Messenger RNA of opsin from bovine retina: Isolation and partial sequence of the *in vitro* translation product

(rough endoplasmic reticulum/precursor protein/hydrophobic NH₂-terminal extra piece/initiator methionine)

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ABSTRACT Opsin, the apoprotein of the visual pigment rhodopsin, is synthesized on membranes of the rough endoplasmic reticulum and subsequently passes through the Golgi apparatus to the rod outer segment. This pathway parallels the early stages of biosynthesis of some secretory proteins and viral membrane glycoproteins. Most of these proteins are initially synthesized as precursor molecules with a short-lived hydrophobic extra peptide segment at the NH₂ terminus. Therefore we investigated whether or not the immediate translation product of opsin mRNA contains a similar short-lived NH₂-terminal extra peptide. The mRNA coding for opsin was isolated from bovine retina polysomes precipitated by antibodies to opsin. The mRNA directed the cell-free synthesis of a protein comparable in size to opsin that was specifically precipitated by anti-opsin antibodies. Sequence analyses of the immunoprecipitated protein labeled with six radioactive amino acids (Met, Asn, Pro, Phe, Tyr, Val) provided the following result: Met-Asn-X-X-X-X-Pro-Asn-Phe-Tyr-Val-Pro-Phe-X-Asn-X-X-X-Val-Val-X-X-Pro-Phe-X-X-Pro-X-Tyr-Tyr-X-X-X-Pro (X is unknown). This partial sequence of the cell-free product corresponds exactly to the published NH₂-terminal segment of native opsin (21 residues long) and extends beyond this region. Met-1 was shown to be the initiator methionine residue, because only the initiator [³⁵S]Met-tRNA^{Met}—not the internal [³⁵S]Met-tRNA^{Met}—donated the NH₂-terminal methionine. This finding essentially rules out the possibility that Met-1 was preceded by a peptide that was rapidly cleaved. Thus opsin, and not a precursor, is the immediate product of opsin mRNA translation.

Rhodopsin is the visual pigment glycoprotein in vertebrate rod photoreceptor cells. Opsin, the apoprotein, is continuously synthesized in the adult retina and assembled into membrane disks in the rod outer segment (ROS) compartment of the cell (1) where it comprises about 90% of the total protein in ROS membranes (2). In the outer segment, opsin is firmly embedded in and spans the disk lipid bilayer (3, 4). Its COOH-terminal region faces the cytoplasmic (interdisk) surface (4, 5). Concanavalin A, which binds to rhodopsin in detergent solution and in ROS membrane fragments (6) is detected exclusively as a ferritin conjugate on the opposite intradiskal surface (7, 8). This binding possibly reflects the orientation of opsin's two oligosaccharide chains, which are attached to asparagine residues in the NH₂-terminal region (9, 10), and suggests that the NH₂-terminal region of opsin is inserted across the ROS membrane. During early stages of its biosynthesis, opsin is also firmly bound to membranes (11). Opsin is synthesized in the rough endoplasmic reticulum (RER) and is subsequently transferred to the Golgi zone (12, 13). This pathway parallels the early stages of biosynthesis of secretory proteins and several membrane proteins (14–16).

The mRNAs coding for many secretory proteins and viral

and bacterial membrane proteins direct the cell-free synthesis of precursor molecules in which short-lived hydrophobic extrapeptide segments (15–29 residues long) precede the NH₂ termini of the mature proteins (15–19). It has been hypothesized that the hydrophobic extra piece favors interaction of the precursor with the RER membrane (20–23). This interaction may be an early step in the binding of polysomes to RER membranes and in the passage of these proteins into the cisternal systems (RER, Golgi, and secretory granules) in the course of secretion and membrane assembly. It is therefore of interest to determine whether or not the immediate translation product of opsin mRNA contains a comparable hydrophobic NH₂-terminal extra piece. For this reason we isolated the opsin mRNA, translated it in a cell-free system, and determined the NH₂-terminal amino acid sequence of the protein product.

MATERIALS AND METHODS

Anti-Opsin Antibodies. Bovine opsin was isolated electrophoretically from sodium dodecyl sulfate (NaDodSO₄)/polyacrylamide gels as described (24). In the presence of 0.1% NaDodSO₄ the protein was mixed with an equal volume of complete Freund's adjuvant and injected at 7-day intervals into a goat. Antibody content of sera was tested by precipitin analysis. The initial opsin solution that contained NaDodSO₄ inhibited precipitin formation. Therefore the solution of NaDodSO₄-solubilized opsin was dialyzed against 1000 vol of 0.15 M NaCl at 20°C. During dialysis a precipitate occasionally formed. The centrifuged supernatant (3000 × g for 10 min) was cooled to 4°C and any additional precipitated material (presumably NaDodSO₄ and aggregated protein) was removed by further centrifugation. Over 80% of the opsin was recovered in the clear supernatant, and it formed specific precipitates with anti-opsin antibodies. The dialyzed opsin solution (1 mg/ml) could also be coupled directly to CNBr-activated Sepharose in 0.1 M NaHCO₃ (25) with binding of 90% of the antigen. The opsin-Sepharose immunoabsorbant was used to prepare purified antibody as described (26). About 140 mg of antibody was recovered from 200 ml of antiserum.

Opsin mRNA. Frozen bovine retinas were obtained from Hormel Corp. (Austin, MN). The mRNA coding for opsin was prepared from retinal polysomes precipitated by antibodies to opsin, followed by chromatography on oligo(dT)-cellulose (26). Yield of polysomes was 20–27 A₂₆₀ units/g of retina. Polysomes precipitated by goat anti-opsin antibodies made up 8–12% of the total polysome population. In the presence of normal goat IgG only 0.9–1.5% of the total polysomes was precipitated.

Abbreviations: RER, rough endoplasmic reticulum; ROS, rod outer segment; NaDodSO₄, sodium dodecyl sulfate.

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[³⁵S]Met-tRNA^{Met}. The two [³⁵S]Met-tRNA^{Met} species that transfer methionine to the NH₂-terminal (initiator Met-tRNA₁^{Met}) and internal (internal Met-tRNA₂^{Met}) positions in proteins were prepared from wheat germ as described (27). Cross-contamination of one tRNA^{Met} species by the other was undetectable when tested in the translation of natural mRNAs and sequence analyses of the cell-free products (27).

Cell-Free Synthesis of Opsin. Translation of opsin mRNA was carried out in the wheat germ cell-free system (28) at 25°C for 4 hr (29). Protein products were labeled by one radioactive amino acid at a time. When [³⁵S]Met-tRNA^{Met} was employed as the sole source of labeling, the reaction mixture was supplemented to 50 μM unlabeled methionine to prevent incorporation of [³⁵S]Met released from the charged tRNA^{Met} species (27). Radioactive amino acids employed were: [³⁵S]methionine (580 Ci/mmol), [³H]proline (43 Ci/mmol), [³H]phenylalanine (15.8 Ci/mmol), [³H]tyrosine (22 Ci/mmol), and [³H]valine (15.3 Ci/mmol) obtained from the Radiochemical Centre (Amersham, Bucks, U.K.); [³H]asparagine (13 Ci/mmol) was obtained from Schwarz/Mann (1 Ci = 3.7 × 10¹⁰ becquerels).

Radioactive Amino Acid Sequence Analysis. The total cell-free products and protein products precipitated by anti-opsin antibodies (27) were analyzed in the Beckman 890C automatic sequencer as detailed elsewhere (30). The radioactive samples were supplemented with 5 mg of sperm whale apomyoglobin carrier. Repetitive yields of the protein carrier and of the radioactively labeled material ranged between 91 and 94%. The amino acid sequences of the immunoprecipitated proteins were determined twice. In the duplicate the pattern of radioactive peaks was identical. To calculate the absolute yields of the radioactive sample whose sequence was being determined, the crude sequence data were corrected for background and "out of step" radioactivity (30). Absolute yields of the various radioactive amino acids were over 55% if one assumes the average amino acid composition of opsin as reported by Plantner and Kean (31).

RESULTS

The mRNA obtained from immunoprecipitated retina polyosomes stimulates protein synthesis in the wheat germ cell-free system. Incorporation of [³⁵S]Met into alkali-resistant trichloroacetic acid-insoluble material is 22-fold larger in the presence of opsin mRNA (1 μg of mRNA in 40 μl of wheat germ extract) as compared to a control without added mRNA (Fig. 1 lanes A and B). The total cell-free products were immunoprecipitated by using the double-antibody technique (27). The percent of radioactively labeled products recovered in the specific precipitate (goat anti-opsin and rabbit anti-goat-IgG antibodies) and nonspecific precipitate (normal goat IgG and rabbit anti-goat IgG antibodies) were 6.5% and 1.6%, respectively. Gel electrophoresis of the specific precipitate (Fig. 1 lane C) shows preferential precipitation of a major protein with molecular weight (*M_r*) of 32,000, and of two additional proteins with *M_r* of about 65,000 and 90,000. It should be noted that opsin often aggregates in NaDodSO₄ gels and migrates as monomer, dimer, and trimer (2). In the nonspecific precipitate the amount of proteins of *M_r* 32,000, 65,000, and 90,000 recovered is negligible (Fig. 1 lane D). These results demonstrate antigenic determinants of opsin in protein products comparable in size to opsin monomer, dimer, and trimer. In addition, goat anti-opsin antibodies and normal goat IgG precipitated low and comparable amounts (1.3% and 1.5%) of the protein products programmed by hemoglobin mRNA in the same wheat germ extract.

When the immune precipitate (see Fig. 1 lane C) was

FIG. 1. Autoradiogram of NaDodSO₄/13% polyacrylamide gels of [³⁵S]Met-labeled cell-free products programmed by bovine opsin mRNA. The protein products were synthesized in the wheat germ cell-free system in the absence (lane A) (2400 cpm) and presence (lanes B, C, D) of opsin mRNA. Lane B, total cell-free products (23,800 cpm in sample); lane C, cell-free products precipitated with goat anti-opsin antibodies and rabbit anti-goat IgG (10,200 cpm); lane D, cell-free products precipitated with normal goat IgG and rabbit anti-goat IgG (7400 cpm). Bars at left side of figure mark the positions of molecular weight markers (top to bottom): ovalbumin, glyceraldehyde phosphate dehydrogenase, chymotrypsinogen, and hemoglobin. Bars at right side of figure mark the positions of monomer, dimer, and trimer of glycosylated opsin isolated from ROS. Gel analyses were done according to Maizel (32).

subjected to sequence analyses, discrete radioactive peaks were obtained at the indicated degradative cycles (Fig. 2): [³⁵S]Met, one peak at cycle 1 and none up to cycle 36; [³H]Asn, cycles 2, 8, and 15; [³H]Pro, cycles 7, 12, 23, 27, and 34; [³H]Phe, cycles 9, 13, and 24; [³H]Tyr, cycles 10, 29, and 30; [³H]Val, cycles 11, 19, and 20. The complete structure of bovine opsin has not yet been determined; the sequence of the NH₂-terminal segment (21 residues long) is published (9). The positions and amino acid residues identified in the cell-free product (Fig. 2) match with the corresponding residues of the NH₂-terminal portion of opsin (Fig. 3). The sequence homology observed establishes the isolation of opsin mRNA and translation with fidelity of the mRNA.

The partial sequence data extend to a region beyond the presently known structure of opsin (Figs. 2 and 3). To ascertain that these additional residues originate from opsin, the radioactivity recovered in peaks derived from a sequencer run of the [³H]Pro-labeled product were replotted on a semilogarithmic plot as described (30). In the semilogarithmic plot all radioactive peaks fall on a straight line (Fig. 4). This provides strong evidence that all proline residues originate from a distinct protein species (30). The semilogarithmic plots of the radioactive peaks

FIG. 2. Radioactivity recovered at each sequencer cycle from cell-free products programmed by bovine opsin mRNA. The total cell-free products were precipitated with antibodies to opsin and the amino acid sequence was determined (numbers in parentheses represent cpm in the sample analyzed): [^{35}S]Met (137,000), [^3H]Asn (13,400), [^3H]Pro (38,600), [^3H]Phe (18,000), [^3H]Tyr (19,500), [^3H]Val (15,200). Background radioactivity obtained from the sequencer run of the control sample without added mRNA was subtracted (29). Cycle zero represents a blank cycle (without phenyl isothiocyanate) that was used to wash out potential radioactive contaminants.

of [^3H]Phe (positions 9, 13, 24) and [^3H]Tyr (positions 10, 29, 30) also fall on straight lines and the slopes of the lines correspond to repetitive yields of about 92% (data not shown). These analyses thus indicate, with a high degree of confidence, that the opsin molecule contains the assigned residues (Pro, Phe, and Tyr) between positions 23 and 34.

To ascertain that mature opsin is the immediate product of mRNA translation, we demonstrated that Met-1 is the initiator residue. For this purpose the mRNA was translated in the presence of [^{35}S]Met-tRNA^{Met} species as the sole source of label, using either the initiator tRNA₁^{Met} or the internal tRNA₂^{Met}.

The cell-free products were allowed to react with antibodies to opsin (6.7–7.2% precipitated with goat anti-opsin, 0.9–1.3% precipitated with normal goat IgG), and the amino acid sequences of the specific immune precipitates were determined. The results (Fig. 5) show that insertion of [^{35}S]Met into the NH₂-terminal position of newly synthesized opsin occurs with the initiator [^{35}S]Met-tRNA₁^{Met} but not with the internal [^{35}S]Met-tRNA₂^{Met}. In a parallel experiment the internal [^{35}S]Met-tRNA₂^{Met} inserted methionine residues at correct positions inside the protein programmed by MOPC-41 immunoglobulin light chain mRNA in the same wheat germ extract (27). The yield data are in complete agreement with the conclusion that Met-1 is the initiator residue. When the amino acid sequence of opsin labeled with the initiator [^{35}S]Met-tRNA₁^{Met} was determined (1500 cpm in sample), 53% of the radioactivity was recovered in cycle 1 (800 cpm). The sample labeled with the internal [^{35}S]Met-tRNA₂^{Met} (8200 cpm in sample) contained 5.5-fold more radioactivity, yet none was recovered in cycle 1. If Met-1 were an internal residue, then 820 cpm [8200/10, assuming 10 Met residues per mol of opsin (31)] should have been recovered in cycle 1 at 100% yield, and 435 cpm at 53% yield. The identification of Met-1 as the initiator residue essentially rules out the possibility that Met-1 is preceded by a peptide that is rapidly cleaved during opsin synthesis.

DISCUSSION

The isolation from frozen bovine retina of translatable opsin mRNA is evidenced by: (i) specific precipitation by anti-opsin antibody of cell-free products corresponding in size to opsin (Fig. 1 lane C); (ii) sequence homology of the immunoprecipitated cell-free product with the NH₂-terminal sequence of opsin (Figs. 2 and 3). The observed M_r of bovine opsin from purified ROS analyzed on the same gel was 36,000. The major protein product specifically precipitated by antibodies to opsin had an apparent M_r of 32,000. The size difference is probably due to the oligosaccharides attached to native opsin (10, 31) that retard electrophoretic mobility (33). Glycosylation of opsin presumably does not occur in the cell-free extract (34, 35). Other protein products programmed by the opsin mRNA preparation (Fig. 1 lane B) may originate from premature termination of mRNA translation that frequently occurs in the wheat germ extract (36) and from nonopsin mRNA species.

The partial sequence of opsin programmed by the mRNA (Fig. 3) extends beyond the presently described NH₂-terminal sequence of opsin, which has been published up to residue 21 (9). This information (residues in positions 23–34, Fig. 3) should be useful to align peptide fragments and to extend the determination of the NH₂-terminal sequence of opsin. In this connection, we noted that two methionine residues were detected at positions 38 and 39 of the cell-free product when the sequence was carried to that point. However, this extended analysis was conducted only once.

The NH₂-terminal methionine of opsin isolated from ROS is blocked by an undetermined group (9). The amino acid sequence of the newly synthesized opsin programmed by mRNA in the wheat germ system could be determined directly (Fig. 2) without chemical steps designed to overcome a block. This indicates that blockage of the methionine may be a posttranslational event. A posttranslational NH₂-terminal acetylation was described for α -crystallin (37) and for ovalbumin when it was translated in a reticulocyte lysate (35).

Many secretory and membrane proteins are initially synthesized as precursor molecules in which short-lived hydrophobic extra peptide segments precede the NH₂ termini of the mature proteins. According to the "signal hypothesis" these

FIG. 3. NH₂-terminal sequence of bovine opsin. Amino acid sequence of the first 21 residues of native opsin is from Hargrave (9). The primary structure of the cell-free product is based on radioactive sequence analyses of the immune precipitated protein molecules translated from opsin mRNA in the wheat germ cell-free system (data from Fig. 2). Amino acid residues identified in the cell-free product are enclosed within circles. Residues in italics indicate amino acids identified in the cell-free product that extend beyond the published sequence of native opsin. X, amino acid not identified.

hydrophobic extra pieces direct polysomes synthesizing the secretory proteins to the endoplasmic reticulum membranes, the growing nascent chains are vectorially discharged across the RER membrane, and the extra pieces ("signal peptides") are then cleaved (15, 16, 22). Because autoradiographic studies have shown that opsin is also synthesized on RER (1, 13), it might be anticipated that the immediate translation product of opsin mRNA would contain a similar short-lived hydrophobic NH₂-terminal extra piece. In contrast to this anticipated result, we found correspondence of the NH₂-terminal sequence of opsin programmed by mRNA *in vitro* and the sequence of opsin isolated from the ROS. The possibility that a short-lived extra piece was cleaved during synthesis in the cell-free extract is unlikely because cleavage of short-lived extra pieces of many different precursors synthesized in the wheat germ extract has not been previously reported unless membranes are added (15–18). Furthermore, we found that the NH₂-terminal methionine of opsin is donated exclusively by the initiator-tRNA^{Met} species (Fig. 5), thus demonstrating that opsin is the immediate product of mRNA translation.

It could be argued that the NH₂-terminal segment of opsin functions as the signal peptide and is not cleaved after inter-

action with the RER. If this were the case, this portion of the molecule should be markedly hydrophobic, like other extra pieces, which are highly enriched (70–82%) with hydrophobic amino acids (17, 18, 23). Inspection of the NH₂-terminal sequence of opsin (21 residues) does not show a clear resemblance to other short-lived extra pieces: it is not strikingly hydrophobic (11 hydrophobic residues, 53%); it contains glutamic acid, lysine, and arginine at positions 5, 16, and 21, respectively; it is devoid of any leucine residues, which are present in large abundance in most extra pieces investigated (17, 18); and it is eventually glycosylated at Asn-2 and Asn-15 (9, 10). Further work is required to clarify what determines opsin synthesis on RER. For example, this localization may be attributed to hydrophobic sequences beyond position 21.

Intermediates involved in the glycosylations of the asparagine residues are probably associated asymmetrically within the RER membrane so that oligosaccharides appear exclusively on the cisternal surface (14, 38). These intermediates may participate in directing the nascent chains to the membranes (39). Concanavalin A-ferritin conjugates bind exclusively to the extracellular surface of the ROS plasma membrane and to the intradiskal surface (7, 8), which is transiently continuous with the plasma membrane extracellular surface before its separation (40). This lectin may be demonstrating the orientation of opsin's oligosaccharides in the disk membranes which are exclusively linked to Asn-2 and Asn-15 (9, 10). To arrive at that orientation, it is most likely that the NH₂ terminal has also penetrated through the RER and Golgi membranes to the cisternal surface. The plausibility of the usual asymmetry is supported by evi-

FIG. 4. Semilogarithmic plot of the radioactive peaks recovered from sequence analysis of bovine opsin labeled with [³H]Pro. The data from Fig. 2 (Pro) were replotted after corrections were made for "background" and "out-of-step" radioactivity (29).

FIG. 5. Radioactivity recovered at each sequencer cycle from immunoprecipitated cell-free products programmed by bovine opsin mRNA and labeled with [³⁵S]Met-tRNA^{Met} species. Numbers in parentheses represent cpm in the sample analyzed: (A) initiator [³⁵S]Met-tRNA^{Met} (1500 cpm); (B) internal [³⁵S]Met-tRNA^{Met} (8200 cpm). Cycle 0, see legend to Fig. 2.

dence that oligosaccharide-polyisoprenoid precursors are intermediate donors of opsin's oligosaccharides; a pathway that parallels that of secreted glycoproteins (41). Moreover, the final oligosaccharide structure $\text{GlcNAc}\beta 1 \rightarrow 2\text{Man}\alpha 1 \rightarrow 3(\text{Man}\alpha 1 \rightarrow 6)\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc} \rightarrow \text{Asn}$ (10) is the same as an intermediate proposed in secreted and membrane glycoprotein synthesis (42).

Although there will be need for more direct evidence of opsin's orientation, these parallels between early stages of opsin biosynthesis (RER site of synthesis and its glycosylation) justify comparison of opsin with other membrane glycoproteins and secreted proteins that share the same early pathways. Several viral membrane glycoproteins apparently have short-lived precursors whose NH_2 -terminal hydrophobic sequences are cleaved (15, 16, 43) and thus resemble most secreted proteins. It will be valuable to determine if other membrane glycoproteins may be synthesized without such precursor peptides on their NH_2 terminus. Similar problems are encountered in understanding of the mechanism of ovalbumin secretion. Palmiter *et al.* (35) have shown that the primary translation product of ovalbumin mRNA is the mature protein plus the initiator methionine residue, which is rapidly cleaved. Ovalbumin has no short-lived NH_2 -terminal extra piece; however, its NH_2 -terminal region or internal sequences may have functional equivalence to the extra pieces of other secretory proteins (44).

The interest in opsin mRNA stems from the fact that it codes for a eukaryotic intrinsic membrane protein that is the apoprotein of rhodopsin, the receptor of light stimuli. *In vivo*, the synthesis of rhodopsin in rod cells appears to be light regulated, because new disk formation is accelerated during the initial hours of a diurnal light cycle (45). The availability of opsin complementary DNA prepared from mRNA would permit a quantitative analysis of opsin mRNA in photoreceptor cells under various conditions of light exposure. This could provide insight into the locus of the effect of light, which may act concurrently or independently upon disk membrane assembly, opsin transport from Golgi to ROS, or relative rates of mRNA translation and transcription.

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