under Materials and Methods. The following amounts of CHAPS-extracted protein were used for each measurement: nontransfected, 106.0 μg; C316stop, 102.0 μg. Spectra are representative of two independent experiments.

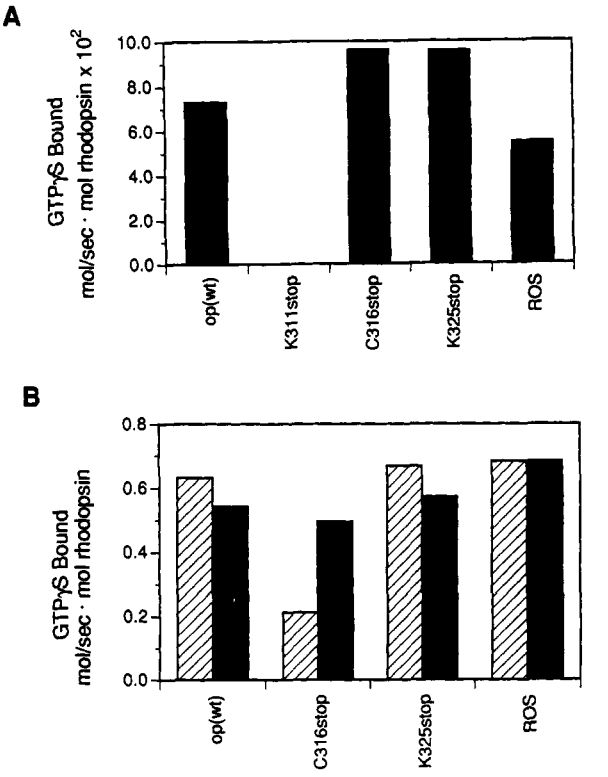

FIGURE 4: Activation of G_t by rhodopsin mutants. (A) Activation of G_t by HEK-293 cell membranes expressing rhodopsin truncation mutants. Rates of activation were normalized to the level of expression of each sample quantified from Figure 2 (lanes 1–8) as described under Materials and Methods. Membranes were used at a final concentration of 6.25 ng/μL (2.8 μg) in each reaction. The rates of activation calculated from these reaction curves are: nontransfected, −0.003 pmol/s; op(wt), 0.169 pmol/s; K311stop, −0.001 pmol/s; C316stop, 0.048 pmol/s; K325stop, 0.101 pmol/s; and ROS, 0.176 pmol/s. All points are duplicate determinations and are representative of two experiments with an average deviation of ±12%. (B) Activation of G_t by CHAPS extracts of HEK-293 cell membranes expressing rhodopsin truncation mutants. Membranes were prepared and the GTPγS-binding assays performed as described under Materials and Methods except that the membranes were first extracted with buffer C containing 2% CHAPS. For each assay, the following amount of membrane protein was used: nontransfected, 2.5 μg; op(wt), 0.25 μg; K311stop, 2.5 μg; C316stop, 1.25 μg; K325stop, 0.25 μg; ROS, 0.0125 μg. The final concentration of CHAPS in the assay mixture was adjusted to 0.01%. The rates of GTPγS binding were normalized to the amount of CHAPS-extracted rhodopsin (hatched bars) measured in Figure 2 or to the amount of rhodopsin estimated from absorbance spectra (solid bars). Measurements represent duplicate determinations of a representative experiment from two independent transfections with an average deviation of ±3%.

activation of G_t using membranes from the transfected cells reconstituted with 11-*cis*-retinal. The rates of GTPγS binding to G_t for each of the expressed constructs were normalized to their levels of expression quantitated by phosphorimage analysis of the Western blot in Figure 2. The level of rhodopsin present in membranes from the transfected cells (Figure 2A, lanes 5–8) was quantitated using ROS rhodopsin as a standard (Figure 2A, lanes 1–3). The results of the GTPγS-binding assay (Figure 4A) demonstrate that the rate of G_t activation for ROS was somewhat lower (30%) than wild-type HEK-293 cell-expressed rhodopsin. In contrast, C316stop and K325stop are approximately 30% more active than wild-type rhodopsin from HEK-293 cells. No activation of G_t could be detected when membranes from nontransfected cells were used in the assay (see Figure 4 legend). In contrast to these other mutants, activity could not be detected for K311stop.

G_t Activation by CHAPS-Extracted Rhodopsin. The studies described above suggest that the membrane-bound form of C316stop is more active than wild-type rhodopsin in its ability to activate its G protein, although only a fraction (43%) of the CHAPS-extracted mutant protein is able to bind retinal. There is an apparent lack of correlation between the ability of C316stop to bind retinal and to activate its G protein. To resolve this conflict, the activation of G_t by the CHAPS-extracted rhodopsin was determined (Figure 4B). The rates of GTPγS binding to G_t for ROS and wild-type-expressed rhodopsin, as well as the K325stop mutant, were approximately 10-fold higher than their activities in membranes. This may be due to an increased mobility of the proteins in detergent solution compared to their mobility within the membrane. CHAPS-extracted wild-type rhodopsin, K325stop, and rhodopsin from ROS demonstrated similar levels of GTPγS binding whether the rates were normalized to the amount of rhodopsin by Western analysis or by absorbance measurements. In constrast, CHAPS-extracted C316stop was significantly less active when normalized to the amount of expressed, extracted rhodopsin according to Western analysis. However, if the rate of G_t activation is normalized to the amount of functional C316stop estimated by absorbance, this mutant is fully functional. These results, as well as the retinal-binding studies, suggest that a specific population of approximately 60% of C316stop may be sensitive to denaturation during detergent extraction although this protein demonstrates greater than normal activity while in the membrane.

DISCUSSION

The carboxyl terminus of G protein-coupled receptors has been implicated in the regulation of both G protein activation and receptor desensitization (Hargrave & McDowell, 1992). However, precise roles for specific amino acids within this domain are not clearly defined. Our laboratory has generated a set of truncation mutants in bovine rhodopsin to address the question of participation by the carboxyl terminus in the activation of G_t. The truncation mutant K311stop, in which the carboxyl terminus is absent, is unable to bind 11-*cis*-retinal and, therefore, unable to stimulate the activation of G_t. However, C316stop, which has only five additional amino acids, is a functional photoreceptor, suggesting that only a very small sequence of amino acids within the rhodopsin carboxyl terminus is essential for the formation of a functional photoreceptor.

It has been reported that a "fourth loop" is formed by palmitoylation of cysteines-322 and -323 in the carboxyl terminus and their association with the membrane (Ovchinnikov et al., 1988). One of these cysteines (Cys322) is highly conserved among many receptors in this multigene family (Hargrave & McDowell, 1992). By removing all but five amino acids from this sequence, we have determined that a large portion of this loop, from C316 to C322, is not critical for contact between G_t and rhodopsin. In our experiments, membranes containing either C316stop or K325stop are able to catalyze the activation of G_t at rates higher than wild-type rhodopsin, suggesting a release from steric restraint. Three laboratories (Kuhn & Hargrave, 1981; Aton & Litman, 1984; Pellicone et al., 1985) have reported that proteolytic removal of the last 12 amino acids (generating a 336 amino acid polypeptide) resulted in elevated activity. Depalmitoylation of membrane-bound rhodopsin has also been shown to enhance the activation of G_t (Morrison et al., 1991). These results are consistent with our observations and suggest that either truncation or removal of the anchor for the fourth cytoplasmic loop enhances activity.

Despite the ability of C316stop to activate G_t at higher than normal rates in membranes, structural weaknesses in this mutant do become apparent when the detergent-solubilized proteins are compared. C316stop is less extractable than either K325stop or the wild-type protein, and only 43% of the protein that is extracted binds retinal. However, when the rates of G_t activation are compared for CHAPS-extracted wild-type and mutant rhodopsins normalized to absorbance, it is clear that C316stop is a fully functional photoreceptor that couples normally to G_t. Additionally, the data suggest that the nine amino acids from C316 to G324 increase the fraction of stable, functional, detergent-extracted protein. Since the loss of activity observed for C316stop is not apparent in the membrane-bound form, the effect of these amino acids on the signaling properties of native rhodopsin is unclear.

Interestingly, a number of laboratories have proposed that amino acids within the proximal region of the carboxyl terminus participate in the activation of G_t by rhodopsin. Experiments using synthetic peptides from the carboxyl terminus as competitive inhibitors of G_t activation suggest that specific amino acids within the sequence 317–339 are involved in the interactions between rhodopsin and its G protein (Takemoto et al., 1985, 1986). A peptide corresponding to amino acids 310–321 (the "fourth cytoplasmic loop") disrupts the stabilization of metarhodopsin II by G_t (Konig et al., 1989). Finally, it has also been suggested that G_t $\beta\gamma$ binds within this region of rhodopsin (Phillips & Cerione, 1992) based on the ability of a peptide corresponding to amino acids 310–324 to interact directly with the $\beta\gamma$ complex and to prevent its interaction with rhodopsin. However, our results demonstrate no reduction in G_t activation in the fraction of the mutant C316stop which is able to bind 11-*cis*-retinal. It may be that the critical amino acids of the carboxyl terminus that interact with the G_t subunits lie within the amino acid sequence 311–315. Alternatively, the peptides used by other laboratories corresponding to a sequence downstream from Asn315 may bind to other important domains within rhodopsin and prevent them from interacting with G_t. In this manner, the carboxyl terminus might serve as a negative regulator of G protein activation by blocking the interaction of other loop sequences with G_t in the dark and undergoing conformational changes to expose G protein-binding domains on those loops in the light. Since C316stop is still light-dependent and not constitutively active, conformational changes in domains other than amino acids 315–348 of the carboxyl terminus must be required for the interaction of G_t with rhodopsin. The lines of evidence which support the altered conformation of loops 2 and 3 include increased susceptibility to CNBr modification and to proteolytic cleavage in the light (Pellicone et al., 1985a,b).

Several potential functions can be envisioned for the five amino acid residues in the proximal region of the carboxyl terminus. They include (1) regulation of the stability or folding of the photoreceptor, (2) control of retinal binding, and (3) sites important for the interaction between rhodopsin and G_t. While the present series of experiments do not rule out (2) or (3), they do suggest that this region is important in maintaining the properly folded conformation of the photoreceptor. Progressive truncation results in altered glycosylation, reduced ability to extract the receptor with detergent, and loss of retinal binding in the case of the shortest mutant, K311stop. These properties may be an indication of proteins that are not properly intercalated into the membrane. Previous studies have identified amino acids within the intradiscal domains as critical for the correct folding of the photoreceptor (Doi et al., 1990). In those studies, mutations in amino acids critical for folding also resulted in altered glycosylation patterns and loss of retinal binding. Our report suggests for the first time the participation of extradiscal sequences at the carboxyl terminus in receptor folding. One possibility is that amino acids K311 through N315 might be part of a stop/transfer sequence. The presence of a stop/transfer sequence in the seventh membrane-spanning domain has been proposed previously (Friedlander & Blobel, 1985). Deletion or alteration of such a sequence might result in the transfer of the seventh transmembrane domain across the bilayer and in the formation an incorrectly folded protein. Alternatively, the interaction of this sequence with chaperones may be important for the proper folding of such polytopic membrane proteins.

Little is known concerning the role played by the carboxyl terminus of other G protein-coupled receptors in the activation of their G proteins. Truncation of the α_1-adrenergic receptor at amino acid 368 by site-directed mutagenesis resulted in a receptor that was equally as efficient as the wild-type protein in activating phospholipase C, despite the loss of all but 17 amino acids of the carboxyl terminus (Cotecchia et al., 1990). Removal of the entire carboxyl terminus of the TSH receptor (Chazenbalk et al., 1990) by site-directed mutagenesis resulted in a 100-fold drop in affinity for ligand and an inability to couple to G_s. The TSH receptor belongs to a different subfamily that possesses extensive extracellular amino-terminal domains for the binding of peptide ligands. This receptor also contains the conserved cysteine which corresponds to Cys322 in rhodopsin, as well as additional sequence

homology in the proximal region of the carboxyl terminus. The reduced ligand binding demonstrated for the truncated TSH receptor mutant suggests that the protein is also folded improperly. Therefore, truncation of the carboxyl terminus leads to loss of ligand binding for receptors that have external sites of interaction as well as those with ligand-binding domains within the plane of the membrane.

In summary, we have defined a five amino acid sequence at the proximal end of the carboxyl terminus which is necessary for the expression of a functional photoreceptor capable of light-dependent G protein activation. This sequence appears to be critical for the proper folding of the rhodopsin molecule. The presence of such a critical domain, its length, and its function in other members of this receptor family remain to be determined. Questions not yet answered for rhodopsin include a potential role for this sequence as a direct contact site for G_t as well as its precise involvement in receptor folding. Additional mutations introduced into the photoreceptor will be used to address these important questions.

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