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Molecular Cloning, Primary Structure, and Orientation of the Vertebrate Photoreceptor Cell Protein Peripherin in the Rod Outer Segment Disk Membrane[†,‡]

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Received June 28, 1989; Revised Manuscript Received January 16, 1990

ABSTRACT: Peripherin, a 39-kDa membrane protein, has been previously localized to the rim region of the vertebrate rod photoreceptor disk membrane by use of monoclonal antibodies and immunocytochemical labeling techniques. As an initial step in determining the structure and function of this protein, we have cloned and sequenced cDNA containing its complete coding sequence. A bovine retinal λgt11 expression library was screened with the antibodies, and a 583 base pair clone was initially isolated. The remaining part of the coding sequence was obtained from subsequent rescreenings of the same library and an independent λgt10 library. A C-terminal CNBr fragment of peripherin was purified by immunoaffinity chromatography and reverse-phase high-performance liquid chromatography. The amino acid sequence of the isolated C-terminal peptide and the N-terminal sequence analysis of immunoaffinity-purified peripherin are in agreement with the cDNA sequence. The cDNA sequence predicts that there are possibly four transmembrane domains. On the basis of immunocytochemical studies and sequence analysis, the hydrophilic C-terminal segment containing the antigenic sites for the antiperipherin monoclonal antibodies has been localized on the cytoplasmic side of the disk membrane. There are three consensus sequences for asparagine-linked glycosylation. Deglycosylation studies have indicated that at least one of these sites is utilized. The possible function of peripherin in relation to its primary structure is discussed.

Rod outer segments (ROS)[1] of vertebrate rod photoreceptor cells are specialized organelles that function in the transduction of light into electrical signals as part of the visual excitation process. These organelles contain hundreds of closely stacked membrane disks that are discontinuous with a surrounding plasma membrane throughout most of the ROS (Rosenkranz, 1977). The photoactive protein, rhodopsin, is the major constituent of both the plasma membrane and the disk membrane. The C-terminus of rhodopsin and the F_1–F_2 loop that joins the fifth and sixth membrane-spanning helices are orientated on the cytoplasmic face of the membranes where they are able to interact with other components of the visual cascade such as rhodopsin kinase and transducin or G-protein (Kuhn,

[†] This research was supported by grants from the National Institutes of Health (EY 02422) and the Medical Research Council of Canada (MT 5822).
[‡] The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02884.

[1] Abbreviations: ROS, rod outer segment; SDS, sodium dodecyl sulfate; Ig, immunoglobulin; DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; Endo H, endo-β-*N*-acetylglucosaminidase H; bp, base pair; Tris, tris(hydroxymethyl)aminomethane.

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1981; Hargrave, 1982). The N-terminal segment, which contains two asparagine-linked carbohydrate chains, is orientated on the extracellular surface of the plasma membrane and in the lumen of the disk (Rohlich, 1976; Hargrave, 1977; Clark & Molday, 1979).

In addition to rhodopsin, the plasma membrane contains a unique set of proteins that are not present in the disk membrane. Several ricin binding glycoproteins were identified that are specific for the plasma membrane (Molday & Molday, 1987a). These proteins were used as markers in the purification of the plasma membrane from the disk membrane with a density perturbation method (Molday & Molday, 1987b). The 63-kDa cGMP-gated cation channel and the 220-kDa sodium–calcium exchanger were subsequently shown to be restricted to the plasma membrane (Bauer, 1988; Cook et al., 1989).

The disk membrane, itself, can be divided into two domains with unique protein compositions. Falk and Fatt (1969) have shown that an osmium tetroxide solution dissolved the lamellae of the disks but not the rims. Papermaster et al. (1978) used a polyclonal antibody to localize a 290-kDa protein to the disk incisures and rims of frog ROS. This polyclonal serum does not, however, cross-react with any component present in the mammalian photoreceptor. Molday et al. (1987) localized a 33-kDa protein, peripherin, specifically to the rim region of the bovine disk membrane using monoclonal antibodies and immunocytochemical labeling techniques. A 220-kDa concanavalin A binding glycoprotein and several minor proteins are also present in the mammalian disk membrane, but they have not yet been localized to either domain (Molday & Molday, 1987b).

Peripherin is not extractable from the disk membrane with chelating and chaotropic agents, but it requires a detergent for solubilization (Molday et al., 1987). The protein migrates as a dimer on an SDS–polyacrylamide gel in the absence of 2-mercaptoethanol, suggesting that it may exist as two subunits held together by one or more disulfide bonds (Molday et al., 1987). In the presence of 2-mercaptoethanol, peripherin migrates as a relatively sharp band directly beneath rhodopsin at a molecular weight of about 33 kDa. The quantification of peripherin has been complicated by the difficulty in purifying it. Peripherin appears, though, to constitute less than 5% of the total disk protein. This could still make it, after rhodopsin, one of the more abundant membrane proteins of the photoreceptor cell.

Although a number of proteins involved in the visual cascade system have been sequenced including transducin (Yatsunami & Khorana, 1985; Medynski et al., 1985), phosphodiesterase (Ovchinnikov et al., 1986), and arrestin or S-antigen (Shinohara et al., 1987), to date rhodopsin and the 63-kDa cGMP-gated channel (Kaupp et al., 1989) are the only integral membrane proteins of ROS for which the amino acid sequence is known. In this paper we have obtained the protein sequence of peripherin from its cDNA sequence as an initial step in determining the structure and function of this unique rim-specific membrane protein of ROS disks.

Experimental Procedures

Screening of Retinal Libraries. A bovine retinal cDNA library that was prepared in the phage expression vector λgt11 (Barrett et al., 1985) was screened with monoclonal antibodies (Young & Davis, 1983). One complete library was plated on *Escherichia coli* Y1090 at a density of 180 phage/cm^2 and incubated at 42 °C for 3 h. Nitrocellulose filters that had been saturated with isopropyl 1-thio-β-D-galactopyranoside were overlaid on the plaques, and the plates were incubated at 37 °C for 5–8 h. The filters were quenched in 3% BSA and then incubated with a mixture of the antiperipherin monoclonal antibodies 2B6 and 3B6 (Molday et al., 1987). The filters were reacted with goat antimouse Ig that had been labeled with ^{125}I at a specific activity of 4×10^9 dpm/mg according to the chloramine T method (Hunter & Greenwood, 1962). Positive signals were plaque purified by four additional rounds of screenings at successively lower phage dilutions. cDNA that was used as probes in later screenings of the same λgt11 library and a λgt10 library (Nathans & Hogness, 1983) was labeled with ^{32}P according to the method of Feinberg and Vogelstein (1983).

Isolation of Phage DNA and Sequencing. Recombinant phage DNA was isolated by the procedure of Maniatis et al. 1982). *Eco*RI-digested λ DNA was fractionated on an agarose gel, and the insert DNA was purified by the method of Vogelstein and Gillespie (1979) using the Gene Clean kit (Bio 101 Inc). Purified insert was ligated into M13mp18 or M13mp19 for sequencing by the dideoxynucleotide chain termination method (Sanger et al., 1977). Both strands of the region coding for the open reading frame were sequenced to completion.

Northern Blot Analysis. Total RNA was isolated from bovine retinas according to the method of Chirgwin et al. (1979). Polyadenylated retinal RNA was purified on an oligo(dT)–cellulose column (Aviv & Leder, 1972). Liver RNA was the generous gift of Walter Funk. RNA was separated on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a Hybond nylon membrane (Amersham). The transfers were hybridized in 5× Denhardts, 9% dextran, 43% formamide, 0.1% SDS, 100 μg/mL salmon sperm DNA, and 5× SSPE (1× SSPE = 180 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4) at 42 °C with 10^6 dpm/mL ^{32}P-labeled cDNA probes. The membranes were then washed at 70 °C in 0.1× SSPE and 0.1% SDS.

Immunoaffinity Column Preparation. The antiperipherin monoclonal antibody 2B6 was purified from ascites fluid by precipitation with 50% ammonium sulfate followed by DEAE-Sepharose chromatography. SDS–polyacrylamide gel electrophoresis indicated that the antibody was essentially pure. The 2B6 monoclonal antibody was covalently coupled to Sepharose CL-2B with a CNBr activation method (Cuatrecasas, 1970). Approximately 2 mg of antibody was coupled to each milliliter of packed beads.

Purification of Peripherin for N-Terminal Sequence Analysis. ROS were prepared from 80 frozen retinas (Hormel) as previously described (Molday & Molday, 1987b) and were resuspended at a protein concentration of 2 mg/mL in a buffer containing 20 mM Tris–acetate, pH 7.4, 100 mM NaCl, and 0.4 mM phenylmethanesulfonyl fluoride. Approximately 8 mg of the resuspended ROS was solubilized with an equal volume of 50 mM octyl glucoside and passed through 1 mL of the 2B6 antibody–Sepharose CL-2B column. The column was washed with 20 volumes of the resuspension buffer containing 25 mM octyl glucoside and then eluted with 0.1 M acetic acid, which also contained 25 mM octyl glucoside. The eluted protein was then dialyzed against 0.1% SDS and sequenced by the Protein Microchemistry Centre at the University of Victoria.

Isolation of a CNBr Peripherin Peptide for Amino Acid Sequencing. After being washed in distilled H_2O, 10 mg of the ROS preparation was resuspended in 0.7 mL of 98% formic acid, and the volume was adjusted to 1 mL with distilled H_2O. CNBr was added to a final concentration of 0.05 g/mL, and the digest was left for 14 h at room temperature in the

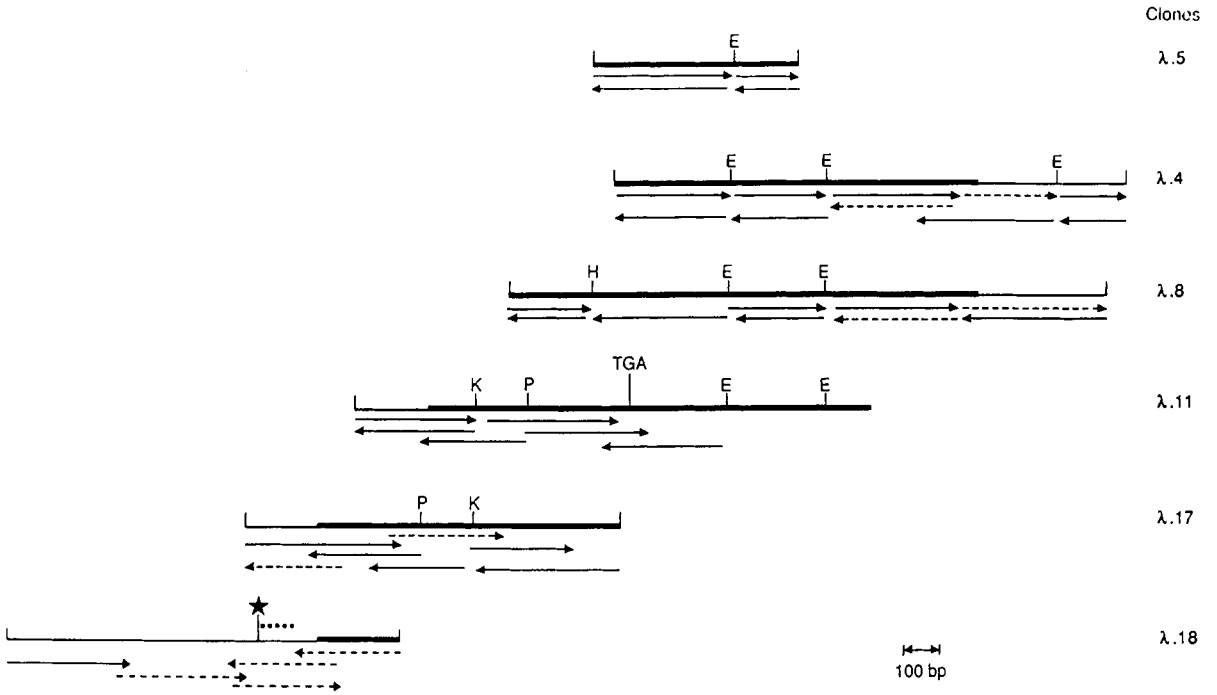

FIGURE 1: Restriction map and sequencing strategy of cloned cDNA encoding peripherin. The restriction map shows only the relevant sites for *Eco*RI (E), *Hpa*II (H), *Kpn*I (K), and *Pst*I (P). The insert from clone λ.5, obtained from screening of the cDNA retinal library with the antiperipherin monoclonal antibodies, was used to rescreen the library. Clones λ.4 and λ.8 were isolated. The 5′ *Eco*RI–*Hpa*II fragment from λ.8 was used to rescreen the same library. Two additional clones, λ.11 and λ.17, were obtained. The 499-bp *Eco*RI–*Pst*I fragment from λ.17 and a degenerate oligonucleotide probe derived from the N-terminal amino acid sequence of peripherin were used to screen an independent λgt10 library. Clone λ.18 hybridized to both probes. Identical sequence overlap among the clones is represented by the bold lines and sequence divergence by the narrow lines. The dashed segment above clone λ.18 represents the sequence that is in agreement with the N-terminal amino acid sequence. The sequence coding for the first five N-terminal amino acids of the mature protein is not present on clone λ.18. The arrows represent the direction and extent of sequence determination. The sequence was determined by the dideoxynucleotide method using the universal M13 sequencing primer (solid arrow) or synthesized oligonucleotides (dashed arrow). The position of the termination codon (TGA) on clone λ.11 and the position of the start of the open reading frame (★) on clone λ.18 are indicated.

dark. The mixture was dried under vacuum, and the pellet was extracted several times with 0.05 M NH_4HCO_3, pH 8.0.

The extracted CNBr-cleaved peptides were passed through 2 mL of the 2B6 antibody–Sepharose CL-2B column, and the column was washed with 15 volumes of 0.05 M NH_4HCO_3, pH 7.0. The column was then eluted with 5 volumes of 0.05 M formic acid, and the eluate was dried under vacuum. The sample was resuspended in 100 μL of distilled H_2O containing 0.05% trifluoroacetic acid and loaded onto a reverse-phase C18 HPLC column (Waters, Millipore Corp.). The HPLC column was eluted with a distilled water–acetonitrile gradient that contained 0.05% trifluoroacetic acid. The gradient ran from 10 to 80% acetonitrile over a period of 100 min. Peptide elution was detected by monitoring of the column effluent at 215 nm. Peak fractions were assayed for the presence of the peripherin peptide by dot blotting onto Immobilon paper (Millipore) and screening with the 2B6 monoclonal antibody as previously described (Molday et al., 1987). The peak fraction assaying positive for 2B6 binding was sequenced.

Deglycosylation of Peripherin. ROS were solubilized at a protein concentration of 3 mg/mL in 2% SDS in the presence and absence of 0.1 M dithiothreitol. The solubilized ROS were diluted 1:9 with either N-Glycanase F buffer (0.2 M sodium phosphate, pH 8.6, 1.5% Nonidet P-40, 0.4 mM phenylmethanesulfonyl fluoride, and 10 mM phenanthroline) or Endo H buffer (0.05 M sodium phosphate, pH 5.5, 1.5% Nonidet P-40, and 0.4 mM phenylmethanesulfonyl fluoride). The diluted ROS were incubated at 37 °C for 24 h with either 9 units/mL N-Glycanase F (Genzyme) or 0.06 unit/mL Endo H (Seikagaku Kogyo). The reaction products were separated on an 8% polyacrylamide gel, and peripherin was detected by Western blotting as previously described (Molday et al., 1987).

Immunocytochemical Localization of Peripherin. Neuraminidase-treated ROS were labeled with ricin–gold–dextran particles (average diameter was 24 nm), washed in 20% sucrose–0.01 M Tris, pH 7.4, buffer, and hypotonically lysed in 0.01 M Tris buffer, pH 7.4, as previously described (Molday & Molday, 1987b). The ROS membranes were adsorbed to the bottom of polystyrene microtiter plate wells by incubation overnight at 4 °C, fixed in 0.25% glutaraldehyde–0.02 M cacodylate buffer, pH 7.0, for 1 h, and labeled with 2B6 culture fluid for 1 h at 25 °C. The wells were then washed in Tris-buffered saline (0.01 M Tris–0.15 M NaCl, pH 7.4) and relabeled with goat antimouse Ig–gold–dextran particles (average diameter was 10 nm). The wells were then washed in 0.02 M cacodylate buffer, pH 7.0, refixed in 1.25% glutaraldehyde–0.1 M cacodylate buffer, pH 7.0, and prepared for electron microscopy as previously described (Molday & Molday, 1987b).

RESULTS

Isolation of Peripherin cDNA Clones. A mixture of two antiperipherin monoclonal antibodies, 2B6 and 3B6, was used to screen the λgt11 bovine retinal cDNA library. Plaque screening of 350000 recombinants led to the isolation of 3 clones that reacted to both antibodies. Clone λ.5, with the largest insert, has a 105-bp open reading frame (Figure 1) that is in frame with the β-galactosidase gene in the λgt11 vector.

The library was rescreened with the complete insert from λ.5. Over 300 positive signals were obtained from the primary screen. Twenty of these positives were chosen at random and plaque purified. Clones λ.4 and λ.8, shown in Figure 1, contain the largest inserts. The two clones have complete sequence overlap with λ.5 but diverge from each other at the 3′ un-

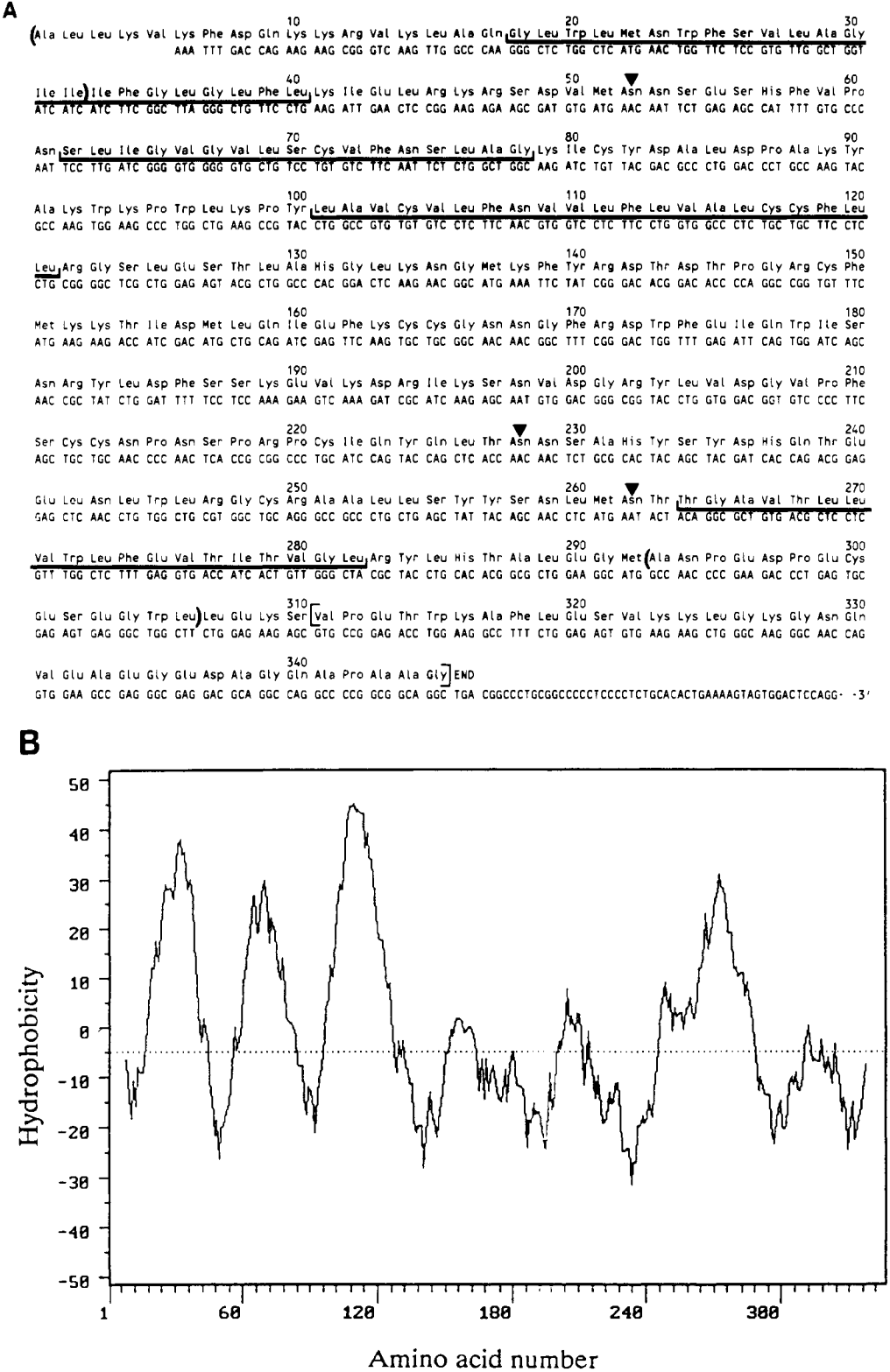

FIGURE 2: Amino acid sequence of peripherin. (A) The amino acid sequence of an isolated C-terminal peptide of peripherin and the N-terminal amino acid sequence of immunoaffinity purified peripherin (parentheses) are in agreement with a conceptual translation of the cDNA sequence. Some of the cysteine and tryptophan residues were destroyed during the protein sequencing and were not detected. The segments that are predicted by the hydrophobicity plot (B) to be possible membrane-spanning domains are underlined. There are three potential asparagine-linked glycosylation sites within the sequence (▼). The part of the sequence containing the antigenic sites for the antiperipherin monoclonal antibodies is bracketed. (B) A Kyte–Doolittle hydropathicity plot was done on the translated sequence. Hydrophobic amino acids are plotted above the dashed line and hydrophilic residues below the line.

translated end. The open reading frame continues to the 5′ end of clone λ.8. The 249-bp *Eco*RI–*Hpa*II fragment at the 5′ end of λ.8 was used to rescreen the same library. Two additional clones, λ.11 and λ.17, were obtained. These clones have complete sequence overlap with the *Eco*RI–*Hpa*II probe but diverge from each other at the 5′ ends (Figure 1). An independent λgt10 bovine retinal library was screened with the 499-bp *Eco*RI–*Pst*I fragment from λ.17 and with a degenerate oligonucleotide derived from the N-terminal sequence analysis of peripherin. From a screen of 500000 recombinants, only one positive signal, clone λ.18 (Figure 1), was obtained that reacted to both probes. Clone λ.18 has complete sequence

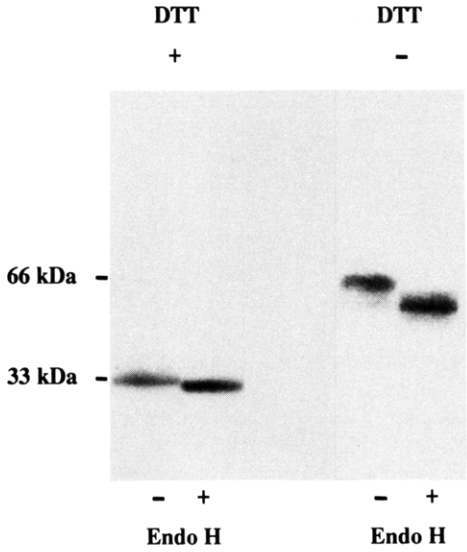

FIGURE 3: Deglycosylation of peripherin. ROS proteins were solubilized in 2% SDS in the presence (+DTT) or in the absence (−DTT) of dithiothreitol. The solubilized ROS were incubated at 37 °C for 24h in the presence (+) or in the absence (−) of Endo H. Samples were separated on an 8% polyacrylamide gel, electrophoretically transferred to Immobilon paper, and labeled with 2B6 antiperipherin antibody followed by ^{125}I-labeled goat antimouse IgG for autoradiography. The mobility shift is more evident when, in the absence of a reducing agent, peripherin migrates as a dimer. Similar results were obtained when ROS were treated with N-Glycanase F.

overlap with λ.17 for 234 bp before diverging from it at its 5′ end.

Amino Acid Sequence of Peripherin. The sequencing strategy used for the different cDNA clones is outlined in Figure 1. The complete amino acid sequence of peripherin is shown in Figure 2. The sequence of the first 32 amino acids of peripherin was obtained from N-terminal sequence analysis (Figure 2). The initial amino acid in the mature protein is alanine, indicating that some form of posttranslational processing occurs. Except for the first five N-terminal amino acids, the coding region of clone λ.18 is in complete agreement with the amino acid sequence data. The remaining amino acid sequence was deduced from the overlapping cDNA clones λ.11 and λ.17. Amino acid sequence data were also obtained directly from a C-terminal CNBr fragment of peripherin. As shown in Figure 2, this sequence is also in complete agreement with that derived from the cDNA clones.

The predicted size of the mature protein is 345 amino acids (Figure 2). There are three potential asparagine-linked glycosylation sites (Hubbard & Ivatt, 1981) within the sequence (Figure 2). Treatment of peripherin with Endo H or N-Glycanase F causes a small decrease in its mobility (Figure 3), indicating the presence of carbohydrate on at least one of these sites.

The hydrophobicity of the predicted peripherin sequence was examined. The Kyte–Doolittle plot predicts that there are four hydrophobic regions that are long enough to be potential membrane-spanning domains (Figure 2). This is in agreement with the solubility properties of the protein which suggest that peripherin is an integral membrane protein (Molday et al., 1987). The same hydrophobic regions were also predicted to be membrane spanning by the method of Rao and Argos (1986) using the parameters proposed in the original paper.

Blot Hybridization Analysis. Northern blot analysis of retinal poly(A) RNA revealed a family of transcripts that hybridized to peripherin cDNA probes (Figure 4). Two predominant bands of 6.5 and 2.9 kb are present. Both the variety and large size of the peripherin transcripts were

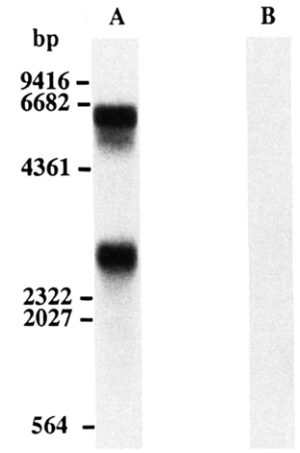

FIGURE 4: Northern blot analysis. RNA isolated from bovine retina (A) and liver (B) were separated by formaldehyde–agarose gel electrophoresis, transferred to a nylon filter, and hybridized with ^{32}P-labeled peripherin cDNA probes as described under Experimental Procedures. The seven *Eco*RI fragments from clones λ.4 and λ.8 (Figure 1) were used individually as the hybridization probes, and they gave similar results. Size markers were denatured *Hin*dIII fragments obtained from λ DNA.

unexpected from the Western blot of ROS, which had indicated that there is only one protein of 33 kDa (Molday et al., 1987). The large 3′-untranslated region and the presence of divergent cDNA clones (Figure 1), however, are in agreement with the hybridization results. No hybridization was seen to liver RNA.

Localization of Peripherin to the ROS Disk Membrane. Immunocytochemical labeling of hypotonically lysed ROS was used to localize peripherin specifically to the disk membrane (Figure 5). Upon hypotonic lysis of ricin–gold–dextran-labeled ROS, inverted plasma membrane vesicles are formed that have the disk membranes radiating out from them (Molday & Molday, 1987b). The 2B6 antiperipherin monoclonal antibody conjugated to gold–dextran particles was shown to bind preferentially to the rim region of these disks. Little, if any, labeling was detected on the inverted plasma membrane vesicles, which contain larger ricin–gold–dextran particles.

Discussion

A cDNA sequence for peripherin, a membrane protein localized in the rim of ROS disks, has been presented. The initial cDNA clones were obtained by screening of a bovine retinal expression library with antiperipherin monoclonal antibodies. These antibodies have been shown to bind to cone photoreceptor membrane as well as to the rod disk membranes.[2] Many of the major photoreceptor proteins are different in the two cell types (Hurwitz et al., 1985; Nathans et al., 1986; Lerea et al., 1986), and it is possible that there could be more than one form of peripherin. The Northern blot analysis is in agreement with this possibility. Since over 90% of the photoreceptor cells in the bovine retina are rod cells, it is more likely that the cDNA sequence presented here encodes the rod form of the protein rather than the cone form. One CNBr-cleavage peptide that bound to an antiperipherin monoclonal antibody was isolated. The amino acid sequence obtained from this peptide is in agreement with that predicted from the cDNA sequence. If there is a different form of peripherin in the cones, either it must be in such a low abundance as not to be detected, or alternatively, it could

[2] D. Hicks and R. S. Molday, unpublished results.

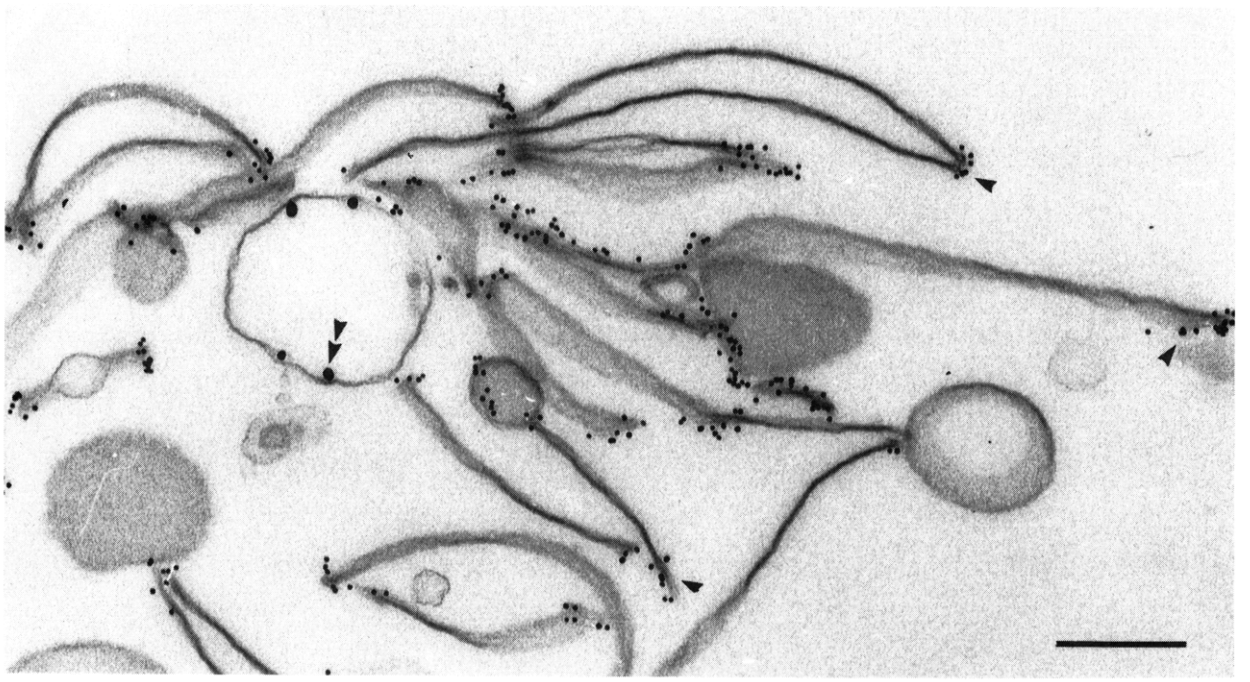

FIGURE 5: Localization of peripherin to the disk membrane. An electron micrograph of a hypotonically lysed ROS preparation. Ricin–gold–dextran particles (24-nm diameter) were used to label the extracellular surface of ROS. Inverted plasma membrane vesicles with attached disks formed after hypotonic lysis of ROS and the ricin–gold–dextran particles were observed on the inner surface of the vesicles (double arrowheads). These membrane preparations were relabeled with the 2B6 antiperipherin monoclonal antibody and goat antimouse Ig–gold–dextran particles (10-nm diameter). Peripherin appears to be almost entirely restricted to the rim region of the disks (single arrowheads). Little, if any, labeling was observed on the inverted plasma membrane vesicles. Bar = 0.20 μm.

diverge from the rod protein at a location further removed from the antigenic site.

Several cDNA clones were isolated that have divergent 5′ ends (Figure 1). If these clones were derived from true mRNA molecules, they would give rise to protein products that are truncated at the N-terminal end. No evidence was obtained for the existence of these truncated products from either N-terminal sequence analysis or from Western blotting. It, therefore, seems likely that these 5′ divergent regions are cloning artifacts.

A model for the organization of peripherin in the disk membrane is outlined in Figure 6. The protein is shown as having four transmembrane domains as predicted from the Kyte–Doolittle hydrophobicity plot. As with rhodopsin each of the hydrophobic segments is bordered on its carboxy-terminal side by a positively charged residue that is capable of interacting with the phospholipid head groups (Nathans & Hogness, 1983). It was possible to orientate the C-terminus of the protein relative to the disk membrane because the two antiperipherin monoclonal antibodies had been previously shown to bind on the cytoplasmic face of the protein (Molday et al., 1987). The antigenic sites for the same antibodies were also localized to the C-terminal portion of the protein by the cDNA clones obtained from the immunological screening of the library. There are three potential asparagine-linked glycosylation sites present on the protein, and in the model presented in Figure 6, all of these sites are located within the lumen of the disk. The use of glycosidases has indicated that there is N-linked carbohydrate on peripherin, which is in agreement with the placement of at least one of these sites within the lumen. The orientation of the other features presented in the model is highly speculative since there is only a small database on which to base the prediction of the presence of a transmembrane segment.

In the model (Figure 6), there are several cysteine residues placed within the lumen of the disk that would have the potential to form intramolecular or intermolecular disulfide bonds. The greater reducing potential of the cytoplasm is thought to inhibit such interactions on the cytoplasmic side of the membrane. Molday et al. (1987) demonstrated that,

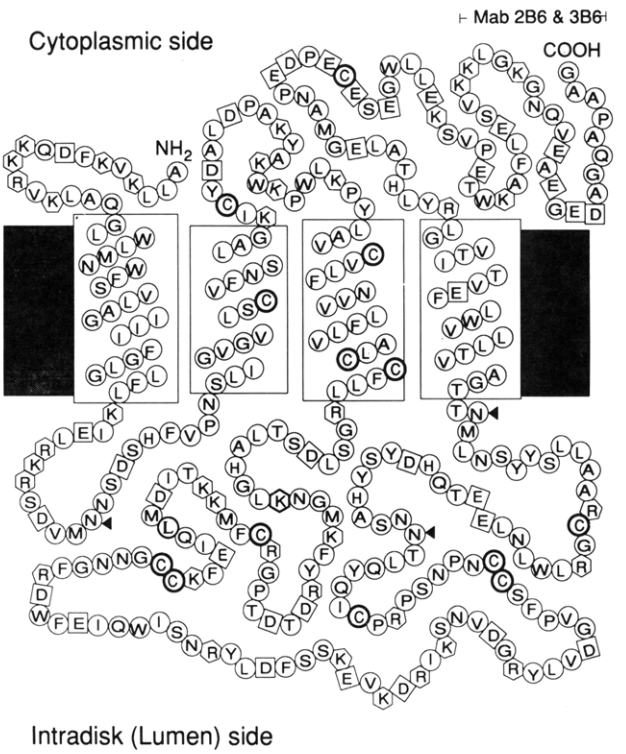

FIGURE 6: Structural model of peripherin. The model shows the orientation of the protein within the disk membrane. The location of the antigenic sites of the antiperipherin monoclonal antibodies (Mab) is indicated. The negatively (□) and positively (○) charged amino acids are highlighted. The two potential sites for asparagine-linked glycosylation (arrowheads) are also shown.

in the absence of a reducing agent, peripherin migrates as a dimer. It is still not yet clear whether peripherin is forming a heterodimer or a homodimer.

The protein encoded by the cDNA sequence of peripherin would have a molecular mass close to 39 kDa. This value is higher than the 33 kDa estimated from SDS–polyacrylamide gel electrophoresis. Peripherin migrates with an apparent molecular mass closer to 35 kDa when rhodopsin is digested with *Staphylococcus aureus* V-8 protease or when it is purified by affinity chromatography. It appears, then, that rhodopsin effects the mobility of peripherin on the SDS–polyacrylamide gel (Molday et al., 1987). In addition, many membrane proteins exhibit anomalous migration on SDS–polyacrylamide gels. Rhodopsin with an actual molecular mass of 39 kDa has been reported to migrate anywhere between 34 and 39 kDa on different gel systems.

The function of peripherin is not known. No significant homology to any other protein was detected in a search of the Swiss protein databank (August 1989 release) with the FASTP algorithm (Lipman & Pearson, 1985). The localization of peripherin to the rim region of the disk membrane is suggestive that it may play a role in anchoring the disks to the cytoskeletal system of the rod cell. The C-terminal cytoplasmic domain of peripherin is highly charged, and it is possible that it may be interacting with a cytoskeletal component. Electron microscopic studies have indicated that there are filamentous structures extending from the rims of the disks (Roof & Heuser, 1982), and a 240-kDa spectrin-like protein has been suggested to play a role in disk–membrane interactions (Wong & Molday, 1986). Alternatively, charge repulsion among adjacent peripherin molecules on the cytoplasmic surface may cause curvature in the disk membrane, and this could aid in the formation of the rim region.

Interactions of the sulfhydryl groups or carbohydrate chains that are present on peripherin within the lumen of the disk may also help to form the disk rim. Disk morphogenesis in *Xenopus* and frog retina has been shown to be disrupted by tunicamycin, an inhibitor of N-linked glycosylation (Fliesler et al., 1984, 1985a). In tunicamycin-treated retina, membrane vesicles are formed in the space between the rod inner and outer segments where disk formation would normally occur (Fliesler et al., 1985a). Fliesler et al. (1985b) suggested that the carbohydrate chains of rhodopsin on the opposing disk membrane faces may interact and cause membrane adhesion. The interaction of the opposing membrane faces appears to be greatest near the rim of the disk. Although rhodopsin and other disk proteins may be involved in membrane adhesion, the localization of peripherin to the rim makes it a prime candidate as the major adhesive molecule in the membrane. Work is in progress investigating this possibility.

Added in Proof

Recently, we (G. Connell, R. Bascom, R. McInnes, and R. Molday) have found that the bovine peripherin sequence is 92% homologous to the mouse rds protein (Travis et al., 1989). This indicates that these are the same protein of different species.

Acknowledgments

We thank Dr. Reudi Aebersold for determining the partial amino acid sequence of the isolated CNBr peptide of peripherin, Laurie Molday for carrying out the electron microscopy, Shu-Chan Hsu for assistance with the protein purification, and Dr. Ross MacGillivray, Dr. Roderick McInnes, and Roger Bascom for helpful discussions. The λgt11 bovine retinal library was generously provided by Dr. Dan Oprian, and the λgt10 library was generously provided by Dr. Jeremy Nathans.

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Comparative Efficiency of Forming m^{4}T·G versus m^{4}T·A Base Pairs at a Unique Site by Use of *Escherichia coli* DNA Polymerase I (Klenow Fragment) and *Drosophila melanogaster* Polymerase α–Primase Complex†

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Received November 8, 1989

ABSTRACT: Synthesis of a 25-mer oligonucleotide template containing O^4-methylthymine (m^{4}T) at a unique site is reported. The sequence used is analogous to that studied previously to determine the mutation frequency of O^6-methylguanine in vitro and in vivo. The templates containing m^{4}T or unmodified T were used in a primer-extension gel assay to determine kinetic parameters for incorporation by DNA polymerases of dGTP and dATP opposite either m^{4}T or T. Both *Escherichia coli* DNA polymerase I (Klenow fragment, Kf) and *Drosophila melanogaster* polymerase α–primase complex (pol α) were used. On the basis of the V_{max}/K_m ratios, the pairing of m^{4}T·G was preferred over that of both m^{4}T·A and T·G by more than 10-fold. The two polymerases gave almost identical values for the frequency of formation of all pairs investigated including m^{4}T·G pairs, suggesting that the 3′→5′ exonuclease activity of the Klenow fragment does not efficiently edit such pairs. Extension beyond m^{4}T·G was demonstrated with both Klenow and pol α. In similar kinetic experiments, bacteriophage T4 DNA polymerase, which has a very high 3′→5′ exonuclease activity, allows stable incorporation of G opposite m^{4}T in contrast to G opposite T. This kinetic approach allows quantitation of the mutagenic potential in the absence of alkylation repair and additionally provides qualitative data on mutagenesis that are in accord with our previous in vivo studies showing that replication of m^{4}T causes T → C transitions.

The established role of O^6-alkyl-G in mutagenesis and initiation of carcinogenesis by alkylating agents has obscured the possible similar role of other alkyl derivatives. Over the last decade there has been increased attention focused on *O*-alkylpyrimidines as also having biological relevance. Several experiments indicated not only that O^4-alkyl-T was mutagenic (Singer et al., 1983, 1984, 1986; Preston et al., 1986, 1987) but that repair was very slow in mammalian cells and tissues (Swenberg et al., 1984; Richardson et al., 1985; Brent et al., 1988), so that this modified base persists for long periods. The presence of O^4-methyl-T and O^4-ethyl-T has been correlated with organ specificity of tumors resulting from *N*-nitroso alkylating agents (Singer et al., 1981; Swenberg et al., 1984; Dyroff et al., 1986; Belinsky et al., 1986; Huh & Rajewsky, 1988).

It is of interest to compare mutation frequency of O^6-alkyl-G with that of O^4-alkyl-T in the same system, using site-directed methods for insertion of the desired derivative. The lability of the O^4-alkyl group on thymine (Singer et al., 1978b) made the synthesis of deoxynucleoside triphosphates and of defined oligonucleotides difficult using established methodology. Using modified techniques, Singer et al. (1983, 1986) were able to demonstrate that O^4-methyl-dTTP could substitute for dTTP in poly[d(A-T)] synthesis by *Escherichia coli* DNA polymerase I. When the resulting polymer, poly[d(A-T,m^{4}T)], was replicated in vitro with the same polymerase, dGTP was incorporated, showing the likely pairing of m^{4}T with G as well as with A. Later, Preston et al. (1986, 1987) used this fact to design a ϕX174 *am*3 genome in which a single m^{4}T was placed opposite A at position 587. Upon replication of this genome in *E. coli* spheroplasts, mutants were generated, all of which contained C at this position, again indicating pairing of m^{4}T with G during replication. In these experiments, no synthetic m^{4}T oligonucleotide was necessary as the modified base was incorporated by polymerase insertion.

† This work was supported by Grant CA 42736 (B.S.), Grant CA 43066 (J.M.E.), and Grants GM 21422 and GM 42554 (M.F.G.) from the National Institutes of Health. Grant CA 42736 was administered by the Lawrence Berkeley Laboratory under DOE Contract DE-AC0376SF00098.

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0006-2960/90/0429-4698$02.50/0