

In Vitro Biosynthesis, Core Glycosylation, and Membrane Integration of Opsin

BARBARA M. GOLDMAN and GÜNTER BLOBEL

Laboratory of Cell Biology, The Rockefeller University, New York 10021

ABSTRACT A membrane-integrated, core-glycosylated form of bovine opsin was synthesized *in vitro* when bovine retina mRNA was translated in a wheat germ cell-free system supplemented with dog pancreas microsomal vesicles; glycosylation and integration of opsin into membranes were coupled to translation. Proteolysis with thermolysin was used to probe the orientation of opsin within the dog pancreas microsomal membrane, and to compare it with that of opsin in rod cell disk membranes isolated from bovine retina. Intact microsomal or disk vesicles were required for production of discrete, membrane-associated thermolysin fragments of opsin; no discrete opsin fragments were detected when membranes were incubated with thermolysin in the presence of the nonionic detergent, Triton X-100. The major opsin fragments produced by thermolysin treatment of intact microsomal vesicles resembled those from disk vesicles in their size, oligosaccharide content, and order of appearance. In each case, the first cleavage of opsin took place at the COOH-terminus, generating a glycosylated fragment, O', which was only slightly smaller than intact opsin. Both the microsomal and disk membrane forms of O' were next cleaved internally; glycosylated fragments of similar sizes in both cases were detected which were derived from the NH₂-terminal portion of O'. Several smaller NH₂-terminal fragments of opsin were detected only in thermolysin-treated microsomal membranes, and not in disk membranes. The data suggest that the topology of opsin integrated into dog pancreas microsomal vesicles is similar to that in rod cell disk vesicles, although not identical. In each case, the glycosylated NH₂-terminal region of opsin is located within the lumen of the vesicle, while discrete COOH-terminal and internal segments of opsin apparently emerge at the outer, cytoplasmic face of the membrane. Thus, opsin in the heterologous microsomal membrane, like its counterpart in the native disk membrane, may cross the bilayer at least three times. The internal domain of the polypeptide that emerges at the outer membrane surface is apparently more highly exposed in the case of opsin in microsomal membranes, evidenced by the additional internal thermolysin cleavage sites detected.

Rhodopsin, an integral membrane glycoprotein of vertebrate rod photoreceptor cells, functions as a visual pigment within the disks of the rod outer segment (ROS).¹ Its apoprotein moiety, opsin, constitutes >90% of disk membrane protein (24). The disk membrane represents the final destination in the biosynthetic pathway of opsin, which is synthesized on rough endoplasmic reticulum (RER) of the inner segment, and is

transferred through the Golgi complex to the base of the outer segment, where it becomes incorporated into newly forming disk vesicles, probably via a plasma membrane intermediate (1, 2, 22, 35, 36). Opsin is membrane-associated at all stages of this pathway (25); however, only the biosynthetically late form of opsin, that localized in disk membranes, has been well characterized. Mature opsin contains two complex oligosaccharides linked to asparagine residues 2 and 15 in the amino-terminal region (9, 14). The polypeptide chain apparently crosses the disk membrane at least three times (11): the glycosylated NH₂-terminal region of opsin extends into the lumen of the disk (10, 11, 30), while the COOH-terminal region and an internal segment are exposed on the disk surface (10, 11, 15,

¹ *Abbreviations used in this paper:* A₂₆₀ (A₂₈₀) unit, the quantity of material giving an absorbance reading of 1.0 at 260 nm (280 nm) with a 1.0-cm light path; Con A, concanavalin A; DTT, dithiothreitol; kd, kilodaltons; RER, rough endoplasmic reticulum; ROS, rod outer segment.

28, 31). Opsin localized in disk membranes undergoes chemical modifications, including light-dependent, reversible retinal attachment, and phosphorylation (for review, see references 16 and 23).

The only early biosynthetic form of opsin that has been characterized to date is the nonglycosylated primary translation product of opsin mRNA generated in the wheat germ cell-free system (32). The goals of our present studies were to identify an RER form of opsin, to synthesize *de novo* a microsomal form of opsin equivalent to the putative RER intermediate, to investigate the mode of opsin integration into the membrane, and to compare the orientation of the polypeptide backbone in the microsomal membrane with that of the disk membrane form of opsin.

MATERIALS AND METHODS

Materials

[³⁵S]Methionine (900–1,200 Ci/mmol) and ¹⁴C molecular weight standards were obtained from New England Nuclear (Boston, Mass.); wheat germ from General Mills (Minneapolis, Minn.); Sephadex G-15, protein A- and concanavalin A (Con A)-Sepharose 4B from Pharmacia (Uppsala, Sweden); and staphylococcal nuclease and thermolysin from Boehringer (Mannheim, W. Germany). Trasylol (apoptinin) was obtained from FBA Pharmaceuticals (New York); SDS from BDH Chemicals, Ltd. (Poole, England); and Triton X-100 from Sigma Chemical Co. (St. Louis, Mo.).

Cell-free Protein Synthesis

Polyadenylated messenger RNA (mRNA) was prepared from bovine retina tissue (obtained from local abattoirs) by SDS/phenol/chloroform extraction and oligo(dT)-cellulose chromatography as detailed elsewhere (8). A 23,000 g supernatant (S-23) fraction was prepared from wheat germ embryos obtained by flotation of commercial wheat germ in carbon tetrachloride:cyclohexane (2.5:1, vol:vol) (20); S-23 preparations were pretreated with staphylococcal nuclease (12.5 U/ml final concentration) for 10 min at 22°C in the presence of 1 mM CaCl₂ to inactivate endogenous mRNA; nuclease treatment was terminated by adding EGTA to a final concentration of 2 mM. Components of the wheat germ cell-free system were essentially as described elsewhere (17), except that we used per milliliter of translation mixture: 2 A₂₆₀ U of retina mRNA, 400–500 μCi [³⁵S]Met, and 16 U of human placental RNase inhibitor (3); incubations were done at 26°C for 1 h. Dog pancreas rough microsomes were isolated and treated with staphylococcal nuclease as previously described (33); membranes were added to translation mixtures to a final concentration of 3 A₂₆₀ U/ml. Bovine retina rough microsomes were prepared according to standard cell fractionation procedures (4), and nascent polypeptide chains were completed in a modified rabbit reticulocyte cell-free system (27, 33) containing 50 A₂₆₀ U/ml rough microsomes and 250 μCi/ml [³⁵S]methionine.

Immunoprecipitation of Opsin from *In Vitro* Translation Products

Ammonium sulfate fractionation (34) was used to prepare an IgG fraction from sheep antiserum raised against SDS-denatured, electrophoretically purified bovine opsin; anti-opsin antiserum was a generous gift of Dr. David Papermaster. Procedures for immunoprecipitation of *in vitro* translation products were essentially as previously described (12). Wheat germ translation mixtures were denatured with SDS (2%, wt:vol) at 100°C, diluted with 4 vol of 25 mM Tris-HCl, pH 7.4, 190 mM NaCl, 6 mM EDTA, 2.5% Triton X-100, 125 U/ml Trasylol, and incubated with antibody for 60 min at 37°C and overnight at 4°C. Antigen-antibody complexes were recovered by binding to staphylococcal protein A covalently linked to Sepharose beads, which were washed several times with 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 100 U/ml Trasylol, 7 μM unlabeled methionine. Immunoprecipitated opsin was released from protein A-Sepharose by incubation at 100°C for 3 min in the SDS-containing loading buffer used in gel electrophoresis (see below).

Proteolysis with Thermolysin

For proteolysis of opsin synthesized *in vitro*, translation mixtures were supplemented with 5 mM calcium, and 0.1 vol of a freshly prepared 3 mg/ml solution of thermolysin (in 10 mM Tris-acetate, pH 7.5, 2 mM CaCl₂) was added.

Incubation was carried out at 26°C for lengths of time specified in figure legends. Thermolysin treatment was terminated by addition of EDTA to 10 mM and of SDS to 2%, followed immediately by incubation for 5 min at 100°C. Immunoprecipitation was performed as described above. For proteolysis of the disk form of opsin, a suspension of unbleached rod outer segment (ROS) membrane vesicles in Tris-acetate/CaCl₂ (see above) containing ~3 mg protein/ml was prepared as described earlier (24), and was incubated with 0.15 mg/ml thermolysin at 26°C in the dark for times specified in the legend to Fig. 2A. Digestion was terminated with EDTA and SDS, as above.

Polyacrylamide Gel Electrophoresis

Samples were prepared for electrophoresis by addition of at least 2 vol of loading buffer (0.2 M Tris base, 3.3% SDS, 11% sucrose, 0.01% bromophenol blue) containing 40 mM dithiothreitol (DTT). After incubation at 100°C for 3 min, proteins were alkylated for 1 h at 37°C with 0.2 M α-iodoacetamide. Electrophoresis was generally on 15% SDS polyacrylamide slab gels containing 8 M urea only in the resolving gel and not in the stacking gel; exceptions are noted in figure legends. Fluorography was done by the method of Bonner and Laskey (6). Molecular weights of polypeptides were estimated from their electrophoretic mobilities (measured from the top of the resolving gel), using a plot of log (molecular weight) vs. mobility derived from the migration of the following ¹⁴C-labeled standards: phosphorylase B, 92 kilodaltons (kd); bovine serum albumin, 68 kd; ovalbumin, 43 kd; carbonic anhydrase, 30 kd; cytochrome c, 12 kd.

RESULTS

Cell-free Synthesis and Core Glycosylation of Opsin

mRNA was extracted from calf retina and translated in a wheat germ cell-free system in the presence of [³⁵S]methionine. The resulting translation products were processed for immunoprecipitation with sheep anti-opsin IgG, and the immunoprecipitate was analyzed by SDS urea polyacrylamide gel electrophoresis. Subsequent fluorography revealed a major polypeptide band of ~30 kd (Fig. 1, lane 1). A partial amino-terminal sequence of this product (double-labeled with [³⁵S]-Met and either [³H]Pro, [³H]Phe, or [³H]Val) was determined by sequential Edman degradation (30 cycles) and was indistinguishable from that of authentic bovine opsin (data not shown); these data confirmed the previously reported finding that the primary translation product of opsin mRNA is not synthesized with a transient NH₂-terminal signal sequence (32).

As a first approach toward the identification of a glycosylated RER form of opsin, we completed nascent opsin chains associated with rod cell RER in a rabbit reticulocyte cell-free system in the presence of [³⁵S]Met. Immunoprecipitation yielded a major band of ~36 kd (Fig. 1, lane 4). This band presumably represented the core-glycosylated RER form of opsin, because it migrated more slowly than nonglycosylated opsin (lane 1) and had an electrophoretic mobility similar to that of the disk form of opsin (see Fig. 2A, lane 1). These data supported the earlier conclusion, based on electron microscope autoradiography of pulse-labeled rod cells, that opsin is synthesized on the RER (for review, see reference 23).

De novo synthesis of a form of opsin equivalent to this RER product was next attempted by supplementing the wheat germ cell-free system with translocation- and glycosylation-competent dog pancreas microsomal vesicles. Translation of retina mRNA in the presence of microsomal membranes generated a new form of opsin (Fig. 1, lane 3, arrow) which migrated more slowly than nonglycosylated opsin (lane 1) and at the same rate as opsin produced by *in vitro* readout of bovine rough microsomes (lane 4), corresponding to a molecular weight of ~36 kd. Synthesis of this presumably core-glycosylated 36 kd form of opsin occurred only when membranes were present during

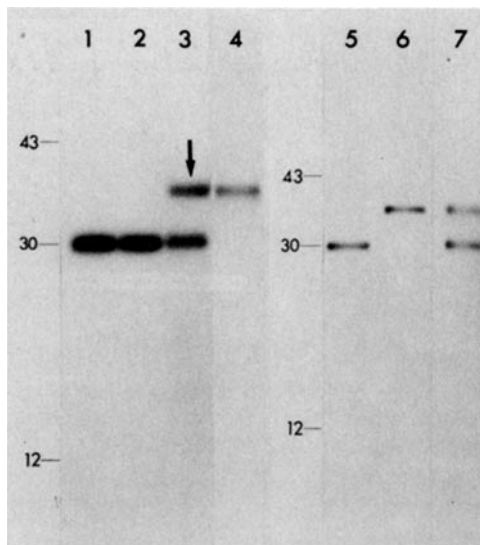


FIGURE 1 Characterization of the *in vitro* synthesized forms of bovine opsin. Immunoprecipitated opsin was analyzed by SDS urea polyacrylamide slab gel electrophoresis and fluorography (see Materials and Methods). Lane 1: bovine retina mRNA was translated in the wheat germ system in the absence of membranes. Lane 2: as in lane 1, with an additional 30-min post-translational incubation in the presence of 3 A_{280} U/ml of nuclease-treated dog pancreas microsomal membranes. Lane 3: mRNA was translated in the presence of the same concentration of membranes as above. Lane 4: bovine retina rough microsomes were incubated in a modified rabbit reticulocyte lysate cell-free system (27, 33). Opsin was immunoprecipitated from 10- μ l (lanes 1-3) or 100- μ l (lane 4) aliquots of translation mixtures. On a separate slab gel (lanes 5-7), aliquots of opsin immunoprecipitated after translation in the presence of membranes (see lane 3) were chromatographed on Con A-Sepharose columns as previously described (18); the anti-opsin immunoprecipitate from 10 μ l of translation mixture was applied to each column. Eluates were concentrated by precipitation with 2 vol of ethanol:ether (1:1) in the presence of 50 μ g/ml carrier yeast tRNA; precipitates were dissolved in loading buffer containing 40 mM DTT and heated for 3 min at 100°C before electrophoresis on a separate polyacrylamide gel. The unbound effluent fraction is seen in lane 5, while lane 6 shows the fraction specifically eluted with α -methylmannoside. Lane 7 contains the unbound fraction obtained when α -methylmannoside was present in all buffers used in packing, equilibrating and running the column. The arrow (lane 3) indicates the 36 kd core-glycosylated form of opsin synthesized in the presence of membranes. The numbers to the left of this and succeeding fluorographs indicate the molecular weights ($\times 10^{-3}$) of 14 C-labeled standards of indicated electrophoretic mobilities. Standards used are given in Materials and Methods.

translation (lane 3), and not when membranes were added after translation was complete (lane 2).

To verify that the differences in mobility observed between the two forms of opsin synthesized in the presence of membranes (lane 3) reflected the presence or absence of Asn-linked oligosaccharides, we analyzed them by chromatography on Con A-Sepharose. The 30 kd opsin band was recovered exclusively in the unbound effluent fraction (lane 5), as expected for a protein lacking core oligosaccharides. The 36 kd band was retained by Con A-Sepharose and was eluted by α -methylmannoside (lane 6), as expected for a core-glycosylated polypeptide. In control experiments, the binding of 36 kd opsin to Con A-Sepharose was blocked by presaturating the column with

α -methylmannoside (lane 7), demonstrating that binding was specific for mannose residues.

In light of evidence that transfer of core oligosaccharides occurs within the RER lumen (13), and assuming that core glycosylation of opsin by the *in vitro* translocation system occurred at the physiologically correct sites, i.e., Asn residues 2 and 15 (9, 14), our data suggested that at least the NH_2 -terminal segment of the 36 kd opsin synthesized *de novo* had been translocated across the heterologous microsomal membrane. We next used controlled proteolysis to assess the extent to which the remainder of the polypeptide had been translocated across the bilayer.

Cotranslational Integration of Opsin into Microsomal Membranes

We chose thermolysin to probe the configuration of opsin in dog pancreas microsomal membranes principally because of data already available characterizing the discrete fragments of rhodopsin that are generated by thermolysin proteolysis of disk membranes (10, 15, 28, 31). A typical pattern of rhodopsin cleavage obtained when total ROS membranes are incubated with thermolysin is shown in Fig. 2A. Thermolysin rapidly cleaves at least two small peptides from the COOH-terminus of rhodopsin (15), converting the 36 kd rhodopsin band (lane 1, arrow) to a slightly smaller (~34 kd) intermediate (lane 2, arrow), termed O' (15). Continued incubation of ROS membranes with thermolysin (lanes 3-5) results in the progressive cleavage of O' at a cytoplasmically exposed internal site (10, 15, 28), generating two large fragments, F₁ and F₂ (lane 6, arrows), both of which remain membrane-bound (10, 15, 28). The 25 kd F₁ fragment (indicated by the upper arrow, lane 6) contains the two Asn-linked oligosaccharides, and thus includes the NH_2 -terminal region of O' (10, 28); in other experiments, F₁ could be resolved as a set of at least two closely migrating fragments (data not shown), confirming an earlier report (29). F₂ was also resolved as a set of at least two bands (lanes 3-5) as noted earlier (15, 29); after an 18-h treatment of disk membranes with thermolysin a major fragment predominated, with molecular weight of ~9 kd (indicated by the lower arrow, lane 6). The F₂ fragment contains the retinal-binding site of opsin (10, 28) and represents the COOH-terminal portion of O' (15, 28). Disruption of ROS membrane vesicles with Triton X-100 before incubation with thermolysin resulted in the degradation of rhodopsin to low molecular weight peptides, largely complete within 1-h incubation with protease (lanes 7-9). Our results therefore suggest that protection of F₁ and F₂ fragments from further cleavage by thermolysin is afforded primarily by the membrane (see also reference 16). Earlier reports that treatment of membranes with nonionic detergent does not result in further cleavage of rhodopsin fragments by thermolysin (10, 11, 28) may be attributable to incomplete disruption of disk vesicles.

The data presented in Fig. 2A provide a useful framework for analyzing data generated by thermolysin treatment of opsin integrated into dog pancreas microsomal membranes. However, while rhodopsin and its major thermolysin fragments are the predominant polypeptides in disk membranes, opsin is not the predominant translation product of retina mRNA; opsin and its thermolysin cleavage products therefore can be clearly identified only after immunoprecipitation. The sheep anti-opsin IgG used in our experiments is directed against antigenic determinants in the NH_2 -terminal moiety of the molecule (D.

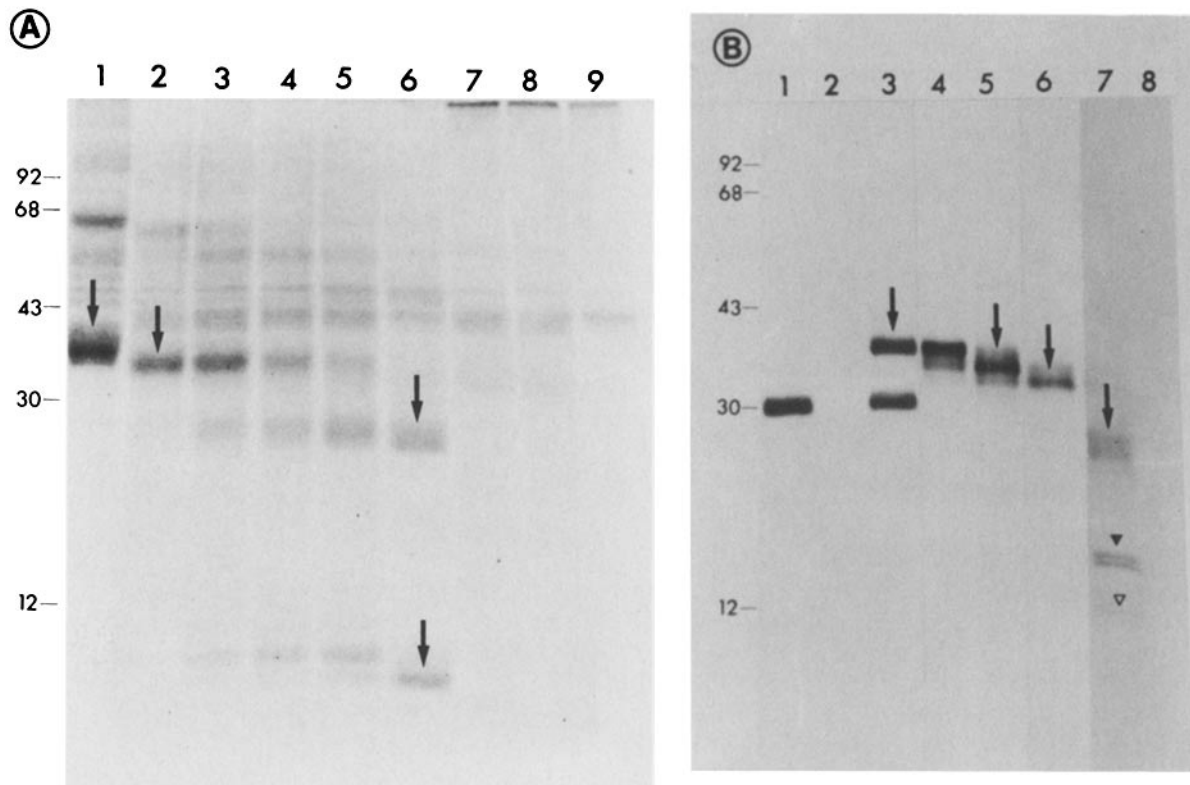


FIGURE 2 Comparison of thermolysin cleavage patterns of (A) bovine rhodopsin in retina disk membranes, and (B) bovine opsin integrated into dog pancreas microsomal membranes. (A) Unbleached ROS membranes were analyzed either before proteolysis (lane 1), or after thermolysin treatment in the absence (lanes 2–6) or presence (lanes 7–9) of 1% (wt:vol) Triton X-100. Thermolysin digestions (see Materials and Methods) were at 26°C for 10 min (lane 2), 20 min (lane 3), 1 h (lanes 4 and 7), 2 h (lanes 5 and 8), or 18 h (lanes 6 and 9). Digestions were terminated by addition of EDTA to 10 mM and SDS to 2%, followed by a 2-min incubation at 100°C. 5 μ l of each sample (~15 μ g protein) were prepared for electrophoresis (see Materials and Methods) on a 15–22% SDS polyacrylamide exponential gradient slab gel containing 8 M urea. Proteins were visualized after electrophoresis by staining with Coomassie Blue. The arrows indicate intact rhodopsin (lane 7) and its thermolysin fragments O' (lane 2), F₁ (upper arrow, lane 6) and F₂ (lower arrow, lane 6). (B) Anti-opsin immunoprecipitates from wheat germ translation mixtures were analyzed by electrophoresis on a 15% SDS polyacrylamide urea slab gel and fluorography; the same fragments were resolved as when an exponential gradient slab gel (see above) was used. Lane 1: Translation of bovine retina mRNA in the absence of dog pancreas microsomal membranes, followed by post-translational incubation with membranes (3 A₂₈₀ U/ml) for 30 min at 26°C. Lane 2: as in lane 1, plus a 1-h incubation at 26°C with 0.3 mg/ml thermolysin. Lane 3: translation of bovine retina mRNA in the presence of dog pancreas microsomal membranes (3 A₂₈₀ U/ml). Lanes 4–8: as in lane 3, plus post-translational incubation with 0.3 mg/ml thermolysin at 26°C for 30 min (lane 4), 1 h (lane 5), 5 h (lane 6) or 18 h (lane 7). Lane 8: as in lane 6, except that Triton X-100 was added to a final concentration of 1% (wt:vol) before incubation with thermolysin. Thermolysin digestions were terminated with EDTA and SDS, as above. Aliquots of translation mixtures (10 μ l in lanes 1 and 2; 15 μ l in lanes 3–5; 30 μ l in lanes 6–8) were processed for immunoprecipitation as described in Materials and Methods. Arrows indicate 36 kD core-glycosylated opsin (lane 3), its initial thermolysin cleavage product (lane 5), and the putative O' (lane 6) and F₁ (lane 7) fragments; arrowheads (lane 7) indicate additional fragments with molecular weights of ~17 kD (closed arrowhead) and 14 kD (open arrowhead). Lanes 7 and 8 are from a longer exposure of the same fluorograph. As previously discussed for the case of secretory proteins (33), protection of processed forms from post-translational proteolysis is not complete, particularly when dog pancreas microsomal vesicles are incubated with protease at room temperature. The reduced recovery of opsin fragments observed after prolonged exposure of microsomal vesicles to thermolysin at 26°C (lanes 6 and 7) probably reflects damage to some vesicles because of proteolysis of dog pancreas microsomal membrane proteins.

Papermaster, personal communication), and therefore should precipitate only those thermolysin fragments of opsin that retain these NH₂-terminal determinants.

No detectable fragments of opsin were immunoprecipitable from thermolysin-treated translation mixtures that had been supplemented with dog pancreas microsomal vesicles only after protein synthesis was completed (Fig. 2B, compare lanes 1 and 2), indicating that the nonglycosylated 30 kD opsin synthesized in the absence of membranes was not integrated into microsomal membranes during a post-translational incubation. In contrast, the 36 kD core-glycosylated opsin synthesized when membranes were present during translation (lane 3, arrow)

underwent a stepwise and time-dependent degradation into discrete fragments (lanes 5–7). Because the nonglycosylated 30 kD opsin also synthesized in the presence of membranes (lane 3) was completely degraded at a time when the glycosylated form was largely intact (lane 4), the fragments detected at later times (lanes 5–7) were clearly derived solely from the 36 kD glycosylated species. The 36 kD band was converted first to a large intermediate fragment (lane 5, arrow) and subsequently to a polypeptide (lane 6, arrow) with electrophoretic mobility similar to that of the O' fragment of rhodopsin (Fig. 2A, lane 2, arrow). Production of O' results from cleavage at two closely spaced sites in the exposed COOH-terminal segment of rho-

dopsin (15), suggesting that the two large fragments shown in lanes 5 and 6 may have been generated from opsin synthesized *in vitro* by cleavages at two similar sites. Prolonged incubation with thermolysin resulted in the disappearance of the putative O' fragment, and the appearance of several smaller fragments, including a set of bands (indicated by the arrow in Fig. 2B, lane 7) resembling F₁ in electrophoretic mobility (Fig. 2A, lane 6, arrow) and in heterogeneity (see above). The other fragments detected, doublet bands at ~17 kd (lane 7, closed arrowhead) and a band at ~14 kd (open arrowhead), were significantly smaller than the F₁ fragment, yet considerably larger than would be expected for a putative F₂ fragment (compare to Fig. 2A, lane 6). We did not detect an opsin fragment of a size comparable to F₂, probably because of the specificity of the anti-opsin antibodies used to immunoprecipitate fragments of opsin synthesized *in vitro* (see above). No discrete opsin fragments were observed when incubation with thermolysin was carried out in the presence of the nonionic detergent Triton X-100 (Fig. 2B, lane 8).

In view of their immunoprecipitability (see above), the putative O' and F₁ fragments shown in Fig. 2B (lanes 6 and 7, arrows) presumably contained the NH₂-terminal region of opsin. Moreover, these fragments should contain the core sugars of opsin (Fig. 1) if they are comparable to the authentic O' and F₁ fragments of rhodopsin, each of which retains the NH₂-terminal Asn-linked oligosaccharides (15). Data in Fig. 3 shows that the specific binding to Con A-Sepharose exhibited by intact opsin (lane 1) was in fact retained by the putative O' (compare lane 3, arrow, with lane 2) and F₁ fragments (compare lane 5, arrow, with lane 4). The fragments smaller than F₁ similarly bound to Con A-Sepharose (lane 5, arrowhead), suggesting that they were generated by further cleavages at the COOH-terminal region of F₁; this novel set of fragments has not been detected so far among those generated by thermolysin treatment of disk membranes.

DISCUSSION

Our data demonstrate that bovine opsin synthesized in the wheat germ cell-free system in the presence of dog pancreas microsomal vesicles underwent translation-coupled core glycosylation and asymmetric integration into the microsomal membrane. A core-glycosylated 36 kd form of opsin was synthesized only when microsomal membranes were present during translation; in the absence of membranes, a 30 kd form of opsin lacking core oligosaccharides was synthesized which could not be glycosylated by post-translational incubation with microsomal vesicles. Transfer of oligosaccharides to Asn residues of a polypeptide reportedly is limited to the luminal side of the microsomal vesicle (13) and thus requires translocation across the membrane of at least that portion of the polypeptide that contains glycosylation sites. Furthermore, the protein translocation system of the RER apparently initiates translocation of nascent chains only, and not of completed polypeptides (for review, see reference 5). The translation-dependent core glycosylation of opsin therefore suggested that translocation of at least the NH₂-terminal segment of nascent opsin across the microsomal membrane had taken place, because the oligosaccharides of authentic rhodopsin are linked to Asn residues near the NH₂-terminal (9, 14). This translocation may be mediated by an uncleaved, internal signal sequence, functionally similar to that of chicken ovalbumin (19), because opsin is not synthesized with a transient NH₂-terminal signal sequence (32).

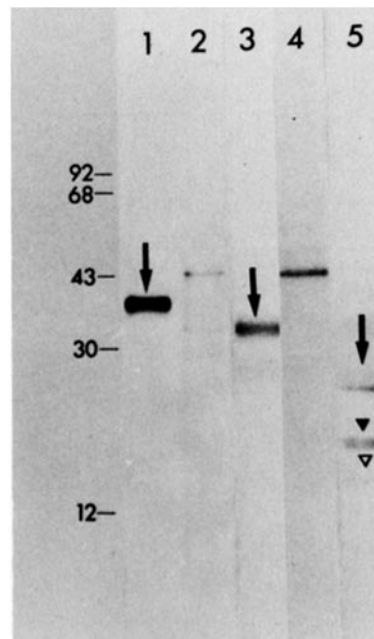


FIGURE 3 Con A-Sepharose chromatography of thermolysin fragments of opsin integrated into microsomal membranes. Retina mRNA was incubated in the wheat germ system in the cotranslational presence of microsomal membranes, and SDS-denatured translation products were reacted with Con A-Sepharose either before proteolysis (lane 1), or after thermolysin digestion for 5 h (lanes 2 and 3) or 16 h (lanes 4 and 5). Bound and unbound fractions were treated with anti-opsin antibodies, and immunoprecipitates were prepared for electrophoresis as described in the legend to Fig. 1. Lanes 1, 3, and 5: bound material specifically eluted with 0.2 M α -methylmannoside. Lanes 2 and 4: unbound material. Lanes 4 and 5 were from longer exposure of the same fluorograph. The bands at 43 kd visible in lanes 2 and 4 resulted from nonspecific entrapment of radioactivity by IgG heavy chains. Arrows indicate Con A-bound opsin (lane 1) and thermolysin fragments O' (lane 3) and F₁ (lane 5).

The topology of newly synthesized opsin within the heterologous microsomal membrane was further explored by controlled proteolysis, using thermolysin as probe. Discrete fragments were generated from opsin integrated into dog pancreas microsomal vesicles that resembled fragments resulting from thermolysin treatment of disk vesicles in their size, the presence of oligosaccharides and the sequence in which they were generated. To illustrate these similarities, data on the derivation of opsin fragments generated by thermolysin treatment of disk vesicles (10, 15, 28, 31) have been schematically summarized in Fig. 4A. The first thermolysin cleavages, at the COOH-terminal sites of opsin, generate the somewhat smaller derivative, O'. Subsequent cleavage in the interior of O' generates two membrane-bound fragments, with F₁ containing the NH₂-terminal region of O', and F₂ retaining the COOH-terminal portion; the apparent heterogeneity of both F₁ and F₂ fragments (15, 29; see also Fig. 2A) raises the possibility that more than one site within this internal segment is cleaved by thermolysin. Like rhodopsin in disk membranes, the 36 kd form of opsin integrated into dog pancreas microsomal vesicles was first cleaved at two closely spaced COOH-terminal sites, generating a fragment comparable to O' in size and, like O', containing oligosaccharides. Subsequent cleavage of this O' fragment generated smaller opsin fragments, including a set of closely migrating bands resembling F₁ in size (~25 kd) and in the

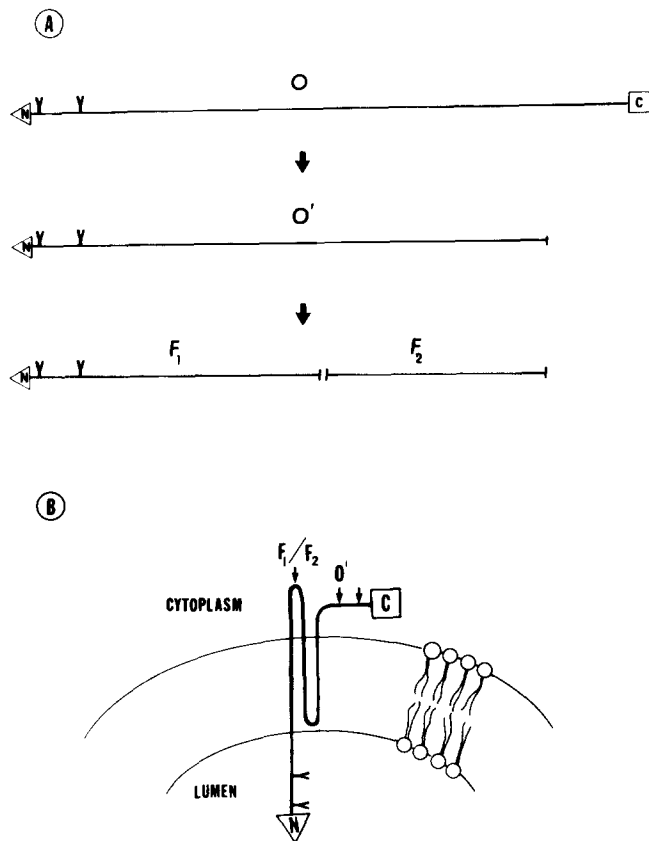


FIGURE 4 Schematic illustration of the derivation of thermolysin fragments of opsin integrated into the disk membrane. (A) Diagrammatic representation of intact opsin (O) and its thermolysin fragments O', F₁ and F₂, indicating the amino-terminus (N), the amino-terminal oligosaccharides (Y), and the carboxy-terminus (C). (B) Simplified model for the topology of rhodopsin in disk membranes, based on that of Fung and Hubbell (11). Symbols are as above; thermolysin cleavage sites are indicated by arrows.

presence of oligosaccharides; the observed heterogeneity of these F₁-like fragments may indicate the existence of several closely spaced cleavage sites within the O' fragment, although possible heterogeneity of the core sugars cannot be ruled out. A putative F₂ fragment, derived from the COOH-terminal portion of O', could not be detected, presumably because of the NH₂-terminal specificity of the sheep anti-opsin antibodies used. Several discrete opsin fragments smaller than F₁ were observed after prolonged (18 h) thermolysin digestion of microsomal vesicles containing opsin, including doublet bands of ~17 kd and a single band of ~14 kd. Because these fragments were immunoprecipitable and contained core oligosaccharides, they were apparently derived by additional cleavages near the COOH-terminal region of F₁, i.e., to the left of the internal cleavage site indicated in Fig. 4A.

Discrete fragments of opsin were generated only when thermolysin digestions were carried out on intact dog pancreas microsomal vesicles or bovine disk membrane vesicles, and not when the nonionic detergent Triton X-100 was present during proteolysis. The susceptibility of discrete sites within opsin to cleavage by thermolysin therefore reflects their exposure to the environment at the outer, cytoplasmic, face of the membrane vesicle. The simplest topology of the polypeptide backbone consistent with the available data is shown in Fig. 4B, which was adapted from a model published earlier (11). The NH₂-terminal, core-glycosylated region of opsin synthesized *in vitro*

was translocated into the lumen of the microsomal vesicle; like the NH₂-terminus of opsin in disk vesicles, it was completely protected from the action of thermolysin. Discrete internal and COOH-terminal segments of opsin apparently were not translocated. The temporal sequence of cleavage at these sites, generating first O' and then F₁ fragments, was the same as that of opsin in disk membranes, and indicates that the COOH-terminal region contained the more highly exposed cleavage sites. The additional sites within the F₁ region of opsin that were exposed at the surface of the microsomal membrane could conceivably reflect incomplete folding or membrane integration of this internal segment, or topological heterogeneity within the population of newly synthesized opsin molecules integrated into microsomal vesicles. Alternatively, the RER form of opsin, which differs structurally from the disk form in its unprocessed core oligosaccharides, absence of retinal and lack of phosphorylation, may have a characteristic topology that is modified slightly from that of the disk form, e.g., with multiple insertions in the F₁ region.

Our data thus indicate that similar regions of opsin are exposed at the cytoplasmic membrane face in both microsomal and disk vesicles. Because the sidedness of isolated disk vesicles is identical to that of native disks in the ROS (7), this suggests that the orientation of opsin with respect to the membrane is not significantly altered during its biosynthetic pathway. Intracellular transport of newly synthesized opsin within the membranes of vesicles (26), fusion of these vesicles with the plasma membrane at the base of the ROS, and invagination of the membrane to form new disks (1, 21, 36), would generate a disk form of opsin with the same orientation with respect to the membrane as the earlier RER intermediate.

We thank Dr. David Papermaster for his generous gift of anti-opsin antiserum, and Dr. Dennis Shields for preparing retina disk membranes. This work was supported in part by a grant from the National Institutes of Health (PHS 27155) to G. Blobel. B. M. Goldman is the recipient of a U. S. Public Health Service postdoctoral fellowship from the National Eye Institute (1-F32-EY05208) and a postdoctoral award from Fight for Sight, Inc., New York.

Received for publication 23 February 1981.

REFERENCES

1. Basinger, S., D. Bok, and M. Hall. 1976. Rhodopsin in the rod outer segment membrane. *J. Cell Biol.* 69:29-42.
2. Basinger, S. F., and M. O. Hall. 1973. Rhodopsin biosynthesis *in vitro*. *Biochemistry* 12: 1996-2003.
3. Blackburn, P., G. Wilson, and S. Moore. 1977. Ribonuclease inhibitor from human placenta. Purification and properties. *J. Biol. Chem.* 252:5904-5910.
4. Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes. II. Reconstitution of functional rough microsomes from heterologous components. *J. Cell Biol.* 67: 852-862.
5. Blobel, G., P. Walter, C. N. Chang, B. M. Goldman, A. H. Erickson, and V. R. Lingappa. 1979. Translocation of proteins across membranes: The signal hypothesis and beyond. Society for Experimental Biology Symposium. C. R. Hopkins and C. J. Duncan, editors. Cambridge University Press, Great Britain. 33:9-36.
6. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labeled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* 46:83-88.
7. Chen, Y. S., and W. L. Hubbell. 1973. Temperature- and light-dependent structural changes in rhodopsin-lipid membranes. *Exp. Eye Res.* 17:517-532.
8. Erickson, A. H., and G. Blobel. 1979. Early events in the biosynthesis of the lysosomal enzyme cathepsin D. *J. Biol. Chem.* 254:11771-11774.
9. Fukuda, M. N., D. S. Papermaster, and P. A. Hargrave. 1979. Rhodopsin carbohydrate: Structure of small oligosaccharides attached at two sites near the amino terminus. *J. Biol. Chem.* 254:8201-8205.
10. Fung, B. K.-K., and W. L. Hubbell. 1978. Organization of rhodopsin in photoreceptor membranes. I. Proteolysis of bovine rhodopsin in native membranes and the distribution of sulfhydryl groups in the fragments. *Biochemistry* 17:4396-4402.
11. Fung, B. K.-K., and W. L. Hubbell. 1978. Organization of rhodopsin in photoreceptor membranes. 2. Transmembrane organization of bovine rhodopsin: evidence from proteolysis and lactoperoxidase-catalyzed iodination of native and reconstituted membranes. *Biochemistry* 17:4403-4410.
12. Goldman, B. M., and G. Blobel. 1978. Biogenesis of peroxisomes: Intracellular site of synthesis of catalase and uricase. *Proc. Natl. Acad. Sci. U. S. A.* 75:5066-5070.

13. Hanover, J. A., and W. J. Lenarz. 1980. N-Linked glycoprotein assembly. *J. Biol. Chem.* 255:3600-3604.
14. Hargrave, P. A. 1977. The amino-terminal tryptic peptide of bovine rhodopsin. A glycopeptide containing two sites of oligosaccharide attachment. *Biochim. Biophys. Acta.* 492: 83-94.
15. Hargrave, P. A., and S.-L. Fong. 1977. The amino- and carboxyl-terminal sequence of bovine rhodopsin. *J. Supramol. Struct.* 6:559-570.
16. Hargrave, P. A., S.-L. Fong, J. H. McDowell, M. T. Mas, D. R. Curtis, J. K. Wang, E. Juszcak, and D. P. Smith. 1980. The partial primary structure of bovine rhodopsin and its topography in the retinal rod cell disc membrane. *Neurochemistry International.* 1:231-244.
17. Katz, F. N., J. E. Rothman, V. R. Lingappa, G. Blobel, and H. F. Lodish. 1977. Membrane assembly *in vitro*: synthesis, glycosylation, and asymmetric insertion of a transmembrane protein. *Proc. Natl. Acad. Sci. U. S. A.* 74:3278-3282.
18. Lingappa, V. R., J. R. Lingappa, R. Prasad, K. E. Ebner, and G. Blobel. 1978. Coupled cell-free synthesis, segregation and core-glycosylation of a secretory protein. *Proc. Natl. Acad. Sci. U. S. A.* 75:2338-2342.
19. Lingappa, V. R., J. R. Lingappa, and G. Blobel. 1979. Chicken ovalbumin contains an internal signal sequence. *Nature (Lond.)*, 281:117-121.
20. McMullen, M. D., P. H. Shaw, and T. E. Martin. 1979. Characterization of poly(A⁺) RNA in free messenger ribonucleoprotein particles and polysomes of mouse Taper ascites cells. *J. Mol. Biol.* 132:679-694.
21. Nilsson, S. E. G. 1964. Receptor cell outer segment development and ultrastructure of the disk membrane in the retina of the tadpole (*Rana pipiens*). *J. Ultrastruct. Res.* 11:581-620.
22. O'Brien, P. J., C. G. Muellenberg, and J. J. Bungenburg de Jong. 1972. Incorporation of leucine into rhodopsin in isolated bovine retina. *Biochemistry.* 11:64-70.
23. O'Brien, P. J. 1978. Rhodopsin: a light sensitive membrane glycoprotein. *In* Receptors and Recognition, Series A. P. Cuatrecasas and M. F. Greaves, editors. J. Wiley & Sons, Inc., New York. 6:109-149.
24. Papermaster, D. S., and W. J. Dreyer. 1974. Rhodopsin content in the outer segment membrane of bovine and frog retinal rods. *Biochemistry.* 13:2438-2444.
25. Papermaster, D. S., C. A. Converse, and J. Siu. 1975. Membrane biosynthesis in the frog retina: opsin transport in the photoreceptor cell. *Biochemistry* 14:1343-1352.
26. Papermaster, D. S., B. G. Scheider, and J. C. Besharse. 1979. Assembly of rod photoreceptor membranes: immunochemical and autoradiographic localization of opsin in smooth vesicles of the inner segment. *J. Cell Biol.* 83(2, Pt.2):275 a (Abstr.).
27. Pelham, H. R. B., and R. J. Jackson. 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. *Eur. J. Biochem.* 67:247-256.
28. Pober, J. S., and L. Stryer. 1975. Light dissociates enzymatically-cleaved rhodopsin into two different fragments. *J. Mol. Biol.* 95:477-481.
29. Pober, J. S., V. Iwanij, E. Reich, and L. Stryer. 1978. Transglutaminase-catalyzed insertion of a fluorescent probe into the protease-sensitive region of rhodopsin. *Biochemistry.* 17: 2163-2169.
30. Rohlich, P. 1976. Photoreceptor membrane carbohydrate on the intradiscal surface of retinal rod discs. *Nature (Lond.)*, 263:789-791.
31. Saari, J. C. 1974. The accessibility of bovine rhodopsin in photoreceptor membranes. *J. Cell Biol.* 63:480-491.
32. Schechter, I., Y. Burstein, R. Zemell, E. Ziv, F. Kantor, and D. S. Papermaster. 1979. Messenger RNA of opsin from bovine retina: Isolation and partial sequence of the *in vitro* translation product. *Proc. Natl. Acad. Sci. U. S. A.* 76:2654-2658.
33. Shields, D., and G. Blobel. 1978. Efficient cleavage and segregation of nascent presecretory proteins in a reticulocyte lysate supplemented with microsomal membranes. *J. Biol. Chem.* 253:3753-3756.
34. Stelos, P. 1967. Isolation of immunoglobulin: Salt fractionation. *In* Handbook of Experimental Immunology. D. M. Weir, editor. Blackwell Scientific Publications, Oxford. 4.
35. Young, R. W. 1967. The renewal of photoreceptor cell outer segments. *J. Cell Biol.* 33:61-72.
36. Young, R. W., and B. Droz. 1968. The renewal of proteins in retinal rods and cones. *J. Cell Biol.* 39:169-184.