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Photoreceptor peripherin is the normal product of the gene responsible for retinal degeneration in the *rds* mouse

(protein sequence analysis/monoclonal antibodies/photoreceptor disk morphogenesis)

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ABSTRACT **Retinal degeneration slow (*rds*) is a retinal disorder of an inbred strain of mice in which the outer segment of the photoreceptor cell fails to develop. A candidate gene has recently been described for the *rds* defect [Travis, G. H., Brennan, M. B., Danielson, P. E., Kozak, C. & Sutcliffe, J. G. (1989) *Nature (London)* 338, 70–73]. Neither the identity of the normal gene product nor its intracellular localization had been determined. We report here that the amino acid sequence of the bovine photoreceptor-cell protein peripherin, which was previously localized to the rim region of the photoreceptor disk membrane, is 92.5% identical to the sequence of the mouse protein encoded by the normal *rds* gene. The differences between the two sequences can be attributed to species variation. Monoclonal antibodies were used with Western blot analysis to localize the wild-type mouse peripherin/*rds* protein to isolated mouse rod outer segments and to show that it, like bovine peripherin, exists as two subunits linked by one or more disulfide bonds. The relative amounts of peripherin/*rds* protein and rhodopsin in retinal extracts of normal and *rds* mutant mice were also compared. Identification of peripherin as the protein encoded by the normal *rds* gene and its localization to membranes of rod outer segments will serve as a basis for studies directed toward defining the role of this protein in the morphogenesis and maintenance of the outer segment and toward understanding the mechanism by which the *rds* mutation causes retinal degeneration.**

Retinal degeneration slow (*rds*) is an inherited retinal degeneration that has been identified in an inbred strain of mice (1). Mice that are homozygous for the *rds* gene fail to develop the outer segment of photoreceptor cells (2–4). Other retinal cells and other parts of the photoreceptor cells, including the inner segment, cell body, synaptic region, and cilium, develop normally during the 3-week postnatal period of development. After this time, however, the photoreceptor cells lacking the outer segments begin to undergo a slow progressive degeneration, and after 12 months few photoreceptor cells remain. Démant *et al.* (5) have localized the *rds* mutation to mouse chromosome 17. More recently, Travis *et al.* (6) have identified a candidate gene in which an insertion of 10 kilobase pairs of foreign DNA into an exon is thought to be responsible for the *rds* defect. Neither the normal gene product nor its localization within the photoreceptor cell was reported.

Outer segments of bovine rod photoreceptor cells contain the membrane protein peripherin (7). Analysis by SDS/polyacrylamide gel electrophoresis in the presence and absence of a disulfide-reducing agent indicates that this protein consists of two subunits of apparent molecular mass 33 kDa linked together by one or more disulfide bonds. Immunogold labeling studies using monoclonal antibodies indicate that peripherin is localized along the rim region of rod outer segment (ROS) disks (7, 8). Recently, the cDNA sequence of bovine peripherin has been determined (8) and found to code for a 346-amino acid polypeptide chain containing four possible transmembrane regions and three consensus sequences for N-linked glycosylation. Deglycosylation studies indicate that at least one of these sites contains an N-linked carbohydrate chain. Amino-terminal sequence analysis indicates that the initiator methionine is absent from bovine peripherin purified from ROS.

In this paper we report that the amino acid sequence of bovine photoreceptor-cell peripherin is 92.5% identical to the protein sequence encoded by the candidate wild-type mouse *rds* gene. Western blot analyses using monoclonal and polyclonal antibodies indicate that the protein encoded by the wild-type mouse gene, like bovine peripherin, is localized in ROS membranes and exists as two subunits linked together by one or more disulfide bonds. On the basis of these results, we conclude that the two proteins are the same. The few differences between the mouse and bovine peripherin sequences can be attributed to species variation.

MATERIALS AND METHODS

Materials. A synthetic peptide having an N-acetylated cysteine-glycine linker and a sequence corresponding to amino acids 308–317 (-Glu-Lys-Ser-Val-Pro-Glu-Thr-Trp-Lys-Ala-NH_2) of mature bovine peripherin and the wild-type mouse *rds* protein (6, 8) was supplied by IAF Biochemicals (Montreal). Mutant *rds* mouse strain (020/A) was kindly provided by James McGinnes (Univ. of California at Los Angeles School of Medicine) from a stock colony maintained by S. Sanyal (Erasmus University, Rotterdam, The Netherlands). Protein concentrations were determined using the bicinchoninic acid (BCA) assay kit as supplied by Pierce. ROS were isolated from freshly dissected bovine or BALB/c mouse retinas by sucrose density centrifugation as described (9).

Generation of Anti-Peripherin Antibodies. Monoclonal antibodies 2B6 against bovine peripherin and 4D2 against rhodopsin have been described (7, 10). Monoclonal antibody 5H2 was obtained from hybridoma cells generated by the fusion of NS-1 myeloma cells with spleen cells from mice immunized with bovine peripherin purified by immunoaffinity chromatography. Polyclonal peripherin antibody Pab(308–317) was produced by repeated injections of the Ac-Cys-Gly-Glu-Lys-Ser-Val-Pro-Glu-Thr-Trp-Lys-Ala-NH_2 peptide conjugated to keyhole limpet hemocyanin by a thiolation reaction (11). Antipeptide antibodies were purified from rabbit serum

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Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; ROS, rod outer segment(s).

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by immunoaffinity chromatography on a corresponding peptide-Sepharose column.

Immunoaffinity Purification of ROS Peripherin. Approximately 10 mg of bovine ROS was washed twice with resuspension buffer (20 mM Tris acetate, pH 7.4/100 mM NaCl/0.4 mM phenylmethylsulfonyl fluoride) and then resuspended in 0.5 ml of the same buffer. The resuspended ROS were added dropwise to 10 ml of 18 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) over a 10-min period with constant stirring. The entire procedure up to and including the ROS solubilization was carried out under dim red light.

Anti-peripherin monoclonal antibody 2B6 (7) purified from mouse ascites fluid by ammonium sulfate precipitation and diethylaminoethyl (DEAE) ion-exchange chromatography was covalently coupled to CNBr-activated Sepharose CL-2B by the method of Cuatrecasas (12). Approximately 2 mg of antibody was coupled to each milliliter of packed beads. The solubilized ROS were diluted with an equal volume of resuspension buffer and incubated with 2 ml of the 2B6 antibody-Sepharose beads for 1 hr. The beads were poured into a column and washed with 15 volumes of resuspension buffer containing 9 mM CHAPS, and bound material was eluted with 0.05 mM formic acid/9 mM CHAPS. The eluted column fractions were neutralized by dialysis against 10 mM Hepes, pH 7.2/100 mM NaCl/9 mM CHAPS.

Solid-Phase Radioimmunoassays. Retinas were dissected from four eyes of 3-week-old normal BALB/c or *rds* mutant mice, washed by centrifugation in 5% (wt/vol) sucrose/20 mM Tris buffer, pH 7.4, and solubilized in 500 μl of 1% (vol/vol) Triton X-100/Tris-buffered saline, pH 7.4. The samples were diluted with 400 μl of Tris-buffered saline and 50-μl aliquots were applied to the wells of a vinyl microtiter plate. For analysis of rhodopsin, the samples were first diluted 1:5 in Tris-buffered saline before applying 50 μl to the wells. The plate was dried overnight at 37°C and rinsed in Tris-buffered saline. Individual wells were incubated in 50 μl of 1:10 diluted anti-rhodopsin monoclonal antibody 4D2, anti-peripherin monoclonal antibody 5H2, or a non-retinal monoclonal antibody as a control. After 1 hr the wells were washed in buffer and 50 μl of ^{125}I-labeled goat anti-mouse immunoglobulin (10^4 dpm/μl) was applied to each well. The wells were washed with Tris-buffered saline after 1 hr and assayed for radioactivity in a Beckman Gamma 8000 counter. Samples were run in triplicate.

SDS/Polyacrylamide Gel Electrophoresis and Western Blots. SDS/polyacrylamide slab gel electrophoresis was carried out as described (9). For Western blot analysis, proteins were electrophoretically transferred to Immobilon membranes (Millipore) and labeled with either 1:10 diluted hybridoma cell culture fluid containing the monoclonal antibody or diluted affinity purified rabbit polyclonal antibody (0.05 mg/ml) for 1 hr. Goat anti-mouse or goat anti-rabbit immunoglobulin labeled with ^{125}I was used as the secondary antibody for autoradiography (7, 9).

RESULTS

Amino Acid Sequence Comparison of Bovine Peripherin and Mouse *rds* Protein. The nucleic acid sequence of bovine photoreceptor-cell peripherin (8) is 87% identical to the sequence of the cDNA proposed to be responsible for the retinal degeneration slow defect (6). At the amino acid level, the two sequences are 92.5% identical (Fig. 1*A*). The 25 amino acid changes that occur are, for the most part, conservative in nature and are distributed throughout the sequence (Fig. 1*B*). The N-terminal amino acid of the mature bovine peripherin is alanine (8), and it is likely that the initiator methionine is also removed from the mature mouse protein. Neither protein has an obvious signal sequence. The Kyte–Doolittle hydropathy plots (8, 13) of the two sequences are essentially identical. There are four predominant hydrophobic regions that are potential membrane-spanning domains. Peripherin appears to span the disk membrane at least once, since the antigenic sites for monoclonal antibodies 2B6 and 3B6 have been localized on the cytoplasmic or interdiskal side (7, 8) and the oligosaccharide chain (8) is expected to be exposed on the lumen or intradiskal surface as in the case of rhodopsin (14). Further studies are required, however, to determine whether peripherin spans the membrane four times

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RDS        - MALLKVKFDQKKRVKLAQGLWLMNWLSVLAGIVLFSLGLFLKIELRKRSE -50
PERIPHERIN-  ALLKVKFDQKKRVKLAQGLWLMNWFSVLAGIIIFGLGLFLKIELRKRSD -49

RDS        - VMNNSESHFVPNSLIGVGVLSCVFNSLAGKICYDALDPAKYAKWKPWLKP -100
PERIPHERIN- VMNNSESHFVPNSLIGVGVLSCVFNSLAGKICYDALDPAKYAKWKPWLKP -99

RDS        - YLAVCIFFNVILFLVALCCFLLRGSLESTLAYGLKNGMKYYRDTDTPGRC -150
PERIPHERIN- YLAVCVLFNVVLFLVALCCFLLRGSLESTLAHGLKNGMKFYRDTDTPGRC -149

RDS        - FMKKTIDMLQIEFKCCGNNGFRDWFEIQWISNRYLDFSSKEVKDRIKSNV -200
PERIPHERIN- FMKKTIDMLQIEFKCCGNNGFRDWFEIQWISNRYLDFSSKEVKDRIKSNV -199

RDS        - DGRYLVDGVPFSCCNPSSPRPCIQYQLTNNSAHYSYDHQTEELNLWLRGC -250
PERIPHERIN- DGRYLVDGVPFSCCNPNSPRPCIQYQLTNNSAHYSYDHQTEELNLWLRGC -249

RDS        - RAALLNYYSSLMNSMGVVTLLVWLFEVSITAGLRYLHTALESVSNPEDPE -300
PERIPHERIN- RAALLSYYSNLMNTTGAVTLLVWLFEVTITVGLRYLHTALEGMANPEDPE -299

RDS        - CESEGWLLEKSVPETWKAFLESFKKLGKSNQVEAEGADAGPAPEAG -346
PERIPHERIN- CESEGWLLEKSVPETWKAFLESVKKLGKGNQVEAEGEDAGQAPAAG -345
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B

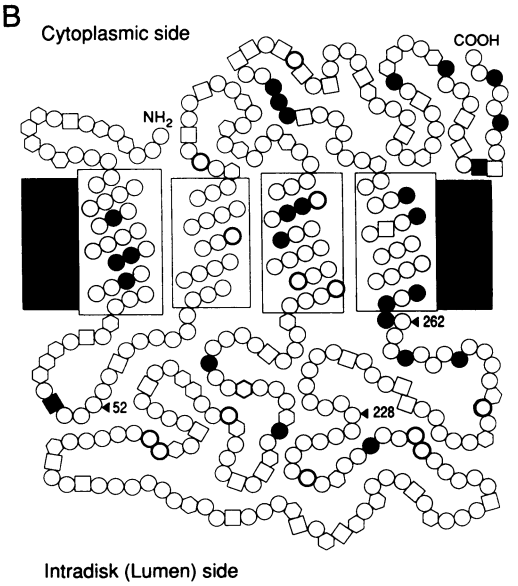

FIG. 1. (*A*) Alignment of the amino acid sequence of bovine peripherin (8) with the proposed normal mouse *rds* protein sequence (6). The sequences have 92.5% identity. The region of the peripherin sequence that contains the antigenic sites for the anti-peripherin monoclonal antibodies 2B6, 3B6, and 5H2 is bracketed (8). (*B*) Structural model of peripherin showing the orientation of the protein within the disk membrane and the location of the amino acid differences between the mouse and bovine sequences (filled symbols). The negatively (□) and positively (○) charged amino acids are indicated. The three potential sites for N-linked glycosylation on the bovine sequence are also shown (arrowheads at positions 52, 228, and 262). The consensus sequence for the N-linked carbohydrate at amino acid 262 is not conserved in the mouse sequence. The 13 cysteine residues that are conserved in both species are highlighted (●).

as depicted in the model in Fig. 1*B*. The 13 cysteine residues and the negative charge on the hyrophilic C-terminal segment are conserved in both species. At amino acid position 343 of the peripherin sequence, an alanine residue is substituted for a glutamic residue in the *rds* sequence; the reverse substitution occurs at position 336. There are three sites on peripherin that have a consensus sequence for N-linked glycosylation (Asn^{52}, Asn^{228}, and Asn^{262}), and at least one of these sites is utilized (8). The consensus sequence for N-linked glycosylation at is not conserved in the mouse *rds* protein sequence (Fig. 1*A*). It is likely that this site, which is close to a putative transmembrane domain, is not utilized in bovine peripherin. The model as depicted in Fig. 1*B* has a large hydrophilic domain of 142 amino acids (amino acids 122–263). This domain contains two consensus sequences for N-linked glycosylation and is expected to be present within the intradisk or lumen space.

Normal Peripherin/*rds* Protein Is Present in Mouse ROS. To determine whether the peripherin/*rds* protein is present in wild-type mouse ROS, polyclonal antibody Pab(308–317) was made against a synthetic peptide corresponding to a segment (amino acids 308–317 in Fig. 1*A*) that is identical in both the bovine and the mouse protein. This antibody labeled the peripherin/*rds* protein of 33 kDa in Western blots of both bovine and normal mouse ROS (Fig. 2).

Anti-bovine peripherin monoclonal antibody 5H2, which binds to an epitope along the 35-amino acid C-terminal segment as indicated by positive immunological screening of clone λ.5 (8), also labeled the mouse peripherin/*rds* protein, although somewhat less intensely than the bovine protein (Fig. 2). In the absence of a sulfhydryl reducing agent, the mouse peripherin/*rds* protein, like its bovine counterpart, migrated as a dimer of 68 kDa (Fig. 2). Two previously developed monoclonal antibodies, 2B6 and 3B6 (7, 8), which also bind to an epitope within the 35-amino acid C-terminal segment of bovine peripherin, did not label mouse peripherin/*rds* protein as determined by Western blotting (Fig. 2). However, the 3B6 antibody did bind strongly to rat peripherin. Recently, the sequence of the rat peripherin protein was predicted from its cDNA sequence (15). Comparison of the 35-amino acid C-terminal sequence of bovine, mouse, and rat peripherin/*rds* proteins indicates that amino acid 340 (see Fig. 1*A*) is a glutamine in both the bovine and the rat protein but is a proline in the mouse protein. Since it is known that

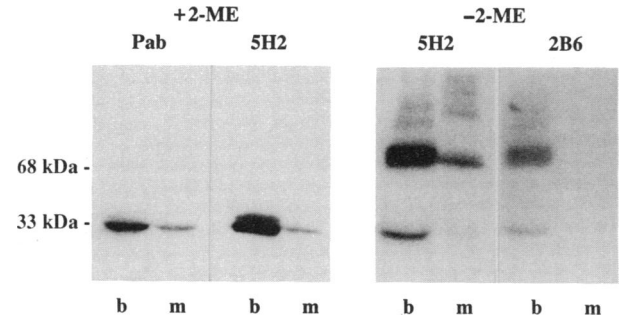

FIG. 2. Western blots of bovine (b) and mouse (m) ROS proteins separated by SDS/polyacrylamide gel electrophoresis in the presence (+2-ME) or in the absence (−2-ME) of 4% (vol/vol) 2-mercaptoethanol. Western blots were labeled either with polyclonal antibody (Pab) raised against a peptide consisting of amino acids 308–317 of the mature sequence or with anti-peripherin monoclonal antibody 5H2 or 2B6. In the presence of the reducing agent (+2-ME) both the Pab and 5H2 antibodies labeled bovine and mouse peripherin having an apparent molecular mass of 33 kDa; in the absence of a reducing agent the 5H2 antibody was observed to preferentially label a dimer of 68 kDa in both mouse and bovine ROS. Monoclonal antibodies 2B6 and 3B6 (7), which were produced against bovine and rat peripherin, respectively, show little, if any, crossreactivity with the mouse protein.

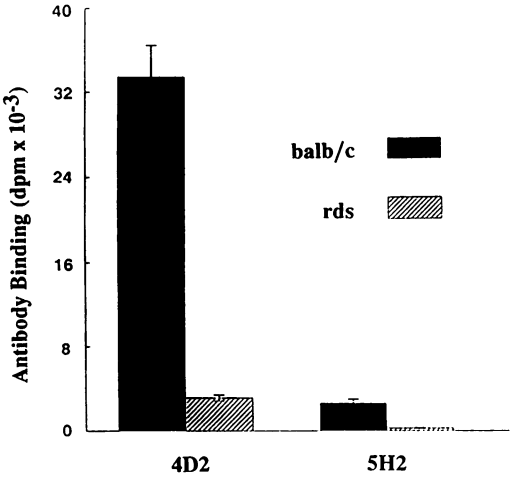

FIG. 3. Expression of peripherin/*rds* protein in normal and *rds* mice. Retinas were dissected from the eyes of two normal BALB/c or two homozygous mutant *rds* mice at 3 weeks of age. The retinal extracts were solubilized in 1% Triton X-100, and 50 μl for peripherin/*rds* analysis and for controls or 50 μl of 1:5 diluted extract for rhodopsin analysis was dried onto the wells of a vinyl microtiter plate. Individual wells were incubated with 50 μl of 1:10 diluted hybridoma culture fluid containing anti-rhodopsin monoclonal antibody 4D2, anti-peripherin monoclonal antibody 5H2, or a non-retinal monoclonal antibody as a control. After 1 hr the wells were washed in buffer and 50 μl of ^{125}I-labeled goat anti-mouse immunoglobulin (10^4 dpm/μl) was applied to each well. After washing, radioactivity of individual wells was measured in a Beckman Gamma 8000 counter. Samples were run in triplicate.

a single amino acid change can reduce or prevent antibody binding (16), it is likely that this Gln^{340} → Pro change is responsible for the low degree of binding of the 2B6 and 3B6 antibodies to mouse peripherin/*rds* protein.

Expression of Peripherin/*rds* Protein in Normal and Mutant *rds* Mice. The expression of rhodopsin and the peripherin/*rds* protein in normal BALB/c and mutant *rds* mouse retinas at 3 weeks of age was measured by solid-phase radioimmunoassay. The rhodopsin level in homozygous *rds* mutant mouse retinal extract was <10% that of the normal BALB/c mouse retinal extract (Fig. 3). This is in agreement with recent studies of Schalken *et al.* (17). The peripherin/*rds* protein was readily detected in normal mouse retinal extract by the 5H2 anti-peripherin monoclonal antibody. In the homozygous *rds* mutant mouse retinal extract, 5H2 antibody binding was only marginally above that for control samples in which an irrelevant antibody was used. It is likely that the low level of 5H2 antibody binding in the *rds* mouse retinal extract is due to nonspecific binding, since the 5H2 epitope is contained within the C-terminal 87 amino acids that would not be expected to be present in the *rds* mouse (6).

DISCUSSION

Using differential and substractive screening procedures, Travis *et al.* (6) identified a candidate gene responsible for photoreceptor-cell degeneration in the *rds* mutant mouse. However, localization or characterization of the protein encoded by this gene was not reported. In this study, we have shown that the protein sequence predicted from the wild-type *rds* cDNA is highly homologous (92.5%) to the sequence of bovine peripherin (8). Furthermore, polyclonal and monoclonal antibodies used with Western blot analysis indicate that the normal mouse *rds* protein, like bovine peripherin, is localized in isolated ROS membranes and exists as two subunits linked together by one or more disulfide bonds. On the basis of these studies, we conclude that the previously

identified outer-segment disk-membrane protein peripherin (7) is the normal product of the *rds* gene. Travis *et al.* (6) have shown that normal-sized mRNA coding for the wild-type *rds* gene product is not produced in the *rds* mutant mouse. It was also shown that in the *rds* mutant mouse, foreign DNA is inserted into the *rds* protein-coding sequence such that, if translated, this abnormal protein would most likely not contain the C-terminal 87 amino acids. On this basis one would predict that binding of C-terminal specific anti-peripherin antibodies to *rds* retinal extract would not be observed. Radioimmunoassays using anti-rhodopsin and anti-peripherin monoclonal antibodies confirm that outer-segment proteins are significantly reduced in quantity in *rds* retinal extract. However, due to the low level of anti-peripherin antibody binding observed in the *rds* retinal extract, it is not possible to confirm, without further studies, the total absence of peripherin in the *rds* mouse as predicted from the genetic analysis.

The function of peripherin/*rds* protein is not known. However, preembedding and postembedding immunogold labeling studies using monoclonal antibodies 2B6 and 3B6 indicate that peripherin is preferentially localized along the rim regions of bovine and rat ROS (7, 8), as well as cone outer segments. The localization of peripherin to the rim region of the disk membrane and the phenotype associated with the *rds* defect suggest that this membrane protein may play a role in the morphogenesis and maintenance of the disk rim structure. It is possible that peripherin may serve to anchor the disk membranes to the cytoskeletal system of the outer segment. The negatively charged C-terminal domain of peripherin, which has been localized on the cytoplasmic side of the disk membrane, may interact with the filamentous cytoskeleton-like structures that have been observed to link the disk rim and plasma membrane (18). Alternatively, the protein or carbohydrate domain within the intradiskal space may interact with other peripherin molecules or other membrane proteins to form and maintain the highly curved disk rim membrane domain. The large intradiskal hydrophilic domain containing possible N-linked glycosylation sites, as suggested in the peripherin model, may serve this function. Fliesler *et al.* (19) have shown that treatment of retina with tunicamycin, an inhibitor of N-linked glycosylation, disrupts normal disk formation. It is possible that a loss of the N-linked carbohydrate of peripherin/*rds* protein, as well as rhodopsin, prevents the formation of the flattened disk-like structures of rod and cone outer segments.

In conclusion, peripherin has been identified as the normal product of the *rds* gene on the basis of sequence analysis. Localization of peripherin to outer segments of mouse photoreceptor cells is consistent with ultrastructural features of retinal degeneration in the *rds* mutant mouse which indicate that the primary defect is localized to the outer segment. The failure of the photoreceptor outer segments to form is likely to result either from the inability of the peripherin/*rds* protein to be expressed in the *rds* mouse or from its expression as an altered protein.

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