SPACRCAN, a Novel Human Interphotoreceptor Matrix
Hyaluronan-binding Proteoglycan Synthesized by Photoreceptors
and Pinealocytes*

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The interphotoreceptor matrix is a unique extracellular complex occupying the interface between photoreceptors and the retinal pigment epithelium in the fundus of the eye. Because of the putative supportive role in photoreceptor maintenance, it is likely that constituent molecules play key roles in photoreceptor function and may be targets for inherited retinal disease. In this study we identify and characterize SPACRCAN, a novel chondroitin proteoglycan in this matrix. SPACRCAN was cloned from a human retinal cDNA library and the gene localized to chromosome 3q11.2. Analysis of SPACRCAN mRNA and protein revealed that SPACRCAN is expressed exclusively by photoreceptors and pinealocytes. SPACRCAN synthesized by photoreceptors is localized to the interphotoreceptor matrix where it surrounds both rods and cones. The functional protein contains 1160 amino acids with a large central mucin domain, three consensus sites for glycosaminoglycan attachment, two epidermal growth factor-like repeats, a putative hyaluronan-binding motif, and a potential transmembrane domain near the C-terminal. Lectin and Western blotting indicate an Mr around 400,000 before and 230,000 after chondroitinase ABC digestion. Removal of N- and O-linked oligosaccharides reduces the Mr to approximately 160,000, suggesting that approximately 60% of the mass of SPACRCAN is carbohydrate. Finally, we demonstrate that SPACRCAN binds hyaluronan and propose that associations between SPACRCAN and hyaluronan may be involved in organization of the insoluble interphotoreceptor matrix, particularly as SPACRCAN is the major proteoglycan present in this matrix.

The light-sensitive photoreceptor inner and outer segments project from the outer retinal surface into a carbohydrate-rich IPM† (1). Several structure-function activities of fundamental importance to vision are thought to be mediated by the IPM, including visual pigment chromophore exchange, metabolite trafficking, retinal adhesion, photoreceptor alignment, and photoreceptor membrane turnover (2–12). Because this matrix resides in such a key location and is putatively crucial in supporting photoreceptor function, additional information is required as to the identity, role, and involvement of specific IPM molecules in mediating these activities.

Early attempts to remove IPM components for subsequent characterization used saline rinses of the outer retinal surface, which succeeded in isolating some soluble molecules. For example, the interphotoreceptor matrix retinoid-binding protein was first isolated from the IPM by rinsing, which also removed a variety of enzymes, some mucins, and immunoglobulins (13–17). The retention of other less soluble molecules following aqueous rinses was not initially appreciated but was clearly documented in the studies of the IPM in Xenopus (18) and rat (19).

Recently, an abundant sialoglycoprotein that is retained in the human IPM following rinsing was characterized and named SPACR (20). A polyclonal antibody prepared against SPACR intensely labels the rod-associated matrix with weaker labeling of the cone matrix (20). Sequence analysis of peptides from purified SPACR revealed 100% identity to the deduced sequence of interphotoreceptor matrix proteoglycan 1 (GenBank™ accession number AF047492) cDNA (also called IPM150) (21). The gene product of interphotoreceptor matrix proteoglycan 1 is listed in GenBank™ as a chondroitin sulfate proteoglycan core protein, but our analysis indicates that it is a glycoprotein and not a proteoglycan (21).

Earlier reports document the presence of another prominent protein in the insoluble IPM. This molecule, initially referred to as IPM200 (8), is clearly a proteoglycan core protein because it will only enter a 7.5% polyacrylamide gel after digestion with chondroitinase ABC (23). Furthermore, it shows intense immunoreactivity in Western blots to a chondroitin ΔDi6S monoclonal antibody (23). Until now the identity of this proteoglycan has remained unknown. In this study we describe the cloning and characterization of the human cDNA encoding this novel proteoglycan in addition to the chromosomal localization of the gene counterpart. The identification of the proteoglycan came about during cloning attempts of the human homolog of PG10.2, a gene that is expressed specifically in rat pineal gland

PNA, peanut agglutinin; HA, hyaluronan; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CFC, cetylpyridinium chloride; EFEMP-1, EGF fibrillin-like extracellular matrix protein-1; EGF, epidermal growth factor; GAG, glycosaminoglycan; Healon, the trade name for highly purified HA used in ophthalmic surgery; Nonidet P-40, nonylpolyoxethanol; PG10.2, rat pineal gland clone 10.2.

This paper is available on line at http://www.jbc.org
and retina (24). The N-terminal sequence of the proteoglycan core protein isolated from the human IPM matched the deduced amino acid sequence of the human PG10.2 homolog. We named this gene and its product “SPACRCAN,” because it is a novel proteoglycan located in the subretinal space, the term used by ophthalmologists for the IPM. We document the expression of the SPACRCAN gene in photoreceptors and pinealocytes and localize SPACRCAN in the IPM where it surrounds both rods and cones. SPACRCAN is retained in the insoluble IPM through its binding to hyaluronan, suggesting that one function of SPACRCAN is to participate with the glycoprotein SPACR in binding and organizing hyaluronan into the primary scaffold of the insoluble IPM (25). Finally, there are homologous regions between the deduced amino acid sequences of human SPACRCAN and the glycoprotein SPACR, which suggests a novel family of IPM-specific molecules.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Avidin-conjugated lectins wheat germ agglutinin and PNA, biotinylated horseradish peroxidase, streptavidin, and goat anti-rabbit IgG were obtained from Vector Laboratories (Burlingame, CA). Nitro blue tetrazolium, 5-bromo-4-chloro-3-indolyl phosphate tablets, iodoacetic acid, and dithiothreitol were purchased from Sigma. Protease inhibitors were from Roche Molecular Biochemicals, (Indianapolis, IN). 3,3′-Diaminobenzidine tablets were from Amresco (Solon, OH). The biotinylated monoclonal antibody prepared against chondroitinase ABC-digested proteoglycan and chondroitin ABC-D6S (from clone 3-B-3) were from Seikagaku Corporation (Ijamsville, MD). Chondroitin ABC (protease free) and Streptomyces hyaluronidase were also from Seikagaku Corporation (Ijamsville, MD). N- and O-glycosidases were from Oxford GlycoSciences, (Wakefield, MA). Cetylpyridinium chloride was obtained from ICN Biomedicals, (Aurora, OH). Immunoblot-P membranes were purchased from Millipore, (Bedford, MA). Healon, Amer- sham-Pharmacia Biotech, was the Gel RNA-mix that was purchased from Pierce. The 22-mer HA oligosaccharides were a gift from Markku Tammi and have been described in previous work (see Ref. 26).

**Tissue Sources**—The 42 human eyes used in this analysis were obtained from the Cleveland Eye Bank, Cleveland, OH. Donor ages ranged from 14 to 77 years, with postmortem times between 2 and 12 h. Seven human pineal glands with donor ages from 20 to 78 years were also used; five were obtained through the Cooperative Human Tissue Network at The Cleveland Clinic Foundation, Department of Anatomic Pathology, and two were from the section of Neuropathology, Clinical Brain Disorders Branch, NIMH, Bethesda, MD.

cDNA Library Screen—Approximately 1 x 10^6 bacteriophage clones from a Agt10 human retinal cDNA library (kindly provided by Dr. Jeremy Nathans, Johns Hopkins University School of Medicine, Baltimore, MD) were screened (27) using a rat PG10.2 cDNA probe. The cDNA probe was derived by PCR using rat PG10.2 sequence-specific forward (5′-TGTTTTGGCCCAAATGATTATGTTTCTCC) and reverse (5′-CCAGGGTGGCATTGCTTTCG) primers and the rat PG10.2 cDNA as the DNA template (24). The PCR product was purified using QIAquick gel extraction kit (QIAGEN, Inc., Valencia, CA) and random primer labeled with [α-32P]dCTP (3000 Ci/mmol, ICN Biomedicals, Aurora, OH) and the Prime-it II kit (Stratagene, La Jolla, CA) and manipulated with IPLab (Scanalytics, Fairfax, VA). The gel was stained with SYB green II (Molecular Probes, Eugene, OR) then scanned using a STORM 860 apparatus (Molecular Dynamics, Inc., Sunnyvale, CA). The gel was blotted and probed with a 462-base pair SPACRCAN probe generated by PCR using the forward (5′-CCGAGGATTAGTTCTTCTCAG) and reverse (5′-CCAGGGATGCA-GATTCTCTCAG) primers designed from human SPACRCAN genomic sequence data (3) and from the hPG10.2.3 cDNA sequence, respectively. The 5′-cDNA region of SPACRCAN was generated using the reverse primer (5′-CAGTAGAGGAGGATGCTTG) for first strand cDNA synthesis. The cDNA amplification was performed using a final 10-min extension step at 72 °C. The genomic PCR products were sequenced using SP6 and T7 RNA polymerases, respectively. Northern Analysis—The cDNA insert of the phage clone, hPG10.2.3, was subcloned into the EcoRI site of the pGEM-4Z vector (Promega, Madison, WI). Sense and antisense riboprobes, labeled with 32P-S-500 (Clonamid, NEW Life Science Products), were generated using SP6 and T7 RNA polymerases, respectively. In situ hybridization histochemistry was performed on fresh frozen human retinal and pineal gland sections cut at 12 μm. These sections were processed as described previously (28) and were dipped in emulsion and developed after exposure for 2 months. Sections were examined with bright and darkfield illumination using a Leitz photomicroscope. Images were digitized using a SenSys-KF1401 CCD camera (Photometris, Tuscon, AZ) and manipulated with IPLab (Scanalytics, Fairfax, VA), Adobe 5.0 (Adobe Systems, San Jose, CA), and Canva 6 (Deneba, Miami, FL) software on a PowerPC Macintosh computer.

**IPM Isolation**—After bisecting the eye, retinas were removed from IPM Isolation—The cDNA insert of the phage clone, hPG10.2.3, was subcloned into the EcoRI site of the pGEM-4Z vector (Promega, Madison, WI). Sense and antisense riboprobes, labeled with 32P-S-500 (Clonamid, NEW Life Science Products), were generated using SP6 and T7 RNA polymerases, respectively. In situ hybridization histochemistry was performed on fresh frozen human retinal and pineal gland sections cut at 12 μm. These sections were processed as described previously (28) and were dipped in emulsion and developed after exposure for 2 months. Sections were examined with bright and darkfield illumination using a Leitz photomicroscope. Images were digitized using a SenSys-KF1401 CCD camera (Photometris, Tuscon, AZ) and manipulated with IPLab (Scanalytics, Fairfax, VA), Adobe 5.0 (Adobe Systems, San Jose, CA), and Canva 6 (Deneba, Miami, FL) software on a PowerPC Macintosh computer.

**Reverse Transcriptase PCR and PCR Amplification**—One microgram of DNase I-treated total RNA extracted from human retinal tissue was used as the template for each first strand cDNA synthesis. To generate the central cDNA region of human SPACRCAN, the reverse primer, 5′-CTCTAGAGGATGCTGTTGTTGTTGTTGTTGTTGTTGTTTCCAG (from the cDNA sequence of the phage clone, hPG10.2.3, was used to synthesize the second strand cDNA. Amplification of the cDNA was performed with forward (5′-CTCTAGAGGATGCTGTTGTTGTTGTTGTTGTTGTTGTTGTTTCCAG) and reverse (5′-CTCTAGAGGAGGATGCTTG) primers designed from human SPACRCAN genomic sequence data and from the hPG10.2.3 cDNA sequence, respectively. The 5′-cDNA region of SPACRCAN was generated using the reverse primer (5′-CAGTAGAGGAGGATGCTTG) for first strand cDNA synthesis. The cDNA amplification was performed using a final 10-min extension step at 72 °C. The genomic PCR products were sequenced using SP6 and T7 RNA polymerases, respectively. The sequencing PCR was repeated at the following temperatures after an initial incubation of 94 °C for 3 min, 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, with a final 10-min extension step at 72 °C. All other PCR procedures were performed as follows: 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 3.5 min for a final 10-min extension step at 72 °C. The first strand cDNA syntheses and PCR steps were performed using Superscript II RNAse H-reverse transcriptase and Platinum Taq DNA polymerase (Life Technologies, Inc.,) respectively, as recommended by the manufacturer.
were denatured in 0.05 m phosphate buffer containing 0.2% SDS and 50 mM dithiothreitol at 100 °C for 5 min. Nonidet P-40 was added to a final concentration of 0.7% in 100 μl of sample volume to which was added 0.3 milliunits in 1 μl of recombinant peptide N-glycosidase F. The digestion was continued overnight at 37 °C. Neuraminidase: 50 μg of total protein was digested in 100 μl of 0.1% SDS, 10 mM sodium cacodylate buffer, pH 6.5. 50 milliunits/μl neuraminidase were added following incubation for 1 h at 37 °C. O-Glycosidase: IPM: sample (50 μg), predigested with neuraminidase as described above, was denatured by boiling in 0.1% SDS, 10 mM sodium cacodylate buffer, pH 6.0. Nonidet P-40 was added in 10-fold excess of SDS by weight followed by 50 milliunits of O-glycosidase overnight incubation at 37 °C.

**Antibody Production**—The insoluble IPM removed with distilled water was isolated as described previously (31). The IPM pellet was obtained by centrifugation at 400 × g for 15 min on a table top centrifuge. The pellet was resuspended in cold 0.1% Tris-buffered saline (pH 8.0 containing 5 mM dithiothreitol). The supernatant was collected after centrifugation (12,000 rpm) in a refrigerated microfuge. The supernatant was diluted 2× with 0.1 mM NaOAc, pH 6.0, and digested with chondroitinase ABC (300 milliunits/ml) for 3 h at 37 °C. At the end of the incubation, an equal volume of SDS sample buffer was added to the IPM extract, the sample was denatured by boiling for 5 min in the presence of 5% 2-mercaptoethanol, and the proteins were separated on 7.5% SDS-PAGE (30 μg of total protein/lane). After electrophoresis the gel was washed with water three times and staining with Gel Code Blue. The gels were destained with water, and the 230-kDa band was removed, minced, and sent to Biodicine Inc., Kennebunk, Maine for immunization in rabbits. Approximately 200 μg of SPACRCAN was injected/boost. Antibodies to SPACRCAN reached a suitable titer for use in Western blotting and immunocytochemical studies following the third boost.

**Western Blotting**—Sample extracts were subjected to SDS-PAGE and electrobotted onto Immobilon-P membranes followed by incubation in PBS containing 2% BSA at pH 7.5 for 30 min. BSA was replaced by the biotinylated lectin (20 μg/ml) in 1% BSA-PBS for lectin blots and incubated for 3 h at room temperature. For Western blots, the membranes were incubated with anti-SPACRCAN (1:1000) or anti-ΔDNA (1:100) in PBS-BSA overnight at 4 °C after BSA blocking. The membranes were washed with PBS-Tween (3 times) and incubated with biotinylated horseradish peroxidase-Avidin complex or alkaline phosphatase-conjugated secondary antibody (1:5000) for 1 h at room temperature. The membranes were washed and the color reaction developed using the substrates 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium or 3,3′-diaminobenzidine.

**Reverse Affinity Chromatography**—IPM extract as prepared above was digested with chondroitinase ABC (3 milliunits/50 μg of protein) in 50 mM Tris acetate buffer, pH 7.3, at 37 °C for 2 h. This was loaded on a PNA-agarose affinity resin (Vector labs) prequilibrated with 0.1% SDS, 10 mM sodium cacodylate buffer, pH 6.0. The column was washed with PBS and eluted with 0.2M lactose.

**CPC Precipitation**—To demonstrate HA binding to SPACRCAN we used a protocol similar to that described (21). Briefly, PNA-agarose-purified SPACRCAN core protein samples (1 μg) in 50 mM Tris acetate buffer (pH 7.3) containing 0.5 M NaCl were incubated for 1 h at room temperature with and without Healon (50 μg). Control samples were digested with Streptomyces hyaluronidase (1 TRU) for 1 h at 37 °C prior to CPC precipitation. Inhibition of endogenous HA binding was performed in the presence of HA oligosaccharides (100 μg/ml). BSA (2 mg/ml) incubated as a negative control. CPC (1.25% in 0.2 M ammonium acetate) was added to the samples and further incubated for 1 h. The CPC pellet and supernatant were obtained by centrifugation (12,000 rpm) of the sample for 15 min at room temperature. The pellets were rinsed twice with 1% CPC in Tris acetate buffer. Pellets were resuspended in 20 μl of water and boiled in SDS-PAGE sample buffer (32) before the proteins in the pellet and supernatant were separated by PAGE. After electrophoresis the proteins were transferred to polyvinylidene difluoride membranes using the Bio-Rad semi-dry blotting system. The membranes were blocked using 2% BSA in PBS before incubating with 1:100 dilution of biotinylated ΔDNA antibody overnight at 4 °C. After incubation with avidin-conjugated horseradish peroxidase (200 μg/ml of BSA), the protein was incubated with the peroxidase color reaction as described previously. BSA was included in the CPC precipitation studies as a negative control (21).

**Immunocytochemistry**—Eyes arrived at the laboratory in eye bank jars on ice. They were gently opened with a razor blade, cutting posterior to the limbus prior to immersion fixation. Freshly isolated eyes and pineal glands used for anti-SPACRCAN immunocytochemistry were incubated in a fixative containing 4% formaldehyde (freshly prepared from paraformaldehyde), in 0.1 M phosphate buffer (pH 7.2). After removal from the fixative, tissues were rinsed in 3 x 10 min changes of 0.1 M phosphate buffer and processed for paraffin microscopy using standard dehydration and infiltration procedures. Tissue sections cut at 7 μm were placed on Superfrost slides, deparaffinized with xylene, and hydrated through graded ethanol prior to enzyme digestion or antibody application.

Chondroitinase ABC digests were performed on deparaffinized tissue sections mounted on microscope slides (Seikagaku Corp., 460 milliunits/ml in 0.1 M Tris acetate, pH 7.3, 37 °C, 1 h). Tissue sections were incubated with 6% BSA in phosphate buffer (0.1 M, pH 7.2) for 30 min to block nonspecific antibody binding. Sections were incubated with the primary SPACRCAN antibody (diluted 1:2000) in 6% BSA, 0.1 M phosphate buffer overnight at 4 °C. After rinsing extensively with 0.1 M phosphate buffer, sections were treated with ABC (Vector Labs., 1:200 dilution) for 1 h at room temperature. Sections were washed with 0.1 M phosphate buffer and incubated in 0.05% 3,3′-diaminobenzidine (Sigma) and 0.03% hydrogen peroxide in the phosphate buffer at room temperature. Sections were examined unstained with transmitted light or Nomarski optics using a Zeiss Axiopt photomicroscope. Images were digitized using a Hamamatsu CCD camera and manipulated with Photoshop software on a Power Macintosh computer.

**RESULTS**

**Cloning of Human SPACRCAN cDNA**—To clone the human SPACRCAN cDNA, phage clones of a human retina cDNA library were screened using a partial cDNA of the rat homolog, PG10.2 (24), as a probe. Six positive clones were isolated and the nucleotide sequence of the largest clone (hPG10.2.3, 1.95 kilobases) was determined. Comparison with the rat PG10.2 cDNA revealed that hPG10.2.3 contained the 3′-half of SPACRCAN’s open reading frame. The remaining SPACRCAN cDNA was obtained by employing reverse transcriptase-PCR using RNA extracted from human retina and by subsequent PCR amplification. The combined nucleotide sequence from the three partially overlapping cDNA clones contains 3989 base pairs (Fig. 1). Two in-frame methionine codons (ATG) are present at nucleotides 15 and 21, and a stop codon (TAA) is evident at nucleotide 3738. At present it is unclear which AUG codon in SPACRCAN mRNA would be preferred for translation initiation of SPACRCAN mRNA. Usually initiation occurs from the first AUG according to the Kozak scanning model (33). However, the second AUG has an A, a purine, in position −3, which is normally critical for function (33). It is also possible that both codons are used.

Downstream to the stop codon at nucleotide 3962 is a consensus polyadenylation motif (AATAAA). If this is the true end of the 3′-untranslated region of SPACRCAN, then a large 5′-untranslated region is likely to exist as the SPACRCAN transcript is approximately 9.0 kilobases (Fig. 2). Finally, SPACRCAN has a 79% nucleotide sequence identity with rat PG10.2 (24), which strongly suggests that these are indeed orthologs.

**Chromosomal Localization of SPACRCAN**—The SPACRCAN gene was localized to chromosome 3q11.2 by using radiation hybrid panel screening and confirmed by fluorescent **in situ** hybridization analysis. When the data were submitted to the
Whitehead/MIT server and tested against the current framework markers, SPACRCAN was localized between markers WI-11447 and WI-16656 (34).

**Tissue Distribution of SPACRCAN Expression—**Northern blot analysis on a variety of human tissues was performed to determine the tissue specificity and the relative levels of expression of SPACRCAN mRNA (Fig. 2). Approximately 5 μg of total RNA was used for each tissue. The blot was probed with a 39-end SPACRCAN-specific cDNA probe. The relative levels of expression were determined by normalizing the SYBR green II-stained 28S ribosomal RNA band to the signal generated by the probe using a STORM 860 phosphoimager (29). The SPACRCAN hybridization signal was observed exclusively in the retina and pineal gland mRNA samples, indicating that the SPACRCAN gene is expressed only in these tissues. The predominant SPACRCAN mRNA appears to be approximately 9 kilobases in length although a smaller and more diffuse signal is also evident at approximately 4.4 kilobases (Fig. 2).

**Cellular Localization of SPACRCAN Expression—**In situ hybridization was performed on fresh frozen sections of the human retina and pineal gland. The 35S-labeled antisense riboprobe signal was localized to the outer retina with a high density of silver grains over the photoreceptor inner segments and a weaker density over the photoreceptor nuclei (Fig. 3, A and B). Both rod and cone photoreceptors were labeled. Retinal sections incubated with the 35S-labeled sense riboprobe were virtually free of silver grains (Fig. 3, C and D).

**Features of the Putative Polypeptide—**The nucleotide sequence of the SPACRCAN cDNA presented in Fig. 1 contains an open reading frame that encodes 1241 amino acids. The deduced amino acid sequence of the SPACRCAN polypeptide is presented in Fig. 4. Several potentially important features are evident. (a) A large mucin-like domain, containing numerous potential O-linked glycosylation sites (76 serine and 35 threonine residues), is located in the central part of the sequence (Thr393-Thr835). (b) Six consensus sites for N-linked glycosylation are present in two clusters, four on the N-terminal side and two on the C-terminal side of the mucin domain (at residues Asn154, Asn 301, Asn 320, Asn 370, Asn 942, and Asn 956). (c) Four consensus sites for GAG attachment are present. The first is at residue Ser603, near the center of the mucin domain, which conforms to the consensus sequence SGXXG (35). The other three are on the C-terminal side of the mucin domain (at residues Ser1007, Ser1031, and Ser1187) and conform to the consensus sequence SGXG (36). Ser1031 is located between two cysteine residues (Cys1025 and Cys1036) and may not be functional. (d) A linear HA-binding motif is present in the deduced
sequence spanning residues Arg<sup>1125</sup>-Arg<sup>1133</sup> (37).<sup>3</sup> (c) Two EGF-like motifs, arranged in tandem, are present near the C terminus (Cys<sup>1014</sup>-Cys<sup>1050</sup> and Cys<sup>1054</sup>-Cys<sup>1092</sup>). EGF-like motifs contain six conserved cysteine residues that participate in the intrachain disulfide bonding required for the structural stability of the motif (38). (f) Also present is a 24-amino acid hydrophobic sequence C-terminally to the EGF-like domain (Ile<sup>1101</sup>-Ile<sup>1124</sup>) suggesting a membrane-spanning region.

Identification of the SPACRCAN Gene Product—The putative GAG attachment sites in the deduced amino acid sequence of SPACRCAN (Fig. 4) and the identification of SPACRCAN’s restricted retinal expression to photoreceptors (Fig. 3, A and B) suggest that SPACRCAN may be a candidate for a singularly abundant chondroitin sulfate proteoglycan core protein in the human IPM that was recently characterized using a ΔDi6S monoclonal antibody following digestion of the human IPM samples with chondroitinase ABC (23). To identify this 230-kDa core protein, the protein band was cut from the gel following SDS-PAGE before and after chondroitinase ABC digestion and stained with Gel Code Blue or the lectin wheat germ agglutinin (Fig. 5). In the undigested sample, SPACRCAN migrated as a broad smear (Fig. 5A, lanes 1 and 3). This apparent molecular mass was estimated using chondroitinase-digested rat chondrosarcoma aggrecan (400 kDa) as the standard (Fig. 5, lanes 1–3). Following digestion SPACRCAN was visible at a lower position at around 230 kDa, and the broad smear, present in the higher molecular weight range of the undigested sample, was absent (Fig. 5A, lanes 2 and 4). When the IPM samples were separated using a 7.5% gel, the high molecular weight components present in the undigested IPM sample only minimally entered the gel and were present at the top of the lane as seen in the Gel Code Blue-stained gels (Fig. 5B, lane 1). Following digestion with chondroitinase ABC (Fig. 5B, lane 2), the high molecular mass band at the top of the lane was no longer present, and a new band was observed just above the 220-kDa marker. This dramatic increase in electrophoretic mobility of the high molecular weight IPM protein following chondroitinase digestions clearly indicates that this molecule represents the core protein of a chondroitin-type proteoglycan.

We also used the ΔDi6S antibody (40, 41) to label SPACRCAN in the IPM. In the Western blot using the ΔDi6S antibody (Fig. 5B, lanes 3–4), no immunostaining was evident in the undigested sample (lane 3), whereas intense immunostaining of the 230-kDa SPACRCAN band was present in the chondroitinase ABC-digested sample (lane 4). Lower levels of kDa IPM chondroitin sulfate proteoglycan core protein characterized previously (23) represents the gene product of the SPACRCAN gene.

Further Carbohydrate Analysis of SPACRCAN—Insoluble IPM extracts containing SPACRCAN were analyzed with 5% SDS-PAGE before and after chondroitinase ABC digestion and stained with Gel Code Blue or the lectin wheat germ agglutinin to estimate the change in SPACRCAN mass (Fig. 5A). In the undigested sample, SPACRCAN migrated as a broad smear with a peak intensity close to 400 kDa (Fig. 5A, lanes 1 and 3). This apparent molecular mass was estimated using chondroitinase-digested rat chondrosarcoma aggrecan (400 kDa) as the standard (39) (Fig. 5A, lane 5). After digestion SPACRCAN was visible at a lower position at around 230 kDa, and the broad smear, present in the higher molecular weight range of the undigested sample, was absent (Fig. 5A, lanes 2 and 4).

When the IPM samples were separated using a 7.5% gel, the high molecular weight components present in the undigested IPM sample only minimally entered the gel and were present at the top of the lane as seen in the Gel Code Blue-stained gels (Fig. 5B, lane 1). Following digestion with chondroitinase ABC (Fig. 5B, lane 2), the high molecular mass band at the top of the lane was no longer present, and a new band was observed just above the 220-kDa marker. This dramatic increase in electrophoretic mobility of the high molecular weight IPM protein following chondroitinase digestions clearly indicates that this molecule represents the core protein of a chondroitin-type proteoglycan.

We also used the ΔDi6S antibody (40, 41) to label SPACRCAN in the IPM. In the Western blot using the ΔDi6S antibody (Fig. 5B, lanes 3–4), no immunostaining was evident in the undigested sample (lane 3), whereas intense immunostaining of the 230-kDa SPACRCAN band was present in the chondroitinase ABC-digested sample (lane 4). Lower levels of
immunoreactivity to the ΔDi6S antibody were also evident in Western blots (Fig. 5, lane 4) in bands at approximately 170–180 kDa and 130 kDa. Absent was any immunoreactivity of the 150-kDa SPACR band, which is apparent from any background staining (Fig. 5B, lane 4), as has recently been reported (23). In contrast, when PNA was used in lectin blotting studies, the 150-kDa SPACR band was the only region of background staining (Fig. 5, lane 4). Most intensely decorated is the blot stained in the undigested IPM sample (Fig. 5, lane 1). In some undigested samples, the antibody did weakly label the 150-kDa SPACR band, other proteins have entered the gel, and the 230-kDa SPACRCAN band with minor labeling of bands at 170–180 kDa. Absent was any immunoreactivity to the ΔDi6S antibody when digested with chondroitinase ABC. Lane 5 contains a chondroitinase ABC-digested aggrecan sample to provide a molecular mass standard of approximately 400 kDa. Note the broad smear centered around the 400-kDa marker in lane 1 and a progressive decrease in mobility of SPACRCAN was observed when digested with chondroitinase ABC digestion in lanes 2, 4, and 6. IPM samples separated with 7.5% acrylamide and lanes 2–4 are lectin-digested samples, lanes 3 and 5 are to the undigested sample, and lanes 2, 4, and 6 are of Gel Code Blue-stained samples. The prominent SPACRCAN band slightly above the 220-kDa marker in lane 2 and the absence of staining in lane 1. In some undigested samples, the antibody did weakly label the material that accumulates at the stacking gel, but labeling of the sample presented in lane 1 is not evident. Lane 3 contains a sample first digested with chondroitinase ABC followed by N-glycosidase digestion. Lane 4 contains a chondroitinase ABC, N-, and O-glycosidase-digested sample. Note the progressive increase in SPACRCAN mobility following removal of the N- and O-linked carbohydrates (lanes 2–4).
image was incubated with the anti-SPACRCAN antibody. Tissue in B surrounding the rod and cone photoreceptors in the IPM in was incubated with preimmune serum. Note the dense immunolabeling horizontal band near the upper and nonimmune-treated tissues (absence of labeling in experimental and control micrographs were photographed and printed of the pinealocytes is evident in inner segments. In the pineal gland, intense anti-SPACRCAN labeling of the pinealocytes is evident in C and absent in D (arrows). Adjacent experimental and control micrographs were photographed and printed at the same magnification. Bar in B and D represents 20 μm.

molecular mass of the remaining core protein was approximately 160 kDa. These glycosidase digestions demonstrate that SPACRCAN contains both N- and O-linked glycoconjugates, consistent with the presence of putative N-linked and O-linked glycosylation sites in the deduced sequence. Additionally, the loss of approximately 240 kDa following carbohydrate removal (from about 400 kDa in Fig. 5A, lanes 1 and 3 to around 160 kDa in Fig. 5C, lane 4), suggests that glycoconjugates account for approximately 60% of the mass of SPACRCAN.

**Tissue Distribution of SPACRCAN Proteoglycan**—The distribution of anti-SPACRCAN immunoreactivity was established in tissue sections of human retina and pineal gland (Fig. 6). The polyclonal antibody to human SPACRCAN, which showed specific binding to the SPACRCAN band in Western blots (Fig. 5C, lane 2), also intensely labels the IPM around both rods and cones (Fig. 6A). In contrast, the IPM in sections treated with preimmune serum was unlabelled (Fig. 6B). These results indicate that in the retina, SPACRCAN is localized to the IPM where it is present around both rod and cone photoreceptors. In pineal sections, the anti-SPACRCAN antibody showed diffuse immunoreactivity with intense staining of the cell bodies of the pinealocytes, but no staining in the septal areas (Fig. 6C). Control sections treated with preimmune serum showed some diffuse background staining but specific pinealocyte labeling was absent (Fig. 6D). These data indicate that in the retina and in pineal gland, SPACRCAN is localized to the IPM, where it is present around both rod and cone photoreceptors and is present in pinealocytes, respectively. These localization patterns therefore reflect the cellular localization of SPACRCAN mRNA expression (Fig. 3).

Function of SPACRCAN—The presence of putative HA-binding motifs in the deduced amino acid sequence of SPACRCAN (Fig. 4) and the earlier reports that HA is present in the IPM (26, 42, 43) suggest that one function of SPACRCAN may be to form associative interactions with HA. To evaluate this possibility we performed experiments using CPC, a detergent that selectively precipitates GAGs and any covalently or noncovalently associated protein (44). We have demonstrated previously (21) that SPACRCAN present in IPM extracts can be selectively precipitated using CPC. This is because CPC efficiently precipitates proteoglycans through interactions with their covalently attached GAGs. When we used a hyaluronan-free preparation of SPACRCAN purified by DEAE-Sepharose ion-exchange chromatography (45) in a CPC precipitation assay, SPACRCAN was found in the pellet fraction both in the presence and absence of hyaluronan.  This confirms our result that the proteoglycan SPACRCAN interacts with CPC in an HA-independent manner.

To directly evaluate the binding of the core protein of SPACRCAN to HA we first digested the IPM extracts with chondroitinase ABC to remove the GAG chains, followed by PNA-agarose chromatography. Such a preparation contains mainly SPACRCAN and SPACR along with some minor PNA-binding proteins (compare Fig. 5B, lanes 5 and 6). When this sample was subjected to CPC precipitation and the pellet and supernatant components were separated with PAGE, blotted, and SPACRCAN detected with the ΔDi6S antibody, a distinct SPACRCAN band was decorated with the antibody in both the supernatant and pellet (Fig. 7, lanes 1). Following the addition of 50 μg/ml Healon, a source of HA, to the sample before CPC precipitation, SPACRCAN was present only in the pellet fraction (Fig. 7, lanes 2). However, when the sample was treated with Streptomyces hyaluronidase before precipitation with CPC, SPACRCAN was present only in the supernatant and not in the pellet (Fig. 7, lanes 3). Preincubation of the sample with HA oligosaccharides (100 μg/ml) before CPC precipitation led to the presence of SPACRCAN only in the supernatant (Fig. 7, lanes 4). In comparison, preincubation of the sample with HA oligosaccharides before the addition of Healon resulted in the presence of SPACRCAN in the pellet and not in the supernatant (Fig. 7, lanes 5). As a negative control, BSA was incubated with Healon before CPC precipitation. BSA was only present in the supernatant and not in the pellet (Fig. 7, lanes 6). Collectively these precipitation studies indicate that SPACRCAN can bind HA in the absence of its GAG chains.

**DISCUSSION**

Expression and Localization of SPACRCAN—The combined results of the Northern and in situ hybridization analyses indicate that SPACRCAN mRNA is present only in the retina and pineal gland and that within these multicellular tissues, SPACRCAN gene expression is restricted to rod and cone photoreceptors of the retina and pinealocytes in the pineal gland. Photoreceptors and pinealocytes are the most abundant cell type in the retina and pineal gland and these highly specialized cells are phylogenetically related (46, 47). A number of molecules expressed by retinal photoreceptors are also expressed in the pineal gland, including interphotoreceptor matrix retinoid-binding protein (48), cone arrestin (49), opsins (50), transducin (51), and several other molecules involved in the phototransduction cascade (52). SPACRCAN, initially cloned from rat pineal (24), can be added to this group of molecules that are expressed by photoreceptors and pinealocytes.

The localization pattern of SPACRCAN gene expression is also comparable to the localization of SPACRCAN protein product as demonstrated by the immunohistochemical data using the anti-SPACRCAN antibody. Furthermore, the anti-SPACRCAN antibody used in the Western blotting and immunohistochemical analyses of chondroitinase-digested human retinal tissues depicts virtually identical patterns of labeling as seen with the monoclonal ΔDi6S antibody described previously (23). These data, together with the N-terminal sequencing analysis presented in this study, demonstrate that the 230-kDa

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cause the N-terminal sequence of the SPACRCAN core protein CPC, and both supernatant (upper panel) and pellets (lower panel) were analyzed by Western bloting using the \( \Delta \)D6S antibody after separation on 7.5% SDS-PAGE. Lane 1 contains a PNA-purified sample precipitated with CPC. Note the immunoreactivity of SPACRCAN in both supernatant (upper lane 1) and pellets (lower lane 1), suggesting the presence of endogenous HA in the sample. Lane 2 contains a sample preincubated with Healon (50 \( \mu \)g/ml) for 1 h at 37 °C before CPC precipitation. SPACRCAN is present in the pellet (lower lane 2) but not in the supernatant (upper lane 2). Lane 3 contains purified SPACRCAN predigested with Streptomyces hyaluronidase for 1 h at 37 °C prior to CPC precipitation. This hyaluronidase degrades only HA, which results in the retention of SPACRCAN in the supernatant (upper lane 3) with none in the pellet (lower lane 3). Lane 4 contains a sample preincubated with a 22-saccharide fragment of HA (100 \( \mu \)g/ml) for 1 h at 37 °C. This HA oligosaccharide is able to prevent CPC precipitation of SPACRCAN, as evidenced by the presence of SPACRCAN in the supernatant (upper lane 4) and the absence of SPACRCAN in the pellet (lower lane 4). The addition of Healon after preincubation of the sample with the HA oligosaccharide led to the accumulation of all SPACRCAN in the pellet only (lower lane 5) with none remaining in the supernatant (upper lane 5). Lanes 1–5 are Western blots stained with the \( \Delta \)D6S antibody. Lane 6 contains a Gel Code Blue-stained gel of a negative control incubation of BSA and Healon prior to CPC precipitation. Note that all BSA is retained in the supernatant (upper lane 6), and none is present in the pellet (lower lane 6).

chondroitin sulfate proteoglycan core protein is indeed SPACRCAN. It should also be noted that the retinal distribution of SPACRCAN immunoreactivity was in the IPM, an extracellular compartment, whereas in the pineal gland, these antibodies show a distribution of immunoreactivity over the pinealocytes. These fundamental differences in localization of SPACRCAN in retina and pineal gland may reflect differences in processing and/or function of this molecule in these two tissues.

**Features of the Deduced Polypeptide of SPACRCAN**—Because the N-terminal sequence of the SPACRCAN core protein isolated from the IPM corresponds to residues Ser\(^{82}\)-Pro\(^{166}\) in the deduced sequence, it is unlikely that residues Met\(^{1}\)-Arg\(^{81}\) are a part of the functional protein and suggests that the functional N terminus of the mature protein begins at Ser\(^{82}\). Met\(^{1}\)-Gly\(^{22}\) is probably the signal peptide involved in the secretion of this molecule, because it has the required tripartate structure, including a central hydrophobic region (Phe\(^{4}\)-Leu\(^{19}\)) flanked by two hydrophilic sequences (Met\(^{1}\)-Met\(^{3}\) and Ile\(^{10}\)-Gly\(^{22}\)). The putative cleavage site of this signal sequence conforms to the −1, −3 rule where −1 (Gly\(^{22}\)) does not have a long side chain and −3 (Ile\(^{10}\)) is not a charged amino acid (53). The remaining sequence (Asp\(^{23}\)-Arg\(^{81}\)) is likely a propeptide cleaved from the functional protein following secretion.

SPACRCAN contains two EGF-like modules near the C terminus of the deduced sequence (Fig. 4). EGF-like modules are characteristic of a number of extracellular matrix proteins and are implicated in cell-matrix interactions (54–56). Some EGF-like domains require calcium for their biological function (57). Interestingly, one of the EGF-like domains in SPACRCAN contains the critical asparagine (Asp\(^{1028}\)), which has been implicated in calcium binding (57), whereas the other domain does not. The potential for calcium binding by SPACRCAN suggests an important physiological role for this molecule in sequestering extracellular calcium released by photoreceptors in response to light (58–60).

SPACRCAN may also participate in calcium binding in the pineal through its EGF-like module. Many mammals, including humans, develop calcified inclusions with increasing age (corpora arenacea or pineal sand) (47). These inclusions consist primarily of hydroxyapatite, calcium phosphate, and an organic component (61, 62). The cause of these concretions is not known, although there are several hypotheses. They include intracellular calcium accumulation and mineralization leading to pinealocyte degeneration and subsequent release of these deposits (63), and the exchange of released polypeptides, combined with a carrier protein, for calcium by the vascular system. The calcium is then supposedly deposited as concretions (64). Whether SPACRCAN binds calcium in the pineal gland and is involved in pineal sand formation remains to be determined.

A short hydrophobic region in the deduced SPACRCAN polypeptide suggests that it could function as a membrane-spanning domain (Fig. 4, Iso\(^{1101}\)-Iso\(^{1224}\)). This region shows an average of 2.5 on the hydrophathy scale, suggesting it may represent a putative membrane-spanning segment (64, 65). A similar putative transmembrane domain is also present in the predicted sequence of the rat homolog PG10.2 and other chondroitin and heparin sulfate proteoglycans, including neuroglycan C and the syndecan family (24, 66–68). It should be noted that the procedures used to isolate SPACRCAN from the IPM (Tris buffer extraction) would not be expected to remove proteins that are anchored to the plasma membrane in this manner. Either this putative membrane-spanning region is non-functional or SPACRCAN may be cleaved N-terminal to this transmembrane region and released to the extracellular compartment. Additional studies will be required to resolve this issue.

**Hyaluronan Binding of SPACRCAN**—The CPC precipitation studies indicate that SPACRCAN can bind HA in the absence of its GAG chains. The finding that CPC can partially precipitate SPACRCAN in the absence of exogenous HA suggests that endogenous HA is in the sample but not at a sufficient concentration to interact with all the SPACRCAN molecules present, because some SPACRCAN remains in the supernatant (Fig. 7, lanes 1). One might argue that the precipitation by CPC was due to the presence of incompletely digested chondroitin sulfate on SPACRCAN. Our finding that CPC precipitation of SPACRCAN was eliminated when the sample was digested with HA-specific hyaluronidase (Fig. 7, lanes 3), clearly indicates that endogenous HA is the GAG responsible for interaction with SPACRCAN in this preparation. We were also able to block CPC precipitation of SPACRCAN by pretreatment with a 22-saccharide fragment of HA (Fig. 7, lanes 4), suggesting that these short HA fragments compete for HA binding of SPACRCAN more efficiently than the endogenous HA. The HA oligosaccharides were not able to prevent binding when exogenous HA was added (Fig. 7, lanes 5), suggesting that higher...
FIG. 8. Optimal global alignment comparison of SPACRCAN and SPACR prepared with the sequence alignment utility and the FASTA algorithm available through the Munich Information Center for Protein Sequences. Double dots between aligned residues indicate absolute identity; single dots indicate homology; no dots indicate no homology; and dashes indicate interruptions in the sequence allowing for the logical alignment of the two molecules. Asparagine residues in bold (N) represent consensus sites for N-linked glycosylation. Serine residues in bold (S) represent consensus sites for xylosylation and GAG attachment. The sequences beginning and ending in arginine or lysine that are underlined represent putative hyaluronan-binding motifs. Note that three of the consensus sites for N-linked glycosylation are in perfect alignment. The conserved cysteine residues in the EGF-like modules are presented in bold (C). Major regions of homology are evident over the first 350 residues of the N terminus and from SPACRCAN 898/SPACR 572 to SPACRCAN 1058/SPACR 727 near the C terminus.
concentrations of HA can more efficiently compete for the HA-binding sites. Collectively, these precipitation studies clearly indicate that SPACRCAN can bind HA. This function may have an important role in organization of the IPM and may be causally responsible for the difficulty in extracting this novel molecule from the IPM using physiological salt solutions. It is likely that SPACRCAN-HA interactions are mediated through the receptor for hyaluronan-mediated motility-type HA-binding motifs identified in the deduced sequence of SPACRCAN (Fig. 4) (37, 69), but confirmation of these regions as binding sites must await experimental analysis.

A New Family of IPM Molecules—SPACR is another novel human IPM molecule recently characterized (20). When the deduced amino acid sequences of SPACRCAN and SPACR are aligned, several homologous regions are evident (Fig. 8). The first 81 residues from the deduced sequence of SPACRCAN and the first 70 in SPACR are not present in the isolated molecules. From N-terminal sequence analysis we know that the functional N terminus of SPACRCAN and SPACR (20) begins at residue Ser81 and Ser71, respectively, showing 54% homology. The four residues immediately preceding the N-terminal sequence in both SPACRCAN (Arg79-Arg81) and homology. (related to drusen formation. Novel molecules present in the EFEMP1 suggests that mutations in other genes coding for the human retina cDNA library, Dr. Ivan Still for his help in identifying the expressed sequence tags used in chromosomal localization of SPACRCAN, Dr. John Hassell for his comments on potential GAG attachment sites in SPACRCAN, Dr. Eva A. Turley and John Kyu Yong Choe for their review of the deduced SPACRCAN sequence and their comments on potential HA-binding motifs, Dr. John W. Crabb for his critical review of a preprint of the manuscript, and Mrs. Karen G. Shadrach for her expert technical assistance.

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