Characterization of SPACR, a sialoprotein associated with cones and rods present in the interphotoreceptor matrix of the human retina: immunological and lectin binding analysis

Shreeta Acharya¹, Mary E. Rayborn and Joe G. Hollyfield
The Eye Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA
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To whom correspondence should be addressed at: Department of Ophthalmic Research (FFb), The Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195

Rod and cone photoreceptors project from the outer retinal surface into a carbohydrate-rich interphotoreceptor matrix (IPM). Unique IPM glycoconjugates are distributed around rods and cones. Wheat germ agglutinin (WGA) strongly decorates the rod matrix domains and weakly decorates the cone matrix domains. This study characterizes the major WGA-binding glycoprotein in the human IPM, which we refer to as SPACR (sialoprotein associated with cones and rods). SPACR, which has a molecular weight of 147 kDa, was isolated and purified from the IPM by lectin affinity chromatography. A polyclonal antibody to SPACR was prepared that labeled and purified from the IPM by lectin affinity chromatography. Analysis of lectin binding allowed us to identify some of the structural characteristics of SPACR glycoconjugates. Complete deglycosylation results in a systematic increase in electrophoretic mobility, indicating the presence of both N- and O-glycosidases. Deglycosylation also results in a reduction in the relative molecular mass of SPACR by about 30%. Analysis of lectin binding allowed us to identify some of the structural characteristics of SPACR glycoconjugates. Treatment with neuraminidase exposes Galβ1–3GalNAc disaccharide as indicated by positive peanut agglutinin (PNA) staining, accompanied by the loss of WGA staining. Maackia amurensis agglutinins (MAA-1 and MAA-2), specific for sialic acid in α2–3 linkage to Gal, bind SPACR, while Sambucus nigra agglutinin (SNA), specific for α2–6 linked sialic acid, does not, indicating that the dominant glycoconjugate determinant on SPACR is the O-linked carbohydrate, NeuAcα2–3Galβ1–3GalNAc. The abundance of sialic acid in SPACR suggests that this glycoprotein may contribute substantially to the polyanionic nature of the IPM. The carbohydrate chains present on SPACR could also provide sites for extensive crosslinking and participate in the formation of the ordered IPM lattice that surrounds the elongate photoreceptors projecting from the outer retinal surface.

Key words: retina/interphotoreceptor matrix/sialoprotein/N-glycosidase/O-glycosidase

Introduction

The interphotoreceptor matrix (IPM) resides in an extracellular compartment bordered by the outer retinal surface on one side, and the apical surface of the retinal pigment epithelium (RPE) on the other. Photoreceptors, the primary sensory receptors of the visual system, extend their inner and outer segments into this carbohydrate-rich IPM (Röhlich, 1970). Several structure–function activities of fundamental importance to vision are proposed to take place within this matrix, including visual pigment chromophore exchange, retinal adhesion, metabolite trafficking, photoreceptor alignment, and cell–cell interactions thought to be involved in photoreceptor membrane turnover (Hageman and Johnson, 1991). The role and degree of involvement of specific IPM components in mediating these activities is not well understood.

Proteins, glycoproteins, proteoglycans, and glycosaminoglycans make up the major subgroups of macromolecules comprising the IPM (Berman and Bach, 1968; Adler and Severin, 1981; Adler and Klucznik, 1982). The polyanionic nature of the IPM was first detected by metachromatic staining of this compartment (Sidman and Wislocki, 1954; Wislocki and Sidman, 1954). Additional details of the structure and composition of the IPM came from electron microscopic (Fine and Zimmerman, 1963; Röhlich, 1970; Feeney, 1973; Johnson, 1977; Hollyfield et al., 1989), immunocytochemical (Porrello and LaVal, 1986), and biochemical analyses (Wortman, 1959; Wortman and Freeman, 1962; Berman, 1964; Berman and Bach, 1968; Berman, 1969; Adler and Severin, 1981; Nakamura, 1981; Adler and Klucznik, 1982; Kaneko, 1987).

The IPM was initially thought to be unstructured and amorphous (Röhlich, 1970). However, studies using lectin cytochemistry (Wood et al., 1984; Johnson et al., 1985; Johnson et al., 1986; Johnson et al., 1987; Sameshima et al., 1987; Hollyfield et al., 1990b), antibodies to chondroitin sulfate (Hageman and Johnson, 1987), and cationic dyes (Varner et al., 1987; Hollyfield et al., 1989) documented the presence of distinctly different matrix domains surrounding rod and cone photoreceptors. Early attempts to isolate IPM constituents for biochemical analysis, using saline rinses of the outer retinal surface (Berman and Bach, 1968; Adler and Severin, 1981; Adler and Klucznik, 1982), removed soluble components, such as interstitial retinoid binding protein (IRBP), a variety of enzymes (acid proteases, acid hydrolases), mucins, and immunoglobulins (for review, see Hageman and Johnson, 1991). We now know that these rinsing procedures leave behind an extensive matrix surrounding rods and cones that resists aqueous extraction with physiological salt solutions (Johnson and Hageman, 1989; Hollyfield et al., 1990a). This aqueous insoluble IPM can be isolated by hypotonic treatment, which frees the IPM from the outer retina as a relatively intact, sheet-like unit (Johnson and Hageman, 1989; Hollyfield et al., 1990a,b). None of the macromolecules of this insoluble IPM have been well characterized, and their function(s) remains unknown.

A prominent feature of the IPM surrounding rod photoreceptors is the presence of WGA-binding glycoconjugates (i.e., oligosaccharides containing terminal N-acetylgalactosamine or
Results

The distribution of WGA-binding in the human IPM and the relative intensity of binding to the domains surrounding rods versus cones can be best appreciated in flat mounts of isolated IPM samples that include the cone-pure fovea (Figure 1A–C). In the center of the fovea, WGA only lightly decorates the narrow matrix surrounding foveal cones (Figure 1A). Near the foveal slope, where some rod photoreceptors are found, the rod-associated matrix stains more intensely than the matrix surrounding cones (Figure 1B). Within 1.5 mm of the foveal center, in regions of higher rod density, the bright WGA fluorescence surrounding the smaller rod compartments is in clear contrast to the weaker fluorescence of the thick-walled matrix of the perifoveal cones (Figure 1C).

As we planned the purification strategy that involved the WGA-binding properties of SPACR, two critical initial steps were included in the protocol. First was the issue of IRBP. This molecule is the major soluble protein of the IPM, it has a similar molecular weight to SPACR (144 vs 147 kDa), it binds WGA, and could possibly be a contaminant in our protein preparation (Fong et al., 1985; Saari et al., 1985; Chader, 1989). In Western blots of the crude extract or of purified SPACR probed with an IRBP antibody (rabbit polyclonal), we determined that the initial PBS rinsing steps were sufficient to eliminate immunodetectable IRBP from our IPM samples (Figure 1D, Lanes 1–3).

Because of an earlier report describing a PNA binding glycoprotein in the IPM with a molecular weight similar to SPACR (Shuster et al., 1987), we first used a PNA-agarose column to remove the PNA binding components from our IPM sample prior to WGA-agarose chromatography. We then utilized the affinity for WGA as an essential step to isolate SPACR from IPM extracts. From ~150 µg of IPM protein that could be obtained from each retina, following elution from WGA-agarose, ~50 µg of total protein was recovered per retina. SPACR represented ~95% of the protein in this eluate, the remainder consisting of several other WGA-binding proteins, which were not prominent in Coomassie blue stained SDS/PAGE gels, but were clearly visible in the WGA blots. SPACR could be freed from other WGA-binding proteins by gradient elution from the WGA column, using GlcNAc in a concentration range of 0.0–0.45 M. SPACR eluted from the column at 0.1 M (Figure 1D, lanes 4–9). Purified SPACR was also subjected to isoelectric focusing where it again migrated as a single band, indicating high purity of the isolated protein (data not shown).

Immunological characterization of SPACR was performed with the polyclonal antibody generated against purified SPACR. Under reducing conditions, the antibody recognized only SPACR in Western blots of the IPM (Figure 1D, lane 10). The antibody did not recognize either purified human or bovine IRBP standards, nor did it cross-react with other WGA-binding proteins that coelute with SPACR (data not shown).

In retinal sections, SPACR immunolabelling was observed only in the IPM surrounding photoreceptors, and was similar to the distribution of WGA labeling (compare Figure 1E and 1F).
Fig. 2. (A–C) Confocal images of the isolated IPM double labeled with WGA-FITC (A), and the SPACR antibody–Texas Red (B). Yellow pattern in the overlays of these two images (Figure 2C) indicates regions of colocalization of WGA-binding and SPACR immunoreactivity. Heaviest labeling with both probes is associated with the rod matrix domains, but weaker labeling of the larger cone matrix compartments is clearly evident (arrows). Bar length in lower left of (B) represents 10 µm. (D) SDS/PAGE of crude IPM extract demonstrating changes in mobility of SPACR after glycosidase treatment (arrow on left and right designate native SPACR position at 147 kDa). Lanes 1–3, Coomassie blue stained gel. Lanes 4–6. Western blot transferred from the gel on the left probed with the SPACR antibody. Lanes 7–9, WGA blot from a gel similar to that shown in lanes 1–3. Native IPM samples are in lanes 1, 4, and 7. IPM samples treated with N-glycosidase are in lanes 2, 5, and 8. IPM samples treated with N- and O-glycosidase are in lanes 3, 6, and 9. Note the progressive increase in mobility of SPACR as N-linked and O-linked glycoconjugates are removed. The antibody recognized deglycosylated SPACR (lane 6), but WGA did not (lane 9). Distance between double arrows on the right represents the increased migratory distance between fully glycosylated SPACR and deglycosylated SPACR. (E) Transfer blots of native IPM samples probed with the lectins indicated at the top of each lane (see legend of Table II for description of abbreviations). Note that SPACR (position indicated by arrows) is recognized by all the lectins used except SNA. (F–H) Staining patterns in sections of the outer retina using the indicated fluorescent tagged lectins. Each lectin brightly decorated the IPM. Asterisks are located in the middle of the retinal pigment epithelium in each figure. Compare with staining patterns in Figure 1E and 1F.

contrast, control sections incubated with preimmune serum did not show labeling of the IPM when the secondary antibody was applied (Figure 1G). In the control sections, only autofluorescence was evident at the level of the age pigment (lipofuscin) present in the RPE, as reported previously (Hollyfield et al., 1985). IPM preparations isolated with distilled water, double labeled with WGA-FITC and anti-SPACR antibody–Texas Red, were analyzed with confocal microscopy (Figure 2A–C). WGA-FITC staining patterns showed bright fluorescence surrounding the smaller rod-associated matrix compartments and weaker, but distinct, staining within the larger cone-associated matrix compartments, as reported previously (Hollyfield et al., 1990b).
distribution of SPACR immunoreactivity (Figure 2B) was similar to the WGA staining pattern. Overlays of the FITC and the Texas Red image showed extensive overlapping of these fluorescent patterns as evidenced by the yellow reticular pattern throughout the IPM (Figure 2C), indicating that WGA-binding and SPACR immunoreactivity colocalize within the IPM. We were unable to obtain N-terminal sequence information from the purified protein, indicating a chemically modified N-terminus. Amino acid analysis of SPACR yielded the composition data presented in Table I. Of particular importance is the high content of serine/threonine residues, which are potential acceptor sites for O-glycosylation.

SPACR displayed a reduction in molecular mass when digested independently with N- and O-glycosidases indicating the presence of both types of carbohydrate linkages (Figure 2D). Each enzyme decreased the M_r by ~25 kDa, suggesting that the glycoconjugates account for at least 30% of the mass of SPACR. The antibody reacted both with the partially and completely deglycosylated forms of the protein (Fig 2D, lanes 4–6). After treatment with N- and O-glycosidases, the M_r of the SPACR polypeptide was approximately 105 kDa. We also attempted chemical deglycosylation using anhydrous trifluoromethanesulfonic acid (TFMS, Pierce Chemicals, Rockford, IL). The protein, after chemical deglycosylation, migrated with an M_r of 105 kDa (data not shown), which is in agreement with the M_r following enzymatic deglycosylation.

Table I. Amino acid composition analysis of SPACR

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Amount (pmol)</th>
<th>Est. # residues</th>
<th>% Content</th>
<th>Note</th>
<th>% Average distribution</th>
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<tbody>
<tr>
<td>Asp + Asn</td>
<td>64.1</td>
<td>64</td>
<td>6.7</td>
<td>Low</td>
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<tr>
<td>Glu + Gln</td>
<td>120.3</td>
<td>120</td>
<td>12.5</td>
<td></td>
<td>10.4</td>
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<tr>
<td>Ser</td>
<td>112.3</td>
<td>112</td>
<td>11.7</td>
<td>High</td>
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<tr>
<td>Gly</td>
<td>100.1</td>
<td>100</td>
<td>10.5</td>
<td>High</td>
<td>7.1</td>
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<tr>
<td>His</td>
<td>8.6</td>
<td>9</td>
<td>1</td>
<td></td>
<td>2.2</td>
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<td>6.9</td>
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<tr>
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<td>63</td>
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<td>7.5</td>
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<tr>
<td>Pro</td>
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<td>63</td>
<td>6.7</td>
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<tr>
<td>Tyr</td>
<td>27.1</td>
<td>27</td>
<td>2.9</td>
<td></td>
<td>3.3</td>
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<tr>
<td>Trp</td>
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<td></td>
<td></td>
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<tr>
<td>Val</td>
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<td>76</td>
<td>7.9</td>
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<tr>
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<td>12</td>
<td>1.3</td>
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<tr>
<td>Cys</td>
<td>NR</td>
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<tr>
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<tr>
<td>Leu</td>
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</tr>
<tr>
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<td>27</td>
<td>2.8</td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td>Lys</td>
<td>27.8</td>
<td>28</td>
<td>2.9</td>
<td>Low</td>
<td>5.8</td>
</tr>
</tbody>
</table>

Total estimated number of residues: 1059.

*, The value obtained in the analysis for Cys indicated a 5.5% content. Since Cys is partially destroyed during the hydrolysis reaction used for the composition analysis, the Cys content is not included in the table but is likely to be significantly higher than the average distribution.

Table II. Analysis of lectin binding to SPACR

<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specificity</th>
<th>Native</th>
<th>N-Glycanase</th>
<th>O-Glycanase</th>
</tr>
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<tr>
<td>WGA</td>
<td>Chitose, sialic acids</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>Succinylated WGA</td>
<td>GlcNAc</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>MAA 1</td>
<td>NeuAc2,3Galβ1,4Glc</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>MAA 2</td>
<td>NeuAc2,3Gal</td>
<td>++</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>SNA</td>
<td>NeuAc2,6Gal</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PNA</td>
<td>Galβ1,3GalNAc</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>PNA (after neuraminidase)</td>
<td>Galβ1,4Glc</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>

The gradings are based on the relative binding intensities observed on lectin blots: ++++, most intense binding; ++, intermediate intensity; +, weak binding; --, no binding. See Figures 1 and 2 for tissue distribution of lectin binding.
Since glycoconjugates represent a significant portion of the mass of SPACR, we were interested in determining the oligosaccharide chemistry in order to gain insight into properties of the carbohydrates that could be important in mediating interactions of SPACR with other molecules in the IPM. Lectin blots and staining patterns in tissue sections are presented in Figures 2 and 3. A summary of the lectin binding analysis to SPACR carbohydrates is given in Table II. It should be pointed out that each enzymatic digestion was performed on the same batch of protein isolate. Equal protein concentrations were loaded on the gels prior to electrophoresis, transfer, and lectin blotting. The efficiency of electrotransfer to the membrane for a particular gels prior to electrophoresis, transfer, and lectin blotting. The protein isolate. Equal protein concentrations were loaded on the SDS-PAGE gels, is known to be inhibited by glycophorin, which contains the O-linked epitope NeuAcα2→3Galβ1→4GlcNAc (Konami et al., 1994). PNA binding of SPACR following neuraminidase digestion was also lost following O-glycanase treatment (Figure 3 B). Since the most potent inhibitor of PNA is T-antigen (Lotan et al., 1975), it seems that the basic O-linked glycoconjugate structure in SPACR is NeuAcα2→3Galβ1→4GlcNAc Ser/Thr. However, S-WGA (which binds GlcNAc, but not sialic acid) recognizes O-deglycosylated SPACR. This binding can occur only through the presence of additional GlcNAc residues on the O-linked chain. This suggests that some of the O-linked chains may be more complex than the simple trisaccharide NeuAcα2→3Galβ1→4GlcNAc.

In other experiments, we compared the lectin binding patterns of SPACR and IRBP. The carbohydrate properties (Fong et al., 1985) and structure of IRBP have been well established (Taniguchi et al., 1986). In our analysis, IRBP bound Con A, WGA, and S-WGA with high affinity. MAA-1 bound IRBP very weakly, in contrast to the strong binding of MAA-1 to SPACR (data not shown).

The mobility of SPACR was not affected by treatment with chondroitinase ABC, indicating that chondroitin sulfate type GAGs were not present in the molecule. However, SDS-PAGE separated IPM samples following chondroitinase treatment showed the appearance of two new bands at M, 170 kDa and 200 kDa, respectively (not shown). Both of these bands bound PNA (Hageman and Johnson, 1991), but neither the 170 kDa nor the 200 kDa band showed immunoreactivity with the anti-SPACR antibody.

Discussion

The binding of WGA to the IPM was first revealed in studies of etched plastic embedded sections of Xenopus laevis eyes (Wood et al., 1984). These authors also reported that when the isolated

Fig. 3. (A) Neuraminidase treated IPM samples separated by SDS/PAGE, followed by blotting with the indicated lectins. Lanes 1, 3, 5, and 7 represent untreated IPM controls. Lanes 2, 4, 6, and 8 are from the neuraminidase digest. Note the loss of WGA and MAA-2 staining following desialylation accompanied by intense staining with PNA. (B) Lectin blots of N- and O-glycosidase digests of IPM stained with the indicated sialic acid binding lectins. Lanes 1, 4, and 7 are IPM control samples. Lanes 2, 5, and 8 are IPM samples digested with N-glycosidase. Lanes 3, 6, and 9 are O-glycosidase digested IPM samples. MAA-1 has a different staining pattern than WGA and MAA-2. See Table II for listing of relative binding intensities.
retina was rinsed extensively with saline prior to fixation, WGA-binding in the IPM was only minimally reduced, suggesting that the bulk of the WGA-binding epitopes was part of an aqueous-insoluble complex. The preferential binding of WGA to the domains surrounding rod photoreceptors was also described in primate retinas, but the relative solubility of the WGA-binding components was not evaluated (Sameshima et al., 1991; Kuehn et al., 1996). Our initial interpretation of these fluorescent patterns was that SPACR and WGA-binding domains may be equally distributed around rods and cones. The images presented in Figure 1A–C demonstrate unequal binding of WGA in the isolated IPM, with more intense binding to the rod-associated matrix than to the cone matrix (Hollyfield et al., 1990b; Tien et al., 1992). Since SPACR was purified from the insoluble IPM isolate and our analysis of WGA-binding patterns was also made from IPM isolates, we used this preparation for double label studies of SPACR immunoreactivity and WGA-binding. In these preparations, we observed similar patterns of WGA-binding and SPACR immunoreactivity, with intense binding surrounding rods and weaker, but distinct binding in the compartments surrounding cones. Our interpretation of these binding patterns is that SPACR is present around both photoreceptor types, but the distribution is not equal; the concentration in the rod-associated IPM is higher than that surrounding cones. Further, most of the WGA-binding in the isolated IPM, both in intensity and distribution, represents the binding of this lectin to the carbohydrides present on SPACR.

It is clear from the data obtained following chondroitinase digestion that SPACR is distinctly different from the two PNA-positive core proteins of chondroitin sulfate-type proteoglycans identified earlier in the IPM (Hageman and Johnson, 1991; Kuehn et al., 1997). When we treated the isolated IPM with chondroitinase, we also observed the appearance of two PNA-binding proteins by SDS/PAGE that migrated with an apparent Mr of 170 and 200 kDa. Although one of these presumed core proteins migrates quite close to SPACR, neither are recognized by our SPACR antibody.

Although the carbohydrates of SPACR resemble those present in mucins, the molecule is smaller than those generally classified as mucins (Allen, 1983; Schachter and Brockhausen, 1992). The lectin binding patterns suggest that the dominant epitope recognized by the sialic acid specific lectins on SPACR is the O-linked trisaccharide NeuAcα2–3Galβ1–3GalNAc. The strong binding of both MAA-1 and MAA-2, coupled with the lack of SNA binding, argues in favor of α2–3- rather than α2–6-linked sialic acid in SPACR. An intriguing feature of the binding analysis was that S-WGA displayed similar binding intensities to native SPACR as well as both the N-deglycosylated and the O-deglycosylated SPACR. This suggests that the lectin has binding sites on both the N- and O-linked chains of SPACR. S-WGA is specific for GlcNAc and does not bind sialic acid (Monsigny et al., 1979). WGA and S-WGA are both reported to have similar substrate specificities, except that S-WGA recognizes only GlcNAc and not sialic acid. The binding of S-WGA to N-deglycosylated SPACR can be explained if the remaining O-linked trisaccharide contains an additional GlcNAc residue. The O-linked sugar chain in SPACR could have a structure similar to that present in bone sialoprotein (Midura and Hascall, 1996) and aggrecan (Nilsson et al., 1982):

\[
\text{NeuAcα2–3Galβ1–4GlcNAc} \quad \downarrow (\beta 1,6) \\
\text{NeuAcα2–3Galβ1–3GlcNAc–O-Ser/Thr}
\]
or the intermediate structure shown below:

\[
\text{GlcNAc} \quad \downarrow \\
\text{NeuAcα2–3Galβ1–3GlcNAc–O-Ser/Thr}
\]

MAA-1, which recognizes NeuAcα2–3Galβ1–4GlcNAc, binds poorly to N-deglycosylated SPACR. If this is a result of steric hindrance, then the former structure would be quite likely to occur in SPACR. Quantitative carbohydrate sequence analysis will be the next step in precisely establishing the identity of the SPACR oligosaccharides.

Analysis of the N-linked sugars revealed several unexpected features. Con A does not bind SPACR, while S-WGA and MAA-1 recognize both the native and O-deglycosylated forms. Among the different types of N-linked chains, Con A binds high mannose and hybrid type oligosaccharide chains with high affinity, and the biantennary type with lower affinity, but does not bind the tri- and tetraantennary chains (Osawa and Tsuji, 1987). The lack of Con A binding to SPACR likely reflects the presence of complex type glycoconjugates. Moreover, WGA exhibits a relatively high binding specificity for the complex type N-linked chains, but low affinity for high mannose-type glycoconjugates (Yamamoto et al., 1978). Since S-WGA does recognize the N-linked sugars in SPACR, this argues for the presence of complex type N-linked glycoconjugates in SPACR.

It is interesting to note the substantial decrease in binding intensity of MAA-1, an α2–3 linked sialic acid specific lectin, to SPACR after removal of the N-linked sugars (Figure 3B, Table II). A potent inhibitor of this lectin is lactose, which is present internally in the N-triantennary matrix. It is possible that MAA-1 has a higher affinity for the structure NeuAcα2–3Galβ1–4GlcNAc, present in the N-linked chains, than to the O-linked trisaccharide NeuAcα2–3Galβ1–3GlcNAc. We compared the lectin binding properties of another IPM glycoprotein, IRBP, to SPACR. Human IRBP has two sialylated N-linked sugar chains with no O-linked sugar (Si et al., 1989). Con A binds extremely well to IRBP and minimally to SPACR, whereas both WGA and S-WGA bind IRBP and SPACR with comparable reactivities. However, MAA-1 binds SPACR more intensely than IRBP. Because IRBP is known to have a hybrid-type oligosaccharide structure (Taniguchi et al., 1986), it is likely that SPACR has a tri- or tetraantennary type N-linked sugar.

Our deglycosylation studies indicate that N- and O-linked glycoconjugates represent a significant portion of the molecular mass of SPACR (at least 30%), suggesting that these carbohydrates may be of fundamental importance to the function of this molecule. While the function of SPACR is yet to be determined, earlier studies on the development of photoreceptors in Xenopus indicate that lactose and certain other galactosyl derivatives are
able to support photoreceptor outer segment disc morphogenesis in the absence of the RPE (Stiemke and Hollyfield, 1994). Although we do not yet know when SPACR is elaborated during retinal development, this molecule may be involved in establishing the appropriate microenvironment required for development and maintenance of normal photoreceptor outer segment membrane structure.

While most of the rod matrix compartments bind WGA, but not PNA unless treated with neuraminidase (Tien et al., 1992), PNA binding to the matrix of a small population of rod photoreceptors (<0.2%) in the human retina has been reported (Iwasaki et al., 1992). This PNA binding is localized to individual rod matrix compartments and may represent unglycosylated SPACR, although PNA binding sites on an entirely different glycoprotein cannot be ruled out at this time. To avoid any molecular heterogeneity in the biochemical analysis, our purification protocol would have removed both asialo SPACR and any other molecules with PNA binding determinants. It seems appropriate to mention that during the natural history of human retinal aging, rod photoreceptors are lost more rapidly than cones (Gao and Hollyfield, 1992). Rod loss is rapid between the second and fourth decades (900 rods lost/year) and slows thereafter (500 rods lost/year). If PNA binding observed in the matrix of rods is due to the presence of asialo-SPACR, these PNA binding domains may reflect early stages in rod degeneration that could target these cells for removal. Such a mechanism has been described for the removal of glycoproteins from the circulation by asialoglycoprotein receptors on hepatocytes (Ashwell and Harvard, 1982; Steer and Ashwell, 1986; Drickamer, 1988).

The identification and partial characterization of SPACR reported in this study opens up a number of fundamental issues regarding the role of this glycoprotein in the IPM. Is SPACR uniquely present in the IPM or is it also found in other matrices outside the eye? What is the significance of the polyanionic nature of SPACR? Are the abundant negative charges contained in SPACR related to the function of the molecule? Are there sialic acid binding proteins present in the IPM that interact with SPACR? How do SPACR molecules associate to form the expansive insoluble IPM complex present around photoreceptors? Do the carbohydrates, the protein, or both play a role in this association? These and other questions will be the subject of future studies on the structure and function of this new IPM molecule.

Materials and methods

Reagents

The following lectins were used: WGA (wheat germ agglutinin); S-WGA (sucinylated wheat germ agglutinin); MAA-1 (Maackia amurensis agglutinin-1); MAA-2 (Maackia amurensis agglutinin-2); SNA (Sambucus nigra agglutinin); PNA (peanut agglutinin); and Con A (Concanavalin A). All lectins, the biotinylated peroxidase conjugated HRP, streptavidin, and goat anti-rabbit IgG were obtained from Vector Laboratories, (Burlingame, CA). WGA-Sepharose, N-acetylglucosamine, lactose, NP-40, and BCIP-NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets) were purchased from Sigma Chemical Co. (St. Louis, MO). Protease inhibitors were from Boehringer Mannheim, (Indianapolis, IN). Immobilon P was from Millipore (Bedford, MA). DAB tablets were from Amresco (Solon, OH). Neuraminidase (Clostridium perfringes) was obtained from Genzyme Diagnostics (Cambridge, MA). N- and O-glycosidases were from Oxford GlycoSciences (Wakefield, MA). Chondroitinase ABC was purchased from Seikagaku Corporation (Ijamsville, MD).

Protein purification

Human eyes were obtained from the Cleveland Eye Bank, Cleveland, OH, with postmortem times ranging from 1.5 to 8 h. Fifty-seven pairs of eyes were used in this analysis. The anterior segment of each eye was removed by a circumferential cut posterior to the limbus. After removing the vitreous, the retina was dissected from the posterior eye cup and isolated after cutting the optic nerve. The isolated retina was rinsed twice in 50 ml volumes of phosphate-buffered saline (PBS) containing protease inhibitors. These initial rinsing steps remove PBS soluble macromolecules (Adler and Klucznik, 1982), including IRBP, a well characterized soluble IPM glycoprotein (Liou et al., 1982; Wiggert et al., 1984). Retinas were then placed in distilled water for isolation of the PBS insoluble IPM (Johnson and Hageman, 1989; Hollyfield et al., 1990a,b). Because of the polyanionic nature of the components that are retained in the IPM after PBS rinses, these extracellular components swell on hydration and detach the IPM from the outer retina, when the retina is placed in distilled water. A detailed description of the time course involved in swelling and detachment of the IPM in distilled water has been described previously (Hollyfield et al., 1990a). After detachment, the swollen IPM has a translucent, diaphanous appearance, in contrast to the chalky, opaque-like appearance of the inner retina. The inner retina debris was manually removed from the preparation and discarded. The insoluble IPM was pelleted by centrifugation at 8000 r.p.m. for 10 min. Several detergents and chaotropic agents were used in an attempt to extract SPACR from the IPM pellet. While it was soluble in SDS (1%) it could not be completely extracted in CHAPS (5%), 4 M guanidinium chloride, or 4 M urea. However, using Tris, a positively charged buffer, and increasing the pH to 8.0, SPACR could be efficiently extracted. This extraction procedure is probably effective due to the negatively charged nature of the SPACR molecule.

After extraction of SPACR with 0.1M TBS (Tris buffered saline) pH 8.0 containing 5 mM DTT, the supernatant was dialyzed against TBS before loading on a PNA-agarose column. The flow-through from this column was applied to a WGA-sepharose column and the protein eluted with 0.25 M GlcNAc. Individual fractions were analyzed for purity by SDS–PAGE, followed by lectin blotting using biotinylated WGA, and stored as individual fractions at -70°C.

Antibody preparation

Crude extracts of IPM proteins, solubilized in 0.5% SDS, were loaded on a PNA-agarose affinity column. The flow-through, which was essentially free of PNA binding components, was separated by preparative SDS/PAGE (10%) (Laemmli, 1970). After staining with Coomassie blue, the band corresponding to SPACR was cut from the gel and destained with 50% methanol, 10% acetic acid. The gel fragment was crushed in a 10 ml plastic tube with a spatula, and the SPACR sample was sent to Biodesign International, Kennebunk, ME, for antibody production in a rabbit. The test bleed after the fourth boost provided an antibody titer suitable for Western blotting and immunohistochemical analyses.
Affinity purified SPACR was blotted on PVDF membranes (Immolon P) and submitted for N-terminal amino acid sequence analysis on an Applied Biosystems Procise 492, and for phenylisothiocyanate derivatization for amino acid composition analysis in the Protein Core Laboratory, using an Applied Biosystems amino acid analyzer.

**Lectin blots and Western blots**

SDS–PAGE separated IPM samples were electroblotted onto Immobilon-P membranes and incubated in 0.1 M TBS pH 7.5 for 30 min. This was replaced by the appropriate biotinylated lectin (20 µg/ml) in 1% BSA (bovine serum albumin)-TBS and incubated for 3 h at room temperature. The membranes were washed with TBS (three times) and incubated with biotinylated HRP (horseradish peroxidase)-avidin complex. The bands were then visualized by the peroxidase reaction product. For Western blots, the membranes were incubated in 1:1000 diluted primary antiserum in TBS overnight at 4°C after BSA blocking. Membranes were washed and incubated with alkaline phosphatase–conjugated secondary antibody (1:1000) for 1 h at room temperature. The membranes were washed and the color reaction developed using the substrates BCIP-NBT.

**Enzyme treatments**

Purified SPACR or crude IPM extracts were used for the deglycosylation experiments. Each enzyme digestion was done as described by the manufacturer.

**Neuraminidase digestion.** Native protein (20 µg) was dissolved in 100 µl of 0.1 M sodium phosphate buffer, pH 6.5. 50 mU/µl of neuraminidase was added followed by incubation for 1 h at 37°C. The reaction was terminated by addition of Laemmli sample buffer. The digest was separated by SDS/PAGE, electroblotted to PVDF membranes and analyzed by lectin staining.

**N-Glycosidase digestion.** Samples (20 µg) were denatured in 0.05 M phosphate buffer containing 0.2% SDS and 50 mM DTT at 100°C for 5 min. NP–40 was added to a final concentration of 0.7% in 100 µl of sample volume, followed by the addition of 0.3 mU in 1 µl of recombinant peptide N-glycosidase F. The digestion was carried out overnight, followed by an analysis of the samples with SDS/PAGE and Western blotting.

**O-Glycosidase digestion.** SPACR was predigested with neuraminidase, as described above, prior to digestion with O-glycosidase. Samples (20 µg) were denatured by boiling in 0.1% SDS, 10 mM sodium cacodylate buffer, pH 6.0. NP–40 was added in 10-fold excess of SDS by weight, followed by 50 mU of O-glycosidase and overnight incubation.

**Chondroitinase digestion.** 50 µl of 1 mg/ml IPM extract was resuspended in 150 µl of acetate buffer pH 6.0, 30 mU in 3 µl of chondroitinase ABC was added and the reaction allowed to proceed at 37°C for 3 h.

**Tissue preparation for microscopy**

Selected areas from the outer wall of human eyes that had been fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4 were placed in CRYOForm (IEC, Needham Heights, MA), frozen in liquid nitrogen, and cryosectioned at 7 µm. Sections were placed on glass slides and allowed to dry at room temperature. Lectins were applied to the sections at concentrations of 100 µg/ml in PBS containing 0.1% BSA for 30 min at room temperature. For antibody staining, rabbit immune or preimmune serum, diluted 1:1000 in PBS, was applied to sections overnight in a cold room at 4°C. After rinsing 2× with PBS, goat anti-rabbit IgG conjugated to Texas Red was applied to the sections for 2 h at room temperature. All slides were washed 4× with the PBS/BSA solution, the slides were gently blotted, and the sections mounted in glycerol and viewed in Zeiss Axiohot epifluorescent microscope (Oberkochen, Germany) using the appropriate filter sets. Images were digitized using a Hamamatsu Color Chilled 3CCD camera (model C5810) and manipulated with Photoshop Software in a Power Macintosh computer (model 7600/132).

The IPM was isolated and prepared for single or double label lectin and anti-SPACR immunocytochemistry as described previously (Hollyfield et al., 1990b). IPM isolation and fixation (4% paraformaldehyde in 0.1 M phosphate buffer on ice at pH 7.4) was done in 3 cm Falcon plastic petri dishes. After three rinses, 5 min each with PBS, the tissue was blocked with BSA/PBS (1 mg/ml) for 20 min at room temperature. The isolates were incubated for 1 h with the SPACR polyclonal antiserum, diluted 1:1000 with PBS. Antibody controls were incubated for the same time with preimmune serum at the same dilution. After three rinses with PBS, 5 min each, goat anti-rabbit IgG conjugated to Texas Red was applied for 1 h. After three rinses, 5 min each with PBS, WGA conjugated to FITC (1:250 dilution in PBS, Vector Labs) was applied for 30 min. After 3 × 5 min rinses with PBS, the fluid was gently withdrawn from the dish, a 22 mm square coverglass was applied to the sample, and the staining patterns were evaluated using epifluorescent microscopy (described in the above paragraph) or a Leica TCS-NT confocal microscope (Heidelberg, Germany).

**Illustrations**

Figures for this study were prepared from digitized images imported into PowerPoint software where they were composed and labeled. Figures were printed on a Codonics Dye Diffusion Printer (NP-1600 Series, Middleburg Heights, OH).

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Abbreviations
BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; CHAPS, (cholamidopropyl)dimethylamonium-1-propane sulfonate; Con A, concanavalin agglutinin; DAB, diaminobenzidine; DTT, dithiothreitol; FITC, fluorescein isothiocyanate; GAGs, glycosaminoglycans; Gal, galactose; GalNAc, N-acetylgalactosamine; GlcNAc, N-acetylglucosamine; HRP, horseradish peroxidase; IEF, isoelectric focusing; IPM, interphotoreceptor matrix; IRBP, intestinal retinoid binding protein; MAA, tetrathiocin; NeuAc, neuraminic acid; NP-40, nonylphenox ypolyethoxy ethanol; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PNA, peanut agglutinin; PVDF, polyvinylidene fluoride; RPE, retinal pigment epithelium; S-WGA, sialylated wheat germ agglutinin; SDS, sodium dodecyl sulfate; SNA, Sambucus nigra agglutinin; SPACR, sialoglycoproteins; TBS, Tris(hydroxymethyl)aminomethane; WGA, wheat germ agglutinin.

References


