

# Localization of Peripherin/*rds* in the Disk Membranes of Cone and Rod Photoreceptors: Relationship to Disk Membrane Morphogenesis and Retinal Degeneration

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**Abstract.** The outer segments of vertebrate rod photoreceptor cells consist of an ordered stack of membrane disks, which, except for a few nascent disks at the base of the outer segment, is surrounded by a separate plasma membrane. Previous studies indicate that the protein, peripherin or peripherin/*rds*, is localized along the rim of mature disks of rod outer segments. A mutation in the gene for this protein has been reported to be responsible for retinal degeneration in the *rds* mouse. In the present study, we have shown by immunogold labeling of rat and ground squirrel retinas that peripherin/*rds* is present in the disk rims of cone outer segments as well as rod outer segments. Additionally, in the basal regions of rod and cone outer segments, where disk morphogenesis occurs, we have found that the distribution of peripherin/*rds* is restricted to a region that is adjacent to the cilium. Ex-

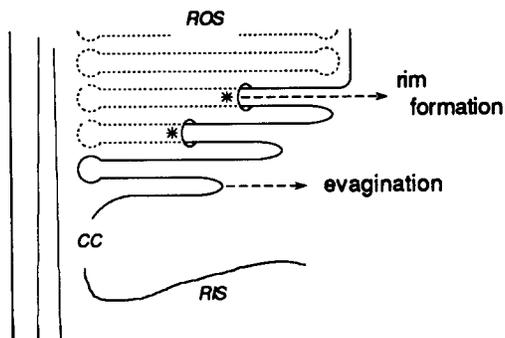
tension of its distribution from the cilium coincides with the formation of the disk rim. These results support the model of disk membrane morphogenesis that predicts rim formation to be a second stage of growth, after the first stage in which the ciliary plasma membrane evaginates to form open nascent disks. The results also indicate how the proteins of the outer segment plasma membrane and the disk membranes are sorted into their separate domains: different sets of proteins may be incorporated into membrane outgrowths during different growth stages of disk morphogenesis. Finally, the presence of peripherin/*rds* protein in both cone and rod outer segment disks, together with the phenotype of the *rds* mouse, which is characterized by the failure of both rod and cone outer segment formation, suggest that the same *rds* gene is expressed in both types of photoreceptor cells.

**V**ERTEBRATE rod and cone photoreceptor cells contain a specialized compartment, called the outer segment, which serves as the site for phototransduction. The outer segment consists of an ordered stack of disk membranes, and is connected to the inner segment of the cell by a thin nonmotile cilium. Based on ultrastructural observations, Steinberg et al. (1980) proposed a model for the morphogenesis of new disk membranes from the ciliary plasma membrane. In their model, disk membranes are predicted to grow by two separate, sequential processes. Firstly, the plasma membrane evaginates to form nascent disks that are "open"; i.e., exposed to extracellular space. Secondly, a rim develops between the upper membrane of a nascent disk and the lower membrane of its more mature (i.e., more distal) neighbor (Fig. 1). Steinberg et al. (1980) proposed that the rim represents the addition of new components from the ciliary plasma membrane, and not just the fusion of the nascent disk edges. In rod outer segments, the rim develops completely, so that the external surface of a nascent disk becomes the intradiskal surface. In cone outer segments, rim development is typically incomplete and cone disks remain "open,"

although in mammalian cones the opening is very small or sometimes absent at the distal end of the cone outer segments (Anderson et al., 1978).

Evidence from experiments in which disk morphogenesis was perturbed in rod and cone cells supports the proposal that the nascent disks form by growing outward from the connecting cilium (Williams et al., 1988; Vaughan and Fisher, 1989), rather than by an invagination as suggested by earlier investigators (Nilsson, 1964). However, there have been no studies that test the validity of the proposed mechanism of rim development.

The rim region of the mature rod outer segment disks contains several proteins that are not detected in the lamellar region of the disk. Papermaster et al. (1978) have shown that the rim region of frog photoreceptor outer segment disks contains a high molecular mass membrane protein of ~290 kD. Preembedding and postembedding immunogold labeling studies, using mAbs, indicate that bovine rod outer segments contain a membrane glycoprotein called peripherin, or peripherin/*rds*, which is localized in the rim of mature disk membranes (Molday et al., 1987). The cDNA for this pro-



**Figure 1.** Model for disk morphogenesis based on the proposal of Steinberg et al. (1980). These are two proposed membrane growth processes: the first is an evagination of the ciliary plasma membrane; the second is the formation of the disk rim, which produces a closed disk. CC, connecting cilium; RIS, rod inner segment; ROS, rod outer segment. \* indicates the leading edge of rim growth.

tein has recently been cloned and shown to code for a protein of 346 amino acids with four putative transmembrane domains (Connell and Molday, 1990).

Travis et al. (1989) have used subtractive hybridization to identify cDNA clones that code for a protein responsible for retinal degeneration in the *rd*s mutant mouse. In the wild-type mouse, the cDNA codes for the normal protein of 346 amino acids; in the mutant *rd*s mouse, insertion of 10 kb of DNA into a protein coding region gives rise to an abnormal mRNA, which, if translated, would result in an abnormal protein product. Sequence analysis indicates that peripherin (now referred to as peripherin/*rd*s) is the product of the wild-type *rd*s gene (Connell et al., 1991). A high degree (>85%) of positional identity has been found between the amino acids of mouse (Travis et al., 1989), bovine (Connell and Molday, 1990), rat (Begy and Bridges, 1990), and human (Travis et al., 1991b) peripherin/*rd*s. Because photoreceptor outer segments fail to develop in the *rd*s mutant mouse, the normal peripherin/*rd*s protein appears to be essential for normal photoreceptor outer segment disk morphogenesis.

In the present study, we have used immunocytochemical methods to study the distribution of peripherin/*rd*s in cone outer segments and the basal region of rod outer segments. The study thus provides new information on rim formation and the sorting of outer segment membrane proteins during disk morphogenesis. It also gives insight into the relationship of peripherin/*rd*s and the degeneration of both rod and cones in the *rd*s mutant mouse.

## Materials and Methods

### Immunochemical Reagents

The following mAbs in the form of hybridoma culture fluids were used: mAb 3B6 produced against the COOH terminus of rat peripherin/*rd*s (Molday et al., 1987; Connell and Molday, 1990); mAb rho 3D6 against the COOH terminus of bovine rhodopsin (MacKenzie and Molday, 1982); and mAb rho 4D2 against the NH<sub>2</sub> terminus of rat rhodopsin (Hicks and Molday, 1986). <sup>125</sup>I-labeled goat anti-mouse IgG was prepared as previously described (MacKenzie and Molday, 1982). FITC-goat anti-mouse IgG was prepared by reacting FITC (0.05 mg/ml) with affinity-purified goat anti-mouse IgG (2 mg/ml) in 0.02 M sodium borate buffer, pH 8.4, for 2 h, and removing unconjugated FITC by Sephadex G-25 gel filtration chromatogra-

phy. Goat anti-mouse IgG conjugated to 5-nm colloidal gold was obtained from Janssen Life Sciences Products (Piscataway, NJ).

### SDS Gel Electrophoresis and Western Blotting

Bovine rod outer segment membranes that had been purified on sucrose density gradients (Molday et al., 1987) and a 13-lined ground squirrel (*Spermophilus tridecemlineatus*) retinal homogenate were solubilized in 0.01 M Tris buffer, pH 7.4, containing 2.5% SDS, 5% 2-mercaptoethanol, and 20% sucrose at a concentration of 2–3 mg/ml and applied to lanes of an 8% polyacrylamide-SDS slab gel. For Western blotting, proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA), blocked, and labeled with 1:30 diluted hybridoma culture fluid and <sup>125</sup>I-labeled goat anti-mouse Ig for autoradiography (Molday et al., 1987).

### Immunofluorescence Microscopy

Immunofluorescence microscopy was carried out on cryosections of acrylamide-embedded retinal tissue, using the procedure of Johnson and Blanks (1984). Briefly, retinas in intact eyecups were fixed in 3% paraformaldehyde-PBS, pH 7.4, for 3 h at 23°C. Pieces of each eyecup were infiltrated with 8% acrylamide for 8 h at 4°C before initiation of acrylamide polymerization with ammonium persulfate. 6–8 μm sections were cut from frozen acrylamide-embedded retinas, incubated in 0.1% Triton X-100 for 30 min, blocked in 10% normal goat serum, and labeled with 1:20 diluted hybridoma fluid for 1 h. After washing in PBS, the sections were labeled with FITC-goat anti-mouse Ig (50 μg/ml) for 1 h. The sections were then washed in PBS again, and examined in a photomicroscope (Axiophot; Zeiss, Oberkochen, FRG). In control experiments, the 3B6 hybridoma culture fluid was incubated with 0.1 mg/ml of a synthetic COOH-terminal peripherin peptide (Gln-Val-Glu-Ala-Glu-Gly-Glu-Asp-Ala-Gly-Gln-Ala-Pro-Ala-Ala-Gly-COOH) for 30 min before labeling the sections.

### Sample Preparation for Electron Microscopy

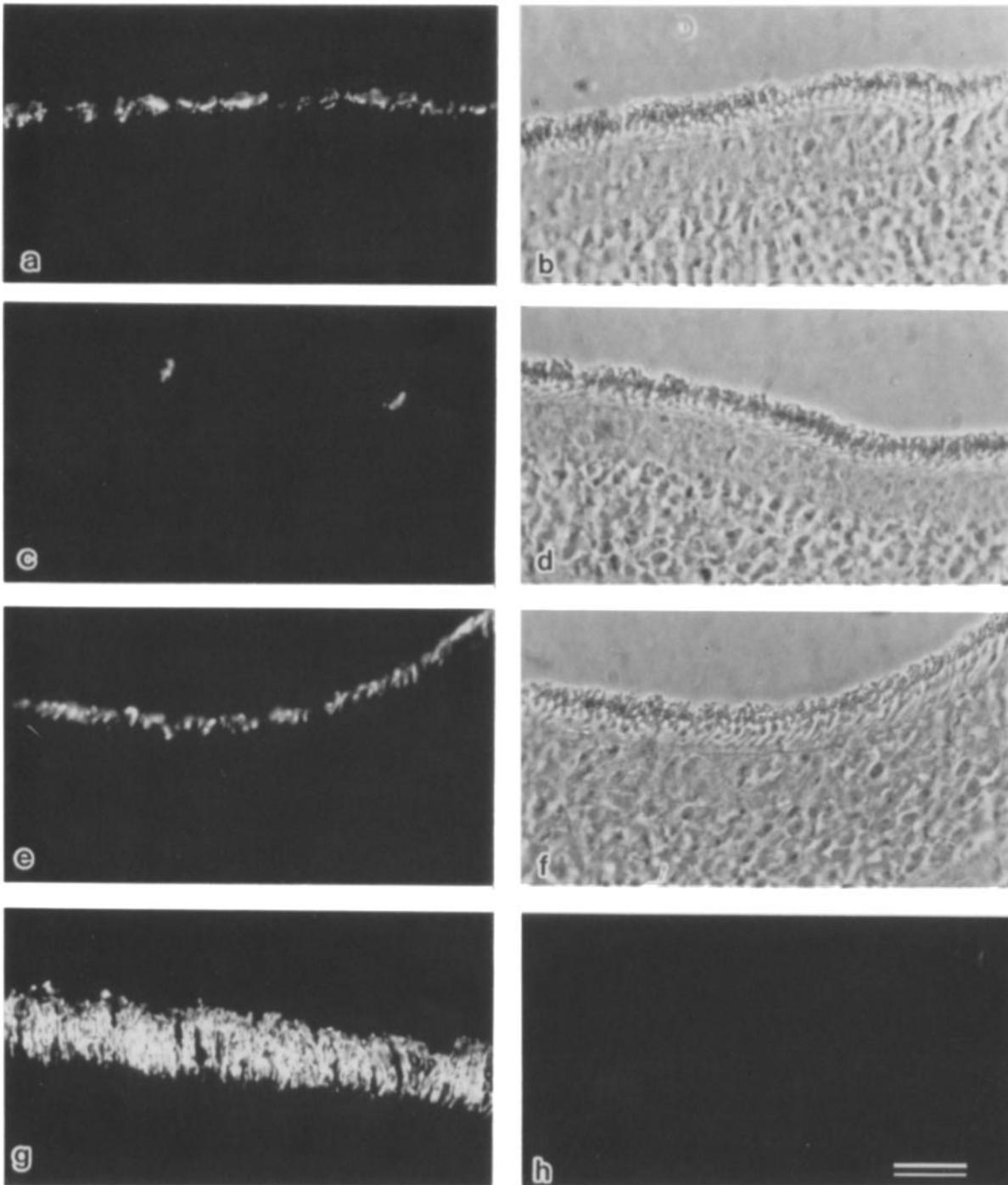
For conventional EM, light-adapted 6-wk-old Long Evans rats were anesthetized and fixed by transcardiac perfusion with 2% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, for ~40 min. The eyes were then enucleated, cut into small pieces, and immersed in the same fixative overnight at 4°C. After rinsing with cacodylate buffer, the tissues were postfixed with 2% OsO<sub>4</sub> in cacodylate buffer for 2 h, dehydrated by a graded methanol series, and infiltrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in an electron microscope.

For immunocytochemistry, the rats were decapitated and the eyes were then enucleated and hemisected. The posterior half of the eyes were fixed by immersing in 0.5% glutaraldehyde plus 4% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 h at 24°C. These eyecups were then cut into small pieces, dehydrated by a graded methanol series, and infiltrated and embedded in L. R. White resin.

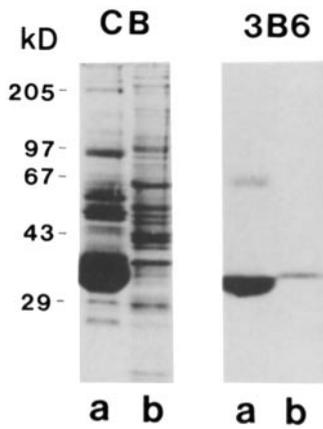
L. R. White-embedded pieces of the superior temporal region of the left retina of a California ground squirrel (*Spermophilus beecheyi*) were generously given to us by Dr. Don Anderson and R. Fariss from the University of California at Santa Barbara (Santa Barbara, CA). The pieces of retina had been fixed by immersion in 1.0% glutaraldehyde plus 1.0% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 h at 24°C, and stained en bloc with 2% uranyl acetate (Erickson et al., 1987). L. R. White-embedded tissues were sectioned with a diamond knife, and the sections were collected on nickel grids.

### Postembedding Immunogold Labeling

The immunolabeling procedure followed that of Arikawa and Williams (1989). Briefly, L. R. White sections were incubated with hybridoma culture fluid (diluted twofold in 1% BSA and 0.5 M NaCl in phosphate buffer) containing the 3B6 primary antibody for 1 h at 25°C, washed in buffer, and then incubated with goat anti-mouse IgG conjugated to colloidal gold (5 nm) for 1 h. The sections were washed again, stained with uranyl acetate, and examined in an electron microscope. For quantification of label intensity, the number of gold particles per unit area was determined from micrographs (at a magnification of 40,000), and the data were expressed as mean ± SEM. Where appropriate, a one-tailed *t* test was used to determine the probability of no significant difference between samples of data.



**Figure 2.** Ground squirrel and bovine retinal tissue sections labeled with mAbs and FITC-goat anti-mouse Ig. (a) Fluorescence micrograph of squirrel retina, labeled with mAb rho 3D6 reactive against both rod opsin and red/green cone opsin. (b) Corresponding phase light micrograph for a. (c) Fluorescence micrograph of squirrel retina, labeled with mAb rho 4D2 reactive against rod opsin. Note that only two photoreceptor outer segments are labeled. (d) Corresponding phase light micrograph for c. (e) Fluorescence micrograph of squirrel retina, labeled with mAb 3B6 reactive against peripherin/*rds* protein. (f) Corresponding phase light micrograph for e. (g) Fluorescence micrograph of bovine retina, labeled with mAb 3B6. (h) Fluorescence micrograph of bovine retina, labeled with mAb 3B6, in the presence of an excess of a 16-amino acid synthetic peptide, corresponding to the COOH-terminus of bovine peripherin/*rds*. In these micrographs the retinal pigment epithelial layer was removed from the retina tissue and is not shown. Bar, 25  $\mu$ m.



**Figure 3.** Detection of peripherin/*rds* protein by Western blot analysis. Isolated bovine rod outer segments (a) and ground squirrel retina homogenate (b) were subjected to SDS-PAGE, and either stained with Coomassie blue (CB), or transferred to Immobilon membrane and labeled with the mAb 3B6 and  $^{125}\text{I}$ -labeled goat anti-mouse Ig for autoradiography.

## Results

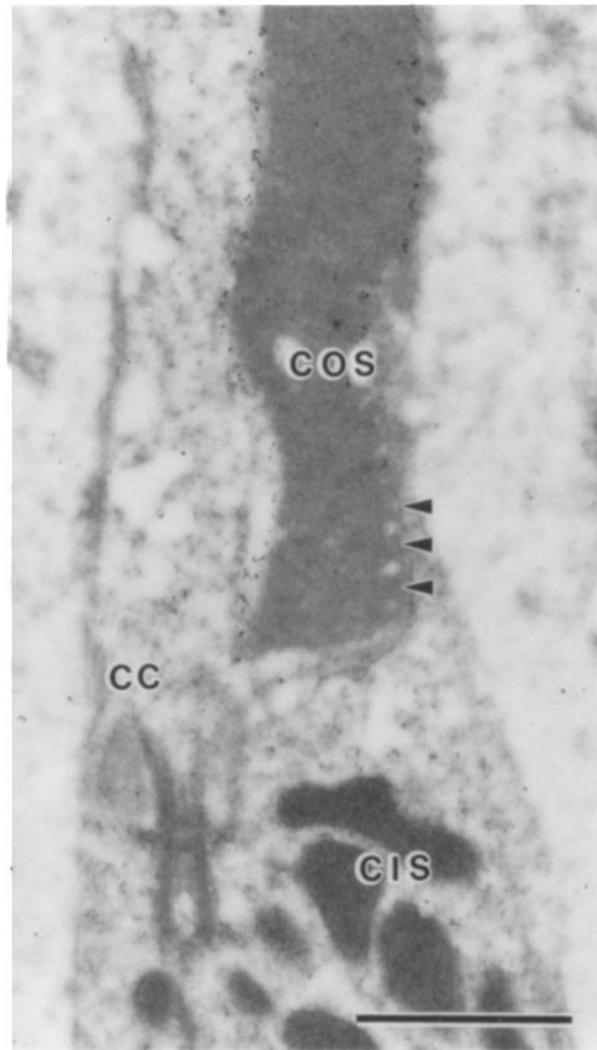
### Immunofluorescence Labeling of Cone and Rod Outer Segments

It has been shown previously by a variety of methods that ~95% of the photoreceptors in ground squirrel retinas are cone cells (e.g., Jacobs et al., 1976). Consistent with this past work, we found (Fig. 2, a–d) that mAb rho 3D6, which binds to both rod cell opsin and red/green cone cell opsin, labeled most of the photoreceptor outer segments in sections of 13-lined ground squirrel retinas (only the blue cone cells would not have been labeled), whereas only a few outer segments in any given section were labeled by the mAb rho 4D2, which binds only to rod cell opsin (Hicks and Molday, 1986; Molday, 1988).

Immunofluorescence microscopy with mAb 3B6 was used to determine the cellular distribution of peripherin/*rds* in squirrel retina. This antibody was generated against the COOH terminus of rat peripherin/*rds* (Molday et al., 1987; Connell and Molday, 1990). As shown in Fig. 2, e and f, the cone-dominant photoreceptor outer segment layer was intensely labeled. Other retinal cell layers were not labeled. The 3B6 antibody also labeled the outer segments of the rod-dominant bovine retina (Fig. 2 g). The specificity of labeling was confirmed by comparing control sections of bovine retinas in which preincubation with a 16-amino acid synthetic peptide, corresponding to the COOH terminus of bovine peripherin/*rds*, completely inhibited the binding of the 3B6 antibody (Fig. 2 h).

### Western Blots of Ground Squirrel Retina

To determine if the peripherin/*rds* protein in cone outer segments has a similar relative molecular weight to peripherin/*rds* found in rod outer segments, Western blots of ground squirrel retinal homogenate and isolated bovine rod outer segments were labeled with mAb 3B6. As shown in Fig. 3, the 3B6 antibody labeled a 35-kD protein in ground squirrel retina, and a 33-kD protein and its dimer in bovine rod outer segments. The apparent lower relative molecular mass observed for peripherin/*rds* in bovine rod outer segments is due to the abundance of rhodopsin in rod outer segments, which prevents efficient transfer of peripherin/*rds*, such that only the leading edge of peripherin/*rds* in rod outer segments is labeled. Bovine peripherin/*rds* migrates with an apparent relative molecular mass of 35 kD when most of the rhodop-

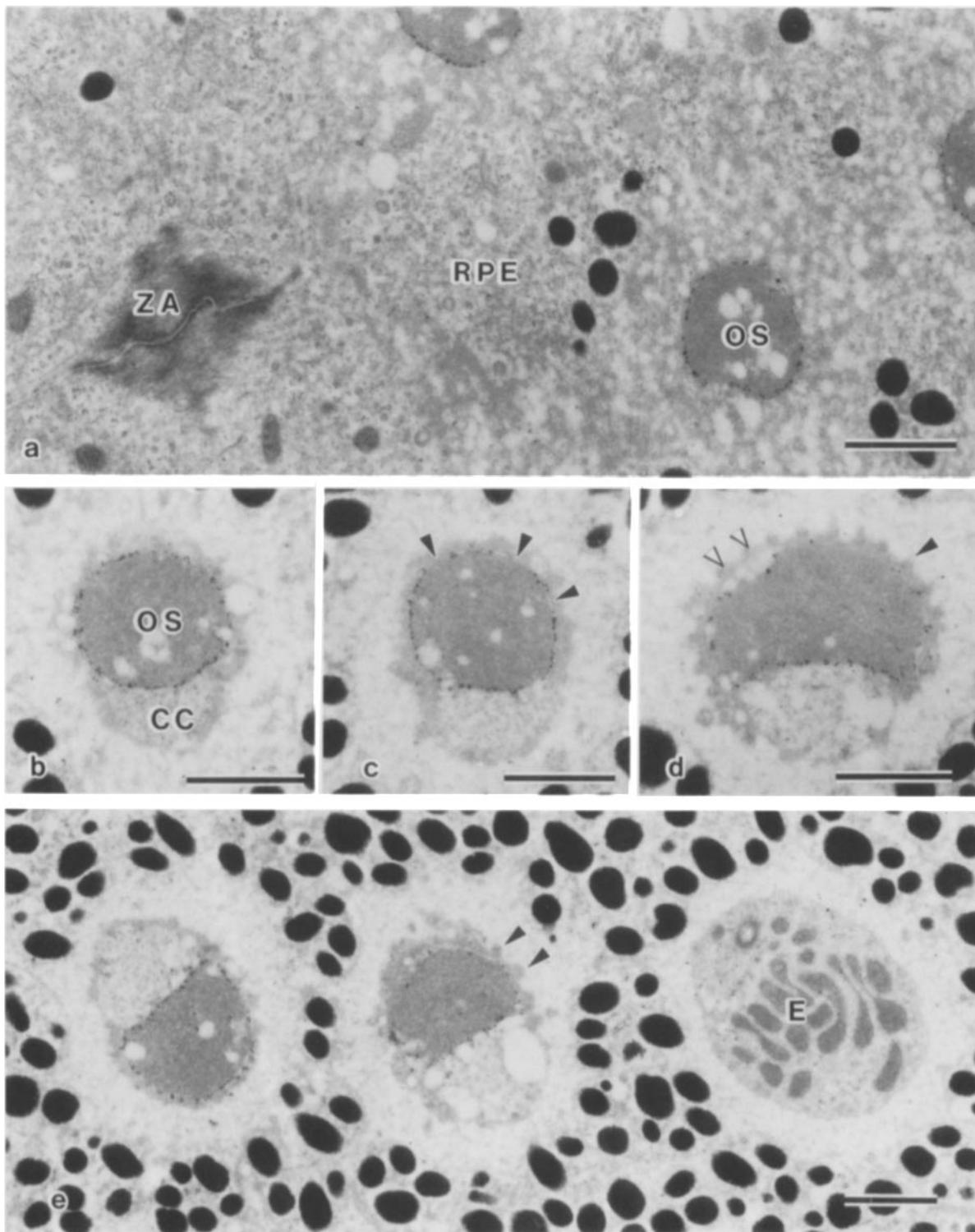


**Figure 4.** Longitudinal section of a cone photoreceptor in a Californian ground squirrel retina, immunogold labeled with antiperipherin (3B6). Label is evident along the margins of the sectioned outer segment, except at the base of the outer segment, opposite the connecting cilium (arrowheads). CIS, cone inner segment; COS, cone outer segment; CC, connecting cilium. Bar, 1  $\mu\text{m}$ .

sin is enzymatically digested (Molday et al., 1987), or when peripherin/*rds* is purified by affinity chromatography.

### Immunogold Labeling of Cone Photoreceptors

Unlike cone outer segments of amphibians, in which all the disks are clearly open, mammalian cone disks, especially at the distal end of the outer segment, appear closed—like rod disks—in most longitudinal sections of the outer segment (Anderson et al., 1978). In ultrathin sections of ground squirrel retina, 3B6 labeling was limited to the outer segments. In ultrathin sections of bovine retinas, 3B6 also labeled the outer segments of both the rods and cones (data not shown). In longitudinal sections of the ground squirrel cone outer segments, labeling was evident only along the sides of the outer segment. Moreover, in sections that included the connecting cilium on one side, labeling appeared restricted to that side in the basal part of the outer segment (Fig. 4).



**Figure 5.** Oblique sections of cone outer segments at different levels in the retina of a Californian ground squirrel, immunogold labeled with antiperipherin (3B6). Label is found entirely around the disks in sections of distal regions. In sections of more proximal regions, label incompletely surrounds the disks. (a) Distal portion of the photoreceptor layer. The outer segment is surrounded by the retinal pigment epithelium. (b–d) Successively more proximal sections of cone outer segments. Arrowheads in c indicate breaks in the labeling. Arrowheads in d each indicate a calycal process, which is an extension from the inner segment that surrounds the base of the outer segment. At the open arrowheads, the disk membrane has been artefactually pulled away from the plasma membrane. (e) An oblique section that passes through the ellipsoid of one cone (right), near the base of the outer segment (comparable to d, arrowheads indicate calycal processes) of another (middle), and through a more distal region (comparable to c) of a third. CC, connecting cilium; OS, cone outer segment; E, ellipsoid; RPE, retinal pigment epithelium; ZA, zonula adherens between two adjacent retinal pigment epithelial cells. Bars: (a and e) 1.0  $\mu\text{m}$ ; (b–d) 0.5  $\mu\text{m}$ .

At the distal end, label was evident on both sides of the outer segment with similar intensity.

In near transverse sections of the distal portion of the cone outer segment, where the disks are surrounded by the retinal epithelium, labeling by 3B6 was found entirely around the margin of the outer segment. At this level, the profile of the disk is completely circular and the connecting cilium is not visible in cross section (Fig. 5 *a*). In the middle of the outer segment, where the disks have a circular profile, but the connecting cilium is visible, 3B6 labeling outlined the whole disk (Fig. 5 *b*). In more proximal sections, where the disks are indented by the connecting cilium, 3B6 labeling was similar except that small gaps in the labeling were sometimes evident opposite the cilium (Fig. 5 *c*). More proximally still, this region opposite the connecting cilium was only sparsely labeled, whereas adjacent to the cilium, label intensity was comparable to that in more distal regions (Fig. 5 *d*). In sections through the base of the outer segment, 3B6 labeling was predominantly on the ciliary side (Fig. 5, *d* and *e*), consistent with the labeling pattern observed with longitudinal sections. Fig. 5 *e* illustrates a very oblique section, where the proximal-distal difference in the extent of labeling around the disks is evident in adjacent photoreceptors, which have been sectioned at different levels.

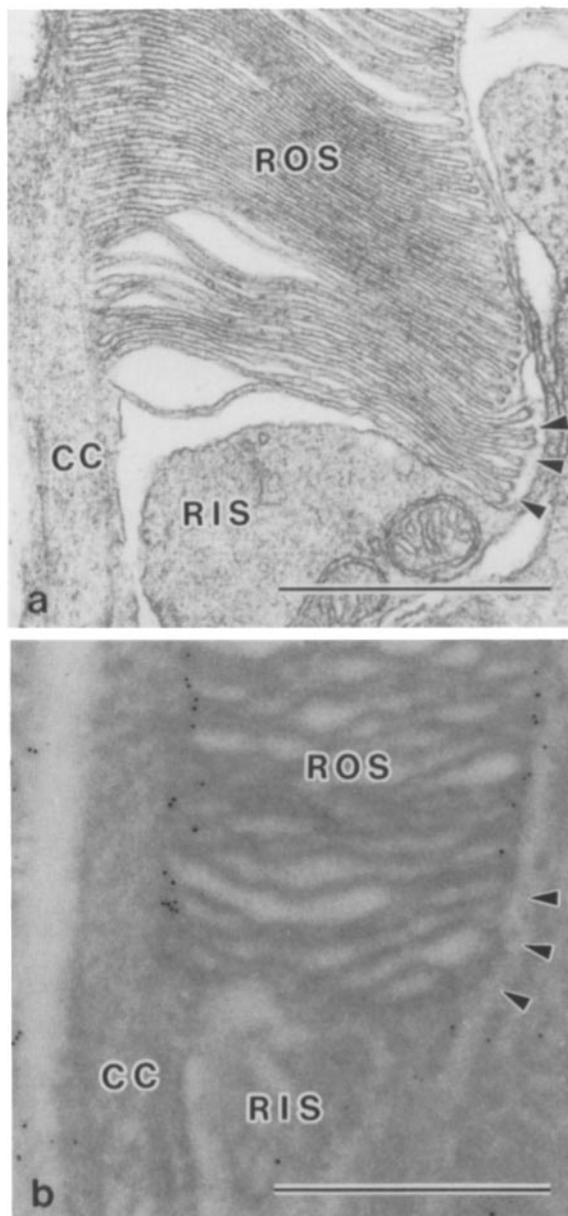
Because the disk rim and the enveloping plasma membrane of the outer segment are so close together, it is not normally possible to distinguish labeling of the disk rim from that of the plasma membrane in sections of intact tissue. By isolating bovine rod disks from the plasma membrane, it has been shown that 3B6 labels the disk rims with little if any labeling of the plasma membrane (Molday et al., 1987; Molday and Molday 1987). In Fig. 5 *d*, part of the disk membrane has pulled away from the plasma membrane. Here, some label is evident on the disk rim but not on the plasma membrane, indicating that in cone outer segments, as in rod outer segments, 3B6 labels only the disk rim.

### Immunogold Labeling of the Nascent Disks in Rod Photoreceptors

In a rat retina fixed by transcardiac perfusion for conventional electron microscopy, 6–10 nascent disks were observed at the base of each rod outer segment. The thickness of the stack of the nascent disks was 0.2–0.3  $\mu\text{m}$  (Fig. 6 *a*). Even with conventional EM, the nascent disks are not always well preserved (Steinberg et al., 1980), so it is not surprising that after processing for immunocytochemistry, in which osmication is omitted and glutaraldehyde fixation is minimal, the nascent disks are not easily resolved and distinguished from the mature disks (Fig. 6 *b*).

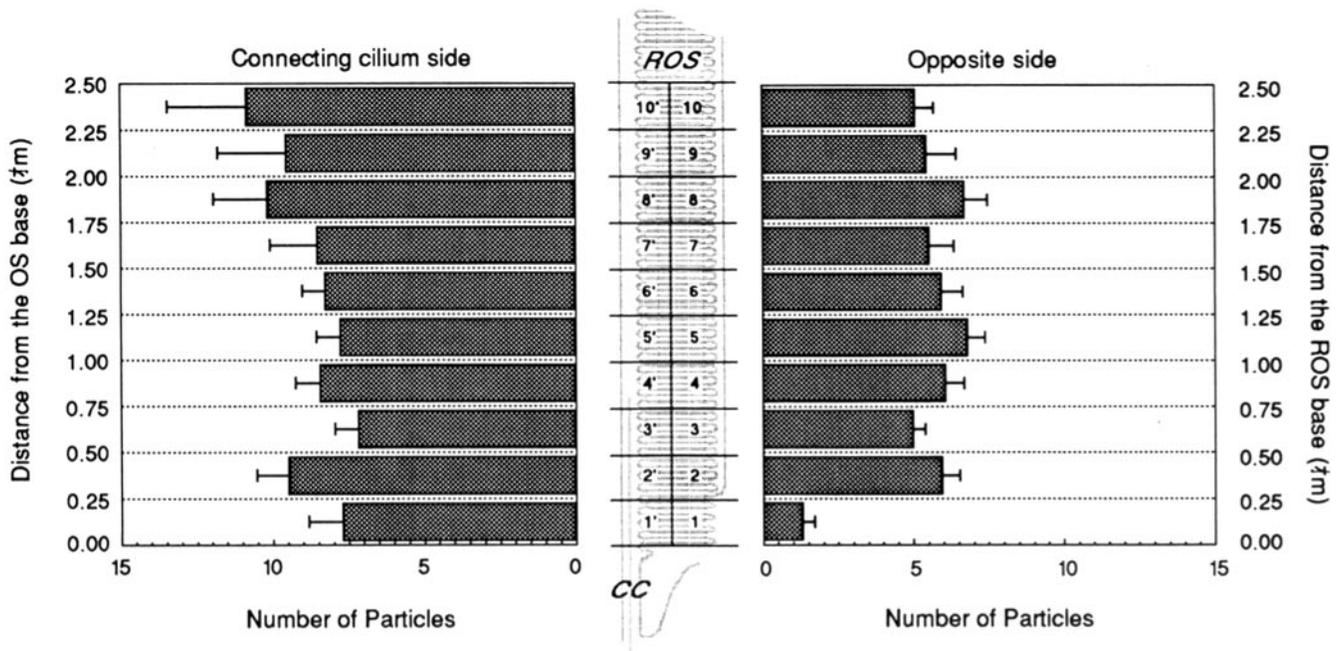
On L. R. White sections of rat retinas, 3B6 labeled the margins of the rod outer segments, as previously reported (Molday et al., 1987). At the base of the outer segment, the margin opposite the cilium was not labeled. The extent of this unlabeled region corresponded to that of the nascent disks (Fig. 6, compare *a* with *b*).

Preferential labeling of the ciliary side of the nascent disks was particularly manifest after quantification of the amount of 3B6 label on each side of rod outer segments that were sectioned longitudinally, with the cilium apparent on one side. The most basal 2.5  $\mu\text{m}$  of each rod outer segment were divided into 0.25- $\mu\text{m}$  segments. The material at the base of the rod outer segment, assumed to be nascent disks, was thus



**Figure 6.** (a) Base of a rat outer segment fixed by transcardiac perfusion and processed for conventional EM. Seven open, nascent disks (arrowheads) are evident. They are  $\sim 0.2 \mu\text{m}$  deep. CC, connecting cilium; RIS, rod inner segment; ROS, rod outer segment. (b) Longitudinal section of rat rod photoreceptor, processed for immunocytochemistry, and labeled with 3B6. Nascent disks are not clearly distinguishable from the mature disks in this lightly fixed tissue. Label is evident along both margins of the sectioned outer segments, except at the base of the outer segment, in a region that corresponds to the nascent disks region (arrowheads). CC, connecting cilium; RIS, rod inner segment; ROS, rod outer segment. Bars, 0.5  $\mu\text{m}$ .

included in the most basal segment. The number of gold particles on each side of each segment was counted. In the most basal segment (i.e., segment 1 in Fig. 7), there was less label on the side opposite the cilium than on the ciliary side ( $P < 0.01$ ). Indeed, this lightly labeled side of segment 1 contained less label than either side of any of the more distal segments ( $P < 0.01$ ) (Fig. 7). In the more distal nine segments, the



**Figure 7.** Histograms showing the amount of 3B6 labeling of rat rod outer segments (ROS). The most basal 2.5  $\mu\text{m}$  of each ROS was divided into 10 equal segments, and the number of gold particles on each side of each segment was counted. Data were collected from 45 ROSs from three retinas. Means  $\pm$  SEMs are shown. CC, connecting cilium.

mean number of particles on the ciliary side of the rod outer segments ( $8.9 \pm 0.4$  per segment) was slightly higher than that on the opposite side ( $5.8 \pm 0.2$  per segment), probably because the fimbriate incisure which extends from the ciliary side into the disk (Pedler and Tilly, 1967) was sometimes included in the section, and 3B6 labels this incisure (Molday et al. 1987).

## Discussion

In the present study, we have found that (a) mAb 3B6 recognizes a 35-kD protein in the ground squirrel retina, a retina in which the photoreceptors are mostly cones; (b) in sections of ground squirrel and rat retinas, 3B6 labels the periphery of the cone and rod outer segments; and (c) 3B6 labeling is absent from regions where the disk membranes are open in both rod and cone outer segments.

### Peripherin/*rds* in Rod Photoreceptors

Peripherin/*rds* protein has been shown previously to be localized along the rims of mature bovine rod disk membranes, using two mAbs, one of which was 3B6 (Molday et al., 1987). We have found the same distribution in mature rat rod disks. Examination of the nascent disks, however, indicates that they are not labeled, except at the connecting cilium. Nascent disks are open to the extracellular space—i.e., the disk rims have not yet formed (Fig. 1; Steinberg et al., 1980)—so that this labeling pattern is consistent with a peripherin/*rds* distribution that is restricted to the disk rims.

### Peripherin/*rds* in Cone Photoreceptors

We have detected peripherin/*rds* in cone outer segments, as well as in rod outer segments, with the 3B6 antibody. The distribution of label in the cone outer segments is also con-

sistent with the presence of peripherin/*rds* only in the disk rims. Along the length of the outer segment, cone disks become closed less abruptly than do rod disks (Cohen, 1961, 1968; Anderson et al., 1978). In other words, development of the disk rim occurs more gradually in cones (Steinberg et al., 1980), so that the nascent disk zone extends further distally. In ground squirrel cones, 3B6 labeling was clearly greater on the ciliary side of the outer segment for most of the proximal half. A similar labeling pattern of frog cone outer segments has been described with an antibody against the 290-kD disk rim protein (Papermaster et al., 1982). In the distal region of ground squirrel cones, where the disks are nearly completely closed, the size and position of the openings are not uniform, although they are positioned away from the cilium, just not centered directly opposite it (Anderson et al., 1978). In ultrathin transverse sections small gaps in 3B6 labeling (as in Fig. 5 c) may correspond to these irregular openings.

### Relationship to Disk Morphogenesis

As described in the introduction, Steinberg et al. (1980) proposed a model for the morphogenesis of new disk membranes, based on conventional electron microscopy. This model has been discussed further by Corless and Fetter (1987). An important point made by these authors is that the disk rims develop by a second growth process. This process begins at the cilium, and is separate from the first growth process in which the disk surfaces develop. The alternative mechanism would be for the disk rims to be formed by reorganization of the nascent disk membranes. Immunolabeling of peripherin/*rds* is consistent with the proposal that rim development is a membrane growth process from the cilium. Observations of cone outer segments are particularly informative, for the gradual development of the rim results in a

larger open disk area, making the immunolabeled sections easier to interpret. In the basal part of the outer segment, 3B6 label was found only at the cilium. Moreover, its concentration at the cilium appeared similar at different positions along the outer segment (Fig. 5, compare *b*, *c*, and *d*), indicating that it is added only as the rim forms. From the amino acid sequence of peripherin/*rds*, it appears that it is a transmembrane protein (Connell and Molday, 1990), so that its addition at the time of rim formation implies the addition of new membrane at this time.

### Sorting of Outer Segment Membrane Proteins

Many of the membrane proteins which are present in rod outer segment plasma membranes are absent in disk membranes. For example, the cGMP-gated channel, the Na<sup>+</sup>/Ca<sup>2+</sup>/K<sup>+</sup> exchanger, ricin binding proteins, and membrane-associated glyceraldehyde-3-phosphate dehydrogenase are only present in the plasma membrane (Cook et al., 1989; Reid et al., 1990; Molday and Molday, 1987; Hsu and Molday, 1990); peripherin/*rds* and the 290-kD rim protein are predominantly, if not exclusively, present in the rim regions of the disk membrane (Molday et al., 1987; Papermaster et al., 1978). Rhodopsin is an exception in that it is abundantly present in both the disk and plasma membranes (Kamps et al., 1982; Molday and Molday, 1987). This segregation leads to the question of how outer segment membrane proteins are sorted into their different membrane domains. Are they transported separately to their final destination, or are both populations mixed first in the nascent disks, and then segregated upon disk closure? Part of the answer comes from our observations of peripherin/*rds* labeling. The results presented here clearly indicate that this disk-specific protein remains associated with the cilium until the stage when the disk membranes actually separate from the plasma membrane, i.e., during rim development. Thus, it appears that peripherin/*rds* is never incorporated into the evaginations of the nascent disk membranes. Elucidation of just what proteins are present throughout the nascent disks should give further insight into this important question of protein sorting.

### *rds* Gene Product

Mice that are homozygous for the *rds* mutation fail to develop rod and cone outer segments during the 3-wk postnatal period (Sanyal and Jansen, 1981; Cohen, 1983; Jansen and Sanyal, 1984). The rest of the photoreceptor cell, including the synaptic region, cell body, inner segment and cilium, and other retinal cells develop normally. However, a slow degeneration of the photoreceptor cells occurs, such that few photoreceptor cells remain one year after birth. Recently, Travis et al. (1989) used subtractive hybridization methods to identify the wild-type gene and the mutation responsible for the *rds* phenotype. Sequence analysis has indicated that the *rds* gene product is peripherin/*rds* (Connell et al., 1991). The immunolabeling studies reported here indicate that peripherin/*rds* is present in cone outer segment disks, as well as rod outer segment disks. This result, together with the earlier findings that cone as well as rod outer segments fail to develop in the *rds* mouse, suggest that the same wild-type *rds* gene may be expressed in both rod and cone photoreceptor cells, and that the same *rds* mutation may be expressed in cone as well as rod cells.

The function of peripherin/*rds* disk rim membrane protein is not known. Ultrastructural studies of *rds* photoreceptor cells indicate that opsin-containing vesicles bud from the cilium and accumulate in the subretinal space, but disk-like structures are not observed (Cohen, 1983; Nir et al., 1990). These studies suggest that peripherin/*rds* may play a direct role in the morphogenesis of disk membranes in rod and cone photoreceptors. From the finding that peripherin/*rds* is localized only along ciliary side of nascent or immature disks, one can speculate that the cytoplasmic domain of peripherin/*rds*, or associated proteins, may interact directly or indirectly with axonemal proteins, to anchor the evaginating nascent disks. As the disks are displaced upward, this interaction is perhaps lost, and peripherin/*rds*, in concert with other rim proteins, may then expand outward to form the rims of the disks. The large intradiskal domain of peripherin/*rds*, suggested by the model of peripherin/*rds* (Connell and Molday, 1990; Travis et al., 1991a), may be important in developing the highly curved region in between the nascent disks adjacent to the cilium, and the highly curved rim region of mature disks. The absence, or the presence of an altered form of peripherin/*rds* in the *rds* mouse might prevent essential interaction between the nascent disks and the axoneme. Accordingly, formation of nascent and mature disks would not occur. Rhodopsin and other outer segment proteins that are transported to the cilium might be expected to be found in irregular membrane vesicles budding from the cilium, as recently described by Nir et al. (1990).

During the preparation of this paper, Travis et al. (1991a) published a report on the distribution of the *rds* gene product. In this study, Lowicryl-embedded ultrathin sections of mouse retina were labeled with a polyclonal antiserum generated against a synthetic peptide that corresponds to a region near the carboxyl terminus of the *rds* gene product. This antiserum labeled the entire rod outer segment at a level just above that of controls. This result is in contrast to the peripheral disk labeling pattern observed in the present and previous studies (Molday et al., 1987; Connell and Molday, 1990) with two mAbs, 3B6 and 2B6. Travis et al. (1991a) have suggested that the epitopes recognized by 3B6 and 2B6 are selectively masked in the nonrim part of the disk. However, the following indicate that this suggestion is unlikely: Firstly, the epitopes recognized by 3B6 and 2B6 are very close to the peptide segment used by Travis et al. (1991a) to prepare the antiserum. Secondly, if the labeling pattern described in the present report is a result of selective masking, epitopes must be masked not only in the nonrim part of a mature disk, but also in an immature (nascent) disk, including its open edge. Furthermore, the epitope for the polyclonal antibody would have to be largely masked in the rim region and not in the lamellar region of disks, for no significant labeling of the disk rim region was observed by Travis et al. (1991a). Thirdly, we find the same peripheral rim-specific labeling pattern by labeling Lowicryl and L. R. White-embedded sections (present and previous [Molday et al., 1987] studies) and cryosections (C. L. Schlamp and D. S. Williams, unpublished results) of retinal tissue from a variety of different species (bovine, rat, mouse, and squirrel), and also by preembedding labeling of unfixed and glutaraldehyde-fixed lysed rod outer segments and isolated disks (Molday et al., 1987; Connell and Molday, 1990). Finally, ROM-1, a protein whose amino acids show 35% positional identity

with those of the peripherin/*rds* protein, has been localized along the rim region of rod outer segment disks (Bascom, R. A., G. Connell, J. Garcia-Heras, L. Collins, D. Ledbetter, R. S. Molday, V. Kalnins, and R. R. McInnes. 1990. *Amer. J. Hum. Genet.* 47:392 (Abstr.).

Mutations in the human homologue of the peripherin/*rds* gene have now been shown to be the basis of a form of autosomal dominant retinitis pigmentosa, in which both the rod and cone photoreceptors degenerate (Farrar et al., 1991; Kajiwara et al., 1991).

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