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Evidence From Normal and Degenerating Photoreceptors That Two Outer Segment Integral Membrane Proteins Have Separate Transport Pathways

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ABSTRACT

Detachment of the neural retina from the retinal pigment epithelium induces photoreceptor degeneration. We studied the effects of this degeneration on the localization of two photoreceptor outer segment-specific integral membrane proteins, opsin and peripherin/*rds*, in rod photoreceptors. Results from laser scanning confocal microscopic and electron microscopic immunolocalization demonstrate that these two proteins, normally targeted to the newly-forming discs of the outer segments, accumulate in different sub-cellular compartments during photoreceptor degeneration: opsin immunolabeling increases throughout the photoreceptor cell's plasma membrane, while peripherin/*rds* immunolabeling occurs within cytoplasmic vesicles. The simplest hypothesis to explain our results is that these proteins are transported in different post-Golgi transport vesicles and separately inserted into the plasma membrane. More complex mechanisms involve having the two co-transported and then opsin finds its way into the plasma membrane but peripherin/*rds* does not, remaining behind in vesicles. Alternatively, both insert into the plasma membrane but peripherin/*rds* is recycled into cytoplasmic vesicles. We believe the data most strongly supports the first possibility. Although the transport pathways for these proteins have not been fully characterized, the presence of peripherin/*rds*-positive vesicles adjacent to the striated rootlet suggests a transport role for this cytoskeletal element.

The accumulation of these proteins in photoreceptors with degenerated outer segments may also indicate that their rate of synthesis has exceeded the combined rates of their incorporation into newly forming outer segment disc membranes and their degradation. The accumulation may also provide a mechanism for rapid recovery of the outer segment following retinal reattachment and return of the photoreceptor cell to an environment favorable to outer segment regeneration. J. Comp. Neurol. 387:148–156, 1997.

Indexing terms: laser scanning confocal microscopy, immunofluorescence, opsin, peripherin/*rds*, retinal detachment

Vertebrate photoreceptors are sensory neurons that have a highly polarized and compartmentalized morphology. Their light sensitive organelle, the outer segment (OS), is composed of a stack of flattened membranous vesicles enclosed by the plasma membrane (Fig. 1; Dewey et al., 1969; Young, 1970). Each disc has two lamellar faces joined at their periphery by a highly curved, electron-dense rim (Papermaster et al., 1978a,b). Integral membrane proteins within the discs occupy morphologically distinctive domains (Papermaster et al., 1982). In rod OS, opsin, a light sensitive, integral membrane glycoprotein is

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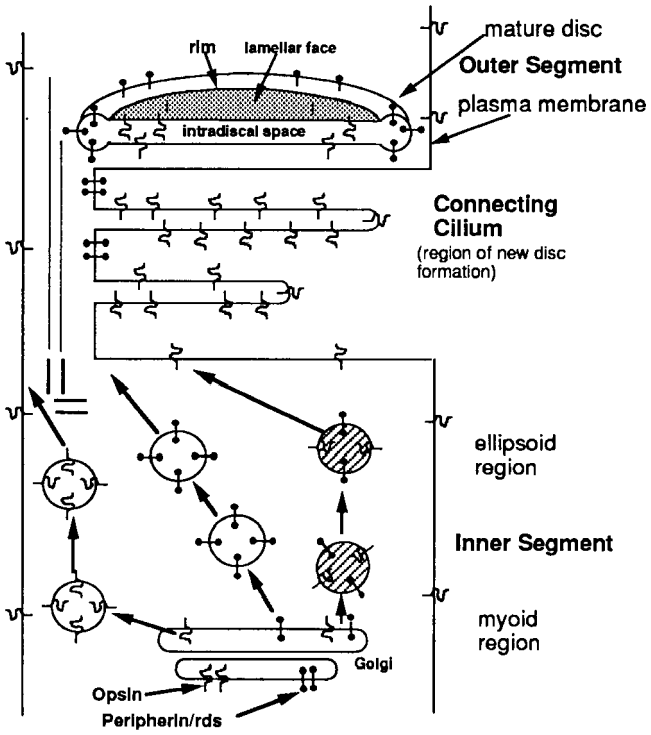

Fig. 1. Two hypothetical pathways for the transport of opsin and peripherin/*rds*. Both proteins are synthesized in the myoid region of the photoreceptor inner segment and are subsequently transported to connecting cilium where they are incorporated into newly formed disc surfaces (the lamellar face in the transected mature disc) or rim. The proteins may be transported together within a single population of vesicles (shaded circles) and sorted during their incorporation into the nascent discs or they may be separated earlier in their transport path into separate vesicles.

restricted to the lamellar face of the discs (Molday et al., 1987). In contrast, another integral membrane glycoprotein, peripherin/*rds*, is concentrated in the region of the rim (Molday et al., 1987; Arikawa et al., 1992).

The OS are dynamic organelles that undergo high rates of renewal. It has been estimated that a rat photoreceptor assembles 0.1 μm^2 of membrane per minute (Young, 1967). Newly synthesized membrane proteins are incorporated into nascent discs at the OS base (Young, 1967; Young and Droz, 1968). Discs are displaced toward the apex of the OS where they are eventually shed and phagocytosed by the adjacent retinal pigment epithelium (RPE; Young and Bok, 1969; Ishikawa and Yamada, 1970). Disc morphogenesis occurs by at least two distinct but coordinated steps. The first is membrane evagination which forms the lamellar surfaces of the disc. The second is membrane fusion in which the rim joins these surfaces (Steinberg et al., 1980; Arikawa et al., 1992). These two processes depend on the transport of the molecular constituents of the discs (opsin and peripherin/*rds* among others) from their site of synthesis in the myoid region of the inner segment (IS) to the area of disc formation (Fig. 1). Arikawa et al. (1992) presented evidence that opsin and peripherin/*rds* are inserted independently into newly forming discs as surface and rim formation proceed. Although sorting of these components must occur since they occupy different domains in the disc, it is unknown if the two constituents are transported together in a common carrier vesicle (Fig. 1, hatched circles) and then sorted as part of the disc morphogenic process, or if they are sorted earlier in the synthesis/transport pathway into independent, specific transport vesicles (Fig. 1, open circles). Although Deretic and Papermaster (1991) have demonstrated a population of opsin-immunopositive vesicles within the IS, peripherin/*rds*-immunopositive vesicles have not been identified in photoreceptors.

A number of studies have shown that a small amount of opsin is detectable in the plasma membrane surrounding the "inner portion" (IS, cell body, axon, and synaptic terminal) of the photoreceptor cell and that OS degeneration increases the opsin content of this particular domain (Nir and Papermaster, 1986; Usukura and Bok, 1987a,b; Jansen et al., 1987; Nir et al., 1987; Bowes et al., 1988; Nir et al., 1989; Nir and Papermaster, 1989; Nir et al., 1990; Lewis et al., 1991; Edward et al., 1993). In the current study, we investigated changes in opsin and peripherin/*rds* immunolocalization during induced OS degeneration with the goal of determining if both proteins accumulate within the plasma membrane. The data, indicating that they do not, provide us with information relevant to the sorting and transport of these integral membrane proteins in photoreceptors.

MATERIALS AND METHODS

Experimental retinal detachment

A portion of the neural retina of the right eye of young adult domestic cats was detached by using the method of Anderson et al. (1986). In this procedure, the vitreous of the lensectomized eye was removed and a 0.25% solution of sodium hyaluronate (Healon™, Pharmacia, Piscataway, NJ) was infused beneath the neural retina separating the retina and RPE. The contralateral eyes of cats used in this study were not subjected to surgical manipulation. Four hours after light onset, cats were euthanized by intravenous injection of sodium pentobarbital 3, 7, or 28 days after retinal detachment and the retinas prepared for immunocytochemistry.

Tissue fixation

Following enucleation of experimental and control eyes, the anterior segment of the eye was removed and its posterior segment fixed by immersion in chilled 4% paraformaldehyde buffered to pH 7.3 with 0.1 M sodium cacodylate buffer. Eye cups were held in fixative for at least 12 hours at 4°C.

Processing of retinal tissue for immunocytochemistry

Retinal samples were prepared for laser scanning confocal microscopic (LSCM) analysis according to the method described by Hale and Matsumoto (1993). Briefly, rectangular pieces of fixed cat retina, or the posterior half of rat eyes, were transferred to chilled 0.1 M phosphate-buffered saline (PBS), pH 7.3. Retinal samples were washed in chilled PBS three times (15 minutes per wash) followed by a 1 hour rinse in chilled PBS to remove residual aldehydes. Retinal samples were then embedded in 5% molten agarose (Type XI, Low gelling temperature, Sigma Chemical Co., St. Louis, MO) in PBS. Agarose-embedded retinal

samples were cooled to 4°C for 30 minutes to ensure complete solidification of the gel matrix in preparation for sectioning. A Vibratome™ tissue slicer (Series 1000, Technical Products International, Inc., St. Louis, MO) was used to cut 100 μm thick sections of agarose-embedded retina. Sections were stored briefly in PBS. To reduce non-specific antibody binding, sections were incubated in normal goat serum (Vector Laboratories, Inc., Burlingame, CA) diluted 1:50 in PBS containing 0.5% BSA (Bovine serum albumin, Fraction V, Sigma Chemical Co., St. Louis, MO), 0.05% sodium azide, and 0.1% Triton X-100 at pH 7.30. All primary and secondary antibody solutions were diluted in this buffer. Following the blocking step, retinal sections were incubated overnight in primary antibody at 4°C.

The monoclonal antibody Per 3B6 was produced against the carboxyl terminus of rat peripherin/*rds* (Molday et al., 1987). This antibody recognizes peripherin/*rds* in rod and cone outer segments in a number of species (Molday et al., 1987; Arikawa et al., 1992) including cat. Two opsin antibodies were used in this study. Rho 4D2, a monoclonal antibody generated against the amino terminus of bovine rhodopsin (Hicks and Molday, 1986) was used at a 1:50 dilution in all single label experiments. Rho 4D2 recognizes rods but not cones in a number of species (Hicks and Molday, 1986; Arikawa et al., 1992), including cats. A rabbit polyclonal antibody generated against purified bovine rhodopsin was used in all double-label experiments at a 1:10 dilution (Usukura and Bok, 1987a,b). After removal from primary antibody solution, sections were washed repeatedly in chilled antibody buffer and incubated overnight in an appropriate secondary antibody (goat anti-mouse or goat anti-rabbit) cyanine-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Following incubation in secondary antibody, sections were washed in chilled antibody buffer and mounted on glass slides. Primary antibodies were omitted from sections used as controls.

Image collection and processing

Immunofluorescence analysis was performed on a modified Bio-Rad 500 LSCM (Bio-Rad, Hercules, CA). All images were stored unprocessed on optical disks. Images selected for publication were digitally processed to improve image sharpness by using local contrast enhancement and gamma curve remap algorithms included in the Comos image acquisition program (Bio-Rad, Hercules, CA). The images selected for publication were imported to Photoshop (Adobe Systems inc., Mountain View, CA) with contrast and sharpness only adjusted to match the images as they appeared on the LSCM monitor.

Pre-embedding electron microscopic immunocytochemistry

The electron microscopic localization of peripherin/*rds* was accomplished by using a pre-embedding labeling technique modified from Griffiths (1993). One hundred micrometer thick sections of agarose-embedded cat retina were incubated overnight at 4°C in the peripherin/*rds* antibody Per 3B6 diluted 1:10. Sodium azide was omitted from all buffers used in this technique to avoid quenching peroxidase activity. After thorough washing, the sections were transferred to horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1:200. Sections were incubated overnight at 4°C, and then washed and pre-incubated in 1 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB; Polysciences, Inc., Warrington, PA) in 0.1 M Tris-HCl buffer, pH 7.6, for 30 minutes. They were then transferred to a DAB/Tris-HCl buffered solution containing 0.02% H_2O_2 for 13 minutes to generate an electron-dense reaction product. Oxidation times were standardized for all experimental and control groups. After post-fixation in 1% paraformaldehyde + 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, overnight and 1% OsO_4 for 1 hour, the sections were prepared for electron microscopy by dehydration in ethanol and re-embedded in Spurr resin (Spurr, 1969). Thin sections were stained sequentially with uranyl acetate (2%) in H_2O and lead citrate and examined using a Phillips CM 10 transmission electron microscope.

Adherence to guidelines on the humane care and use of laboratory animals

All procedures using animals were conducted according to guidelines described in the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care Council of the University of California, Santa Barbara.

RESULTS

Immunolabeling results in normal retina

The patterns of immunofluorescence for both the rhodopsin and peripherin/*rds* antibodies by LSCM in normal cat retina are shown in Figure 2A,B. As expected, labeling

Fig. 2. Confocal microscope immunofluorescence images of normal (A,B) and detached (C–H) cat retina. Images have been paired to allow comparison of immunolabeling patterns for opsin (A,C,E,G) and peripherin/*rds* (B,D,F,H). **A**: Normal retina. The Rho 4D2 antibody to opsin intensely labels the rod outer segment (OS) and lightly labels the plasma membrane surrounding photoreceptor cell bodies in the outer nuclear layer (ONL). The brackets indicate an area of fluorescent signal arising in the myoid of the inner segment (IS). The arrowhead indicates three small punctate structures in the ellipsoid region of the IS labeled with the antibody. **B**: Normal retina. The peripherin/*rds* antibody Per 3B6 intensely labels normal photoreceptor OS where it is restricted to disc rims as shown in the cross sectional view (arrowhead, inset). Punctate labeling also occurs within the myoid region of the IS (arrowheads). **C**: Three-day detachment. Some photoreceptors begin to show an increase in the intensity of opsin immunolabeling of the plasma membrane surrounding their IS and cell bodies. The IS labeling is now diffuse and poorly defined compared to that in normal retina. **D**: Three-day detachment. There is a noticeable increase in the intensity of peripherin/*rds* immunolabeling in the IS, and punctate, labeled structures now occur in the photoreceptor cell bodies in the ONL(arrowheads). **E**: Seven-day detachment. OS are now very short and there is a significant increase in the magnitude and intensity of opsin immunolabeling associated with the plasma membrane of the photoreceptors so that the whole layer of cell bodies (ONL) is intensely labeled and individual cell processes can be seen extending to synaptic terminals on the lower boarder of the ONL. **F**: Seven-day detachment. Although the photoreceptors in this region have longer OS than those in E, they have peripherin/*rds* labeling distributed more broadly throughout the IS and ONL (arrowheads) than in normal or 3-day detached retina. **G**: Twent-eight-day detachment. Opsin immunolabeling is greatly elevated throughout the entire photoreceptor plasma membrane. **H**: Twenty-eight-day detachment. Peripherin/*rds* immunolabeling is visible across the ONL where it occurs as punctate deposits. OS are almost undetectable in this region. A,B,D–H = ×1,750; C = ×3,150; scale bar = 5 μm; B inset, ×5250, scale bar = 1 μm.

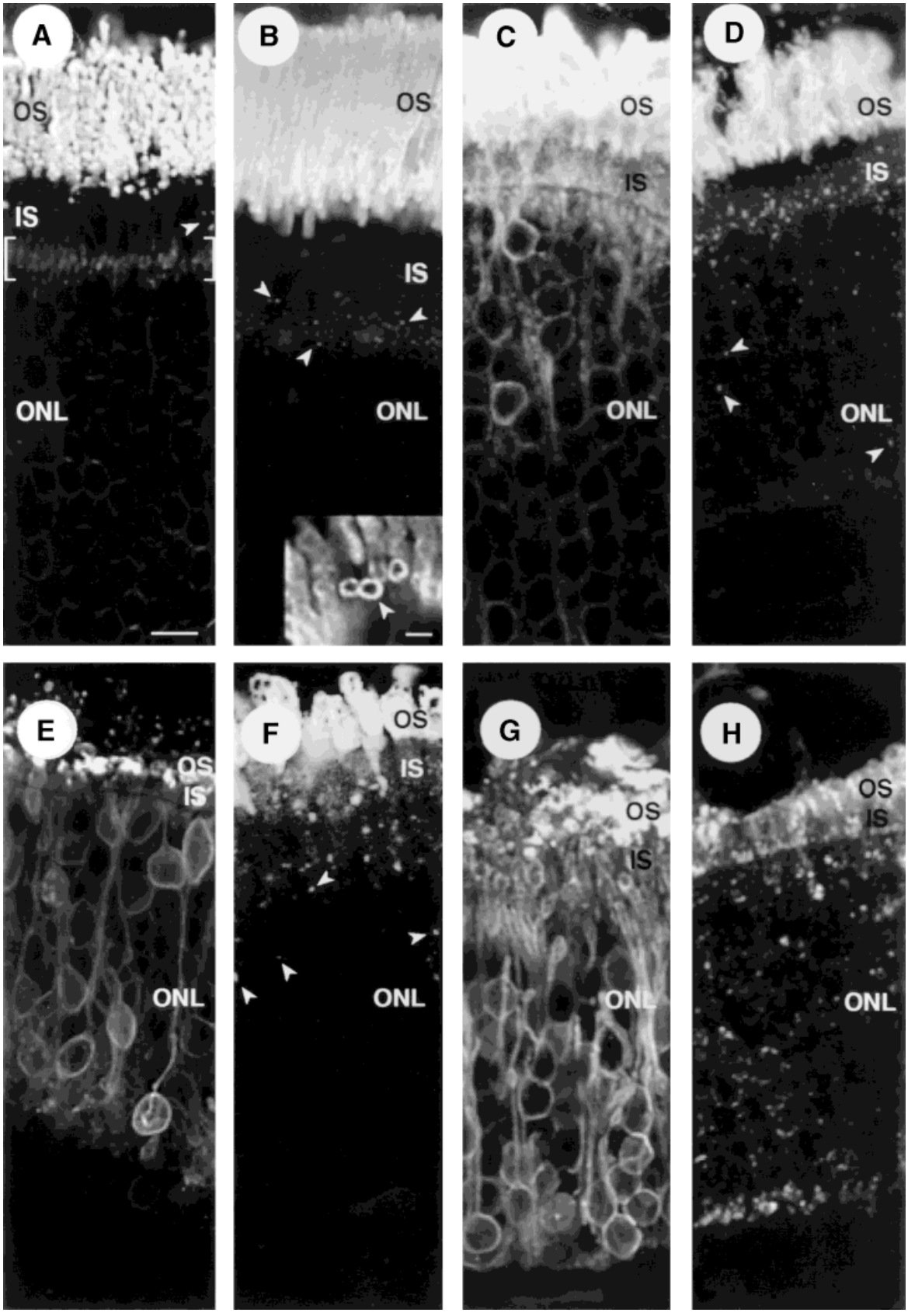
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Figure 2

with the opsin antibody (Fig. 2A) is most intense in the OS. Next in intensity is the labeling present as a compact repeated pattern across the layer of inner segments (between the brackets in Fig. 2A). This labeling occurs within the myoid region of the IS in the area where the RER and Golgi are located. We occasionally observed faint punctate labeling with the opsin antibodies between the area of staining in the myoid and the outer segment (arrowhead, Fig. 2A). There is also faint, but reliable labeling of the plasma membrane surrounding the IS and the cell bodies of the outer nuclear layer (ONL).

The monoclonal antibody against peripherin/*rds*, Per 3B6, intensely labels rod OS (Fig. 2B). Labeling patterns obtained with LSCM when the outer segments are viewed in cross-section (Fig. 2B inset) and by electron microscopic immunolocalization (data not shown) demonstrate that labeling is restricted to the OS disc rims and incisures as reported in other species (Molday et al., 1987; Arikawa et al., 1992). In the same location within the myoid region where opsin immunolabeling shows a compact pattern, the antibody to peripherin/*rds* produces a fainter, loose, punctate pattern (arrowheads, Fig. 2B).

Immunolabeling results in detached retina

Changes in the patterns of opsin and peripherin/*rds* immunolabeling are apparent within 3 days of detachment (Fig. 2C). In the case of opsin, there is an increased intensity of labeling of the plasma membrane surrounding the IS and cell bodies in the area of detachment. Outer segments are undergoing degeneration at this time and they appear shorter than normal but they remain intensely opsin-immunoreactive. Subtle changes occur in the intracellular labeling pattern in the IS where labeling no longer forms compact reticular-appearing structures as in Figure 2A but appears much more widely distributed and diffuse within the shortened IS (Fig. 2C). Changes in peripherin/*rds* antibody labeling are more subtle in the 3 day detachments where there is a slight, but consistent increase in the intensity of the punctate labeling within the IS and the punctate structures now appear for the first time within the cell bodies of the ONL (arrowheads, Fig. 2D).

At 7 days post-detachment, opsin immunoreactivity continues to increase in both extent and intensity within the plasma membrane, now extending from the OS to the synaptic terminals (Fig. 2E). Peripherin/*rds* labeling retains its punctate appearance but the labeled deposits appear larger and more widely dispersed within the cells (Fig. 2F).

In the detachments of 28 days duration, all photoreceptor cells within the zone of detachment show very high levels of opsin immunoreactivity throughout their plasma membrane (Fig. 2G). In these detachments the punctate peripherin/*rds* labeling occurs throughout the ONL (Fig. 2H). As shown in our earlier morphological studies, in general the longer the detachment time, the greater the level of OS degeneration (Erickson et al., 1983; Anderson et al., 1983; Guérin et al., 1993) but significant variation can occur within a detachment and between animals with detachments. For example, the photoreceptors illustrated in Figure 2E,H retain only very short OS compared to those in a neighboring area of detached retina (shown in Fig. 2F,G). As shown in these figures, even the shortest, most disrupted OS continue to label with antibodies to both opsin and peripherin/*rds*. Figure 3A shows an example of a 28 day detachment in an area where OS degeneration is less extensive than in Figure 2H, and hence the amount of punctate label in the ONL is also reduced. In this case, the peripherin/*rds* labeling pattern in a single photoreceptor can be discerned and intense labeling occurs in the cytoplasm around the nucleus and as a line of punctate structures extending through the axon (arrowheads, Fig 3A) to the cell's synaptic terminal (arrow, Fig. 3A).

Observations by higher magnification LSCM (Fig. 3B) and by pre-embedding immuno-electron microscopy (Fig. 3C) show the intracellular nature of the peripherin/*rds* labeling pattern and its apparent association with clusters of membrane-bound vesicles. Although the electron-dense reaction product generated by the interaction of DAB and HRP diffuses and often obscures the membranes, our impression is that it is, in fact, always associated with vesicles 50–130 nm in diameter (Fig. 3C and inset). When post-embedding immunogold procedures were used in an attempt to further define this intracellular labeling, we found gold particles only over membrane-bound vesicles. However, this technique proved unreliable because of the low incidence of labeling, probably due to the paucity of antigen available for binding to the primary antibody in the surface-labeling technique. By LSCM we often observed thin strands of fluorescence with the peripherin/*rds* antibody extending from the base of the OS into the IS (arrowheads, Fig. 4A). By electron microscopic immunolocalization we could also find a similar pattern consisting of a strand of labeled vesicles lying along the striated rootlet—a cytoskeletal structure that extends from the basal body of the connecting cilium into the IS (Fig. 4B). Since this pattern was observed in several instances by both techniques, we assume that it represents the labeling of the same structures.

DISCUSSION

Physical trauma to the eye or any one of several diseases can cause the separation of the neural retina from the RPE, a condition that leads to extensive OS degeneration in all of the photoreceptor cells in the affected area (Anderson et al., 1983; Erickson et al., 1983). In this study we performed a series of experiments to evaluate changes in the localization of two proteins targeted to two highly specific and separate domains within the rod OS during this induced OS degeneration. Clinically these studies are significant because they provide new information about the pathophysiology of photoreceptor degeneration. In addition, studies like this one using photoreceptors with degenerating outer segments as a model can provide insight into the process of protein trafficking in these cells, probably because OS degeneration effectively interrupts the normal target of the OS-specific membrane proteins.

Rod OS membrane renewal is a complex process in vertebrates. Besides occurring at exceptionally high rates (Young, 1967; Young and Droz, 1968), the newly synthesized OS proteins must move from their site of synthesis in the myoid to the apical end of the cell body where they are then inserted into forming disc membranes (Papermaster et al., 1985; Young, 1968). As the discs mature, their rims form and they are separated from the plasma membrane to form the mature disc stack within the plasma membrane of the OS. Thus, there is both a highly directed translocation of the disc membrane proteins and a highly ordered

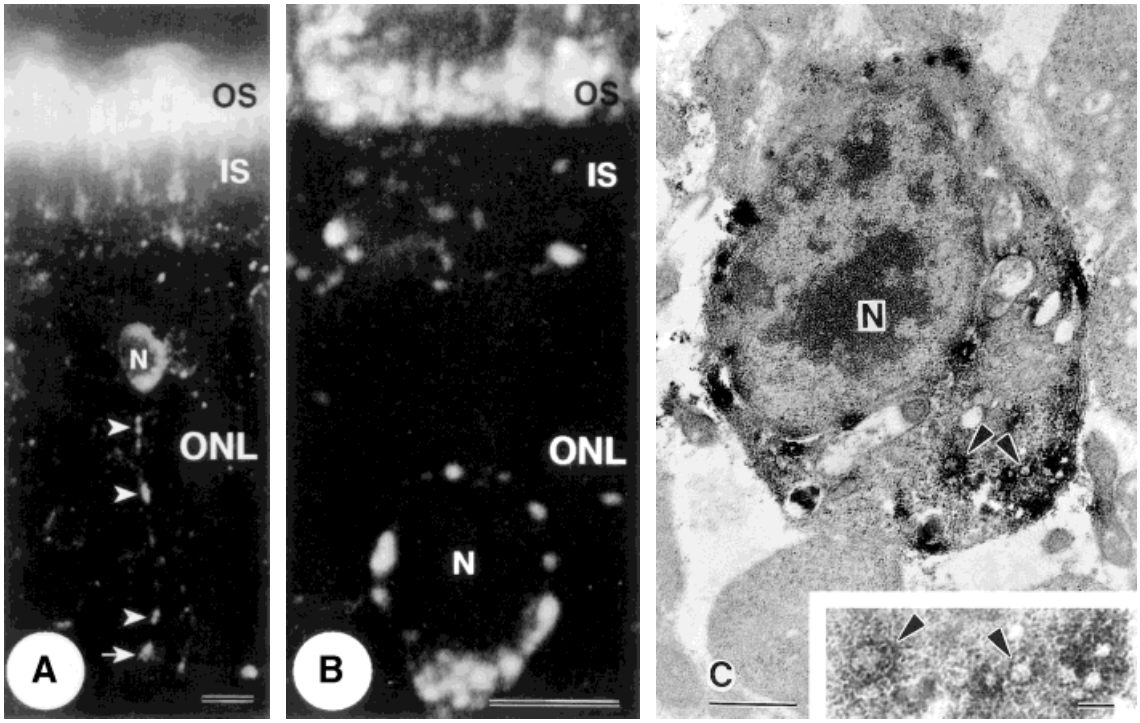

Fig. 3. Confocal immunofluorescence (A,B) and electron microscopic (C) immunolocalization of peripherin/*rds* in photoreceptors detached for 28 days. **A**: In this region, OS are less degenerated than in the example shown in H, and there is less of the punctate label across the ONL. This allows the visualization of label in a single photoreceptor cell where it occurs most heavily as deposits in the body around the nucleus (N), and as punctate structures along the axon (arrowheads) and in the synaptic terminal (arrow). **B**: Higher magnification image showing labeling of the shortened OS as well as distinctive granular structures in the perinuclear cytoplasm around the nucleus (N). **C**: Electron microscopic immunocytochemistry demonstrates that the peripherin/*rds* observed in the cell body by immunofluorescence is located in the perinuclear cytoplasm of the photoreceptors, where it appears to be associated with clusters of vesicles (arrowheads, and inset). The two arrowheads are indicating the same vesicles in the figure and the inset. A, ×1,200, scale bar = 5 μm; B, ×3,400, scale bar = 5 μm; C, ×11,200, scale bar = 1 μm; inset ×24,000, scale bar = 1 μm.

insertion of them into the forming discs (Steinberg et al., 1981).

Opsin and peripherin/*rds* segregate into different compartments

In this study, we sought to determine if two of the disc-specific membrane proteins would come to occupy the same or different subcellular domains when the OS is induced to degenerate. This was accomplished by using the imaging capabilities and increased sensitivity of LSCM along with pre-embedding immunocytochemistry without specimen dehydration and embedment (Hale and Matsumoto, 1993). In the past, high-resolution localization of proteins within cells has generally relied upon electron microscopy of thin sections (e.g., immunogold), which employs preparative techniques that reduce antibody binding due to fixation and dehydration of the tissue (Griffiths, 1993). Additionally, these techniques restrict antibody-antigen interactions to the surface of the section (Nir et al., 1984). Together, these characteristics limit the assay's sensitivity when attempting to localize proteins that are not particularly abundant. In contrast, pre-embedding immunocytochemistry permits a greater degree of antibody binding and thus increased sensitivity. The combination of the relatively gentle fixation, the retention of an aqueous embedding milieu to support protein-protein interaction, a pre-embedding technique, which gives antibodies the potential of binding to antigens throughout the interior of detergent treated cells, and the resolution afforded by both LSCM and electron microscopy allowed us to follow the differential segregation of opsin and peripherin/*rds* as OS degeneration proceeds. Furthermore, by LSCM, we also found subtle differences in the subcellular localization patterns of the two in the IS region of normal photoreceptors.

In normal OS, opsin and peripherin/*rds* immunoreactivity occurred in the same pattern by LSCM as by immunoelectron microscopy (Molday et al., 1987; Arikawa et al., 1992) inasmuch as labeling with the antibodies to opsin occurred over the entire OS, while the peripherin/*rds* antibody labeled the rims of OS discs. Since opsin constitutes 90% of the dry protein mass found in the OS (O'Brien et al., 1972; Basinger and Hall, 1973), the other OS integral membrane proteins such as peripherin/*rds* must be present in relatively small amounts (Papermaster et al., 1978a,b; Goldberg and Molday, 1996). Because all OS disc proteins are synthesized in the IS (O'Brien, 1977a,b; Papermaster et al., 1986), it might be assumed that the relative intensity of antibody labeling for opsin and peripherin/*rds* in the IS would reflect the great disparity between these two in the OS. In the myoid region, where synthesis occurs and the Golgi/endoplasmic reticulum (GERL) compartment is located, we find moderate labeling with the opsin antibodies. The label is present in an extensive network consistent with the organization of the GERL in these cells as described by Schmied and Holtzman (1989). Labeling with the peripherin/*rds* antibody on the other hand produced in this same area only a pattern

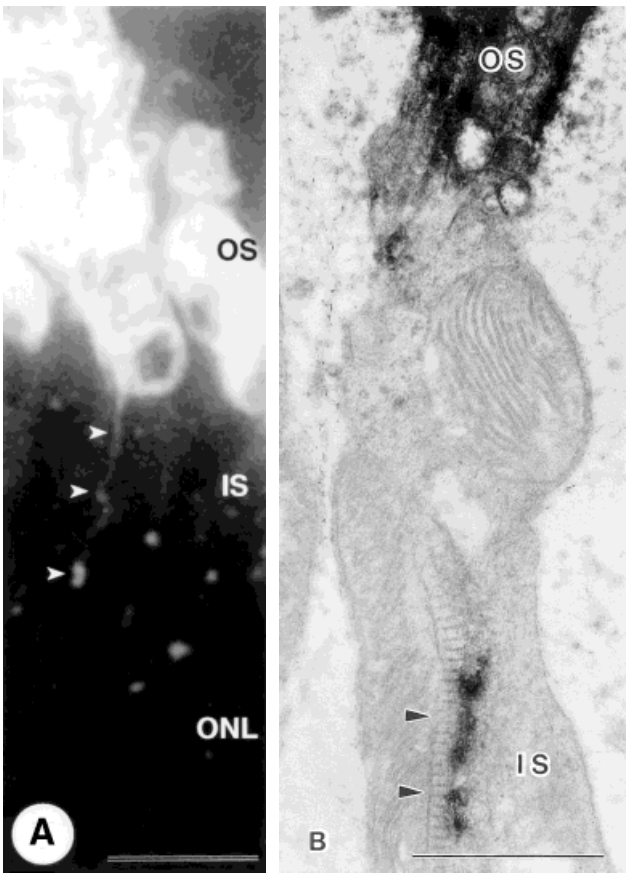

Fig. 4. Confocal immunofluorescence and electron microscopic immunolocalization of peripherin/*rds* in detached photoreceptors. **A**: Strands of peripherin/*rds* immunopositive material (arrowheads) often extend from the base of the OS into the IS. **B**: By electron microscopy, similar deposits of peripherin/*rds* immunolabeling are clearly visible adjacent to the striated rootlet (arrowheads). A, ×5,200, scale bar = 5 μm; B, ×32,000, scale bar = 1 μm.

of scattered, small, punctate structures. In general, the pattern that we observe for opsin is what would be expected for proteins that are produced in GERL by these cells (St. Jules et al., 1993). Thus, we may not be detecting a reticular pattern of peripherin/*rds* staining in the GERL because of its low abundance there (Goldberg and Molday, 1996). The punctate structures may represent a consolidation of this protein into small post-Golgi transport vesicles. The appearance of these structures is much like those described in the cytoplasm of cultured cells heterologously expressing peripherin/*rds* (Goldberg et al., 1995), and similar to the peripherin/*rds*-positive structures that progressively accumulate in the cytoplasm of the cells with degenerating OS. On the other hand, we did not observe large numbers of structures that could represent similar opsin-bearing vesicles. Although this may seem somewhat surprising given the abundance of this protein in rod photoreceptors, the result is consistent with numerous other studies reporting a relative paucity of presumed opsin transport vesicles in the IS (Bird et al., 1988; Eckmiller, 1989; Deretic and Papermaster, 1991). Plasma membrane labeling provides another clear difference between the antibody localization results for these two proteins. A low level of opsin immunoreactivity is always found in the plasma membrane around the proximal portion of normal photoreceptors, whereas labeling with the peripherin/*rds* antibody was never observed in this compartment. This held true during OS degeneration as well when there is a tremendous increase in the amount of opsin detected in the plasma membrane. Double-label experiments (data not shown) confirmed the non-overlap of signals for labeling with antibodies to the two proteins. Although we cannot rule out the possibility that some low level of peripherin/*rds*, undetectable by the current techniques, occurs in the plasma membrane, these results are consistent with those reported when the two proteins are expressed in cultured transfected cells (Oprian et al., 1987; Goldberg et al., 1995) and are thus consistent with the interpretation that, in general, peripherin/*rds* is prevented from entering the plasma membrane.

Detachment causes two events that may be relevant to the actual accumulation of these proteins normally destined for the OS. The first is the rapid degeneration of the OS and the inability of the cells to construct an OS in the absence of reattachment (Kroll and Machemer, 1968; Anderson et al., 1983). The second is the disruption of the normal route for OS membrane recycling—the shedding of old disc membranes from the OS and their phagocytosis and breakdown by the RPE (Young and Bok, 1969; Ishikawa and Yamada, 1970). The simple degeneration of the OS would not explain the accumulation of these two molecular species as reported here because it occurs via the breakdown of the OS into membranous debris that remains in the subretinal space until it is scavenged by macrophages (Anderson et al., 1986; Guérin et al., 1989; Thanos, 1992). The progressive accumulation of the OS disc proteins however can be explained if there is continued biosynthesis in the cell body, but a significant reduction in their incorporation into the OS, and if the cells lack an effective mechanism for removing these proteins other than through the normal shedding and phagocytosis process. Studies, both in vivo and in vitro, have shown that isolated photoreceptors continue to have high activity for opsin synthesis (Basinger and Hall, 1973; Bok et al., 1974; O'Brien and Muellenberg, 1975). During OS degeneration from genetic diseases, opsin biosynthesis is also maintained (Carter-Dawson et al., 1978; Agarwal et al., 1992). In autoradiographic studies, photoreceptors in detached retinas continue to show incorporation of radiolabeled amino acids into proteins which are then delivered to the greatly shortened OS (Lewis et al., 1991), but there is evidence that the rate of the OS renewal process is significantly reduced (Guérin et al., 1993; Kaplan et al., 1990; Hale et al., 1991). Thus the evidence favors a continued biosynthesis of OS disc components in the face of a greatly reduced ability of the cell to incorporate these molecules into a normal OS. In invertebrate photoreceptors, membrane protein recycling is via the usual mechanism of pinocytotic uptake of membrane components (Blest et al., 1979; Blest and Price, 1981), but since vertebrate photoreceptors have evolved another mechanism, one in which they are dependent upon the RPE for this function, they themselves may not have an effective mechanism for the lysosomal degradation of excessive amounts of OS-specific proteins. A certain amount of autophagy has been demonstrated to occur in the cell body of photoreceptors (Remé, 1981; Remé et al., 1986), and so the photoreceptors may have a mechanism for the breakdown of opsin that is recycled back into the cell body (because some is found in the plasma membrane of normal cells outside of the OS) but probably do not have such a mechanism for

peripherin/*rds* (if it is excluded from entering the plasma membrane). Thus, it seems reasonable to conclude that the accumulation of opsin and periperin/*rds* in proximal compartments of the photoreceptor cells is a result of the proteins being "trapped" in this location due to a greatly reduced capacity of the cells to form a normal OS in the face of continued biosynthesis of the molecules, and the cells not having an effective degradative pathway for these proteins.

Implications for the transport of opsin and peripherin/*rds*

Our results show a clear differentiation of the compartments into which opsin and peripherin/*rds* accumulate. Thus, our original hypothesis predicting that both would accumulate as has been shown for opsin in degenerating photoreceptors (Usukura and Bok, 1987; Nir et al., 1989; Lewis et al., 1991), that is, in the plasma membrane, is clearly not correct. Furthermore, the patterns seen for both proteins in the cells with degenerated OS is essentially an extension of the pattern seen in normal cells. We found no evidence that the peripherin/*rds*-positive vesicles also contain significant amounts of opsin, nor did we ever find evidence of significant peripherin/*rds* immunoreactivity in the plasma membrane. If these proteins accumulate into these compartments because their production continues but disc morphogenesis is significantly slowed, then the simplest interpretation of the data is that the two are transported in different vesicles. Opsin-bearing vesicles fuse with the plasma membrane and this molecule accumulates there; peripherin/*rds*-bearing vesicles cannot fuse with the plasma membrane, and hence they accumulate in the cytoplasm. There are, of course, other more complex interpretations. The molecules may be co-transported, but this requires either that opsin is inserted into the membrane while peripherin/*rds* remains behind in the vesicles, or that, contrary to the results in cultured cells, the peripherin/*rds* is inserted into the membrane but then specifically re-captured in pinocytotic vesicles. While these cannot be definitively ruled out, neither would they seem to be the most parsimonious interpretation of the data. The presence of peripherin/*rds* immunoreactivity along the striated rootlet provides intriguing new evidence that this organelle may be involved in the transport of the peripherin/rds vesicles.

Implications for OS recovery

It is conceivable that the accumulation of OS proteins by rod photoreceptor cells is advantageous in allowing the OS to recover rapidly from degeneration. Clearly photoreceptors do not lose the ability to produce OS components (see above) and, in fact, exhibit a well-documented ability to recover their OS from degeneration induced by vitamin A deficiency (Dowling, 1966; Dowling and Wald, 1981; Fain and Lisman, 1993), light damage (LaVail et al., 1991; Wen et al., 1995) or detachment (Kroll and Machemer, 1969; Anderson et al., 1986; Guérin et al., 1989). In amphibians, light-stimulated membrane assembly in rod OS is independent of protein biosynthesis, the cells apparently utilizing a pool of pre-formed membrane constituents (Matsumoto and Bok, 1984; Bird et al., 1988). The accumulation of opsin and peripherin/*rds* in detached photoreceptors may in fact represent a pool of membrane constituents maintained by the cell during the period of OS degeneration, and thus available for immediate assembly into new OS discs when favorable conditions for recovery (e.g., retinal reattachment) occur.

In summary, the observations indicate a distinct segregation of these two molecules in the cell. Their progressive accumulation could be explained by several mechanisms, but we favor the interpretation that the two are transported separately, and that opsin by virtue of its ability to fuse with the plasma membrane enters that domain, whereas peripherin/*rds*, which lacks this ability, remains in the cytoplasm in its transport vesicles.

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