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Glycoproteins specific for the retinal rod outer segment plasma membrane

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Two ricin-specific glycoproteins have been identified on neuraminidase-treated rod outer segment plasma membranes of bovine retinal photoreceptor cells. Ricin-gold-dextran particles were observed by electron microscopy to densely label the surface of neuraminidase-treated rod outer segments. Western blotting of proteins separated by SDS-gel electrophoresis indicated that two ricin-binding glycoproteins of M_r 230000 and 110000 are specific for the plasma membrane and are not found in disk membranes. These glycoproteins can serve as specific probes for the purification of the rod outer segment plasma membrane.

Rod outer segments are specialized organelles of vertebrate retinal rod photoreceptor cells which function in the primary events of visual excitation. They consist of a stack of approximately 1000 closed disk membranes surrounded by a plasma membrane which is separated from the disk membrane over most of the outer segment. Recent studies indicate that photobleaching of rhodopsin in disk membranes leads to a decrease in the cGMP-sensitive conductance of the rod outer segment plasma membrane as part of the visual process [1,2]. Despite its importance in the visual transduction process and in the rod outer segment renewal process, few studies have been reported on the isolation and analysis of the molecular composition and properties of the rod outer segment plasma membrane in relation to disk membranes 3,4]. This is due to the low amount of the plasma membrane (2–5%) relative to the disk membranes and to the inability to identify a plasma membrane specific marker which can be used as a guide for the purification of the plasma membrane. In this paper we report that neuraminidase treatment of rod outer segments exposes *Ricinus communis* agglutinin-binding sites on several membrane glycoproteins which are specific for the rod outer segment plasma membrane.

In initial studies, retina tissue samples dissected from bovine eyes under dim red light were fixed in 1.25% glutaraldehyde in phosphate-buffered saline (0.01 M sodium phosphate/0.15 M NaCl (pH 7.4)), exposed to light and incubated overnight at 4°C in Tris-buffered saline (0.01 M Tris/0.15 M NaCl (pH 7.4)) in the absence or presence of neuraminidase. Tissue pieces were directly labeled with *Ricinus communis* agglutinin (RCA-60 or RCA-120) conjugated to 8 nm gold-dextran particles [5] for visualization by electron microscopy. As illustrated in Fig. 1a, neuraminidase-treated rod cells showed dense labeling with ricin-gold-dextran particles along the extracellular surface of the rod outer segment and cilium connecting the outer segment to the inner segment. The rod inner segment was also labeled, but to a much lower degree. Retina tissue which had not been treated with neuraminidase was only sparsely labeled with ricin gold-dextran markers on both the surface of outer and inner segments (Fig. 1b) as previously

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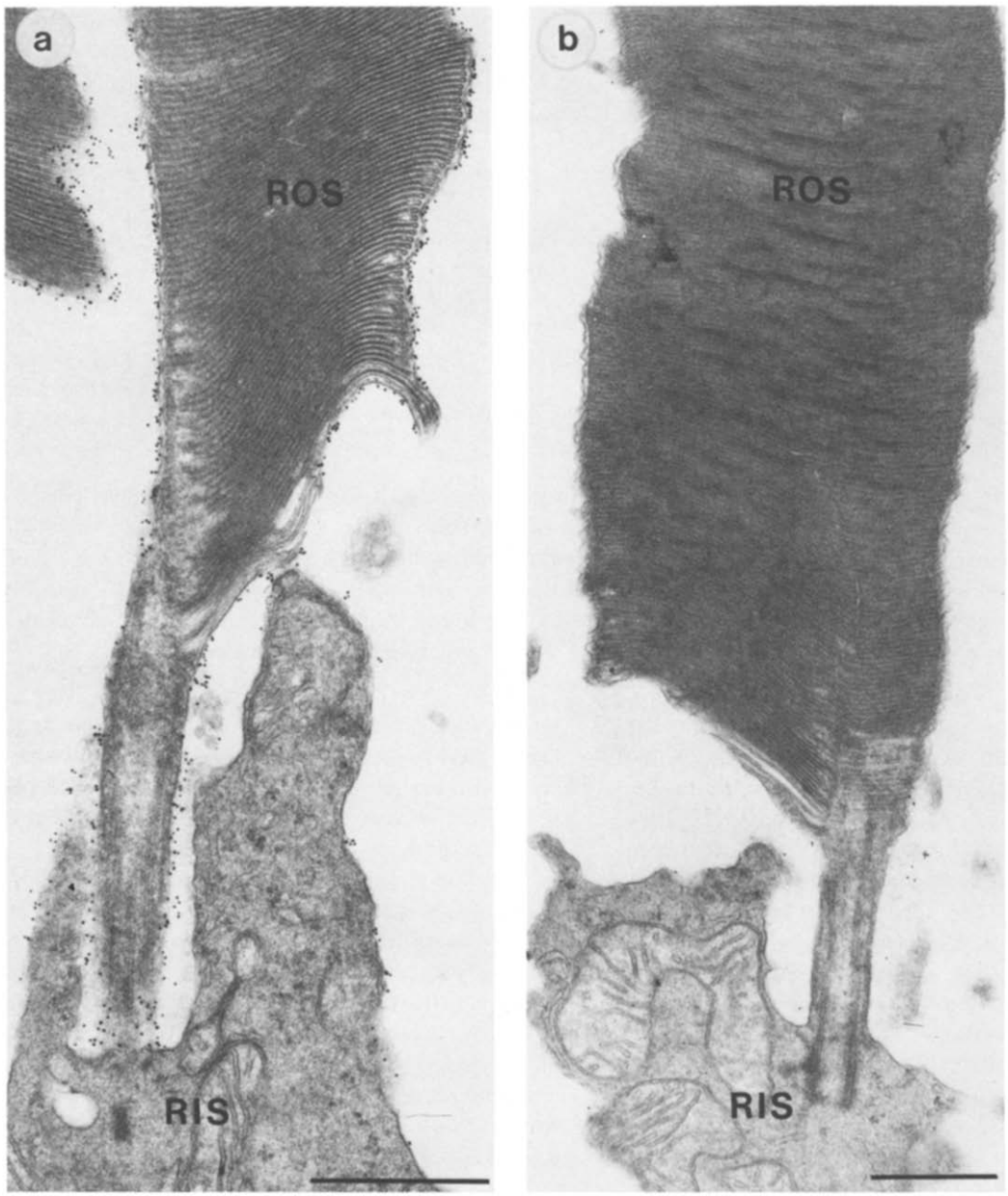


Fig. 1. Transmission electron micrographs of neuraminidase-treated and untreated bovine rod photoreceptor cells labeled with ricin-gold-dextran particles (8 nm diameter). Bovine retina tissue was fixed in 1.25% glutaraldehyde-0.1 M sodium cacodylate buffer (pH 7.2) containing 5% sucrose for 1 h and subsequently washed in Tris-buffered saline (0.01 M Tris/0.15 M NaCl (pH 7.4)). One piece of tissue (1 mm^2) was incubated with 0.1 units of neuraminidase (*Arthrobacter ureafacieus*) in Tris-buffered saline at 4°C overnight and another piece was incubated under the same conditions in the absence of neuraminidase. The tissue was then washed in Tris-buffered saline and treated with 0.3 ml, of ricin-gold-dextran particles [5] for 4 h at 25°C. Finally the tissue pieces were washed in buffer and prepared for electron microscopy by conventional procedures. (a) Neuraminidase-treated rod cell labeled with ricin-gold-dextran particles. Note the dense labeling of the rod outer segment (ROS) and connecting cilium. The rod inner segment (RIS) was also labeled, but less intensely. (b) Untreated rod cell labeled with ricin-gold-dextran particles. Only a few gold particles are seen on the cell surface. Bar, 0.5 μm.

reported [5,6]. Ricin-gold-dextran labeling was specific since galactose, an inhibitor of ricin eliminated labeling on both untreated and neuraminidase-treated retinal rod cells.

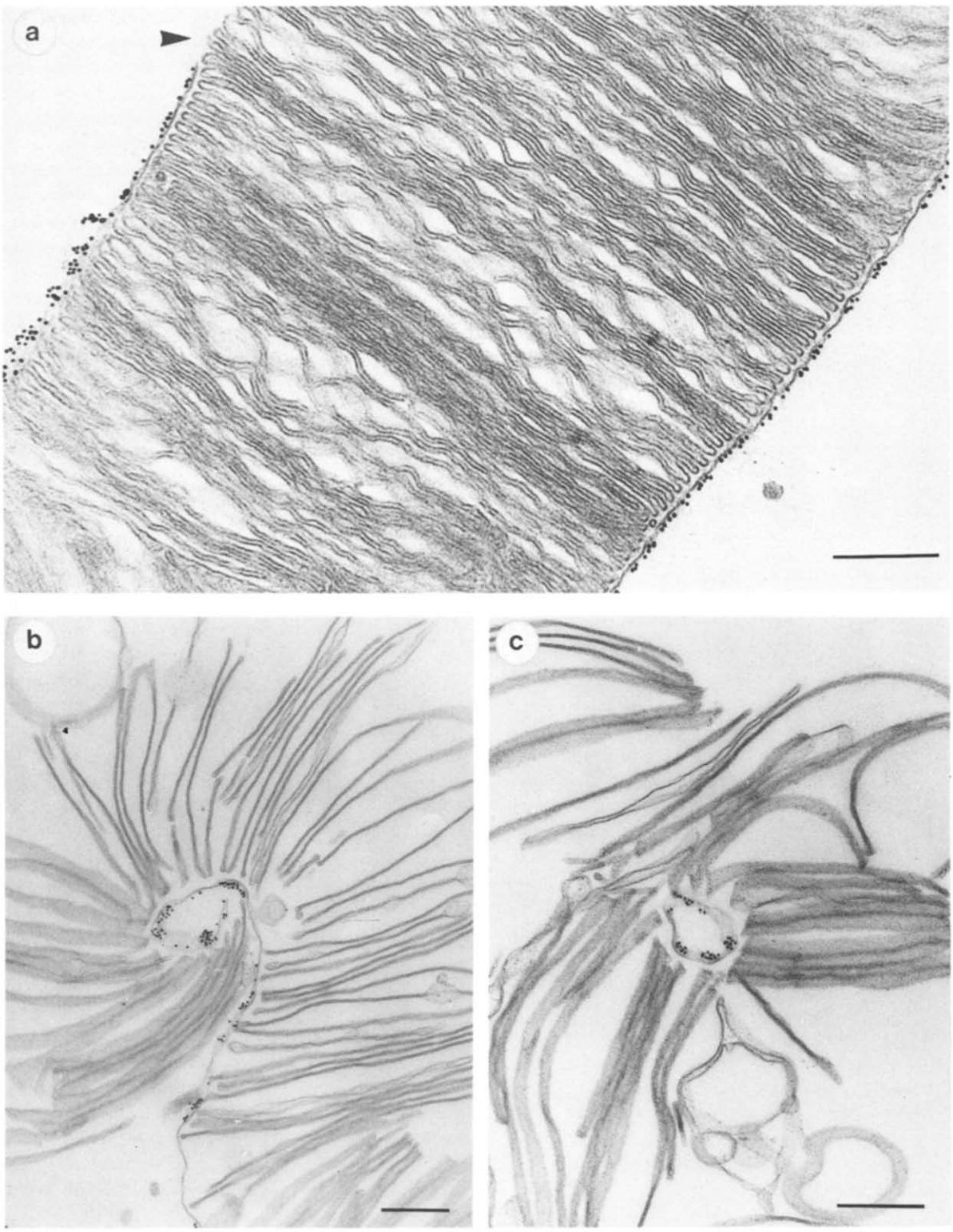
Unfixed rod outer segments purified by sucrose gradient centrifugation [7] could also be densely labeled with ricin gold-dextran particles after neuraminidase treatment (Fig. 2a). Gold particles lined the external surface of the rod outer segment plasma membrane. No labeling was observed on exposed disk membranes in broken rod outer segment fragments. When neuraminidase-treated rod outer segments which had been labeled with ricin gold-dextran particles were incubated in 0.02 M Tris buffer (pH 7.4) in the absence of sucrose, lysis of the outer segments occurred. Structures in which disks radiated from gold-labeled rod outer segment plasma membrane fragments or vesicles were frequently observed under the electron microscope (Figs. 2b, c) along with stacks of disks. Gold particles were sequestered along the inner surface of the plasma membrane vesicles. These results suggest that hypotonic lysis of rod outer segments results in the formation of inside out or inverted plasma membrane vesicles which are still associated with disk membranes possibly through a filamentous cytoskeletal system [9,10].

Ricinus communis agglutinin binding glycoproteins of untreated and neuraminidase-treated rod outer segments were analyzed by Western blots of rod outer segment proteins separated by SDS-polyacrylamide gel electrophoresis. Prior to electrophoresis and Western blotting rod outer segments stabilized in 20% sucrose were (1) incubated overnight at 4°C (untreated sample); (2) incubated overnight at 4°C in the presence of neuraminidase (neuraminidase-treated sample); or (3) solubilized in 1% Triton X-100 and incubated overnight at 4°C in the presence of neuraminidase (Triton X-100 solubilized, neuraminidase-treated sample). Results are shown in Fig. 3 for gels stained with Coomassie blue and Western blots labeled with ^{125}I-labeled ricin.

The Coomassie blue-stained gels confirmed the high purity of the rod outer segment fraction from the sucrose gradient. Rhodopsin appeared as the major component with an apparent M_r of 34000; other proteins including subunits of transducin (M_r 37000 and 39000), phosphodiesterase doublet (M_r 88000–90000) and the high molecular weight concanavalin A-binding glycoprotein rod outer segment 1.2 (M_r 220000) were also observed along with other minor bands [11,12]. The high purity of rod outer segments fractionated on sucrose gradients have also been confirmed by other laboratories [7,13]. No difference in the Coomassie blue-staining pattern was observed for the untreated, neuraminidase-treated or Triton X-100-solubilized neuraminidase-treated samples as shown in Fig. 3.

In Western blots of untreated rod outer segments, ricin faintly labeled bands of apparent M_r 34000 and 68000 (gel a′). In neuraminidase-treated rod outer segment samples ^{125}I-labeled ricin intensely labeled two proteins of apparent M_r 230000, and 110000 (gel b′). The band at 110000 appeared as a doublet. In addition, several bands were labeled more faintly in the M_r range of 120000–160000 as well as bands of M_r 34000 and 68000. When rod outer segments were solubilized in Triton X-100 or CHAPS and treated with neuraminidase in order to expose the interior surface of the disks as well as the surface of the rod outer segment plasma membrane to neuraminidase, ricin was observed to label the same bands of M_r 230000 and 110000 with the same intensities as observed in the unsolubilized neuraminidase-treated sample (gel c′). An additional major band of 160000 M_r was also labeled with ricin in detergent-solubilized, neuraminidase-treated rod outer segments. The 230000 M_r ricin-binding glycoprotein is different than the concanavalin A binding 220000 M_r protein of rod outer segments disk membranes [11] as indicated by a slight difference in their mobility on SDS-gels of neuraminidase-treated rod outer segments (not shown).

It is unlikely that the neuraminidase-sensitive 230000 and 110000 M_r ricin-binding proteins are derived from contaminating membranes of the rod inner segment or other cell types. The rod outer segment fraction from continuous sucrose gradients is highly pure as analyzed by electron microscopy and SDS-gel electrophoresis in this study and in previous reports [7,11,12]. Membrane fragments from inner segments and other cell types are predominantly found as a broad white band at a higher density. The protein composition of this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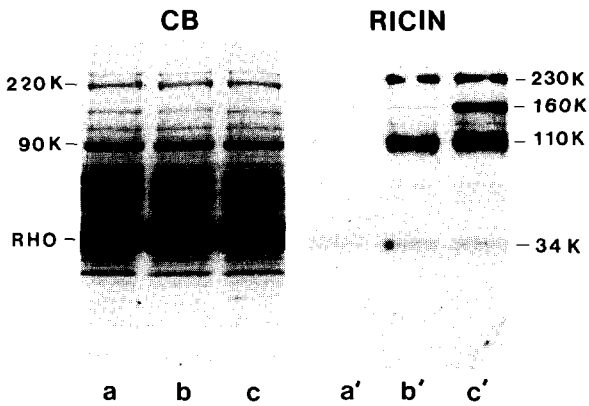

Fig. 3. Identification of ricin binding glycoproteins of rod outer segment plasma membranes by SDS gel electrophoresis and Western blotting. Rod outer segments were isolated from bovine retina by centrifugation on continuous 25–50% sucrose gradients. Samples were incubated at 4°C overnight in 20% sucrose – 20 mM Tris (pH 7.4) overnight (untreated sample, gel a, a'), 20% sucrose – 20 mM Tris (pH 7.4) containing 0.1 units of neuraminidase (neuraminidase-treated sample, gel b, b') or 20% sucrose – 20 mM Tris (pH 7.4) containing 1% Triton X-100 and 0.1 units of neuraminidase (neuraminidase-treated Triton X-100 sample, gel c, c'). The samples were then subjected to SDS-gel electrophoresis and either stained with Coomassie blue (CB) or transferred onto nitrocellulose paper and treated with ^{125}I-labeled ricin. Coomassie blue-stained gels were similar; ^{125}I-labeled ricin gels showed faint bands at M_r 34000 and 68000. Neuraminidase-treated sample and neuraminidase-treated Triton X-100 samples both contained intense bands at 230000 and 110000. No significant difference in intensity of these bands was observed in either of these samples. The neuraminidase-treated Triton X-100 sample contained an additional intense band at 160000. Several minor bands were also observed.

dense fraction is significantly different from that of the less dense rod outer segment fraction. In comparison to the rod outer segment fraction, ricin binds only weakly to proteins of apparent M_r 34000, 150000, 175000 and 230000 when this dense white fraction is treated with neuraminidase in the presence of Triton X-100 (not shown). Furthermore, ricin-gold-dextran labeling of inner segments is considerably less intense than that for the outer segment of neuraminidase-treated retina. This suggests that the ricin-binding glycoproteins are probably less abundant on the rod inner segment plasma membrane. Bovine cone photoreceptor cells which bind ricin-gold conjugates in the absence of neuraminidase [5] make up only a small fraction of bovine photoreceptor cells. Accordingly, cone membranes would not account for neuraminidase-sensitive ricin-binding proteins observed by Western blotting.

These results indicate that the 230000 M_r and 110000 M_r proteins are exposed on the surface of rod outer segment plasma membranes. Neuraminidase treatment of rod outer segments hydrolyses sialic acid residues from these glycoproteins exposing galactose residues which then can be labeled either with ricin-gold dextran as visualized by electron microscopy or with ^{125}I-labeled ricin as analyzed by SDS-polyacrylamide gel electrophoresis. These glycoproteins (or at least their unmasked galactose residues) do not appear to be present on the intradisk or lumen side of disk membranes since no increase in intensity of ^{125}I-labeled ricin binding to these proteins was observed in detergent-solubilized neuraminidase treated rod outer segment membranes in which both the plasma membrane and disk membrane glycoproteins are made accessible to neuraminidase. The additional band at 160000 M_r which labeled with ricin in these detergent-solubilized, neuraminidase-treated rod outer segment membranes may represent a disk-specific glycoprotein or alternatively, a rod

Fig. 2. Transmission electron micrographs of bovine rod outer segment membranes treated with neuraminidase and labeled with ricin-gold-dextran markers. Rod outer segments purified on a 25–50% sucrose gradient were resuspended in 20% sucrose – 20 mM Tris (pH 7.4) containing 0.1 units of neuraminidase. After 14 h the rod outer segments were washed in 20% sucrose by centrifugation, resuspended in 0.2 ml of 20 mM Tris – 20% sucrose and treated with 0.2 mM of ricin-gold-dextran solution at 25°C. After 1 h the ricin-gold-dextran-labeled rod outer segments were washed by repeated centrifugation (20000 × g 15 min). One sample was resuspended in 20 mM Tris containing 20% sucrose and processed for electron microscopy. Another sample was resuspended in 20 mM Tris in the absence of sucrose. After 20 min at 25°C this sample was fixed with 1.25% glutaraldehyde – 0.1 M sodium cacodylate buffer (pH 7.0) and processed for electron microscopy. (a) In the presence of sucrose rod outer segment fragments consisting of stacks of disks surrounded by ricin gold-dextran labeled plasma membrane are observed (bar, 0.5 μm). In region where the plasma membrane was peeled away (arrow), no labeling of disks was observed. In the absence of sucrose the rod outer segments lyse and ricin-gold labeled plasma membranes are observed either (b) in the process of forming inside-out vesicles or (c) as inside-out vesicles containing gold particles. Unlabeled disks are generally seen to radiate from the inside-out plasma membrane vesicles (bar, 0.5 μm).

outer segment plasma membrane protein made accessible to neuraminidase digestion by detergent solubilization.

Previous studies of ricin-labeling of bovine and monkey retina by fluorescent [6] and transmission electron microscopy [5] indicates that intense ricin binding is observed over the basal region of the rod outer segment. In this region disks are continuous with the plasma membrane. Ricin-binding in this region may reflect the presence of galactose containing proteins which undergo subsequent modification as the plasma membrane and disk membrane segregate further along the outer segment.

Electron microscopic studies of ricin-gold-dextran labeled rod outer segments subjected to hypotonic lysis indicate that the plasma membrane vesiculates under these conditions with an inverted orientation. Identification of a specific rod outer segment plasma membrane marker is significant in that it now enables one to monitor purification of the plasma membrane for analysis of the structure and function of this membrane and its components. A strategy can be developed in which neuraminidase-treated rod outer segments labeled with dense ricin-gold dextran conjugates are subjected to hypotonic lysis. Inverted rod outer segment plasma membrane vesicles loaded with gold particles are separated from dissociated disks on the basis of differences in density by gradient centrifugation. The ricin-binding glycoprotein and electron microscopy can be used to evaluate purification. Enriched rod outer segment plasma membrane preparations will be highly useful in characterizing components involved in light-regulated cationic conductance as well as components involved in recognition of rod outer segments by retinal pigment epithelial cells during membrane renewal. Finally, interesting questions arise as to how membrane proteins in the continuously folded basal region of the rod outer segment differentially segregate to the plasma membrane and disk membrane when these membrane systems physically separate further up the outer segment.

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