SPACR, a Novel Interphotoreceptor Matrix Glycoprotein in Human Retina That Interacts with Hyaluronan*

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SPACR (sialoprotein associated with cones and rods), is the major 147–150-kDa glycoprotein present in the insoluble interphotoreceptor matrix of the human retina. Immunocytochemistry localizes SPACR to the matrix surrounding rods and cones (Acharya, S., Rayborn, M. E., and Hollyfield, J. G. (1998) Glycobiology 8, 997–1006). From affinity-purified SPACR, we obtained seven peptide sequences showing 100% identity to the deduced sequence of IMPG1, a purported chondroitin 6-sulfate proteoglycan core protein, which binds peanut agglutinin and is localized to the interphotoreceptor matrix. We show here that SPACR is the most prominent 147–150-kDa band present in the interphotoreceptor matrix and is the gene product of IMPG1. SPACR is not a chondroitin sulfate proteoglycan, since it is not a product of chondroitinase ABC digestion and does not react to a specific antibody for chondroitin 6-sulfate proteoglycan. Moreover, the deduced amino acid sequence reveals no established glycosaminoglycan attachment site. One hyaluronan binding motif is present in the predicted sequence of SPACR. We present evidence that SPACR has a functional hyaluronan binding domain, suggesting that interactions between SPACR and hyaluronan may serve to form the basic macromolecular scaffold, which comprises the insoluble interphotoreceptor matrix.

The IPM resides within the outer eye wall, at the interface between the neural retina and the retinal pigment epithelium. Projecting from the neural retina outer surface, the elongate photoreceptor inner and outer segments penetrate into and are surrounded by the IPM (1). A number of activities of fundamental importance to vision are thought to be mediated by the IPM, including retinal adhesion, visual pigment chromophore exchange, metabolite trafficking, photoreceptor alignment, and membrane turnover (2). The specific IPM molecules that participate in these activities are not well defined.

The distribution of wheat germ agglutinin (WGA)-binding domains in the IPM surrounding rod and cone photoreceptors has previously been documented (3). These matrix components are relatively stable and resist extraction with physiological salt solutions. A major WGA-binding glycoprotein, glycoprotein 147, was identified in extracts of the insoluble IPM from human retina (4). More recently, this protein was purified from the insoluble IPM and characterized (5). The glycoprotein was renamed SPACR, which is an acronym for “sialoprotein associated with cones and rods” (5). A polyclonal antibody prepared against SPACR intensely labels the rod-associated matrix with weaker labeling of the matrix surrounding cones (5). This pattern and intensity of immunoreactivity mimics the differential distribution of WGA binding to the rod and cone matrix domains. Using a variety of lectins in conjunction with neuraminidase and glycosidase digestions, we established that (a) the molecule is heavily sialylated, (b) both N- and O-linked glycoconjugates are present in the molecule, and (c) glycoconjugates account for approximately 30% of the molecular mass (5).

To obtain sufficient amino acid sequence from purified SPACR, which would permit cloning of the gene, SPACR peptides were sequenced and found to be identical to predicted peptides encoded by the cDNA of IMPG1 (also called IPM150, accession number AF047492). The gene product of IMPG1 is reported to be a chondroitin sulfate proteoglycan core protein present in the IPM (6–10). Although both IMPG1 and SPACR are located in the IPM, our earlier studies demonstrated that SPACR is a glycoprotein and not a proteoglycan (5). Could SPACR and IMPG1 be products of the same gene, differing only in post-translational modifications? In this study we present evidence demonstrating that the gene product of IMPG1 is not a proteoglycan, but instead is the IPM glycoprotein SPACR. Furthermore, we demonstrate that one function of SPACR is to bind and organize HA in the IPM.

EXPERIMENTAL PROCEDURES

Reagents—The lectins WGA and peanut agglutinin (PNA), biotinylated horseradish peroxidase, streptavidin, and goat anti-rabbit IgG were obtained from Vector Laboratories (Burlingame, CA). Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate tablets, iodate-acid, and dithiothreitol were purchased from Sigma. Protease inhibitors were from Boehringer Mannheim. Diaminobenzidine tablets were from Anocor (SOLON, OH). Biotinylated chondroitin 6-sulfate A-disaccharide-specific antibody (3B3), chondroitinase ABC (protease-free), chondroitinase ACII (protease-free), and Streptomyces hyaluronidase were purchased from Seikagaku Corp. (Ijamsville, MD). Sequencing grade trypsin was from Boehringer Mannheim. Endoprotease Lys-C and endoprotease Glu-C were purchased from Wako Bioproducts (Richmond, VA). Cetylpyridinium chloride was obtained from ICN Biomedicals (Aurora, OH). Immobilon P membranes were purchased from Millipore (Bedford, MA). Healon (Amersham Pharmacia Biotech, Uppsala, Sweden) was the source of HA. Gel Code stain was obtained from Pierce.

IPM Isolation—Sixty-five human eyes were obtained from the Cleveland Eye Bank (Cleveland, OH) for use in this study. Donor ages ranged

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from 4 to 70 years, with postmortem times between 2 and 12 h. Retinas were isolated and washed extensively with phosphate-buffered saline containing protease inhibitors to remove soluble components, followed by detachment of the insoluble IPM with distilled water (3, 11, 12). The insoluble IPM was collected (5) after centrifugation, and the pellet was solubilized in 0.1 M TBS, pH 8.0, containing 5 mM dithiothreitol. The supernatant was dialyzed against the same buffer before loading on a PNA-agarose column. The flow-through from this column was then applied to a WGA-Sepharose column, and the protein was eluted with 0.25 M N-acetyl-D-glucosamine. Individual SPACR preparations were analyzed for purity by SDS-PAGE, followed by lectin blotting using biotinylated WGA, and stored as individual fractions at −70 °C.

**Chondroitinase ABC/ACD Digestion**—50 ml of 1 mg/ml IPM extract was resuspended in 0.1 M Tris acetate buffer, pH 7.3, and 3 milliunits of Streptomyces hyaluronidase (2–5 turbidity reducing units). The reaction was allowed to proceed at 37 °C for 3 h.

**Streptomyces Hyaluronidase Digestion**—IPM extract (100 µg of total protein) was incubated in 50 mM sodium acetate buffer, pH 6.0, for 2 h at 37 °C with Streptomyces hyaluronidase (2–5–turubility reducing units). The reaction products were analyzed by staining with Gel Code stain after SDS-PAGE.

**Western and Lectin Blotting**—Equal protein concentrations were loaded onto gels prior to electrophoresis, transfer, and hybridization. The efficiency of electrophoresis to the membrane in the enzyme-treated preparation was assessed by comparing the SPACR staining intensity with Coomassie Blue in the gel before and after transfer. We found no significant difference in the transfer efficiency of the IPM before or after enzymatic treatment in any of these studies. SDS-PAGE-separated IPM samples were electrophoretically transferred onto Immobilon-P membranes and incubated in TBS, pH 7.5, for 30 min. TBS was replaced by the appropriate biotinylated lectin (20 g/ml) in 1% BSA-TBS and incubated for 3 h at room temperature. The membranes were washed with TBS (three times) and incubated with biotinylated horseradish peroxidase-avidin D complex, and the bands were visualized by the peroxidase reaction product. For Western blots, the membranes were incubated in 1:1000-diluted primary anti-SPACR antiserum (5) in TBS overnight at 4 °C before 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 was developed using the substrates 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

**Endoproteinase Lys-C, Trypsin, and Endoproteinase Glu-C Digestions**—These digests were carried out as described by Stoe et al. (13). Twenty micrograms of freeze-dried, purified SPACR was reduced and alkylated with iodoacetamide. The enzyme (1:100 by weight) was added to SPACR in sodium bicarbonate buffer, pH 8.0, and incubated overnight at 70 °C. The peptides were separated by standard reverse phase HPLC on a C-18 column using a gradient of acetonitrile in 0.1% trifluoroacetic acid. Sequencing of the isolated peptides was done in the protein core facility of The Cleveland Clinic Foundation.

**Cyanogen Bromide Cleavage**—SPACR was N-deglycosylated as described (5), and 20 µg was dissolved in 200 µl of 70% trifluoroacetic acid. Cyanogen bromide (200 µg) in acetonitrile was added to the tube, flushed with nitrogen, and incubated in the dark at an ambient temperature for 24 h. After diluting with 20 volumes of distilled water, the mixture was lyophilized. The peptides were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, and subjected to sequencing by Edman degradation.

**Mass Spectrometry**—Affinity-purified SPACR was further separated on a 7.5% SDS-PAGE gel. The gel was stained with Coomassie Blue and the band was excised, and then the gel was desiccated and sent to the Harvard Microchemistry Laboratory (Harvard University, Cambridge, MA) for digestion by trypsin and separation by liquid chromatography/mass spectrometry. Four of the resultant peptides were sequenced by tandem mass spectrometry. All peptides were subjected to a BLAST search, which displayed 100% identities with peptides from the deduced sequence for IMPG1. The cDNA sequence of IMPG1 was submitted by M. H. Kuehn and G. S. Hageman to GenBank™ (accession number AF047492), using single letter amino acid codes. The deduced protein contains 771 amino acids, and the N-terminal signal peptide contains 79 residues (single underline). Seven potential N-linked glycosylation sites (in boldface type) are present in two clusters on either side of a mucin-like domain containing numerous potential O-linked glycosylation sites (dotted underline from residue Gly250 to Pro303). An EGF-like domain (single underline from residue Asp259 to Cys279), containing sulfated cysteine residues (in boldface double underline) is present near the C terminus. An HA binding motif (double underline) is located at residues Lys250–Arg253. No consensus sites for GAG attachment are present in this deduced sequence. All SPACR peptides show >100% homology to IMPG1.

**Image Capture and Quantitation**—Images of SDS-PAGE, immuno- blots, and lectin blots were digitized at 300 dots per inch on a flat-bed Scanscan III scanner, using ScanWizard 3.0.7 software from Micro- technology laboratories (Redondo Beach, CA). Band area and pixel density were measured using Kodak Digital Science software (Eastman Kodak Co.) on a Power Macintosh 7600/132 (Apple Computer, Cupertino, CA). Mean density and S.D. values were calculated from the pixel density in the bands of interest. The data were normalized to adjust for unequal background staining throughout the lane. The background staining throughout the lane was subtracted from the stained gels and lectin blots. At least three separate measurements were made for each treatment, and comparisons were made between treatments using a two-tailed Student’s t test. All figures were assembled and labeled with PowerPoint 4.0 software (Microsoft Corp., Redmond, WA) and printed on a Codonics (Middleburg Heights, OH) NC 1600 dye substitution printer.
RESULTS AND DISCUSSION

Sequence Analysis—SPACR was purified on a WGA-Sepharose column after removal of all PNA-binding molecules to ensure a homogeneous population of WGA-binding protein (5). The WGA-binding fraction was further separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes, and the SPACR band was excised for sequence analysis. N-terminal sequencing was attempted on four separate occasions using increasing amounts of protein (from 2 to 20 pmol), which revealed that the majority of this protein is blocked (5). However, using 20 pmol of protein, a low level signal (less than 1 pmol), corresponding to S AffFPTGVKVK was obtained.

Seven peptides were obtained from solution digests of purified SPACR or in gel digests of SPACR separated by SDS-PAGE accounting for 9.1% of the protein sequence. Endoproteinase Glu-C digest yielded peptides EILDNTL and EFAVLEE. Cyanogen bromide cleavage yielded the peptide MQKIFKKN. Tandem mass spectrometric analysis, following trypsin digestion, yielded the peptides S AffFPTGVK, VC-QEAVWEAYR, AELADSQSPYQQELAGK, and QLEIILNFR. The sequence of these seven peptides matched the predicted amino acid sequence of IMPG1 (AF047492) with the primers 1F and 1R (1F, 5′-TCAGTCTGGCTGTGCTAACATGG; 1R, 5′-TGCTACACAC-TACATTCCAT). The upper blot shown is the result from a 12-h exposure to the Europium screen. The 28 S band presented in the lower panel was determined by normalizing the PhosphorImager response to the measured fluorescence of the SYB green II stained RNA before the gel was blotted. The relative levels of IMPG1/SPACR expression presented as histograms in the middle panel is a direct SYB green II-stained RNA before the gel was exposed to the Europium screen. The 28 S band presented in the middle panel is a direct SYB green II-stained RNA before the gel was blotted. The relative levels of IMPG1/SPACR expression presented as histograms in the lower panel were determined by normalizing the PhosphorImager response to the measured fluorescence of the SYB green II stained 28 S band. The calculations were performed using ImageQuant (Molecular Dynamics) and Excel (Microsoft) software.

Tissue Expression—The tissue distribution of SPACR expression was determined by Northern blot analysis using a variety of monkey and human tissues. To test for expression within the retina, 5-mm trephine isolates from different areas of the monkey retina were taken and the RNA isolated. Probes were designed from the cDNA sequence (see Fig. 2 for details). SPACR mRNA was found in nearly equal amounts in macula and peripheral retina but was barely detectable in the pigmented epithelium/choroid (Fig. 2). This low level of expression may be due to contamination from the neural retina, which is difficult to cleanly separate from the retinal pigment epithelium during tissue dissection. This is consistent with our data from human multissue Northern blots, where the retina was the only tissue with detectable SPACR expression (Fig. 3). The mRNA is approximately 3.5 kb in length, consistent with the size of the cDNA sequence of IMPG1 (AF047492). The minor signal around 8 kb observed in the monkey retina lanes (Fig. 2, lanes 1 and 3) may represent an alternative polyadenylation site in the mRNA. These results demonstrate the high level of specificity of this IPM protein to retina.

Features of the Deduced Sequence—The deduced amino acid sequence of IMPG1/SPACR is presented in Fig. 1. Several interesting features are observed. Residues 1–70 represent a likely N-terminal signal peptide involved in the secretion of this IPM molecule. The natural N terminus of the mature protein was found to begin at position Ser71 by N-terminal amino acid analysis of purified SPACR. The tryptic peptides derived from SPACR, which were analyzed by mass spectrometry, did not contain the peptides expected from the first 70 amino acid residues in the deduced sequence, confirming that this is a signal sequence. Thus, the mature SPACR protein contains 701 amino acid residues.

A large mucin-like domain, containing numerous potential O-linked glycosylation sites, lies in the central part of the protein (Gly326–Pro395). Seven consensus sites for N-linked glycosylation are located in two clusters flanking either side of the mucin domain (at residues Asn143, Asn191, Asn215, Asn592, Asn616, Asn630, and Asn648, shown in boldface type in Fig. 1). This confirms and extends our previous direct glycoconjugate analysis of purified SPACR indicating that it contained both N- and O-linked glycoconjugates accounting for approximately 30% of the molecular mass (5).

A linear HA binding motif of the RHAMM type (20, 21) is located at residues Lys286–Lys288. This motif conforms to the
sequence pattern BX-B, where B represents lysine or arginine residues separated by seven amino acids, excluding aspartic or glutamic acid. This site is flanked by two proline residues (Pro277, Pro289), which are known to be strong &beta;turn promotors, and may be required to present this motif in the appropriate conformation for HA binding.

An EGF-like motif is present on the C-terminal side of the mucin-like domain (Asp687-Cys730). EGF-like domains are characteristic of a number of extracellular matrix proteins and are implicated in protein-protein interactions (22–25). The six conserved cysteine residues present in this domain are involved in intrachain disulfide bonding that is required for the structural stability of this motif (26). Some EGF-like domains require calcium for their biological function (27). Interestingly, the EGF-like domain in IMPG1/SPACR contains the critical asparagine (Asn702), which has been implicated in calcium binding (27). The potential for calcium binding by SPACR could point toward an important physiological role for this IPM protein in sequestering extracellular calcium released by photoreceptors in response to light (28, 29).

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Glycoprotein or Proteoglycan?—No documented consensus sites for GAG attachment are present in the deduced amino acid sequence of IMPG1/SPACR (Fig. 1). Our biochemical analysis of SPACR indicates that this molecule is a glycoprotein (4, 5). Based on electrophoretic mobility, the molecular mass of SPACR was defined as 147 kDa (4, 5), which corresponds closely to the 150 kDa ascribed to IMPG1 after chondroitinase digestion (8–10). If IMPG1 is the core protein of a chondroitin 6-sulfate proteoglycan, as claimed (8–10), it should not enter a 7.5% SDS-PAGE unless the chondroitin sulfate chains are degraded. This, however, was not the result we observed when these comparisons were evaluated (Fig. 4A, lanes 1–3). A prominent SPACR band is present in both chondroitinase-digested (Fig. 4A, lane 1) and undigested (Fig. 4A, lane 2) samples. The relative staining densities of the SPACR bands are not significantly different (see histogram in Fig. 4A). The similarities in staining densities of the SPACR bands in these two groups indicate that additional protein with a molecular mass of 150 kDa is not released when the IPM is digested with chondroitinase.

In contrast, dramatic changes are observed in the higher molecular weight proteins. The high molecular weight components, which only minimally enter the gel in the undigested sample (Fig. 4A, lane 2), are no longer observed after chondroitinase digestion (Fig. 4A, lane 1). Instead, two new bands, one just above and the other below the 220-kDa marker, are now present (Fig. 4A, lane 1). Since these bands are seen neither in the untreated sample (Fig. 4A, lane 2) nor in the enzyme control (Fig. 4A, lane 3), they could only represent the core proteins of chondroitin or dermatan sulfate proteoglycans.

A polyclonal antibody to SPACR recognizes both native SPACR and SPACR treated with N- and/or O-glycosidases (5). If the core protein of IMPG1 and SPACR represent the same gene product, the anti-SPACR antibody should recognize any IMPG1 molecules released after chondroitinase digestion, resulting in an increased intensity of immunostaining of the 150-kDa band. We used this antibody in Western blotting analysis (Fig. 4B, lanes 1–3) to determine whether increased im-

FIG. 4. A, IPM samples following 7.5% SDS-PAGE and stained with Coomassie Blue. Lane 1, chondroitinase ABC-treated sample; lane 2, untreated sample; lane 3, enzyme control sample. The thick line between the 97- and 220-kDa molecular mass markers represents the position of SPACR at 147–150 kDa. The dense band opposite the 67-kDa marker in lanes 1 and 3 is BSA present in the chondroitinase ABC buffer. The quantitative data displayed in the histogram on the right are the relative density values measured in the SPACR band in the indicated lanes (hatched bar, chondroitinase-digested; open bar, undigested). The mean densities presented with S.D. values were obtained from digitized images. The relative staining density of the SPACR bands are not significantly different (p > 0.1) between chondroitinase-digested (lane 1, shaded bar) and undigested (lane 2, unshaded bar) sample. B, IPM proteins in a transfer blot from a gel similar to that shown in A, which were probed with a rabbit polyclonal anti-SPACR antibody. Quantitative immunostaining of the SPACR bands (at the level of the thick line between the 97- and 220-kDa markers) is presented in the histogram. The number above each bar reflects the lane number in the adjacent blot from which the density of the SPACR band was measured. SPACR immunoreactivity is not significantly different (see histogram) in lanes 1 (hatched bar) and undigested (lane 2, open bar) sample. No immunoreactivity is evident in the enzyme control lane (lane 3).
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Fig. 5. A, WGA-lectin blot of IPM samples digested with chondroitinase (lane 1) or undigested (lane 2). The quantitative data presented in histograms show the relative staining densities of the distinct band above the 220-kDa marker (**), the band below the 220-kDa marker (*), and the SPACR band (at the level of the thick marker line). The histogram on the right presents the quantitative values of the WGA-staining densities in these bands. Two- to 3-fold increases in WGA staining density occur after chondroitinase digestion (hatched bar) over the density in the undigested samples (open bar) of the two higher molecular weight bands (p < 0.01 for comparisons between both bands), but no significant difference is evident between the SPACR band densities (p > 0.1). B, PNA-lectin blot of IPM samples digested with chondroitinase (lane 1) or undigested (lane 2). The PNA blots shown and the density values were collected as described for A. Note the prominent new bands that occur above and below the 220-kDa marker. Quantitative data presented on the right show that SPACR staining with PNA is not significantly different before and after chondroitinase digestion (p > 0.1).

munoreactivity could be detected in the 147–150-kDa band following chondroitinase digestion. Similar levels of SPACR immunoreactivity are observed in chondroitinase-digested (Fig. 4B, lane 1), and undigested samples (Fig. 4B, lane 2) with no significant differences in the relative staining densities observed when the blots were quantitated (see histogram in Fig. 4B). Additionally, in the undigested sample (Fig. 4B, lane 2), no SPACR immunoreactivity is observed at the top of the lane where the high molecular weight proteoglycan bands are observed in the Coomassie Blue-stained gel (Fig. 4A, lane 2). The two new bands present on either side of the 220-kDa marker, released after chondroitinase digestion (Fig. 4A, lane 1), also fail to show SPACR immunoreactivity (Fig. 4B, lane 1). These data indicate that no additional protein with epitopes recognized by the anti-SPACR antibody is released following chondroitinase digestion.

WGA was the principal lectin used for SPACR identification (3–5). We used this lectin to determine whether additional WGA binding molecules are released following chondroitinase digestion. WGA blots of enzyme-treated and control IPM samples are presented in Fig. 5A, lanes 1 and 2. It should be noted that equal amounts of IPM protein (25 μg of total protein) were loaded for all these comparisons. As previously reported (5), SPACR is the most prominent WGA staining band in the IPM. The intensity in WGA staining of the SPACR band does not change with chondroitinase digestion (Fig. 5A, lane 1) from levels of staining in the undigested IPM sample (Fig. 5A, lane 2). Although there is a general increase in background staining in the region between SPACR and the top of the lane after chondroitinase digestion (Fig. 5A, lane 1), distinct increases in WGA staining of a band just above the 220-kDa marker and a broader, diffuse region below the 220-kDa marker are present. Lower levels of WGA staining occur in these regions of the undigested sample (Fig. 5A, lane 2). The relative staining densities of SPACR and the two higher molecular weight bands were measured. Evident in the histogram presented in Fig. 5A, the higher molecular weight bands show a 2–3-fold increase following chondroitinase digestion, but the densities in the SPACR bands are not significantly different. The enzyme control (not shown) was free of WGA binding.

Although SPACR is predominantly a WGA-binding glycoprotein (5), a minor fraction also binds PNA. In the sample digested with chondroitinase (Fig. 5B, lane 1), no significant increase in PNA staining of SPACR is observed over that present in the undigested IPM sample (Fig. 5B, lane 2). This indicates that a PNA-positive molecule with a mass of 147–150 kDa is present before chondroitinase digestion and that no new PNA-positive molecules with this mass are liberated after chondroitinase digestion. However, higher molecular weight WGA binding bands are generated by this enzyme. New PNA-staining regions are present above and below the 220-kDa marker (Fig. 5B, lane 1). The former is a distinct PNA band, whereas the latter is a broad smudge, composed of at least two closely spaced bands. These new PNA-positive bands are distinct and well separated from the 150-kDa SPACR band (Fig. 5B, lane 2) as evident in the histograms presented in Fig. 5B.

In an important series of papers, Hageman, Blanks, and Johnson (30–32) first reported that the cone-associated IPM compartments contain glycoconjugates that bind PNA, a feature initially thought to be absent in the IPM surrounding rods. Chondroitin sulfate proteoglycans (31, 33) were also found to be uniquely localized to the IPM surrounding cones but not rods.
Further studies by Hageman and collaborators led to the description of two molecules from chondroitinase-digested IPM, which were referred to as IPM150 (now IMPG1) and IPM200. Although a complete biochemical characterization of IPM150 and IPM200 is not available in the peer-reviewed literature, some information is available in several abstracts (6–9) and a review article (2). A key feature is that neither IPM150 nor IPM200 will enter SDS-PAGE unless the IPM sample is digested with chondroitinase. In contrast to the results summarized above, we always observe a PNA-positive band in the 150-kDa region of both control and chondroitinase-treated samples of the human IPM. The new PNA binding band above 220 kDa (Fig. 5B, lane 1) may represent the previously described IPM200 (2, 6–9).

What is responsible for the PNA binding property of this minor fraction of SPACR? Carbohydrate analysis indicates that the dominant glycoconjugate determinant of SPACR is the O-linked carbohydrate NeuAcα2–3Galβ1–3GalNAc, which binds WGA and Maackia amurensis agglutinin-1 and -2 (5). While most of the rod-associated matrix binds WGA and M. amurensis agglutinin-1 and -2 (4, 5), PNA binding to the matrix surrounding a small population of rods in the human retina has also been documented (34). PNA binding to SPACR can be efficiently achieved by removing the terminal sialic acid residues with neuraminidase, which exposes Galβ1–3GalNAc residues that now readily bind PNA (4). Thus, it is likely that the PNA-binding fraction of SPACR represents asialo-SPACR or desialylated SPACR, which in the tissue may be responsible for the PNA binding to the matrix surrounding a small population of rods (34).

The distribution of chondroitin 6-sulfate immunoreactivity in IPM samples digested with chondroitinase ABC was evaluated using 3B3, a monoclonal antibody, which is highly specific for chondroitin 6-sulfate 3-disaccharide (17). Aggrecan (120 ng) from human knee cartilage, predigested with chondroitinase ABC, resulted in strong 3B3 binding (Fig. 6A). When aggrecan was double-digested with chondroitinase ABC and then ACII to remove residual 3-disaccharides from the linkage oligosaccharide (35), it no longer showed reactivity to 3B3, demonstrating the specificity of this antibody. Dot blots using increasing concentrations of total IPM protein, with and without chondroitinase ABC, displayed only faint 3B3 reactivity (Fig. 6B). No differences were found between the digested and undigested IPM samples, and this staining intensity was only slightly greater than that observed using untreated aggrecan, which is known to have a 3B3 mimetope (17). When similar concentrations of IPM were immunoblotted with the same antibody after separation on SDS-PAGE and transblotted, no SPACR reactivity with the 3B3 antibody was observed in samples either before or after chondroitinase digestion (Fig. 6C). Altogether, these data convincingly demonstrate that SPACR, the gene product of IMPG1, is not a chondroitin 6-sulfated proteoglycan core protein. Additionally, none of the higher molecular weight bands that appear after chondroitinase digestion are recognized by this highly specific chondroitin 6-sulfate antibody, inconsistent with reports suggesting that these molecules are chondroitin 6-sulfated proteoglycan core proteins (2, 7).

If the gene product of IMPG1 is the core protein of a proteoglycan, as has been stated in earlier reports (2, 6–9), the predicted amino acid sequence should contain at least one consensus sequence required for GAG attachment. GAGs are linked to serine residues in the core protein by way of a trisaccharide bridge, consisting of a xylose residue attached to serine, followed by two galactose residues (36). Serine residues capable of functioning as xylosylation sites require the consensus sequence SGX, where X represents either glutamic acid or aspartic acid (37, 38). The deduced amino acid sequence of IMPG1 does not contain an SGX consensus site. Although no other sequence for xylosylation has been documented in any mammalian proteoglycan core protein, two reports implicate the sequence EGSAD for GAG attachment in two avian proteoglycans (39, 40). Such a sequence is not present in IMPG1. Mutagenesis studies, converting an SGD site to a TGD sequence, indicate that a threonine-substituted site can be xylosylated but with an efficiency of less than 10% that of SGD sites (41). IMPG1 contains a TGD site (Thr120–Glu122), but not a SGD site and should therefore be considered a proteoglycan only with supporting biochemical evidence. Our biochemical evidence indicates that there are no GAG chains on SPACR.

Hyaluronan Binding—CPC is a detergent that can, under nondissociative conditions, selectively precipitate GAGs along with any associated protein (42). Recent studies demonstrate that HA is a normal constituent of the IPM (43). To explore the possible association of SPACR with HA, we used CPC to pre-
Fig. 7. Co-precipitation of SPACR and hyaluronan from IPM extracts using CPC. Twenty-five micrograms of IPM protein in TBS, containing naturally abundant HA (43), was incubated with 1.25% CPC in 0.1 M Tris acetate buffer, pH 7.3, for 1 h at room temperature as described under “Experimental Procedures.” Lane 1 contains 25 μg of IPM protein from the original extract without CPC. Samples in lanes 2 and 3 were incubated in the absence of enzyme, whereas samples in lanes 5 and 6 were predigested with Streptomyces hyaluronidase before CPC precipitation. Lanes 2 and 4 are pellet fractions; lanes 3 and 5 are the supernatant fractions. Note the co-precipitation of SPACR (at the level of the thick marker line, arrow) as well as a high molecular weight fraction at the top of the lane and in the comb depression at the top of the stacking gel (SG) in lane 2 and the absence of these proteins in lane 4. Lanes 6 and 7 represent the CPC pellet samples obtained when different amounts of total IPM protein were utilized (25 μg in lane 6; 100 μg in lane 7). Note the increased staining density of SPACR precipitated in lane 7 over that in lane 6. BSA incubated with Healon (lane 8) was used as a negative control, which did not precipitate with CPC.  

The IMPG1/SPACR gene is located on chromosome 6q (10). A number of macular dystrophies have been linked to this locus in humans (44–46). Although no specific mutations have yet been identified (44), the gene for this abundant IPM protein is a potential candidate locus for inherited retinal disorders. In summary, the peptide sequence information we obtained from the human glycoprotein SPACR is 100% homologous to the predicted amino acid sequence of IMPG1. The predicted polypeptide for IMPG1/SPACR has no established consensus site for GAG attachment, and the protein is not a product of chondroitinase digestion and displays no immunoreactivity to an anti-proteoglycan chondroitin 6-sulfate antibody. We conclude that the cDNA sequence of IMPG1 is not the core protein for a chondroitin 6-sulfate proteoglycan but instead is the gene for the IPM glycoprotein we describe as SPACR (5). SPACR has a functional binding domain for HA, suggesting that interactions between SPACR and HA may serve to organize the basic macromolecular scaffold, which comprises the insoluble IPM.

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SPACR, a Novel Hyaluronan-binding Glycoprotein

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