scopio-immunohistochemical studies demonstrate that the 50-1B11 antigen is associated with the lamellae of the cone outer segments in the monkey. Further experiments will be required to determine what kind of association the antigen has with the plasma membrane. For example, is it an integral or peripheral membrane protein?

Although the 50-1B11 antigen has not yet been characterized biochemically, it is clear that this antibody will be a useful reagent for studying the distribution of a unique class of cells in the retina. It should also permit in vitro and transplantation studies on factors that influence photoreceptor differentiation and survival. Finally, this antibody should be of value in studies on differences between rods and cones in various pathological conditions.

Key words: monoclonal antibodies, cones, human retina, chick retina

Acknowledgments. The author thanks S. Burns for a helpful discussion, F.M. de Monasterio for advice on the procion yellow experiments, J. Lund and G. Blasdel for performing the injections into the monkey eyes and E. Flatley of the Medical Eye Bank of Western Pennsylvania for the human tissue. The author also thanks J. Hailey for expert technical assistance.

From the Department of Neurobiology, Anatomy, and Cell Science and The Center for Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania. Supported by Research Grant R01-EY05285 from the National Eye Institute. Submitted for publication: May 21, 1985. Reprint requests: Vance Lemmon, Department of Neurobiology, Anatomy, and Cell Science, University of Pittsburgh, Pittsburgh, PA 15261.

References

Immunocytochemical Localization of Opsin in the Inner Segment and Ciliary Plasma Membrane of Photoreceptors in Retinas of rds Mutant Mice

Izhak Nir* and David S. Papermaster

Homozygous 020/A mutant mice bearing the rds gene for slow inherited retinal degeneration have been observed to develop normal photoreceptor inner segments connecting cilia and synaptic contacts but fail to form outer segments. Their retinas are responsive to light, however. In order to assess the sources of these physiological responses we investigated the distribution of opsin in photoreceptors by means of immunoelectron microscopy. Opsin was detected in the inner segment plasma membrane and the distal ciliary plasma membrane. Antibody also bound to lamellar and vesicular membranes in the interphotoreceptor space and, in a small fraction of the photoreceptors, to membranes projecting from the distal cilium. These membranes may represent abortive formation of rod discs in this form of retinal degeneration. Failure to form an organized outer segment may contribute to the persistence of opsin in the inner segment plasma membranes of adult mutant mice. Invest Ophthalmol Vis Sci 27: 836–840, 1986

In the developing retinas of newborn 020/A mutant mice bearing the rds/rd mutation, outer segments fail to differentiate while the development of other retinal layers is normal. By 3 wk of age, the photoreceptors consist of normal appearing inner segments and cilia extending to their maximum length. Subsequently, photoreceptors die at a slow rate and may be removed by macrophages in the subretinal space.1–4 The retinas of these mice are responsive to light despite the absence of outer segments. Reduced ERG responses and a limited light-evoked decline in cyclic nucleotide levels have been noted.3,4 Although opsin was not detectable spectrosopically,3 it was detectable by use of a sensitive immunoassay with antiopsin antibodies.5 Its distribution in photoreceptors has not yet been reported. The possibility that opsin was present in the photore-
Receptor plasma membrane was raised to account for these physiologic and immunochemical observations. 3-5 Opsin is largely confined to the discs and plasma membrane of the outer segment of adult photoreceptors. In developing rat retinas we detected opsin in the inner segment and ciliary plasma membrane of immature photoreceptors prior to outer segment formation. 6 During subsequent stages, opsin was progressively restricted to the outer segment disc and plasma membranes and the distal plasma membrane of the connecting cilium. The morphologic appearance of photoreceptors of adult rds mutant mice resembles that of immature normal mouse rods. We therefore sought to test the postulate that in the absence of outer segments, opsin might accumulate and persist in the inner segment and ciliary plasma membrane of rds mutant mouse rods.

**Materials and Methods.** Retinas were isolated from 10-21-day-old 020/A (rds/rds) mutant mice from a colony maintained on cyclic light 12 hr light:12 hr dark. The colony was established from breeding pairs that were descendants of the original colony described by Sanyal. 1 Opsin was localized on the photoreceptor cell membrane by a pre-embedding immunocytochemical approach described previously. 7 Mice were deeply anesthetized by chloroform prior to enucleation. Retinas were separated from the pigment epithelium, rinsed in cold phosphate-buffered saline (PBS), and fixed in 1% glutaraldehyde in 0.15 M phosphate buffer pH 7.4 for 90 min at room temperature. The retinas were sequentially rinsed in 0.05 M glycine and 2% bovine serum albumin in PBS and incubated with biotinyl-sheep antibovine opsin at 100 µg/ml in PBS for 60 min at room temperature. After rinsing in PBS for 60 min, bound antibodies were detected by avidin–ferritin (0.2 mg/ml) in PBS for 90 min at room temperature followed by a final rinse in PBS prior to postfixation in 1% OsO4 in 0.1 M phosphate buffer pH 7.0. Tissues were dehydrated, embedded in epon, and thin sections were viewed with a Philips (Eindhoven, the Netherlands) EM 300 electron microscope at 60 kV. All procedures involving mice were performed in accordance with the ARVO Resolution on the Use of Animals in Research.

**Results.** Photoreceptor inner segments and cilia were observed in the outer retinal layers of 10-21-day-old retinas affected by the rds mutation. Inner segment mitochondria were normal in appearance and distribution beneath the cilium. No normal outer segments were observed, but numerous vesicles and flattened membrane fragments were observed in the subretinal space. The distal cilium of some cells occasionally bore a few round membrane profiles or projected a flat membrane from the ciliary tip. These observations completely paralleled the earlier descriptions of the retinal degeneration of these mice 1-4 and indicated that our colony was expressing the degeneration appropriately.

Antiopsin binding was observed along the inner segment and ciliary plasma membranes. Inner segment plasma membrane labeling extended proximally to the level of the outer limiting membrane. Labeling density of the inner segment was, however, lower in comparison to the distal cilium (Fig. 1). This may indicate limited capacity of these cells to form a polarized distribution of opsin such that their mature state is comparable to that seen in developing photoreceptors of normal 3-5-day-old immature rats. 6

The distal ciliary labeling was dense and continuous. As in photoreceptors of other species, 6-9 the plasma membrane of the proximal cilium was relatively unlabeled although labeling density in this domain was variable (Figs. 1-2). On some cilia, labeling extended nearly to the base on one side while appearing to bind only to distal sites on the other side (Fig. 1). Similar variability of labeling along the proximal ciliary plasma membrane has been observed in other species 6,9 and may reflect pathways of opsin transport to the distal cilium along the plasma membrane. 10

Membrane vesicles and flattened membrane fragments in the subretinal space were densely labeled with antiopsin (Figs. 1-3) Some of these membranes were found in close proximity to the ciliary plasma membrane (Fig. 2). These membranes occasionally formed lamellae or spheres and may represent abortive attempts to generate outer segment discs (Fig. 3).

**Discussion.** This initial study was devoted to retinas of 10-21-day-old rds/rds mice because the photoreceptors of mice at this age have reached their maximum size 1,2 and have been the subject of considerable physiologic, biochemical, and immunochemical study. 3-5 These earlier studies clearly indicated the presence of photosensitive pigments in these retinas. Electrophysiograms from 4-wk-old rds mice demonstrated light evoked potential changes albeit at low amplitude. 6 This result correlated with the observation of normal synaptic contacts of rods and cones with bipolars and horizontal cells. 7 Although earlier spectroscopic studies failed to detect opsin, more recent antiopsin immunochemical reactions conducted by Schalken et al 5 demonstrated opsin content that approached 3% of the normal control mice of comparable age. These retinas also initiate the light evoked hydrolysis of cyclic nucleotides characteristic of phototransduction in the outer segment. 3 Since the outer segment is absent, Cohen postulated that opsin might be present in the plasma membrane of the photoreceptor and that it was somehow coupled to the transduction cascade. 3 Immunochemistry provided a useful approach to test some aspects of this postulate. The results suggest that
opsin in the inner segment and ciliary plasma membrane may account for the ERG response and the light evoked decline in cyclic nucleotides. If this is so, the elements of the phototransduction system may not require the organization of an outer segment to evoke light sensitivity, at least to a limited extent.

Our results support the biochemical and immunohistochemical studies by Cohen and Schalken et al and localize opsin in the plasma membrane of the photoreceptor connecting cilium and inner segment. Opsin was also detected immunocytochemically on membranes projecting from the distal cilium and on small vesicles and flattened membranes in the subretinal space. These membrane fragments in the subretinal space have been previously described by Jansen and Sanyal and Cohen. The origin of these opsin-laden membranes is not established. Their close proximity to the distal ciliary plasma membrane and the appearance of flattened and spherical membranes associated with the tips of the cilia may indicate a limited formation of outer segment discs at this site.

The distribution of antiopsin binding observed on the photoreceptor plasma membranes in the rds retinas resembles the labeling of photoreceptors at intermediate stages of development of normal mammalian retinas. In immature rats, opsin is also detected in the inner segment. As the cells mature, opsin becomes concentrated in the distal ciliary plasma membrane. The labeling densities become more disparate as the cells mature so that by 2–3 wk of age, opsin is nearly confined to the outer segment and distal cilium. The inner segment is nearly unlabeled in the normal adult rat retina. The greater density of label of the distal cilium when compared to the inner segment observed in the rds mutant mouse retina indicates that the mutants retain some capacity to generate a gradient of opsin concentration above the basal portions of the connecting cilium. The failure of these mutant mice to form normal outer segments may account for their failure to develop a polarized distribution of opsin that is characteristic of the normal adult mouse photoreceptor (Nir and Papermaster, unpublished data). Limited opsin-laden disc formation at the distal cilium is observed, however. Thus the defect in disc formation in this mu-
Fig. 2. Formation of incomplete disc membranes at the tip of the ciliary plasma membrane in a 13-day-old rds mutant mouse retina. The membranes are curled back on the cilium and are densely labeled by antiopsin. At this age, a normal mouse retina forms orderly parallel discs in the outer segment (×77,000).

Fig. 3. Cilium and vesicular membranes in the interphotoreceptor (subretinal) space of a 10-day-old rds mutant retina. The vesicular membranes (V) and distal ciliary plasma membrane (C) are labeled at high density by antiopsin. Some of the membranes are flattened and resemble portions of rod disc. Nearly all of the vesicles in the subretinal space are labeled by antiopsin. Unlabeled membranes (arrow) arise from Müller cell fibers. The inner segment (IS) of an adjacent cell is labeled less than the distal cilium (×56,000).
tant is incomplete and is not a consequence of complete failure to distribute opsin to an appropriate site.

Key words: rds mice, opsin, antibodies, immunocytochemistry, electron microscopy

Acknowledgments. We thank Lillimore Wallmark for technical assistance and Dr. Janet Blanks, University of Southern California, Los Angeles, California, for providing breeding pairs of mice.

From the Technion Faculty of Medicine, Haifa, Israel* and Department of Pathology, VA Medical Center, West Haven, Connecticut and Yale Medical School, New Haven, Connecticut. This research was supported by NIH grants EY–845, EY–3239 and the Veterans Administration. Submitted for publication: September 24, 1985. Re-print requests: Dr. Izhak Nir, Technion Faculty of Medicine, P.O. Box 9649, Haifa, Israel.

References


Immunocytochemical Localization of Fibronectin to the Retinal Pigment Epithelium of the Rat

Richard M. Pino

Fibronectin is an anionic asialoglycoprotein that is found on a variety of cell types. This study was undertaken in an attempt to localize fibronectin to the rat retinal pigment epithelium with ultrastructural immunocytochemistry. Using Fab–HRP conjugates specific for fibronectin, reaction product was localized on the surface of the apical processes and within the cell to GERL. After treatment of tissue by the biotin–avidin method employing ferritin–avidin, ferritin particles marked the apical processes in a quasi–regular distribution. Tufts of particles were separated by a linear distance of 65–85 nm. Fibronectin was not localized to rod outer segments. Invest Ophthalmol Vis Sci 27:840–844, 1986

Rod outer segments (ROS) are phagocytized and degraded by the retinal pigment epithelium (RPE) in the process of photoreceptor renewal. It is probable that membrane receptors are involved in this process. Thus, a knowledge of membrane-associated substances of the RPE may be important to the understanding of the internalization of photoreceptors in normal animals and the dysfunction seen in retinal degeneration in which RPE phagocytosis of ROS is markedly reduced.

Anionic (negative) domains of glycoproteins and glycolipids of the RPEs glycocalyx have been considered as possible recognition molecules of ROS.1 Although high levels of anionic sites have been demonstrated on the RPE surface with cationic ferritin,2 the chemical composition of these sites is not fully known. The binding of cationic ferritin was not altered by prior digestion with a number of enzymes3 that had been used to remove anionic sites other locations.2 Subsequent work using lectin-affinity cytochemistry with ferritin markers revealed the presence of sialic acid3 on the RPE. However, the number of sialic acid sites identified was less than the total number of anionic sites marked by cationic ferritin.

Fibronectin (FN) is an anionic (pI 5.5–6.3) asialoglycoprotein that is found in plasma and on the surfaces of many cell types.4 Two of the many functions attributed to FN are cell–cell attachment and the opsonization of particles prior to phagocytosis.5 Recently, chick RPE has been shown to synthesize FN in vitro.5 FN, as assayed by biochemical methods, has not been