Molecular Cloning and Characterization of Chick SPACRCAN*

Yoko Inoue, Masahiko Yoneda, Jinsong Zhao, Osamu Miyaiishi, Akiko Ohno-Jinno, Takuya Kataoka, Zenzo Isogai, Koji Kimata, Masayoshi Iwaki, and Masahiro Zako

From the Departments of Ophthalmology and Pathology and the Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan, the Aichi Prefectural College of Nursing and Health, Nagoya, Aichi 463-8502, Japan, and the Department of Dermatology, National Center for Geriatrics and Gerontology, Aichi 474-8511, Japan

MY-174, a monoclonal antibody that reacts with specific sialylated O-linked glycoconjugates of chick SPACR (sialoglycoprotein associated with cones and rods), also recognizes another molecule of 300 kDa. Here, we verified that this 300-kDa molecule is chick SPACRCAN (sialoheparan sulfate proteoglycan associated with cones and rods), another member of a novel interphotoreceptor matrix family. Screening for chick SPACRCAN was carried out by plaque hybridization using a probe for chick SPACR. Specific polyclonal antibodies raised against chick SPACRCAN were used for the following experiments. To determine whether the 300-kDa molecule detected by MY-174 was identical to 300-kDa chick SPACRCAN, the migrations of these bands were examined after various glycosidase digestions. Furthermore, the expression levels were measured during retinal development and compared with those of chick SPACR. The results demonstrated that the 300-kDa molecule recognized by MY-174 was chick SPACRCAN, and we further identified it as a proteoglycan with chondroitin sulfate chains. SPACRCAN had heavily sialylated N- and O-linked glycoconjugates, and its MY-174 antigenicity was abolished by O-glycanase treatment after neuraminidase treatment, as observed for chick SPACR. During retinal development, the mRNA and core protein expression levels, MY-174 antigenicity, and hyaluronan binding ability of SPACRCAN peaked around embryonic day 17 and then gradually decreased, whereas the corresponding expression levels of SPACR simply increased, but not its hyaluronan binding ability. The MY-174 reactivity of SPACRCAN in the adult retina was decreased compared with that in the newborn retina, whereas that of SPACR was increased. The decreased hyaluronan binding of SPACR was induced by an inhibitory effect of the excess of sialic acids in the adult stage. Thus, with similar core protein structures and specific sialylated glycoconjugates but distinct chondroitin sulfate chains, SPACRCAN and SPACR may have separate roles in the retina due to their differing expression profiles during development.

The interphotoreceptor matrix (IPM) resides in an extracellular compartment between the outer limiting membrane of the neural retina and the apical surface of the retinal pigment epithelium and is composed of proteins, glycoproteins, proteoglycans, and glycosaminoglycans (1, 2). Projecting from the neural retina outer surface, elongated photoreceptor inner and outer segments penetrate and are surrounded by the IPM (3). The IPM is thought to be involved in several important functions, including retinal adhesion, retinoid transport, intercellular communication, matrix turnover, regulation of neovascularization, cell survival, and photoreceptor differentiation and maintenance. However, specific roles for molecules found in the IPM have not been clearly defined, with the exception of a correlation between the expression level of SPACR (sialoglycoprotein associated with cones and rods) and retinal adhesiveness (4, 5).

Both human SPACR (a glycoprotein) and SPACRCAN (sialoheparan sulfate proteoglycan associated with cones and rods; a proteoglycan) are localized in the IPM of the retina (6, 7), are synthesized and secreted by photoreceptor cells (4), and bind to hyaluronan (8). In contrast to human SPACR, the SPACR orthologs in non-primate mammals, such as mice, rats, and bovines, are proteoglycans (9), whereas the SPACRCAN orthologs are chondroitin sulfate proteoglycans in both primate and non-primate mammals (10, 11). Previously, we revealed that chick SPACR is a glycoprotein that resides in the IPM, similar to the case for human SPACR, and that the coding regions of chick, mouse, and human SPACRs show a high degree of homology (5). Homologous regions are also detected between the deduced amino acid sequences of human SPACRCAN and SPACR, suggesting that they are members of a novel family of IPM-specific molecules (7). However, it remains unclear whether these molecules with similar core proteins also contain similar glycoconjugates and whether or not their expression profiles differ during development to accomplish their roles.

In a recent study (5), we noted that MY-174, a monoclonal antibody raised against specific sialylated O-linked glycoconjugates of SPACR, also detected another molecule of 300 kDa in Western blot analyses. Furthermore, MY-174 specifically stained the matrix surrounding the photoreceptors, suggesting that the 300-kDa molecule resides in the same place as SPACR. SPACRCAN was previously shown to be expressed in this region in human, bovine, mouse, and rat retinas (10). If the 300-kDa molecule is SPACRCAN, this implies that SPACRCAN has the same specific sialylated O-linked glycoconjugates as SPACR. Furthermore, chondroitin sulfate proteoglycans are generally expressed and play roles in the early stages of embryonic development (12–15), suggesting the possibility that SPACRCAN, previously reported to be a chondroitin sulfate proteoglycan, may be expressed and play specific roles in earlier embryonic stages compared with SPACR. However, the expression profiles of SPACRCAN and SPACR have not yet been compared within the same species during retinal development.

In this study, we determined that the 300-kDa molecule recognized by MY-174 in the photoreceptor layer was chick SPACRCAN. The expression profile of SPACRCAN during development revealed that its major expression period is limited to the late embryonic stages. All of the characteristics of SPACRCAN were compared with those of

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) AB 204951.

1 To whom correspondence should be addressed: Dept. of Ophthalmology, Aichi Medical University, Nagakute-cho, Aichi-gun, Aichi-ken 480-1195, Japan. Tel: 81-52-264-4811; Fax: 81-561-63-7255; E-mail: zako@aichi-med-u.ac.jp.

2 The abbreviations used are: IPM, interphotoreceptor matrix; E, embryonic day; PBS, phosphate-buffered saline; P, postnatal day.
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SPACR, and the significance of the glycosylation chains on SPACR for hyaluronan binding was also examined.

EXPERIMENTAL PROCEDURES

Materials—Fertilized eggs from White Leghorn chickens were maintained in a humidified incubator at 37 °C. Embryos at different developmental stages were obtained according to the standard criteria of Hamburger and Hamilton (16), and retinas after embryonic day (E) 12 were used for experiments. Retinas before E12 were not used because they were too difficult to isolate from the chick eye. Retinas from adult White Leghorn chickens were also used.

Mouse monoclonal antibody MY-174 (17) was purified by E-Z-SEP (Amersham Biosciences AB, Uppsala, Sweden). Peroxidase-conjugated goat IgG fractions to mouse immunoglobulins (IgG, IgA, and IgM) were obtained from Organon Teknika Corp. (Durham, NC). Affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit IgG was purchased from TAGO (Burlingame, CA). IgG fractions from non-immunized rabbit serum were obtained from Zymed Laboratories Inc. (South San Francisco, CA) and used as controls for immunohistochemical studies.

Chondroitinase ABC (protease-free) and endo-α-β-acetylgalactosaminidase (O-glycanase) were purchased from Seikagaku Corp. (Tokyo, Japan). Recombinant N-glycanase and neuraminidase (sialidase) were obtained from Roche Applied Science (Tokyo). A newborn chick retinal cDNA library was established by Clontech using neural retinas prepared from 20 newborn chicks (5) and the pTriplEx vector.

Cloning of Chick SPACRCAN—Based on the nucleotide sequence of chick SPACR (nucleotides 1992–2551; GenBankTM accession number AB070714), forward (5’-TGCCAGGCGCTTTCTCCGACC-3’, nucleotides 1992–2011) and reverse (5’-GGTCTGCCATGACTGCGGC-3’, nucleotides 2551 to 2532) primers were designed to make a cDNA strand. PCR was performed on the newborn chick retinal cDNA library, and the cDNA obtained was inserted into the pUC118 DNA vector (Takara Biomedicals, Kyoto, Japan) for use as a probe. Next, we used a plaque hybridization technique and the chick SPACR probe to screen for molecules other than SPACR. The corresponding plasmid DNA was purified from the plaque, and both strands were sequenced using an ABI sequencer (Applied Biosystems, Foster, CA). Finally, we tried to obtain the full-length nucleotide sequence of a candidate for chick SPACRCAN using PCR 5’- and 3’-extension methods.

Antibodies against Chick SPACRCAN—The DNA sequences obtained were analyzed using GENETYX-MAC computer programs (Software Development Co. Ltd., Tokyo). According to the predicted amino acid sequence, the polypeptide 817LTSTVAFSVE–TPVPS (Software Development Co. Ltd., Tokyo). According to the predicted SPACRCAN using PCR 5

Western Blotting—PBS-insoluble IPM samples were prepared from chick retinas according to previously reported procedures (3, 18). The high molecular mass fractions separated on a Sephacryl S-300 HR column (Amersham Biosciences) were used for SPACRCAN analyses. A 30-μl aliquot of each retinal sample was digested with enzymes in the presence of protease inhibitors. Chondroitinase digestion was performed with chondroitinase ABC in 50 mM Tris-HCl (pH 8.0) in the presence of protease inhibitors for 2 h at 37 °C. N-Glycosidase digestion was performed with N-glycanase in 50 mM Tris-HCl (pH 8.0) for 2 h at 37 °C. Acylneuraminyldolhiohydrolyase digestion was performed with neuraminidase (sialidase) in 50 mM Tris-HCl (pH 6.8) for 1 h at 37 °C. O-Glycosidase digestion was performed with O-glycanase in 0.1 M citrate buffer (pH 4.5) for 3 h at 37 °C. All samples were subjected to electrophoresis on 3–8% SDS-polyacrylamide gels, which allowed high molecular mass molecules to enter the gel. Separated proteins on the gels were blotted onto nitrocellulose membranes, and the membranes were incubated with MY-174, anti-SPACRCAN antibodies, polyonal antibody O46-F for chick SPACR (5), or biotinylated hyaluronan. The localization of MY-174, anti-SPACRCAN antibodies, O46-F, or biotinylated hyaluronan was detected using a peroxidase-conjugated secondary antibody (Amersham Biosciences), horseradish peroxidase-conjugated protein A (Zymed Laboratories Inc.), or peroxidase-conjugated streptavidin (Amersham Biosciences), as appropriate. The molecular masses of the protein bands on the SDS-polyacrylamide gels were estimated from the migration positions of protein standards (Invitrogen, Groningen, Netherlands). The NIH Image analysis program was used to measure the expression of SPACRCAN and SPACR in each sample.

Immunofluorescence Microscopy—Chick eyes were cut into two pieces and then fixed in 3.7% (w/v) formaldehyde solution neutralized with calcium carbonate in 0.1 M sodium phosphate buffer (pH 6.8) for 1 h at room temperature with gentle shaking. The fixed retinas were rinsed overnight in PBS containing 0.1% (w/v) glycine at 4 °C, embedded in OCT compound (Miles Scientific, Naperville, IL) on petroleum ether/dry ice, and cut into sections (6–8 μm thick) using a cryostat. Next, the sections were incubated with the anti-SPACRCAN antibodies in 10% (w/v) normal swine serum for 30 min at room temperature. After three washes with PBS for 3 min each, the sections were incubated with fluorescein isothiocyanate isothiocyanate-conjugated goat anti-rabbit IgG in PBS containing 1% (w/v) normal swine serum. Finally, the sections were rinsed in PBS, mounted in mounting medium (Shandon Lipshaw, Detroit, MI), observed under an Olympus fluorescence microscope, and photographed using Kodak Tri-X Pan film. Immunohistochemical staining was performed on 20 different eyes, and reproducible results were obtained. As a control for nonspecific staining, the primary antibody was replaced with non-immune rabbit serum at the same protein concentration. No staining was observed in the control sections.

Northern Blot Analysis—An aliquot (10 μg) of total RNA from each retinal sample was prepared and transferred to a nylon filter as described previously (19). Reverse transcription was performed using SuperScript II reverse transcriptase (Invitrogen) to obtain newborn retinal cDNA templates. A cDNA probe (0.6 kb, nucleotides 3517–4119; GenBankTM accession number AB204591) corresponding to the C terminus of chick SPACRCAN was amplified from the newborn retinal
FIGURE 1. Nucleotide and deduced amino acid sequences of chick SPACRCAN (GenBank™ accession number AB204591). The deduced protein contains 1423 amino acids. Seven potential N-linked glycosylation sites are underlined. Numerous potential O-linked glycosylation sites are present. Two epidermal growth factor-like domains (in **boldface** from Cys1200 to Cys1236 and from Cys1240 to Cys1278) are present near the C terminus. Six RHAMM-type hyaluronan-binding motifs are shaded. Two SEA domains (in *italics* from Thr245 to Tyr354 and from Thr1078 to Lys1201) are present near the N and C termini, respectively. One region (from Leu817 to Val1087) was selected for raising anti-SPACRCAN polyclonal antibodies (dash-underlined). Consensus sites for glycosaminoglycan attachment are boxed. The probe used for the Northern blot analyses was created by PCR amplification using forward (5′-TGCAACACTGCATATCACAC-3′) and reverse (5′-GGATGTCTCTGCACTGGTAG-3′) primers (double-underlined).
cDNA templates with forward (5'-TGCAACACTGATACACAC-3', nucleotides 3517–3536) and reverse (5'-GGATGTCTCTGACCTGGTAG-3', nucleotides 4119 to 4130) primers and used for hybridization. Similarly, a cDNA probe (0.4 kb) corresponding to the N terminus of chick SPACR was amplified from a newborn retinal cDNA template with forward (5'-ATGCATTTGAAAACTGGATT-3') and reverse (5'-TTTCCCTCTGGCAGGCAGTA-3') primers and used for hybridization as described previously (5).

Quantitative Analysis—The NIH Image software program was used to analyze the expression level of each sample on the membranes.

Statistical Analysis—All results were repeated three times and are presented as means ± S.E.

RESULTS

Molecular Cloning of Chick SPACRCAN—Screening of a chick retinal cDNA library using a plaque hybridization technique identified a clone for a molecule that is similar to chick SPACR. We finally obtained a molecule with a 4888-bp open reading frame that encodes a 1423-amino acid protein (Fig. 1). A BLAST analysis of public data bases revealed that the molecule shows the highest homology to human SPACRCAN.
because it has 51.4 and 52.9% nucleotide sequence identities to mouse and human SPACRCANs, respectively. The amino acid sequence contains seven N-linked glycosylation sites, numerous potential O-linked glycosylation sites, and two epidermal growth factor-like domains near the C terminus, similar to the case for chick SPACR.

Fig. 2 shows a comparison of the deduced primary amino acid sequence of chick SPACRCAN with those of mouse and human SPACRCANs. The deduced amino acid sequence shares 38.5 and 64.4% similarities with the mouse and human sequences, respectively, suggesting that they are indeed orthologs.

Establishment of Anti-SPACRCAN Antibodies—Polyclonal antibodies against a synthesized peptide of chick SPACRCAN were newly established as described in “Experimental Procedures” and used for Western blot analyses of PBS-insoluble IPM samples after chondroitinase ABC treatment. A distinct band of 300 kDa was detected (Fig. 3A, arrow). After being stripped, the same membrane was reprobed with anti-SPACRCAN antibodies.MY-174 also stained a 180-kDa band corresponding to O-glycosylase itself, as reported previously (5). Horizontal bars indicate the positions of the original 300-kDa band. B, characterization of chick SPACRCAN. After being stripped, the same membranes shown in A were reprobed with the anti-SPACRCAN antibodies (1:4000 dilution). SPACRCAN has 20-kDa sialylated glycocconjugates. The migration of the 300-kDa molecule detected by MY-174 was completely identical to that of chick SPACRCAN in all digestions examined, indicating that the 300-kDa molecule recognized by MY-174 corresponds to chick SPACRCAN.

Next, we examined the localization of chick SPACRCAN in radial sections of newborn retina by immunohistochemical staining with the anti-SPACRCAN antibodies. Bright fluorescence was specifically detected in the photoreceptor layer (PL, arrow). After digestion with hyaluronan (arrows), indicating that the anti-SPACRCAN antibodies did not cross-react with SPACR. 8, localization of SPACRCAN in the retina. A radial section of an adult retina stained with the anti-SPACRCAN antibodies is shown. Fluorescence was specifically detected in the photoreceptor layer (PL, arrow). A section stained with hematoxylin and eosin (H&E) is shown on the left. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bar = 100 μm.

FIGURE 4. Glycosyl modifications of the 300-kDa band detected by MY-174 and anti-SPACRCAN antibodies. A, characterization of the retinal MY-174 antigen. Western blot analyses were performed on PBS-insoluble IPM samples before and after digestion with chondroitinase ABC. Samples treated with chondroitinase ABC were also stained with MY-174 before and after further digestion with N- or O-glycosylase or neuraminidase. The size of the 300-kDa band did not change after O-glycosylase treatment alone, but was decreased by 20 or 50 kDa after N-glycosylase or neuraminidase treatment, respectively. A smear of 250-kDa band detected after neuraminidase treatment was no longer detected after further treatment with O-glycosylase. MY-174 also stained a 180-kDa band corresponding to O-glycosylase itself, as reported previously (5). Horizontal bars indicate the positions of the original 300-kDa band. B, characterization of chick SPACRCAN. After being stripped, the same membranes shown in A were reprobed with the anti-SPACRCAN antibodies (1:4000 dilution). SPACRCAN has 20-kDa sialylated N-linked glycosylconjugates and 50-kDa sialic acids. The 250-kDa band detected after neuraminidase treatment was decreased to 230 kDa after further digestion with O-glycosylase. SPACRCAN also has 20-kDa O-linked glycosylconjugates. The migration of the 300-kDa molecule detected by MY-174 was completely identical to that of chick SPACRCAN in all digestions examined, indicating that the 300-kDa molecule recognized by MY-174 corresponds to chick SPACRCAN.

FIGURE 3. Establishment of anti-SPACRCAN antibodies. A, Western blot analyses of PBS-insoluble IPM samples stained with the newly prepared anti-SPACRCAN antibodies, MY-174, and biotinylated hyaluronan after digestion with chondroitinase ABC. A single 300-kDa band was detected by the anti-SPACRCAN antibodies, MY-174, and biotinylated hyaluronan (arrows). A 150-kDa band corresponding to SPACR was not detected by the anti-SPACRCAN antibodies, but was detected by MY-174 and biotinylated hyaluronan (arrowheads), indicating that the anti-SPACRCAN antibodies did not cross-react with SPACR. B, localization of SPACRCAN in the retina. A radial section of an adult retina stained with the anti-SPACRCAN antibodies is shown. Fluorescence was specifically detected in the photoreceptor layer (PL, arrow). A section stained with hematoxylin and eosin (H&E) is shown on the left. NFL, nerve fiber layer; GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium. Scale bar = 100 μm.
FIGURE 5. Occurrences of the chick SPACRCAN mRNA and core protein, sialylated glycoconjugates recognized by MY-174, and hyaluronan binding property during retinal development: comparisons with SPACR. A, the chick SPACRCAN and SPACR mRNA expression levels were analyzed by Northern blot analysis. A band for SPACRCAN mRNA was detected at E12, and its intensity increased with development to reach a peak at E16 and decreased thereafter. The densities of the background and the band in the E16 retina are

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SPACRCAN antibodies were examined. Retinal samples were subjected to membrane blot analyses before and after digestion with chondroitinase ABC. After treatment with chondroitinase ABC, the intensity of the 300-kDa band detected by MY-174 increased, suggesting that it is a chondroitin sulfate proteoglycan (Fig. 4A). Following the digestion with chondroitinase ABC, some samples were further digested with N- or O-glycanase or neuraminidase and then subjected to Western blot analyses with MY-174. After treatment with O-glycanase alone following chondroitinase ABC digestion, the size of the 300-kDa band did not change significantly. However, after treatment with N-glycanase or neuraminidase following chondroitinase ABC digestion, the size decreased by 20 or 50 kDa, respectively. Further treatment with O-glycanase after neuraminidase treatment abolished detection of the 300-kDa band altogether. This effect of O-glycanase treatment was not observed without neuraminidase predigestion, and this requirement has been well documented for other glycoproteins (22).

Next, we stripped the above-mentioned membranes and reprobed them with the anti-SPACRCAN antibodies (Fig. 4B). After treatment with chondroitinase ABC, the intensity of the 300-kDa band detected by the anti-SPACRCAN antibodies increased, as shown in Fig. 4A. After treatment with O-glycanase alone following chondroitinase ABC digestion, the size of the 300-kDa band did not change significantly. However, after treatment with N-glycanase or neuraminidase following chondroitinase ABC digestion, the size decreased by 20 or 50 kDa, respectively, as observed for the staining with MY-174.

Because MY-174 and anti-SPACRCAN antibodies recognized completely identical molecules in both immunohistochemical and immunoblot analyses, we finally concluded that the 300-kDa molecule recognized by MY-174 corresponds to chick SPACRCAN. The band that became undetectable after treatment with O-glycanase following neuraminidase treatment in the staining with MY-174 was detected at 230 kDa by staining with the anti-SPACRCAN antibodies (Fig. 4), suggesting that the molecular mass of the O-linked glycoconjugates was 20 kDa. Thus, chick SPACRCAN and SPACR are modified by the same specific glycoconjugates, which are recognized by MY-174.

SPACRCAN and SPACR Expression during Retinal Development—The SPACRCAN and SPACR mRNA expression levels during retinal development were quantified by Northern blot analysis (Fig. 5A). Total RNAs isolated from chick retinas at various stages (from E12 to newborn) were electrophoresed, transferred to nylon filters, and hybridized with radiolabeled cDNA probes for chick SPACRCAN and SPACR. The mRNA expression levels of SPACRCAN and SPACR in each sample were measured using the NIH Image program. A faint band for SPACRCAN mRNA was detected at E12, and its intensity increased with development up to a peak at E16 and then decreased. A band for chick SPACR mRNA was first detected at E15, and its intensity increased with development as shown previously (5).

Immunoblot analyses using the anti-SPACRCAN antibodies, MY-174, and biotinylated hyaluronan were performed to assess the amounts of the chick SPACRCAN and SPACR core proteins, the specific glycoconjugates recognized by MY-174, and the hyaluronan binding property, respectively. Samples of PBS-insoluble IPM were obtained from chick retinas at various developmental stages (from E12 to newborn and postnatal day 2 (P2)) and from adult retinas. After digestion with chondroitinase ABC, the retinal samples were electrophoresed on 3–8% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and incubated with the anti-SPACRCAN antibodies, MY-174, and biotinylated hyaluronan. A single band of 300 kDa was detected at E12 in all staining procedures, and its intensity increased with development to reach a peak at E17–E18 and then gradually decreased toward the adult stage (Fig. 5, B–D). To measure SPACR expression, the membrane was stained with O46-F and MY-174. Distinct 150-kDa bands corresponding to the chick SPACR core protein and the glycoconjugates recognized by MY-174 were detected. A single band of 150 kDa was first detected at E16, and its intensity subsequently increased with development (Fig. 5, B and C). The hyaluronan binding ability of chick SPACR was also measured and compared with the corresponding profile of chick SPACRCAN. The hyaluronan binding property of chick SPACR was first detected at E16, and it increased with development to reach a peak at P2 and then substantially decreased toward the adult stage (Fig. 5D).

Involvement of SPACR Glycosylation in Hyaluronan Binding—The expression levels of SPACR, especially for the glycoconjugates recognized by MY-174, were higher in the adult than at P2, whereas the hyaluronan binding ability was significantly decreased in the adult. To assess this apparent inconsistency, the effects of the glycosylation modifications of SPACR on its binding to hyaluronan were examined by measuring the amounts of bound hyaluronan after digestions with glycosidase enzymes such as O-glycanase, N-glycanase, and neuraminidase. The hyaluronan binding ability of SPACR was much higher at P2 than in the adult without any enzymatic digestions (Fig. 6, lanes 1 and 2), and no change was detected after N-glycanase treatment (lane 4). However, the hyaluronan binding ability in the adult was significantly enhanced after neuraminidase treatment (Fig. 6, lane 3). Furthermore, treatment with O-glycanase following neuraminidase treatment produced a similar enhancement of neuraminidase treatment alone (Fig. 6, lane 5).

FIGURE 6. Involvement of glycoconjugates on SPACR in hyaluronan binding. Samples from P2 and adult retinas were assessed for their hyaluronan binding properties via their affinities for biotinylated hyaluronan. Neuraminidase treatment with or without O-glycanase treatment significantly enhanced hyaluronan binding, whereas N-glycanase treatment did not, suggesting that the presence of numerous sialic acids in adult SPACR may inhibit hyaluronan binding. Lane 1, P2 retina without any treatment; lane 2, adult retina without any treatment; lane 3, adult retina after neuraminidase treatment; lane 4, adult retina after N-glycanase treatment; lane 5, adult retina after O-glycanase treatment following neuraminidase treatment.
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DISCUSSION

As mentioned above, alignment of the deduced amino acid sequences of human SPACRCAN and SPACR reveals several homologous regions (7). Searches for homologous regions between the deduced amino acid sequences of chick SPACRCAN and SPACR show 39.7% homologous regions. This result clearly demonstrates the high degree of homology between these different molecules; and furthermore, both contain seven C-terminal glycosylation sites, numerous potential O-linked glycosylation sites, an epidermal growth factor-like domain, and two SEA domains.

A high degree of homology among the coding regions of chick, mouse, and human SPACRs has been reported previously (5, 23). The homology among the coding regions of chick, mouse, and human SPACRCANs was also assessed. The nucleotide sequences show a high degree of homology, with chick SPACRCAN showing 51.4 and 52.9% identities to mouse and human SPACRCANs, respectively. Furthermore, the deduced amino acid sequence of chick SPACRCAN shares 38.5 and 64.4% similarities with the mouse and human sequences, respectively. More important, chick SPACRCAN contains important features that are also present in mouse and human SPACRCANs (4, 7), including several O-glycosylation sites, glycosaminoglycan attachment sites, two epidermal growth factor-like domains, and several hyaluronan-binding motifs. The high sequence homology to mouse and human SPACRCANs indicates that this chick gene is indeed the ortholog of mouse and human SPACRCANs. The presence of multiple functional domains in the protein suggests that SPACRCAN may play important and complex roles in retinal growth and development (4). The similarities among the chick, mouse, and human SPACRCANs suggest similar functional roles for this molecule in these species.

It is interesting that SPACRCAN and SPACR were both modified by heavily sialylated O-linked glycoconjugates, including the specific glycoconjugates recognized by MY-174. Treatment with O-glycanase following neuraminidase digestion abolished the MY-174 antigenicity of both chick SPACR and SPACRCAN. Molecules with similar core protein structures are generally recognized to have similar functions, and our experiments produced the novel result that these two molecules with similar core proteins also have similar glycoconjugates. Although these molecules had similar core protein structures and similar sialylated glycoconjugates, they may play different roles due to distinct modifications of their chondroitin sulfate chains and their differing expression profiles during development.

Both SPACR and SPACRCAN in the human and mouse IPM commonly have hyaluronan-binding domains containing RHAMM-type hyaluronan-binding motifs (3, 7, 8, 23). Here, we have revealed that chick SPACRCAN also has RHAMM-type hyaluronan-binding motifs, similar to chick SPACR. Hyaluronan is a prominent constituent of the IPM in all species studied to date, except for mice (24, 25), and has been detected in the chick IPM using a biotinylated hyaluronan-binding protein (data not shown). Chen et al. (8) have shown that the RHAMM-type hyaluronan-binding motifs found in mouse SPACRCAN also bind to chondroitin sulfate. Thus, the functional complex of SPACRCAN and SPACR with hyaluronan and/or chondroitin sulfate in the matrix between the neural retina and retinal pigment epithelium may have some important roles via protein-carbohydrate and/or carbohydrate-carbohydrate interactions. Further investigations are necessary.

During the development of the retina as well as the maintenance of its functions, the hyaluronan-binding properties of SPACR and SPACRCAN may be necessary for cells to proliferate and migrate in the extensive matrix enriched with hyaluronan (3, 7, 8, 26). Chondroitin sulfate molecules generally tend to have more numerous chondroitin sulfate chains during the embryonic period (14, 12). Chondroitin sulfate and hyaluronan on the cell surface may cooperate to ensure consistency as the extracellular matrix increases and then matrix stability, thereby promoting the formation of the extracellular matrix. Once retinal formation is completed, SPACRCAN may become redundant; consequently, the volume of the extracellular matrix decreases. SPACRCAN is potentially the embryonic equivalent of SPACR and may be involved in organogenesis, rather than homeostasis, of the retina.

Finally, we have shown that SPACR expression, and its MY-174 antigenicity in particular, increased during retinal development, whereas the hyaluronan binding ability in the adult retina was significantly decreased. It is interesting that the hyaluronan binding ability of SPACR was significantly enhanced after neuraminidase treatment, regardless of O-glycanase treatment. It is possible that the number of sialic acids increases with age and that they stericly coat the hyaluronan-binding motif of SPACR, thereby inhibiting hyaluronan binding. Although the essence of this phenomenon remains unclear, it is possible that changes in the sialic acid residues on SPACR during aging may underlie some of the age-related diseases of the retina due to a decrease in its hyaluronan binding ability in the IPM.

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