Interphotoreceptor Retinoid-Binding Protein in the Cone Matrix Sheath

Electron Microscopic Immunocytochemical Localization

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Using a monoclonal antibody to chondroitin 6-sulfate, a major constituent of the cone matrix sheath, the location of the cone matrix sheath and the relative distribution of interphotoreceptor retinoid-binding protein (IRBP) was examined by light and electron microscopic immunocytochemistry. Chondroitin 6-sulfate immunoreactivity was localized to regions surrounding cone outer and inner segments, extending from the pigment epithelium to the outer limiting membrane. Only a background level of chondroitin 6-sulfate immunoreactivity was found around rods. Ultrathin sections reacted with antibodies to IRBP and chondroitin 6-sulfate showed the presence of both in the matrix surrounding the cones. Chondroitin 6-sulfate immunoreactivity extended from cone inner and outer segments to the adjoining interphotoreceptor matrix (IPM) surrounding the rods, but only background labeling was observed around the rods. The label for IRBP was found in the region containing chondroitin 6-sulfate and the IPM surrounding the rod photoreceptors. The label for IRBP and chondroitin 6-sulfate appeared to be associated with the filamentous reticulum of the IPM. These findings not only show that the cone matrix sheath has not collapsed and that IRBP is present in the cone matrix sheath, but they also demonstrate retention of structural components of the IPM.

The space between the retinal pigment epithelium (RPE) and neural retina is occupied by an extracellular matrix called the interphotoreceptor matrix (IPM). It is composed of proteins, glycoproteins, and glycosaminoglycans. These components are not distributed uniformly throughout the IPM. For example, in the rat, sialoglycoconjugates are concentrated in the apical region of the IPM, and chondroitin sulfates A and C are concentrated in basal regions. Another constituent of the IPM is seen in a distinct domain surrounding cone photoreceptors, which selectively binds the lectin peanut agglutinin (PNA). This PNA-positive region, called the cone matrix sheath, contains proteoglycans, such as chondroitin 6-sulfate. The IPM constituent, interphotoreceptor retinoid-binding protein (IRBP), which may be involved in the transfer of retinoids or serve as a buffer protein, is concentrated at the apical region of the RPE in light-adapted retinas.

Recent light microscopic analysis of silver-enhanced immunogold-labeled sections showed heterogeneity in the distribution of IRBP around rods and cones in the monkey retina. IRBP labeling was dense around rod outer segments but lighter around cone photoreceptors. This region of light labeling corresponded in position to the cone matrix sheath. It was not clear from the light microscopic study whether this region represented labeling in an intact cone sheath or whether it reflected the distribution of label after the collapse of the cone sheath.

We examined the location of IRBP relative to chondroitin 6-sulfate, a major constituent of the cone matrix sheath. We used a light microscopic immunogold procedure with silver enhancement to examine the distribution of chondroitin 6-sulfate in the monkey retina. Ultrathin sections labeled with antibodies to chondroitin 6-sulfate and IRBP were studied to determine whether the region of light IRBP labeling surrounding cones represented labeling inside or outside the cone matrix sheath.
Materials and Methods

Eyes from two monkeys (Macaca fascicularis) were provided by Dr. M. L. J. Crawford (University of Texas Health Science Center, Houston) after vascular perfusion with a mixture of paraformaldehyde 2% and glutaraldehyde 0.5% in 0.1 M phosphate buffer (pH, 7.3-4; the monkeys were killed in conformity with the ARVO Resolution on the Use of Animals in Research). The anterior segments were removed, and the eyecups subsequently were fixed in a mixture of paraformaldehyde 4% and glutaraldehyde 2% for 1 hr on ice and then overnight in paraformaldehyde 4% at 4°C. Small segments were removed from the peripheral superior temporal retina along the horizontal meridian, washed 1 hr in sucrose buffer 4% (0.1 M phosphate buffer, 0.15 mM CaCl2), followed by a 30-min wash in sodium borohydride 1% in sucrose buffer. The tissue was dehydrated in a graded series of methanol (50-100%), infiltrated, and embedded in LR white (Polysciences, Warrington, PA) polymerized at 56°C.

Antisera

Mouse monoclonal antibodies to chondroitin 6-sulfate (mouse monoclonal immunoglobulin M anti-proteoglycan, ADi 6S) were purchased from ICN Immunobiologics (Lisle, IL). This antibody is specific for the ADi-unsaturated disaccharide units of chondroitin 6-sulfate left after digestion with chondroitinase ABC (Sigma, St. Louis, MO).23 Rabbit anti-monkey IRBP polyclonal antibodies were a generous gift from Drs. G. Chader and B. Wiggett (National Eye Institute, Bethesda, MD). Previous studies in our laboratory using this antibody showed IRBP immunoreactivity confined to the IPM and the Golgi apparatus of photoreceptors.24 The same pattern of labeling was observed with affinity-purified or nonaffinity purified IRBP antibodies. The immunoreactivity was abolished after preadsorption of the antisera with the purified protein.

Light Microscopic Immunocytochemistry

We digested 1-μm LR white sections with chondroitinase ABC dissolved in 0.1 M Tris HCl (1 unit/ml, pH 8.0) for 90 min at 37°C in a humidified chamber to expose the antigenic sites. The sections were rinsed twice for 10 min in phosphate-buffered saline (PBS I; 137 mM NaCl, 2.7 mM KCl, 5 mM Na2HPO4, 1.5 mM KH2PO4; pH 7.2), incubated in goat serum 5% in PBS II with bovine serum albumin 0.1% and sodium azide 0.05%) to block non-specific binding, and in primary antibody (ADi 6S) diluted 1:10 in PBS II containing goat serum 1% for 2 hr. The sections were rinsed twice in PBS I and incubated with goat anti-mouse immunoglobulins G and M conjugated to 5-nm gold (1:25 in PBS II; Janssen, Piscataway, NJ) for 1 hr. All sections were washed, dried, and silver enhanced for 10 min using Janssen's IGSS kit. Control samples consisted of undigested sections incubated with primary and secondary antibodies and sections treated with chondroitinase ABC followed by mouse ascites fluid and secondary antibodies.

Electron Microscopic Localization of Chondroitin 6-Sulfate and IRBP

Ultrathin sections were collected on Butvar-coated (BioRad, Cambridge, MA) 0.5-mm slot nickel grids. The sections were treated with chondroitinase ABC (1 unit/ml) for 20 min at 37°C, rinsed twice in PBS I, and incubated in goat serum 5% in PBS II for 15 min. They were incubated with chondroitin 6-sulfate antibody (1:10 in PBS II containing goat serum 1%) 15 min, washed twice each for 5 min, followed by a 15-min incubation in a 5-nm gold conjugate of goat anti-mouse immunoglobulins G and M (1:25 in PBS II). After washing in PBS I and distilled water, the sections were dried and processed for IRBP localization. For IRBP localization, the sections reacted with chondroitin 6-sulfate antibody were incubated in goat serum and, as described, in rabbit anti-monkey IRBP immunoglobulin G (0.009 μg/μl in PBS II) overnight in a humidified chamber at room temperature. The sections were washed in PBS I and incubated with a 15-nm gold conjugate of goat anti-rabbit immunoglobulin G (1:25 in PBS II) for 2.5 hr. All sections were washed as described, stained in aqueous uranyl acetate for 5 min, washed in distilled water, dried for 15 min, and exposed to OsO4 vapors. One control consisted of sections incubated with chondroitin 6-sulfate antibody and rabbit immunoglobulin G (without prior chondroitinase ABC digestion), followed by incubation in both secondary antibodies and goat anti-rabbit immunoglobulin G 15-nm and goat anti-mouse immunoglobulins G and M 5-nm gold conjugates. A second control consisted of sections treated with chondroitinase ABC followed by exposure to mouse ascites fluid, rabbit immunoglobulin G, and both secondary antibodies as described for the first control.

Results

Light microscopic examination of silver-enhanced sections of monkey retina incubated with the monoclonal antibody to chondroitin 6-sulfate showed heavy labeling around the inner and outer segments of cone photoreceptors (Fig. 1). The immunoreactivity extended from the RPE to the external limiting membrane. Only a background level of label was seen.
connecting cilium (Fig. 3). Control sections showed only background labeling for both antigens (Fig. 4).

**Discussion**

Light microscopic immunocytochemistry showed chondroitin 6-sulfate immunoreactivity confined to a region around the cone photoreceptors. No label was present around the rod photoreceptors. These findings are consistent with those previously reported in monkey retina.\(^1\)

Previous studies of the extracellular space surrounding the cone photoreceptors showed that no apparent structural elements were seen in this space unless special cationic stains were used during fixation.\(^1\) Our results show the retention of some struc-

Fig. 1. (A) A light micrograph of silver-enhanced immunogold labeling localizing chondroitin 6-sulfate, a major constituent of the cone matrix sheath. The IPM around cones, cone interphotoreceptor matrix (CIPM), shows immunoreactivity extending from the retinal pigment epithelium (RPE) surrounding the inner and outer segment regions of cones. Only background labeling is seen around rods. CIS, cone inner segment; bar = 10 \(\mu\)m. (B) Control section of monkey retina shows background labeling. (This section was treated with chondroitinase ABC, mouse ascites fluid, and goat anti-mouse IgG + IgM conjugated to 5 nM gold followed by silver enhancement). RPE, retinal pigment epithelium; bar = 10 \(\mu\)m.

Electron microscopic immunocytochemistry confirmed the localization of chondroitin 6-sulfate to a region surrounding cone photoreceptors (Figs. 2, 3). Label for chondroitin 6-sulfate appeared to be associated with the electron-dense filamentous reticulum surrounding the cones. This labeled reticulum was closely apposed to the IPM surrounding the rod photoreceptors, but little to no label was seen in the IPM around the rods (Fig. 2).

Label for IRBP was present in the IPM surrounding the cone photoreceptors, along with the label for chondroitin 6-sulfate. In addition, IRBP labeling was seen in the IPM surrounding the rod photoreceptors (Figs. 2, 3). The amorphous material along cone outer segments was labeled heavily for IRBP, but it was devoid of label for chondroitin 6-sulfate (Fig. 3). The region adjacent to the cone outer segment was not labeled. This area presumably is an extension of the

Fig. 2. Electron micrograph of immunogold labeling for chondroitin 6-sulfate (5 nm gold particle) and IRBP (15 nm gold particle) immunoreactivity in monkey retina. This section, through rod outer segments (ROS) and cone outer segments (COS), shows label for chondroitin 6-sulfate predominately in the interphotoreceptor matrix (IPM) around cones (CIPM). Only background labeling is seen in the IPM around rods. Label for IRBP is seen in the chondroitin 6-sulfate positive region and around rod outer segments. Bar = 0.5 \(\mu\)m.
tural elements of the IPM. A reticulum of fibrillar-like material was seen surrounding both the rod and cone photoreceptors. Retention of IPM structural elements also was observed in monkey retina without using cationic dyes.27

The label for chondroitin 6-sulfate, a major component of the cone sheath, appeared to be associated with the filamentous reticulum of the IPM surrounding the cones. This labeled reticulum extended from the cone inner and outer segments to the adjoining IPM surrounding the rods. This finding clearly showed that the chondroitin 6-sulfate component of the cone matrix sheath had not collapsed, but rather it remained in position under the tissue-processing conditions we used.

The localization of IRBP and chondroitin 6-sulfate on the same ultrathin section showed the presence of immunolabel for IRBP along with that for chondroitin 6-sulfate around the cones. These findings demonstrated the presence of IRBP in the intact cone matrix sheath. Therefore, the light labeling for IRBP seen in our previous study22 was within the intact cone matrix sheath and did not represent the redistribution of label after the collapse of the sheath.

The distribution of chondroitin 6-sulfate in monkey retina was dramatically different from that seen in the rat. Others26 showed chondroitin 6-sulfate immunoreactivity to be highest at the apical RPE zone and the basal zone near the junction of inner and outer segments. Immunoreactivity was present in other regions of the IPM, but the density of labeling was lower. No distinct differences were reported in the distribution of chondroitin 6-sulfate in the IPM around the rods and cones in the rat, but in the monkey, chondroitin 6-sulfate was seen only around the cones.
This difference in distribution of chondroitin 6-sulfate in the IPM of the rat and monkey may be functionally significant; however, the significance of the differential distribution requires further analysis.

Our results also confirmed our previous light microscopic findings and the electron microscopic immunocytochemical findings of others\(^7\) of a dense band of label for IRBP along the cone outer segments. This heavily labeled amorphous band of material may represent a structural and biochemical entity that sequences IRBP for use in buffering or transporting retinoids during the bleaching and regeneration of cone visual pigments. We could not exclude the possibility that the dense band of IRBP around the cones represented constituents precipitated from the cone sheath during tissue processing. Further analyses are required to determine whether this dense band of label for IRBP is a true structural entity.

**Key words:** interphotoreceptor matrix, interphotoreceptor retinoid-binding protein, cone matrix sheath, chondroitin 6-sulfate, immunogold, monkey

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**References**