Interphotoreceptor Matrix Domains Ensheath Vertebrate Cone Photoreceptor Cells

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The retinal interphotoreceptor matrix (IPM) occupies the space between the neural retina and the retinal pigmented epithelium (RPE), two neuroectoderm-derived epithelia. While the IPM appears to be a major route by which photoreceptor cells receive vital metabolic factors, relatively little is known concerning its structure and function. The studies reported here describe the presence of specialized domains of the IPM that ensheath cone, but not rod, inner and outer segments in pig, monkey, and human retinas. These cone extracellular matrix sheaths are chemically and structurally distinct from the remainder of the IPM as revealed by their specific binding of the lectin peanut agglutinin (PNA) and their structural stability during physical dissociation of the retina. Biochemical studies suggest that the PNA-binding components of the cone matrix sheaths are trypsin-sensitive glycoproteins. These structures may play a role in establishing a specialized microenvironment for cone photoreceptors, maintaining proper orientation of cone outer segments, and/or facilitating cone-RPE interactions. Invest Ophthalmol Vis Sci 27:129-135, 1986

Important developmental and physiological roles have been established for components of extracellular matrices. The extracellular matrix that surrounds the apical processes of retinal photoreceptor cells, the interphotoreceptor matrix (IPM), is unique in that it occupies a compartment that is bordered by the apical surfaces of two epithelia, the neural retina and the retinal pigmented epithelium (RPE). Relatively little is known regarding the function of the IPM, although it is a major route by which nutrients and metabolites pass between photoreceptor cells and their major vascular supply, the choroidal vessels. Most IPM components appear to be synthesized by either the RPE or photoreceptor cells.1 In contrast to the extracellular matrix material of connective tissues, neither fibronectin nor collagen has been reported to be present in the IPM.2,3 The soluble fraction of bovine IPM has been shown to contain a high proportion of protein and glycoprotein (98%) compared to glycosaminoglycan (2%),1>2 a property that also differs from connective tissue extracellular matrices.

Much interest in the retinal IPM has been generated by the recent characterization of the interstitial retinol binding protein (IRBP) as one of its major constituents. This glycoprotein mediates transport of the vitamin A derivative, retinol, between the RPE and photoreceptors.4-7 IRBP is reportedly synthesized by photoreceptor cells8-11; however, immunocytochemical localization shows it to be concentrated in the region of the IPM directly adjacent to the RPE cell layer.7,11-13 Changes in the amount and distribution of IRBP accompany the degeneration of photoreceptor cells in animals with inherited retinal dystrophy.11-13 In addition, degeneration-associated changes in the distribution of interphotoreceptor matrix proteoglycans have been reported.14 Biochemical characteristics and functional roles for other components of the retinal IPM remain to be elucidated.

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Our recent studies of lectin binding in the retina\textsuperscript{15-17} as well as those of others,\textsuperscript{18-21} reveal the presence of carbohydrate-containing molecules in the IPM, some of which appear to be selectively associated with cone photoreceptors. In these studies, peanut agglutinin (PNA), a lectin with high binding affinity for galactose-galactosamine disaccharide linkages\textsuperscript{22,23} has been observed to selectively label cone inner and outer segment regions, cone synaptic pedicles, and the inner synaptic layer. The degree of resolution afforded by previously utilized techniques has made it difficult to attribute cone inner/outer segment-specific PNA binding to the IPM, cone cell membrane, or a combination of both. In sectioned retinae cone inner and outer segment membranes most often are not distinctly labeled by PNA. Rather, more diffuse and somewhat irregular PNA binding is observed in closely associated regions. In the present study we have applied improved methods of tissue preparation to investigate this aspect of PNA binding in pig, monkey, and human retinae and have found it to be primarily associated with chemically and structurally distinct domains of the IPM that form cylindrical sheaths around cone inner and outer segments.

Materials and Methods

Reagents

Fluorescein-conjugated peanut agglutinin (FITC-PNA) was obtained from Vector Laboratories (Burlingame, CA) or EY Laboratories (San Mateo, CA); various lots obtained from each of these commercial sources yielded similar results. The hapten sugars D-galactose, a-lactose, and 1-O-methyl-a-D-galactopyranoside and the enzymes chondroitinase ABC (Proteus vulgaris), chondroitinase AC (Arthrobacter aurescens), and heparinase (Flavobacterium heparinum) were obtained from Sigma Chemical Co. (St. Louis, MO). Hyaluronidase (bovine testis) and L-(tosylamido 2-phenyl)ethyl chloromethyl ketone-treated trypsin (bovine pancreas) were obtained from Worthington Biochemical Corp. (Freehold, NJ). Acrylamide and other reagents utilized for embedding were obtained from Bethesda Research Laboratories (Gaithersburg, MD).

Fixation, Embedding, and Lectin Binding

Human retinae were obtained from eye bank donors within 6 hr of death and porcine eyes from a local slaughterhouse within 30 min of death. Monkey eyes were enucleated immediately after death by an overdose of pentobarbital. Experimental animals were treated in conformity with the ARVO Resolution on the Use of Animals in Research. Eyecups were fixed for 2–4 hr by immersion in 4.0% formaldehyde (freshly generated from paraformaldehyde) in 100 mM sodium cacodylate buffer, pH 7.2. Superior morphological preservation is attained when cacodylate buffer is used in place of the phosphate buffer employed in our prior studies.\textsuperscript{16,17} Following fixation, the eyecups were rinsed for a minimum of 6 hr in cacodylate buffer, embedded in acrylamide and sectioned on a cryostat at \(-20^\circ\text{C}\) as previously described.\textsuperscript{17} Sections were exposed to FITC-PNA at 200–500 \(\mu\text{g}/\text{ml}\) in 10 mM phosphate buffered saline containing 1 mM MgCl\(_2\), 1 mM CaCl\(_2\) (PBS-CM), and 1 mg/ml bovine serum albumin (BSA) using published procedures.\textsuperscript{16,17} Specificity of PNA binding was confirmed by competitive inhibition of hapten sugars at 50–200 mM. Sections were examined by epifluorescence and differential interference contrast optics on an Olympus BHS microscope (Olympus Corp. of America; New Hyde Park, NY) and photo-

\[\text{Fig. 1. Fluorescence micrograph of a meridional section of acrylamide-embedded human eye demonstrating the binding of FITC-PNA. Regions associated with cone, but not rod, photoreceptor inner and outer segments (IS, OS) are heavily labeled. Less intense labeling is observed in discrete patches associated with cone photoreceptor synaptic pedicles (arrowheads) within the outer synaptic layer (OSL). Diffuse labeling of the inner synaptic layer (ISL) is also apparent (\(\times700\)).}\]

\[\text{Fig. 2. Nomarski differential interference contrast (A) and fluorescence (B) micrographs of the same meridional section of acrylamide-embedded monkey (Macaca fascicularis) retina exposed to FITC-PNA. Regions devoid of cellular constituents (asterisks) are visible surrounding cone photoreceptor inner (IS) and outer (OS) segments in (A). These cylindrical domains within the interphotoreceptor matrix are responsible for the majority of cone-specific PNA binding, as seen in the fluorescence micrograph of the same field (B). These cone matrix sheaths extend from the outer limiting membrane (arrows) through the subtretinal space (SS). Regions occupied by inner (IS) and outer (OS) segments show reduced levels of fluorescence. (C) and (D) are fluorescence and Nomarski differential interference contrast micrographs of the same oblique section through the photoreceptor layer of this monkey retina. The plane of section passes from a region near the outer limiting membrane in the lower portion of each micrograph past the tips of the photoreceptor outer segments in the upper portion of each micrograph. FITC-PNA binds intensely to acellular domains (asterisks) that surround cone inner (IS) and outer (OS) segments. In the distal portions of these extracellular cone matrix sheaths, a central region with reduced PNA binding and lacking a detectable cellular component is often visible. Note the distinct lack of FITC PNA binding to regions occupied by rod photoreceptor inner and outer segments (A, B \(\times890\); C, D \(\times920\)).}\]

\[\text{Fig. 3. Fluorescence (A) and Nomarski differential interference contrast (B) micrographs of an intact cone extracellular matrix sheath isolated from procine retina following exposure to FITC-PNA. It is of the same dimensions as the cone matrix sheaths identified in sectioned retinae, as well as having similar PNA-binding characteristics. The regions previously occupied by the now absent cone inner and outer segments can be identified in the fluorescence micrograph (arrows in A) (\(\times1,000\)).}\]
Isolation of Cone Matrix Sheaths

Unfixed porcine retinae were dissected free from the pigmented epithelium in ice cold PBS–CM and exposed intact to FITC–PNA at 250 μg/ml in PBS–CM containing 1 mg/ml BSA for 60 min at 4°C. Following three 15-min rinses in ice cold PBS–CM, the retinae were partially dissociated in the same buffer by vigorous trituration with a Pasteur pipette. After allowing larger fragments to settle, aliquots of the suspension were removed and examined by fluorescence and differential interference contrast microscopy without fixation, embedding, or sectioning.

Enzyme Treatments

Unfixed porcine retinae were dissected free from the pigmented epithelium in PBS–CM and subsequently exposed to 0.25% trypsin, 100 U/ml hyaluronidase, 10 U/ml chondroitinase ABC, 10 U/ml chondroitinase AC, or 10 U/ml heparinase in the same buffer for 15 min at room temperature with gentle agitation. Controls were treated similarly in PBS–CM without enzyme. Following enzyme treatment, retinae were rinsed in PBS–CM, fixed, embedded in acrylamide, sectioned and exposed to FITC–PNA (see Fixation, Embedding, and Lectin Binding section above).

Results

As described previously, PNA binds heavily in regions associated with cone inner and outer segments, less intensely in discrete patches associated with cone synapses in the outer synaptic layer, and diffusely within the inner synaptic layer (Fig. 1). PNA-binding material occupies areas surrounding cone inner and outer segments and its labeling obscures lectin binding that may be directly associated with photoreceptor membranes. The distribution of this extracellular PNA-binding material appears to be restricted to the interphotoreceptor space at the level of the outer limiting membrane.

A better appreciation for the restricted distribution of extracellular cone-specific PNA-binding material can be gained from examination of fluorescence and differential interference contrast micrographs of the same meridional (Figs. 2A, B) and oblique (Figs. 2C, D) sections of monkey retina. These sections clearly reveal the presence of regions devoid of cellular constituents surrounding the inner and outer segments of individual cone photoreceptor cells. These cylindrical domains of the interphotoreceptor matrix extend from the outer limiting membrane beyond the tips of the outer segments and are responsible for the majority of cone-specific PNA-binding. There is an absence of FITC–PNA binding in central regions occupied by cone photoreceptor inner and outer segments. In the distal portions of the cone matrix sheaths, this negatively stained region often does not contain a detectable cellular component (Figs. 2C, D).

The cone extracellular matrix sheaths are structural entities that can be isolated using a physical, non-enzymatic method. By trituration of isolated retinae previously exposed to FITC–PNA, a limited number of structures analogous in size, shape, and PNA-binding properties to the cone matrix sheaths observed in sectioned retinae can be identified (Fig. 3). Cone inner and outer segments appear to be absent from the isolated sheaths (Fig. 3B); however, the regions previously occupied by these cellular components are obvious due to their lack of staining by FITC–PNA (Fig. 3A).

Studies designed to assess the biochemical nature of the PNA-binding components of the cone matrix sheaths suggest that they are glycoproteins. Treatment of isolated retinae with trypsin or hyaluronidase eliminates or greatly reduces subsequent PNA-binding to the extracellular matrix domains surrounding cones (Figs. 4A–C). However, under the same conditions, relatively intense PNA binding remains associated with cone photoreceptor inner and outer segment cell membranes. Treatment with chondroitinase ABC (Fig. 4D), chondroitinase AC (not shown), or heparinase (not shown) does not alter PNA binding to cone matrix sheaths, suggesting that the glycosaminoglycans chondroitin sulfate and heparan sulfate are not major sheath components.

Discussion

These studies document the presence of chemically and structurally distinct domains within the retinal IPM that are specifically associated with cone inner and outer segments. These specialized extracellular domains, termed cone matrix sheaths, are chemically distinct from the remainder of the IPM, as indicated by their specific labeling with peanut agglutinin (PNA), a lectin with high binding affinity for galactose–galactosamine disaccharide linkages. Other lectins with binding affinities for galactose or galactosamine, as well as those which bind other carbohydrate moieties, do not show similar binding specificities within the IPM. This characteristic suggests that cone matrix sheaths contain carbohydrate groups that are significantly different in composition, concentration and/or lectin accessibility from those present elsewhere in the IPM. In glycoproteins, galactose-galactosamine linkages are most commonly found in carbohydrate groups coupled to serine or threonine residues via O-glycosidic
Fig. 4. Fluorescence micrographs of enzyme-treated porcine retinae following exposure to FITC-PNA. (A) Control retina incubated for 15 min in PBS in parallel with enzyme-treated retinae (×790). (B) Retina incubated in PBS containing 0.25% trypsin for 15 min. FITC-PNA-binding to the cone extracellular matrix sheaths is eliminated by this procedure. However, following this treatment, FITC-PNA does bind to cone photoreceptor outer segments and to cone inner segments and cell bodies (×940). (C) Retina incubated in PBS containing 100 U/ml hyaluronidase for 15 min. This treatment reduces but does not eliminate FITC-PNA binding to the cone extracellular matrix sheaths (×830). (D) Retina incubated in PBS containing 10 U/ml chondroitinase ABC. No marked effect on the binding of FITC-PNA to cone matrix sheaths is observed (×790). OS: cone outer segment; IS: cone inner segment; OSL: outer synaptic layer.

bonds. Examples of molecules identified as containing such carbohydrate units include antifreeze glycoprotein of antarctic fish, human immunoglobulin A, β-subunit of human chorionic gonadotropin, fetuin, glycoporin, submaxillary mucins and human gastric mucin.24,25

Due to their trypsin-sensitivity, it appears likely that PNA-binding glycoproteins are major components of the cone matrix sheaths or that trypsin-sensitive molecules are responsible for maintenance of their structure. The sheaths appear to be structurally distinct entities within the IPM as evidenced by the fact that they can retain morphological integrity after physical dissociation of the retina. However, they are not analogous to the cellular sheaths shown to surround cone outer
segments in the cat.\textsuperscript{26-28} These sheaths are formed by elaborate sheet-like processes which emanate from the apical surfaces of RPE cells and wrap concentrically around cone outer segments. Similar cellular encasement of cones is not present in the human,\textsuperscript{29} monkey,\textsuperscript{30} or porcine\textsuperscript{31} retina where cones are surrounded by larger spaces and thin, villous processes from the apical RPE surface that extend along only the distal half of the cone outer segment. Based on the observations presented here, the relatively large extracellular spaces present surrounding cone inner and outer segments in these species appear to be maintained by specialized components of the IPM.

The techniques applied in previous examinations of PNA binding in the retina\textsuperscript{15-21} have not afforded resolution sufficient to conclusively associate the lectin-binding with cellular membranes and/or associated extracellular material of cone photoreceptors. Our use of cacodylate as opposed to phosphate buffer during fixation in addition to improved acrylamide embedding and sectioning techniques has afforded better morphological preservation and increased resolution. This, in conjunction with the use of retinace from species in which cones are large and morphologically distinct from rods, has facilitated the characterization of the extracellular cone matrix sheaths. These structures are primarily responsible for the observed PNA binding to cone inner/outter segment regions. This conclusion is compatible with previously published observations\textsuperscript{15-21} and suggests that cone extracellular matrix sheaths may be common among the retinace of higher vertebrates. However, isolated retinace of the frog\textsuperscript{18} and rabbit,\textsuperscript{16} studied as whole-mounts, do not appear to have extracellular PNA-binding material associated with cone outer segments. Rather, PNA binding is distinctly associated with the inner\textsuperscript{16} and outer\textsuperscript{16,18} segment membranes. Perhaps this difference is related to the presence of elaborate RPE cell processes that surround cone (type II) photoreceptors in the rabbit\textsuperscript{18} and extensive RPE processes that extend to the level of the inner segment in the frog.\textsuperscript{33} Alternatively, these observations may represent species differences in PNA-binding glycoconjugates in the IPM or by the loss of IPM material during preparation of whole-mounts. In the latter case, the absence of extracellular PNA-binding material apparently allows the lectin access to cone inner and outer segment membranes which are then labeled. Interestingly, we do not find a significant loss of PNA-binding cone matrix sheath components when whole-mounts of porcine retinae are prepared (Johnson and Hageman, unpublished results).

The molecules responsible for PNA binding to cone matrix sheaths remain to be identified definitively. Interstitial retinol binding protein appears not to be a major PNA-binding molecule; it contains 8.4% carbohydrate,\textsuperscript{10} but galactosamine is not present at significant levels.\textsuperscript{2,10} The specific binding of PNA to the cone matrix sheaths is distinct from the immunocytochemical staining of IRBP.\textsuperscript{3,11-13} Lectin blot analyses of PNA-binding glycoproteins in a variety of vertebrate retinae\textsuperscript{34} also indicate a lack of significant PNA binding by IRBP. We have identified 5–7 major PNA-binding glycoproteins ranging in molecular weight from 30 to 88 kilodaltons in extracts of chick, turkey, rat, dog, and pig retinae, as well as those of the human and monkey.\textsuperscript{34} Specific antibody probes are currently being developed against each of the PNA-binding molecules so that the biochemical composition, structure, and function of the cone matrix sheaths may be characterized more completely.

Potential functional roles for cone matrix sheaths are numerous. These chemical and structural domains of the interphotoreceptor matrix may serve to create a specialized extracellular microenvironment for cone photoreceptors, insulating them from physical or chemical interaction with surrounding rods. Alternatively, the sheaths may serve to maintain a precise orientation for cone outer segments that is important in photoreception and transduction. The orientation of the sheath may in turn be stabilized by surrounding rods. They may also be involved in the establishment and/or maintenance of cone-RPE interactions. We have observed that the cone matrix sheaths extend beyond the tips of the cone outer segments and that in the distal regions, the sheaths surround apical cell processes of the RPE that are associated with the tips of cone outer segments (Hageman and Johnson, unpublished results). It thus appears likely that these novel extracellular structures may contribute significantly to the proper functioning of retinal photoreceptors. Analyses of their composition and structure should lead to a better understanding of photoreceptor function and the role of the interphotoreceptor matrix.

Key words: retina, interphotoreceptor matrix, cone photoreceptors, peanut agglutinin, lectin

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References