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Biochemistry

Expression, stability, and membrane integration of truncation mutants of bovine rhodopsin

(membrane protein biogenesis/glycosylation/G protein-coupled receptor/retinitis pigmentosa/folding)

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ABSTRACT **Premature termination of protein synthesis by nonsense mutations is at the molecular origin of a number of inherited disorders in the family of G protein-coupled seven-helix receptor proteins. To understand how such truncated polypeptides are processed by the cell, we have carried out COS-1 cell expression studies of mutants of bovine rhodopsin truncated at the first 1, 1.5, 2, 3, or 5 transmembrane segments (TMS) of the seven present in wild-type opsin. Our experiments show that successful completion of different stages in the cellular processing of the protein [membrane insertion, *N*-linked glycosylation, stability to proteolytic degradation, and transport from the endoplasmic reticulum (ER) membrane] requires progressively longer lengths of the polypeptide chain. Thus, none of the truncations affected the ability of the polypeptides to be integral membrane proteins. C-terminal truncations that generated polypeptides with fewer than two TMS resulted in misorientation and prevented glycosylation at the N terminus, whereas truncations that generated polypeptides with fewer than five TMS greatly destabilized the protein. However, all of the truncations prevented exit of the polypeptide from the ER. We conclude that during the biogenesis of rhodopsin, proper integration into the ER membrane occurs only after the synthesis of at least two TMS is completed. Synthesis of the next three TMS confers a gradual increase in stability, whereas the presence of more than five TMS is necessary for exit from the ER.**

A number of inherited disorders have been traced to mutations in the family of seven-helix, G protein-coupled receptors (1–3). Often, these mutations result in proteins with substitutions, deletions, or insertions of amino acids without significantly altering the length of the polypeptide. However, in several instances (4–7), the mutations create stop codons that terminate protein synthesis prematurely. The resulting mutant proteins generally lack one or more segments of the polypeptide that are essential for proper folding and ligand binding. Understanding the effect of the truncations on the processing of the polypeptide by the cellular machinery is a problem of fundamental interest in determining the molecular origins of these diseases.

The family of visual rhodopsins provide an excellent model system for such studies. First, several examples of mutations in human rhodopsin (including point mutations, deletions, and truncations) that lead to the degenerative disease retinitis pigmentosa have been documented (8–11). Second, rhodopsin has two N-terminal glycosylation sites that provide markers for the correct orientation of the first transmembrane segment (TMS), because *N*-linked glycosylation occurs exclusively in the lumen of the endoplasmic reticulum (ER; refs. 12, and 13). The nature of glycosylation also indicates the subcellular localization of opsin, because the carbohydrate chains are modified as the protein is transported from the ER membrane through the Golgi to the cell surface (14, 15). Third, rhodopsin is one of the best characterized members of the family of G protein-coupled receptors in terms of its biochemical and structural properties, allowing a more incisive understanding of the effects of the mutations. Finally, wild-type rhodopsin as well as a large number of point mutants have been expressed in tissue culture, and characterized biochemically, providing a powerful database on the structure and function of rhodopsin.

In this work, we have carried out COS-1 cell expression studies of the membrane integration, glycosylation, stability, and intracellular transport of a series of mutants of bovine opsin generated by truncation after the first 1, 1.5, 2, 3, or 5 TMS of the 7 present in wild-type opsin. We have chosen bovine rhodopsin for these studies because it is the most well studied rhodopsin, and we have chosen COS-1 cells because they are especially convenient for studies of the cellular localization and stability of the expressed proteins. Furthermore, because bovine rhodopsin is ≈95% homologous to human rhodopsin, the results with bovine rhodopsin are likely to be fully relevant to human rhodopsin.

MATERIALS AND METHODS

Chemicals. The enhanced chemiluminescence detection kit and the horseradish peroxidase-conjugated sheep anti-mouse IgG were from Amersham; poly(vinylidene difluoride) membranes were from Bio-Rad. *n*-Dodecyl β-D-maltoside (DM) and deoxycholate were from Boehringer Mannheim; protease inhibitors (phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, leupeptin, pepstatin, and aprotinin), L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, tunicamycin, and Igepal-CA630 were from Sigma. Restriction endonucleases were from New England Biolabs, and Texas Red-conjugated donkey anti-mouse IgG and fluorescein isothiocyanate-conjugated sheep anti-rabbit IgG were from Jackson. Biomax MR film was from Eastman Kodak.

Transient Transfection in COS-1 Cells. The COS-1 cell line (1650-CRL, American Type Culture Collection, Rockville, MD) was grown as described (16) on 100-mm culture dishes to 70–80% confluence before transfection by using the DEAE–dextran method (17). For transfection, 10–15 μg of purified plasmid DNA was applied per dish. Cells were cultured for 48 hr. When used, tunicamycin (in DMSO, 0.8 μg/ml) was added 4 hr after transfection.

Pulse–Chase Labeling of Opsins in COS-1 Cells. Opsin mutants were transiently transfected into COS-1 cells growing on 60- mm-diameter dishes and cultured for 18–20 hr. The cells were washed with PBS and then incubated in cysteine- and

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Abbreviations: TMS, transmembrane segment(s); DM, *n*-dodecyl β-D-maltoside; ER, endoplasmic reticulum.

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methionine-free DMEM (starving medium, GIBCO/BRL) for 15 min at 37°C. The medium was then replaced with starving medium containing the equivalent of 0.1 mCi (1 Ci = 37 GBq) of L-[^{35}S]methionine and L-[^{35}S]cysteine (Pro-mix, Amersham) per dish, and cells were incubated for 5 min at 37°C. Pulse labeling was terminated by incubating the cells in chase medium (10% fetal bovine serum in starving medium containing 3-fold excess of unlabeled methionine and cysteine) for the indicated time period. The cells were washed with PBS and solubilized in RIPA buffer (1% Igepal CA-630, 1% deoxycholate, 0.1% SDS, 0.15 M NaCl, 1 mM EDTA, 10 mM Tris[chemp]HCl, pH 7.4, and protease inhibitors) for 10 min on ice. After centrifugation for 5 min at 16,000 × *g* in a Microcentrifuge, supernatants were first incubated with the 1D4 antibody for 10 hr at 4°C and then with *Staphylococcus aureus* cells (Pansorbin cells, Calbiochem) for 20 min at 4°C. Precipitates were washed with ice-cold RIPA buffer and eluted in SDS sample buffer.

DM Extraction. Forty-eight hours after transient transfection cells were washed with ice cold 10 mM NaH_2PO_4 (pH 7.0) containing 150 mM NaCl (PBS) and treated on ice with 10 mM Tris·HCl (pH 7.4), 15 mM NaCl, and 1 mM $MgCl_2$ (swelling buffer) in the presence of 1 mM phenylmethylsulfonyl fluoride. They were then scraped off, pelleted, and extracted at 4°C for 2 hr in 1% (DM) in buffer containing 50 mM Tris, 100 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 μg/ml leupeptin, 1 μg/ml pepstatin, 2 μg/ml aprotinin, 10 μg/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride, pH 6.8. The suspension was centrifuged at 220,000 × *g* for 1 hr at 4°C. Pellet and supernatant fractions were analyzed by SDS/PAGE.

Preparation of COS-1 Microsomes. Opsin mutants were transiently transfected into COS-1 cells and expressed for 48 hr. Dislodged cells (see DM extraction) were resuspended in swelling buffer containing 9.5% sucrose, and microsomes were prepared by Dounce homogenization (60 strokes) on ice. After removal of nuclei and cellular debris, homogenates were spun through a 1 M sucrose cushion for 1 hr at 200,000 × *g* at 4°C in a TLS-55 swinging bucket rotor (Beckman). The enriched microsomal membrane fraction was gently resuspended in 1 ml of PBS (pH 7.3) and adjusted to 2 mM $CaCl_2$.

Urea and Alkali Extraction. Microsomal membranes were prepared from COS-1 cells transiently transfected with the M1 and M1+ constructs, and extraction was performed with either 4 M urea or 90 mM Na_2CO_3, pH 11.5 (18, 19). Pellet and supernatant fractions were analyzed by SDS/PAGE.

SDS/PAGE. Whole-cell extracts and membrane fractions were resolved on SDS polyacrylamide gels (5–17% gradient, unless otherwise noted) under reducing conditions and then electrophoretically transferred to a poly(vinylidene difluoride) membrane. Detection of opsin by enhanced chemiluminescence was carried out according to the manufacturer's instructions (Amersham).

Immunocytofluorescence Labeling. Indirect immunofluorescence labeling of transiently transfected COS-1 cells was carried essentially out as described (20). The only difference here was that cells were fixed in 2% formaldehyde, permeabilized by 0.2% saponin, labeled with 1D4 or B6–30N monoclonal antibodies (1:3,000 dilution), and visualized by either Texas Red- or fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (1:400 dilution).

Trypsin Treatment of COS-1 Microsomes. Aliquots of microsomal membranes were incubated at 37°C with trypsin (84 μg/ml final concentration) for the indicated time. Proteolysis was terminated by the addition of soybean trypsin inhibitor to a final concentration of 350 μg/ml followed by incubation for 15 min on ice. After precipitation with 20% TCA, samples were washed, solubilized in reducing SDS sample buffer, and subjected to gel electrophoresis.

RESULTS

Construction of Deletion Mutants. Mutants of bovine opsin M1, M1+, M2, M3, and M5 in which the sequence included the first 1, 1.5, 2, 3, or 5 TMS, respectively, were constructed by systematic deletion of appropriate regions from the synthetic gene for wild-type bovine opsin (Fig. 1). In each construct, no changes in the amino acid sequence other than the deletion were introduced. In each case, the N-terminal portion remained unaltered, and the C-terminal 16 amino acids of full-length bovine opsin were spliced to the site of the deletion. This scheme ensured that the N- and C-terminal regions of native bovine opsin were retained in all mutants. The presence of the intact N- and C-terminal regions allowed each mutant polypeptide to be probed with monoclonal antibodies B6-30N (23), specific for the N terminus and 1D4 (24), specific for the C terminus. Wild-type opsin has a charge asymmetry between C-terminal and N-terminal sides. Because the net charge difference is thought to be a major determinant for the orientation of a membrane protein (25), the mutants were designed to retain this asymmetry (i.e., the putative cytoplasmic side was kept positive with respect to the putative lumenal side).

Immunofluorescence Labeling Shows that all Mutant Opsins are Localized to the ER. Indirect immunofluorescence microscopic studies showed that wild-type opsin was uniformly distributed over the cell surface (Fig. 2) as previously reported (26). In contrast, each of the mutant opsins displayed a perinuclear staining pattern. Although there are slight morphological differences in each case, the reticular pattern observed resembles that of the ER network, indicating that these opsins are localized to the ER. This was confirmed by double-labeling experiments with antibodies to heavy-chain binding protein, an ER-resident protein (data not shown). Thus, the presence of even five TMS is not sufficient for exit from the ER. These findings suggest that the mutant polypep-

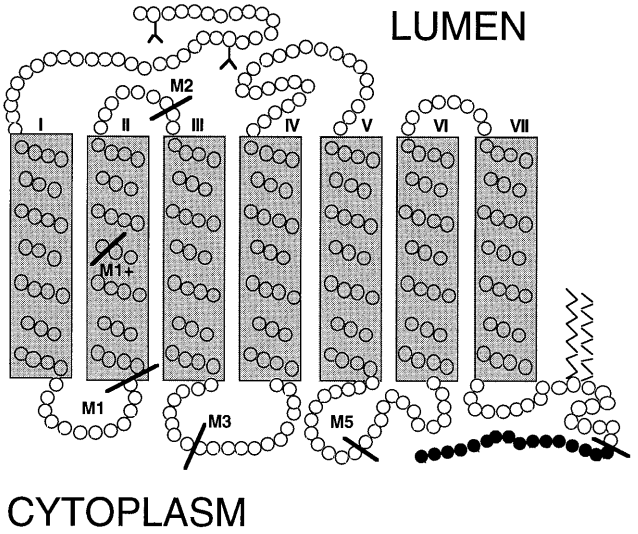

FIG. 1. Secondary structural map of bovine opsin (21) showing the locations at which deletions were carried out. Sites of deletion are indicated by bars. Solid gray boxes represent the approximate boundaries of the TMS (I through VII) and the Y symbols indicate *N*-linked oligosaccharides. All mutants were tagged with the C-terminal 16 amino acids (black) of bovine opsin, which is the epitope recognized by the 1D4 monoclonal antibody. The lengths of the constructs, restriction sites within the bovine opsin gene (22) used to generate the mutant genes, and linker amino acid sequences introduced at the C-terminal portion of the protein preceding the 1D4 tag are: WT (348 a.a.), *Eco*RI–*Sal*I, none; M5 (255 a.a.), *Eco*RI–*Pst*I, AQQQE; M3 (157 a.a.), *Eco*RI–*Pvu*I, ERYVVVCK; M2 (122 a.a.), *Eco*RI–*Nco*I, HGYFVFG; M1+ (103 a.a.), DLFMV; M1 (90 a.a.), *Eco*RI–*Hin*dIII, KLRTPLNY, respectively.

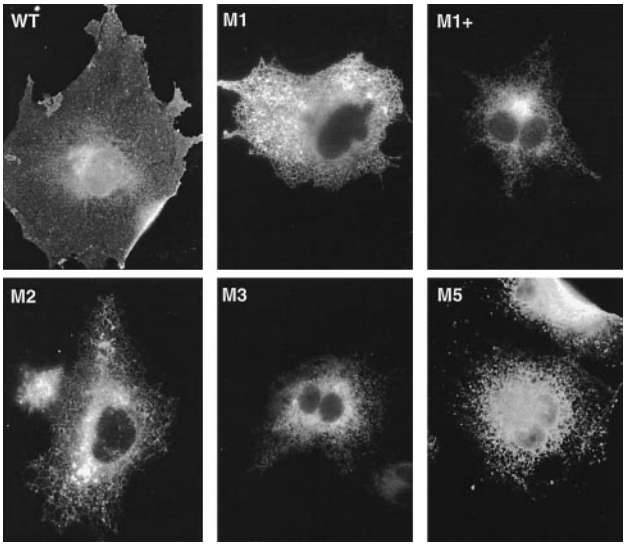

FIG. 2. Immunolocalization of deletion mutants in transiently transfected COS-1 cells. Cells were fixed and permeabilized 18–20 hr after transfection and stained with the 1D4 antibody followed by labeling with a fluorescent secondary antibody.

tides are either misfolded, aggregated, or both, and therefore cannot exit the ER. To further characterize the properties of the mutant opsins, their glycosylation status was investigated.

Wild-Type Opsin displays a complex Glycosylation Pattern. Consistent with previous reports (16), an immunoblot of wild-type opsin expressed in COS-1 cells displayed a complex band pattern (Fig. 3*I*). Glycosylated proteins generally migrate slower than unglycosylated proteins on SDS/PAGE gels. The unglycosylated form of opsin migrates at ≈31 kDa, as verified by the observation of only this band in cells treated with tunicamycin, an inhibitor of *N*-linked glycosylation. The majority of the protein was present in a diffuse band, starting with the band at ≈40 kDa, which represents the diglycosylated form of opsin. The unresolved smear (16) is a result of varying degrees of oligosaccharide processing. A range in the extent of sugar processing is expected, because the cells contain a mixture of species including newly synthesized protein, protein at various stages in the secretory pathway, and fully processed protein at the cell surface.

Mutants with fewer than two TMS do not become Glycosylated. Wild-type opsin shows oligosaccharide processing consistent with passage through the secretory pathway. In contrast, the M3 and M5 mutants displayed a three-band pattern in the immunoblot (Fig. 3*II*). Tunicamycin treatment (Fig. 3) and endoglycosidase H treatment (data not shown) showed that in each mutant the band with the highest mobility corresponded to the unglycosylated form, and that the slower migrating bands represented glycosylated forms of the protein. Because the addition of one *N*-linked oligosaccharide is expected to alter the apparent molecular mass of a protein by ≈3 kDa (14), the two bands with lower mobility observed in the M3 and M5 mutants most likely represent polypeptides carrying either one (middle band) or two oligosaccharides (upper band). As in the case of wild-type bovine opsin, the presence of glycosylation demonstrates that the N terminus of the M3 and M5 mutants is translocated into the ER lumen.

The immunoblot of the M2 mutant shows a 3-band pattern when expressed in the absence of tunicamycin, and a single band when expressed in the presence of tunicamycin (Fig. 3*III*). Of all the mutants studied, the extent of glycosylation observed in the M2 mutant varied the most from experiment to experiment. The reason for this variation is not known. Nevertheless, the immunoblot clearly indicates that the M2 mutant behaves essentially like the M3 and M5 mutants with

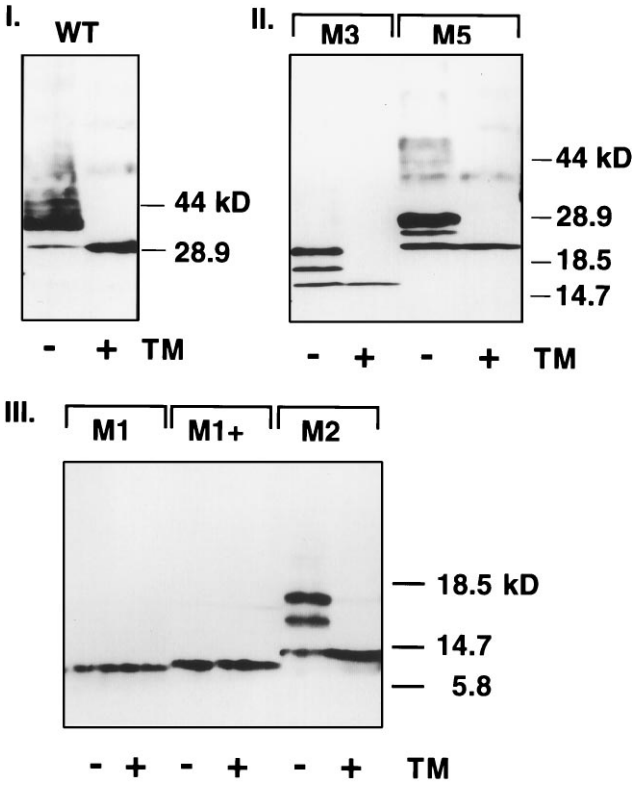

FIG. 3. Immunoblot analysis of wild-type opsin, *I*, of the M3 and M5 mutants, *II*, and of the M1, M1+, and M2 mutants *III*, transiently expressed in COS-1 cells in the absence (−) and presence (+) of tunicamycin (TM). DM (1% extracts of whole cells were used for wild-type opsin and the M2, M3, and M5 mutants. Because the M1 and M1+ mutants were not significantly extracted by 1% DM, the DM insoluble membrane pellet was used instead. Immunoblots were analyzed with the 1D4 antibody (24) which recognizes the C-terminal tag (*I* and *II*) or the B6–30N antibody (ref. 23; *III*) which recognizes an epitope at the N terminus which includes at least one of the two consensus glycosylation sites, the N2 and N15.

respect to glycosylation. Surprisingly, the M1 and M1+ mutants were not glycosylated; the immunoblot of the M1 and M1+ mutants showed only a single band when expressed either in the presence or absence of tunicamycin. The same band pattern was observed in mutants lacking the added C-terminal 16-amino acid tag (data not shown). Because the M1 and M1+ mutant polypeptides were probed using the B6-30N antibody, which recognizes the N-terminal domain containing the consensus glycosylation sites (see legend to Fig. 3), these results demonstrate that absence of glycosylation is an

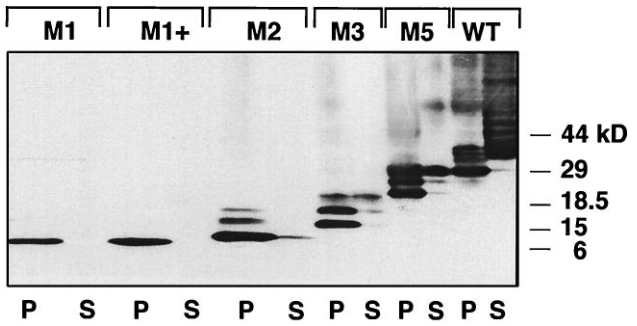

FIG. 4. Immunoblot analysis of pellet (*P*) and supernatant (*S*) fractions of DM extracts of microsomal membranes of COS-1 cells transiently expressing wild-type or mutant opsin polypeptides. Extraction was performed as described in *Materials and Methods*. Immunoblots were analyzed with the 1D4 antibody.

intrinsic property of the M1 and M1+ polypeptides and is not an artifact due to proteolytic cleavage at the N terminus.

Detergent Solubilization Correlates with Glycosylation. To further characterize the different opsin polypeptides, microsomal membranes of COS-1 cells expressing wild-type or opsin mutants were treated with the nonionic detergent DM. DM is a mild detergent that solubilizes a number of proteins in their native state (27). Because glycosylation is known to promote solubility and folding of a polypeptide (28), we reasoned that the differences in glycosylation observed in the mutant opsins might be reflected in their extractability by DM. Over a 2-hr incubation period, wild-type opsin and the M2, M3, and M5 mutants showed significant extraction by DM (Fig. 4). Interestingly, in the M3 and M5 mutants, the glycosylated forms were preferentially extracted, with the diglycosylated form more efficiently extracted than the monoglycosylated form. This finding suggests that these glycoproteins are not grossly misfolded although they are localized to the ER. When extracted for 16 hr, significant extraction of even the unglycosylated form was observed (data not shown). The M2 mutant always showed a lower extent of glycosylation and poorer extraction compared with the M3 and M5 mutants. The M1 and M1+ mutants were not significantly extracted by DM over a 2-hr extraction period, suggesting that they are either aggregated or that they form a tight complex with other ER-resident polypeptides.

Together, the immunofluorescence experiments (Fig. 2), the expression studies in the presence and absence of tunicamycin (Fig. 3), and the DM solubilization experiments (Fig. 4) identify the minimum requirements for two important events that occur in the course of the biogenesis of opsin: (*i*) N-terminal glycosylation, which requires the presence of at least two TMS, (*ii*) exit from the ER, which requires more than 5 TMS. Interestingly, the mutants that were not glycosylated (M1 and M1+) were not efficiently extracted by DM, whereas the ability of the other mutants (M2, M3 and M5) to be extracted by DM correlated strongly with the extent of glycosylation.

Pulse–Chase Labeling Experiments show that Longer Polypeptides are more Stable. To further investigate the biochemical properties of the mutants (M2, M3, and M5) that displayed at least some level of glycosylation, we analyzed their stability by pulse–chase labeling of transfected COS-1 cells with [^{35}S]methionine and [^{35}S]cysteine, followed by immunoprecipitation of opsin with the 1D4 antibody (Fig. 5). When the cells were examined immediately after a 5-min labeling pulse, two bands were observed for each mutant, corresponding to the unglycosylated and doubly glycosylated forms of the polypeptide. In the M2 and M3 mutants, a gradual loss in intensity was observed in both bands over a 4-hr period, implying loss of the protein by degradation in the ER. In both mutants, a third band corresponding to monoglycosylated opsin gradually increased in intensity over the same time period. In the M5 mutant, only a faint monoglycosylated band was observed at all times (it is more readily visible in the dimer bands). No significant change in intensity was observed in the unglycosylated and doubly glycosylated bands. The three-band pattern observed in the pulse–chase studies with all three mutants is approximately similar to that seen when the polypeptides were analyzed under steady-state conditions (Fig. 4).

The M1 and M1+ Mutants are Integral Membrane Proteins, but are Misoriented. The stability of the M1 and M1+ mutants could not be analyzed because their poor extractability in nonionic detergents prevented analysis by immunoprecipitation. One reason for the lack of glycosylation (and possibly their insolubility) could be that these mutants are not inserted into the lipid bilayer. To investigate whether the mutant polypeptides were integral or peripheral membrane proteins, microsomal fractions of COS-1 cells were treated

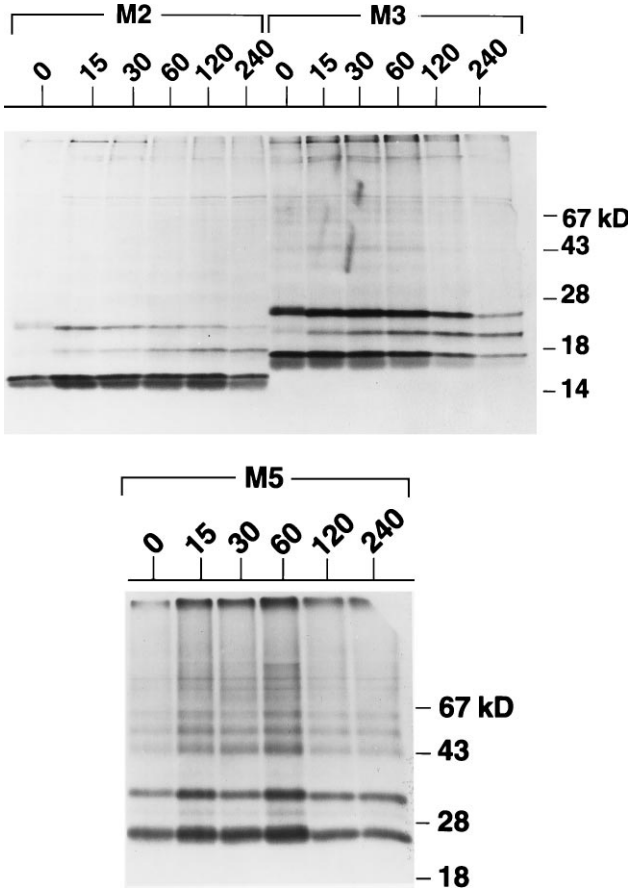

FIG. 5. Pulse–chase labeling of the M2, M3, and M5 mutants. COS-1 cells transiently transfected with mutant opsins were labeled for 5 min with [^{35}S]methionine and [^{35}S]cysteine and incubated in unlabeled methionine and cysteine for indicated chase time in minutes above each lane. Opsin was immunoprecipitated with the 1D4 antibody, resolved by SDS/PAGE (10% for M5; 12.5% for M2, and M3), and visualized by fluorography.

with either urea or sodium carbonate at alkaline pH. Under these conditions, peripheral proteins are expected to be released into the aqueous phase, whereas integral membrane proteins are retained in the membrane fraction (18). Immunoblot analysis (Fig. 6*I*) showed that the M1 and M1+ opsin polypeptides were not released into the aqueous phase upon treatment with either urea or under alkaline conditions suggesting that these mutant opsins are probably to be integral membrane proteins. We conclude that the presence of the first transmembrane segment is sufficient for targeting and insertion into the ER membrane. Our finding is at variance with the report by Ridge *et al.* (29) that short truncated opsin polypeptides could be partially extracted into the aqueous phase under alkaline conditions. However, those polypeptides were considered shorter and contained only the first 63 or 68 amino acids of bovine opsin.

Because the M1 and M1+ mutants are integral membrane proteins, we next investigated the orientation of these polypeptides by limited proteolysis with trypsin. The products of proteolysis were then subjected to SDS/PAGE followed by immunoblotting to analyze the presence of intact C- and N-terminal epitopes (Fig. 6*II*). As a control, we first analyzed the tryptic digest of microsomes isolated from cells expressing the M5 mutant. Because pronounced glycosylated bands are observed with this mutant (Figs. 3 and 4), a majority of the polypeptide must be oriented with its N terminus facing the lumen. As expected, the glycosylated N terminus was protected from cleavage by trypsin (see legend to Fig. 6 for details). The

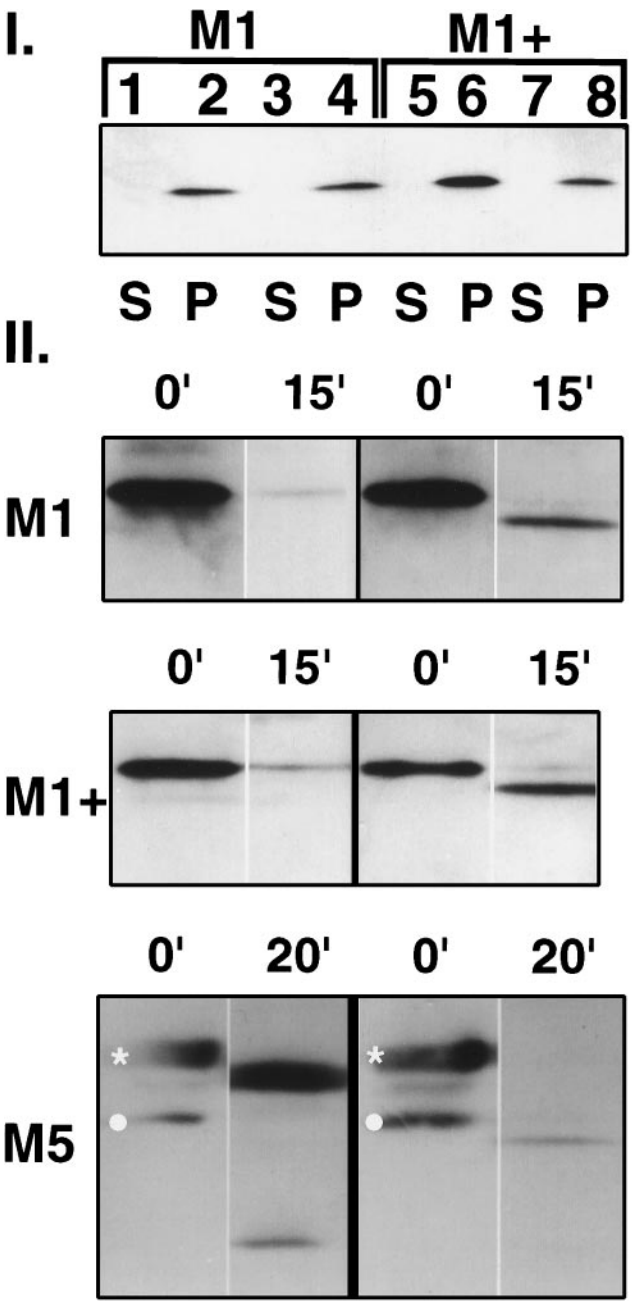

FIG. 6. *I*. Immunoblot showing pellet and supernatant fractions following treatment of microsomal membranes isolated from COS-1 cells transiently expressing the M1 (lanes 1–4) and M1+ (lanes 5–8) mutants with either 4 M urea or sodium carbonate at pH 11.5. For urea-treated samples, supernatant fractions are in lanes 1 and 5, and pellet fractions are in lanes 2 and 6. For sodium carbonate-treated samples, supernatant fractions are in lanes 3 and 7, and pellet fractions are in lanes 4 and 8. *II*. Trypsin treatment of mutants M1, M1+, and M5 in microsomes prepared from transiently transfected COS-1 cells. The immunoblots were probed with monoclonal antibodies specific to either the N terminus (B6–30N, *left* side) or the C terminus (1D4, *right* side). The protease accessible side of the microsomal membrane preparation is equivalent to the cytoplasmic side of the ER membrane. The predominant species present at the end of the reaction in the M5 mutant is derived from proteolysis at the C terminus. In contrast, the predominant species in the M1 and M1+ mutants are derived from proteolysis at the N terminus of the polypeptide, indicating a reversed orientation in the membrane. The minor bands observed in the M5 mutant at 20 min have mobilities which are consistent with N- and C-terminal fragments derived from cleavage of the doubly glycosylated species (*) in the loop region between TMS 1 and TMS 2, but may also be derived from cleavage of the unglycosylated species (○).

sugar moieties do not inhibit proteolysis, because inclusion of Triton X-100 resulted in complete digestion. Similar experiments with the M1 and M1+ mutants showed a large decrease in signal intensity in the blot probed with the N-terminal antibody, indicating loss of the N-terminal epitope. In contrast, in the blots probed with the C-terminal antibody, a new band with a lower apparent molecular weight was detected, indicating that the C terminus was protected from proteolysis relative to the N terminus. In each case, the mobility of the new band was consistent with the expected polypeptide mass generated by loss of the N-terminal region. When the digestion was carried out in the presence of Triton X-100, complete loss of signal was observed with both antibodies. Thus, the proteolysis experiments lead to the surprising result that the absence of glycosylation in the M1 and M1+ mutants is because their orientation in the membrane is reversed compared with that of the first transmembrane segment in full-length opsin.

DISCUSSION

The biogenesis of a correctly folded molecule of rhodopsin involves a series of steps including synthesis, insertion into the ER membrane, and passage from the ER to its final destination. In most seven-helix receptors, all seven TMS are required for ligand (or retinal) binding and G protein activation. However, some of the early and intermediate steps in the biogenesis are expected to occur even when the polypeptide has not been synthesized completely. In this study, we have characterized the biochemical properties of truncated polypeptides of varying lengths not only to better understand the molecular origins of diseases resulting from truncated receptors, but with the idea that understanding the biogenesis of the truncated receptors may also provide molecular "snapshots" of steps in the biogenesis of the full-length protein.

Our analysis suggests that rhodopsin truncation mutants that are unable to exit the ER fall into at least two distinct classes. Mutants such as M2, M3 and M5 are already properly integrated into the ER membrane, and are easily solubilized in nonionic detergents implying that they are not irreversibly aggregated. In contrast, mutants such as M1 and M1+ lack the minimal length requirements to be properly integrated into the ER membrane, are poorly extractable by nonionic detergents, and represent a more serious defect in cellular processing. Thus, although none of the mutants were able to exit the ER, the M1 and M1+ mutants are clearly less advanced along the biogenesis pathway as compared with the M2, M3, and M5 mutants.

Within the set of M2, M3, and M5 mutants, a gradation was observed in biochemical properties depending on the number of TMS that are present. The M3 and M5 mutants display significantly higher levels of glycosylation than the M2 mutant, whereas the M5 mutant is significantly more stable than the M2 and M3 mutants. The sharp increase in stability in the M5 mutant may originate from formation of the disulfide bond (30) between Cys-110 (in the loop between the second and third TMS) and Cys-187 (in the loop between the fourth and fifth TMS), residues which are highly conserved in the family of G protein-coupled receptors. In the pulse–chase experiments with the M2 and M3 mutants, over the same period that the doubly glycosylated band and unglycosylated band decrease in intensity, a gradual increase in intensity of the monoglycosylated species is observed. The appearance of the monoglycosylated species could reflect either the gradual deglycosylation of the doubly glycosylated species or the posttranslational glycosylation of the unglycosylated species. At present, we cannot distinguish between these possibilities.

The most remarkable finding from our expression studies is the apparent reversal of membrane orientation in the M1 and M1+ polypeptides. This is especially surprising given the evidence that membrane proteins are generally oriented such

that there is a net-positive charge on the cytoplasmic side (25). Comparison of the charge distribution in the different mutant constructs, including M1 and M1+, shows that in all mutants the cytoplasmic side is net positive, as in wild-type opsin. Thus, the opposite orientation deduced for the M1 and M1+ mutants cannot be attributed simply to differences in charge asymmetry. Although the reason for the misorientation is not clear, an attractive hypothesis to account for the dramatic difference between the M1, M1+, and the M2–M5 series of constructs is suggested by recent estimates of the size of the channel in the ER membrane that is involved in protein translocation. Imaging studies (31) with negatively stained and frozen hydrated specimens of the sec61p complex in mammals and in yeast indicate a central cavity with a diameter of ≈20 Å. Because the radius of an α-helix is ≈10 Å, this pore size is large enough to accommodate approximately two TMS in an α-helical conformation. Thus, it is conceivable that the first stage in the cotranslational insertion of opsin into the ER membrane involves "filling" the channel with the first two N-terminal TMS which, therefore, represents the minimal length requirement for efficient integration of opsin into the ER membrane.

The three-band pattern observed in the gels of the M3 and M5 mutants is similar to that observed in COS-1 expression studies (26, 32) of a number of point mutants of rhodopsin that have been implicated in retinitis pigmentosa. In most cases, these proteins do not bind retinal efficiently to regenerate a chromophore and appear to be misfolded. One can think of the misfolding in these mutants as originating from a defect in the association of correctly folded domains in a seven-helix receptor (33, 34) comprising sets of secondary structural elements such as the first three or the first five TMS. Although all seven TMS are present in the point mutants, the similarities observed with the truncated mutants may be due to the insertion of the proteins as unassociated domains. The experiments reported here argue that the number of TMS present in such folding domains may greatly influence the biochemical properties of the corresponding mutants, and suggest a structural framework to understand molecular origins of degenerative diseases such as retinitis pigmentosa.

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