Spacrcan Binding to Hyaluronan and Other Glycosaminoglycans

MOLECULAR AND BIOCHEMICAL STUDIES*

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The interphotoreceptor matrix (IPM)\(^1\) is a carbohydrate-rich extracellular compartment (Ref. 1, for review see Ref. 2) sur-

rounding light sensitive photoreceptor cells. This matrix lies between the outer limiting membrane of the retina and the apical surface of retinal pigment epithelium (RPE). It was first described as an amorphous periodic acid Schiff (PAS)-positive material (3). We now know that this unique structure is based on the presence of glycosaminoglycans (GAG) in the form of hyaluronan (HA), chondroitin sulfate (CS) proteoglycans, and N- and O-linked oligosaccharides present in proteoglycans and glycoproteins such as SPACRCAN\(^2\) (4) and SPACR (5). The change in a conserved amino acid residue between two proteins has been found to be related to a type of macular degeneration, the benign concentric annular macular dystrophy (BCAMD, Ref. 6). Such a change may be able to form a stronger \(\beta\)-sheet in SPACR. Why this change can cause BCAMD is unknown.

SPACRCAN is a proteoglycan with a chondroitin sulfate GAG side chain. Interaction of GAGs with proteins and other factors may be fundamental for IPM structure, formation, and function in vision, including its role in retinal adhesion (7–9), intercellular communication (10, 11), membrane turnover (12–14), regulation of neovascularization (13, 14), cell survival (15, 16), photoreceptor differentiation, and maintenance (17–19). However, little is known of the molecular mechanism involved in protein-GAG interactions in the IPM.

Based on its location, it is conceivable that this matrix in the IPM can at least confine the cylindrical photoreceptor outer segments to preserve nearest neighbor associations and can set the precise alignment of photoreceptors to the optical light path (20). The IPM matrix can also form an adhesion bridge to hold the retina to the outer eye wall. When the adhesion bridge formed by this matrix is disturbed, retinal detachment occurs (7, 8). If left untreated, blindness is the most obvious complication from retinal detachment. Currently the prevalence of retinal detachment is estimated to be 0.3% in general population and higher with patients following cataract surgery, high myopia, and trauma (21). Although several techniques are available for repairing a retinal detachment, the degree of recovery varies with individual cases. Thus understanding the mechanisms involved in retinal adhesion could potentially be important in managing retinal detachment.

Studies to date suggest that the adhesion between retina and RPE requires the interaction between photoreceptors and RPE. Although no direct cell-cell contacts are involved, the IPM resides in a critical location and is thought to be involved in

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¶ The abbreviations used are: IPM, interphotoreceptor matrix; SPACR, sialoprotein associated with cones and rods; SPAPRCAN, sialoproteoglycan associated with cones and rods; RPE, retinal pigment epithelium; GAG, glycosaminoglycan; HA, hyaluronan; CS, chondroitin sulfate; RHAM, receptor for HA-mediated motility; HABM, hyaluronan binding motif; CPC, cetylpyridinium chloride; ELISA, enzyme-linked immunosorbent assay; HSPG, heparan sulfate proteoglycan;

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**GST, glutathione S-transferase; NF-HA, non-functional HA binding motif**

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rounding light sensitive photoreceptor cells. This matrix lies between the outer limiting membrane of the retina and the apical surface of retinal pigment epithelium (RPE). It was first described as an amorphous periodic acid Schiff (PAS)-positive material (3). We now know that this unique structure is based on the presence of glycosaminoglycans (GAG) in the form of hyaluronan (HA), chondroitin sulfate (CS) proteoglycans, and N- and O-linked oligosaccharides present in proteoglycans and glycoproteins such as SPACRCAN\(^2\) (4) and SPACR (5). The change in a conserved amino acid residue between two proteins has been found to be related to a type of macular degeneration, the benign concentric annular macular dystrophy (BCAMD, Ref. 6). Such a change may be able to form a stronger \(\beta\)-sheet in SPACR. Why this change can cause BCAMD is unknown.

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were confirmed by automatic sequencing in a CEQ 2000XL DNA analysis enzyme. Colonies were selected for DNA preparation for the experiments to be conducted later. Since SPACRCAN is a chondroitin sulfate proteoglycan, we have also evaluated the possibility that these RHAMM-type HA binding motifs may also bind chondroitin GAGs. In addition, since heparan sulfate proteoglycan, we have also evaluated the possibility of heparin with these RHAMM-type HA binding motifs (HABMs) present in the de-ducted amino acid sequence of Spacrcan. Sparc will be analyzed in experiments to be conducted later. Since Spacrcan is a chondroitin sulfate proteoglycan, we have also evaluated the possibility that these RHAMM-type HA binding motifs may also bind chondroitin GAGs. In addition, since heparan sulfate proteoglycans (HSPGs) containing RHAMM-type HABMs express in the retina and may have roles in guiding the neural retina cell growth (i.e. syndecan-3, Refs. 23–25), we also evaluated the potential interaction of heparin with these RHAMM-type HA binding motifs.

**EXPERIMENTAL PROCEDURES**

**Construct of Spacrcan cDNA Fragments—**Spacrcan cDNA fragments were PCR-amplified using the following oligonucleotide pairs: HA2-F (AAGTCCCGGGGCTTGTCAACATGGGAGCTGCTGTG) and HA2-B2 AAGTGGATCCAGACAACTTGCAGCTGTTGTCG (for pQC43; HA3-F (AAGTCCCGGGCTTGTCAACATGGGAGCTGCTGTG) and HA3-B2 AAGTGGATCCAGACAACTTGCAGCTGTTGTCG (for pQC55, pQC56, pQC57, pQC58, pQC59, and pQC56), respectively. For pQC49, pQC55, pQC56, pQC57, pQC58, pQC59, and pQC60 were constructed to inoculate in a 3-ml 2xYT culture for overnight growth at 37 °C. After adding Triton X-100 to a final concentration of 1%, the homogenate was rotated for 30 min at 4 °C or immediately resuspended in ice-cold 1× phosphate-buffered saline (PBS), centrifuged as above, and either stored at −80 °C or immediately resuspended in ice-cold 1× PBS plus 1 μM phenylmethylsulfonyl fluoride and 1× bacterial protease inhibitor mixture (P8465, Sigma). The resuspension was sonicated on ice with 60% energy output. After adding Triton X-100 to a final concentration of 1%, the homogenate was rotated for 30 min at 4 °C and centrifuged for 10 min at 12,000 × g for 10 min at 4 °C. The pellets were resuspended in ice-cold 1× phosphate-buffered saline (PBS), centrifuged as above, and either stored at −80 °C or immediately resuspended in ice-cold 1× PBS plus 1 μM phenylmethylsulfonyl fluoride and 1× bacterial protease inhibitor mixture (P8465, Sigma). The resuspension was sonicated on ice with 60% energy output. After adding Triton X-100 to a final concentration of 1%, the homogenate was rotated for 30 min at 4 °C and centrifuged for 10 min at 12,000 × g for 10 min at 4 °C. The pellets were resuspended in ice-cold 1× PBS plus 1 μM phenylmethylsulfonyl fluoride and 1× bacterial protease inhibitor mixture (P8465, Sigma). The resuspension was sonicated on ice with 60% energy output. 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RESULTS

Expression of Recombinant Proteins Containing RHAMM-type Binding Motifs—We have previously reported that mouse Spacrcan contains five potential RHAMM-type HA binding motifs (29, 30). To determine if these motifs are functional in HA binding, cDNA fragments containing these HA binding motifs were subcloned into pGEX-2TK vector to make fusion proteins with GST at their N terminus. As shown in Fig. 1A, each clone contains about 100 amino acid residues. Clones prQC43 and prQC44 each contain one HA binding motif. Clone prQC45 contains two HA binding motifs located in tandem with only 2 amino acids separating them. In addition, there is an HA-like binding motif present at the C-terminal end. This motif contains several internal acidic amino acids, which have been suggested to dramatically decrease its HA binding affinity (30). The motif was included in prQC45 to test its function in HA binding. We will refer to it as a non-functional HA binding motif (NF-HA binding motif).

The expressed recombinant protein from each construct was shown in Fig. 2. As predicted based on the nucleotide sequence, recombinant GST fusion proteins prQC44 and prQC45 and their corresponding mutant proteins were about 40 kDa in size. However, prQC43 and each of its corresponding mutant proteins (prQC49, -55, and -57) were calculated to have molecular masses of about 50 kDa, which was about 10 kDa larger than the predicted molecular mass based on mobility in the SDS-polyacrylamide gel. Such an increase was not caused by subcloning duplicate cDNA fragments into the expression vector, because each subclone was confirmed by nucleotide sequencing using primers flanking the cloning site. Furthermore, the identity of the recombinant protein prQC43 was confirmed by mass spectrometric analysis, in which a prQC43-corresponding peptide of YSEELRDPSALYR was identified. Therefore, it is possible that the apparent higher molecular mass of prQC43 and each of its mutants was caused by post-translational modification or anomalous migration because of the protein charge.

As shown in Fig. 1A, prQC43 is a subclone from the mucin-like domain of Spacrcan where many predicted eukaryote O-glycosylation sites were found (29). It has been shown that the predicted eukaryote O-glycosylation site can be functional in bacteria (31). In addition, several immunodominant antigens expressed in Escherichia coli have been found to exhibit molecular masses ranging from 1.6 to 2 times larger than what was predicted mainly because of O-glycosylation (32, 33). Since serine and threonine are the main O-glycosylation sites (32, 33). Since serine and threonine (29), O-glycosylation may have caused the increase in prQC43 molecular mass and each of its mutants was caused by post-translational modification or anomalous migration because of the protein charge.
bands (data not shown). Therefore, those bands may be degradation products of expressed fusion proteins. However, the faint 70-kDa band did not react with anti-GST antibody. Therefore, this probably represented an E. coli protein similar to the product of dnaK (34).

**HA Binds to Recombinant Proteins**—To determine whether the recombinant proteins bind HA, CPC precipitation analysis was carried out in which binding of a cationic detergent, CPC, to anionic GAGs, like HA, leads to co-precipitation of proteins interacting with the GAGs. As shown in Fig. 3, each of the three recombinant proteins could be precipitated by CPC after incubating with HA. In the absence of HA, they could not be precipitated. When the HA-recombinant protein complex was digested with hyaluronidase before CPC treatment, precipitation of the recombinant proteins was abolished. This suggests that each of the three recombinant proteins could bind to HA.

To determine the relative binding affinity of each protein for HA, we employed an ELISA using biotinylated HA-coated plates. Because a portion of the purified proteins was degraded (Fig. 2) and may not contain HABMs, this portion would not be effective in HA binding. To determine the effective concentration of proteins, we scanned the density of protein bands on SDS-polycrylamide gels with a densitometer to obtain the relative density of each band. We then incorporated the effective protein concentration in the ELISA analysis. Fig. 4 shows that binding of proteins prQC43, -44, and -45 to HA was concentration-dependent. The binding increased with increasing protein concentration. In all concentrations tested, more prQC45 was bound to HA than was prQC43 and prQC44.

Because the ELISA was performed using biotinylated HA-coated plates, a competition experiment was included to determine if non-biotinylated HA was able to compete the proteins from binding to the HA plates. As shown in Fig. 5, increasing the concentration of competitor HA did lead to a decrease in the binding of all three proteins (prQC43, -44, and -45) to the HA plates. The competition was stronger with prQC44 and prQC45 than prQC43.

**Mutant Effects on HA Binding**—RHAMM-type HA binding motifs consists of B\(_{1,2}X\_Y\)B, where B is a basic amino acid residue, either Arg or Lys, and \(X\_Y\) corresponds to any seven amino acid residues preferably non-acidic. The presence of basic amino acids in \(X\_Y\) enhances HA binding, while the presence of acidic amino acids reduces the HA binding (30). To test whether the predicted RHAMM-type HA binding motifs in mouse Sparcrcan bind HA, a series of mutations were introduced to alter the flanking B residues in the HA binding motifs of prQC43, prQC44, and prQC45 to either His or one of the neutral and polar amino acids. As shown in Fig. 1B, a single amino acid mutation for the HA binding motif in prQC43 was made in prQC49 and prQC55. Double amino acid mutations were generated in prQC57. For prQC44, single amino acid mutations were generated in prQC58 and prQC59. For prQC45, multiple amino acid residues were mutated in prQC56. As a result, both HA binding motifs were affected. To distinguish their biological effect, the C-terminal HA binding motif was mutated in prQC60, in which both flanking basic amino acids were mutated. To test the effects of NF-HA binding motifs at the C-terminal end, the flanking basic amino acids were mutated in prQC61 (Fig. 1).

Using ELISA, the mutant effects on HA binding were analyzed and are shown in Fig. 6, A–C. Fig. 6A shows the effects of the HA binding motif in prQC43. At a protein concentration over 50 \(\mu\)g/ml, all mutant proteins had significantly lower binding affinity to HA compared with the wild-type prQC43. Similar to prQC43, mutating either the N-terminal (prQC58) or the C-terminal (prQC59) Arg residue to Glu in prQC44 HABM significantly reduced the HA binding (Fig. 6B).

In prQC45, two HA binding motifs are present in tandem with only two amino acid residues separating the two motifs. To determine if both motifs participate in regulating HA binding, we first mutated the C-terminal motif leaving the N-terminal HA binding motif unchanged (prQC60). As shown in Fig. 6C, HA binding of prQC60 was dramatically lower than the wild-type prQC45. This suggests that the C-terminal motif was involved in HA binding. When both HA binding motifs were mutated (prQC56), HA binding was further reduced. Therefore, both motifs appeared to be functional in HA binding. The presence of the two motifs may thus account for the higher level of HA binding in prQC45 compared with prQC43 and prQC44 (Fig. 4).

In addition to the two putative HA binding motifs in prQC45, there is another BX-B motif located at the C-terminal end of prQC45 (KEEIQERM). The presence of several acidic residues (Glu) in the middle of this motif suggests it is a weak candidate for HA binding. To test its involvement in HA binding, we mutated both flanking basic residues, i.e. Lys to Asn and Arg to Gly (prQC61). ELISA analysis showed that alteration of the residues in prQC61 did not affect HA binding when compared with its wild-type prQC45. This is consistent with previous findings of the RHAMM-type HA binding motif, in which the presence of multiple acidic residues within the seven internal amino acids reduces HA binding (30).

**Heparin Binds to Recombinant Proteins**—Heparan sulfates and chondroitin sulfates are two other major negatively charged GAGs present in and around the IPM. The ionic interaction with positively charged amino acid residues could support their interaction with surrounding proteins. To test whether the recombinant proteins prQC43, prQC44, and prQC45 can bind heparin, ELISA was performed on plates precoated with the biotinylated heparin. Fig. 7A shows that all three proteins bind heparin. At lower concentrations, less than 40 \(\mu\)g/ml, more prQC44 was bound to heparin. Increasing protein concentration to over 50 \(\mu\)g/ml did not increase heparin binding to prQC45, but led to a decrease in the binding of prQC43 and prQC44. Therefore, at concentrations over 50 \(\mu\)g/ml, more prQC45 bound to heparin than did the other two proteins.

The heparin binding domain in RHAMM has been located in a 35-amino acid segment that contains two HA binding motifs (35). To determine if the HA binding motifs in the recombinant proteins in this study also participate in heparin binding, mutant proteins shown in Fig. 1B were used for ELISA on the
biotinylated heparin coated plates. If the flanking basic amino acids were involved in heparin binding, then mutating these amino acids would lead to a decrease in heparin binding similar to that observed in HA. For prQC43, mutating either basic amino acid (prQC49 and prQC55) did not decrease heparin binding (Fig. 7B). At a protein concentration over 50 μg/ml, the mutant proteins bound heparin with higher affinity than the wild-type prQC43. Mutating both basic amino acids (prQC57) led to a significant increase in binding to heparin. These results suggest that the Arg residues of the HA binding motif in prQC43 are not necessary for heparin binding. And excessive HA binding motifs may inhibit heparin binding because the binding of prQC43 with heparin decreased at concentrations above 50 μg/ml.

As with prQC43, mutating the HA binding motif in prQC44 (prQC58 and prQC59) did not lead to a decrease in protein binding to heparin (Fig. 7C). This suggests that the basic amino acids of the HA binding motif in prQC44 are not involved in heparin binding.

The effects of mutagenesis on heparin binding of prQC45 are more complicated because this protein contains several motifs. Mutating the basic amino acids in the NF-HA binding motif (prQC61) leads to an increase in binding to heparin (Fig. 7D). This suggests that the basic amino acids in this motif may not be required with heparin binding. The effects of mutation in the tandem arranged HA binding motifs (prQC56 and prQC60) changed according to the protein concentration. At lower concentrations, less than 30 μg/ml, mutagenesis leads to an increase in heparin binding. At higher concentrations, over 30 μg/ml, mutagenesis leads to a decrease in heparin binding. These results suggest that at higher protein concentrations the tandem arranged HA binding motifs may be involved in heparin binding. Because there was no difference in heparin binding between prQC56 and prQC60 and both proteins carry a mutation on Arg-1028 (29), it is possible that Arg-1028 plays a role in heparin binding.

Chondroitin Sulfate A (CSA) and Chondroitin Sulfate C (CSC) Bind to Recombinant Proteins—Binding to CSA and CSC was analyzed by competition experiments using plates precoated with biotinylated HA. Using the control in which no competitor GAG was added, changes in binding in the presence of competitor GAGs CSA or CSC were determined by the relative absorbance from the control. As shown in Fig. 8, both CSA and CSC significantly competed for protein binding to HA with each of the three recombinant proteins (prQC43, prQC44, and prQC45). This suggests that all three proteins can bind to CSA and CSC.

To analyze the effects of HA binding motifs on CS binding, proteins carrying mutated HA binding motifs were also tested. If an HA binding motif is involved in the binding, then mutating the motif should abolish CS competition with HA. As summarized in Table II, the prQC43 mutants (prQC49, prQC55, and prQC57) abolished the capacity of CSA to compete for HA binding, whereas in the case of the prQC44 mutants (prQC58 and prQC59) CSA did not compete with HA. This suggests that an HA binding motif in prQC43 was involved in CSA binding, whereas that in prQC44 was not. For motifs in prQC45, because prQC60 did not affect competition by CSA, whereas prQC56 and prQC61 did, only the N-terminal HA binding motif and the NF-HA binding motif are involved in CSA binding.

Involvement of the HA binding motif in CSC binding is different from that of CSA. Mutating the HA motifs in prQC44...
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and prQC45 abolished the ability of CSC to compete with HA. However, for the motif in prQC43, only mutating the C-terminal basic amino acid abolished the competition, whereas mutating the N-terminal basic amino acid did not affect competition by CSC with HA. These data suggest that the HA binding motifs in prQC44 and prQC45 were involved in binding to CSC. However, only the C-terminal Arg in prQC43 is involved in such binding. The N-terminal Arg may not be involved in CSC binding.

Because mutations in prQC55 lead to an increase in binding to HA in the presence of CSA, whereas prQC49 did not (Fig. 8), this suggests that in the presence of CSA the N-terminal Arg in prQC43 may not have as good a binding affinity to HA as the N-terminal His. Similarly, a double mutation in prQC43 HABM (prQC57), a single mutation at the N-terminal Arg in prQC44 (prQC58) and the double mutation in the NF-HA binding motif in prQC45 (prQC61) lead to an increase in binding to HA in the presence of CSC. Therefore, in the presence of CSC, both Arg residues in prQC43, the N-terminal Arg residue in prQC44, and the NF-HABM in prQC45, may not have as good an HA binding affinity as their corresponding mutants.

Taken together, these data suggest that HA binding motifs are involved in binding to chondroitin sulfate A and C at various levels. For the motif in prQC43, the C-terminal Arg residue is involved in binding to both CSA and C, while the N-terminal Arg is only involved in binding to CSA. In the presence of CSA, it may play an inhibitory role in HA binding. The motif in prQC44 is involved in CSC binding but not in CSA binding. For motifs in prQC45, both HA binding motifs are involved in CSC binding, but only the N-terminal motif is involved in CSA binding. The NF-HA binding motif is involved in both CSA and CSC binding, and in the presence of CSC it may be inhibitory to HA binding.

Relative Binding of the Recombinant Proteins to HA, Heparin, CSA, and CSC—To test the relative binding to HA, heparin, CSA, and CSC, ELISA competition experiments using each of the GAGs were performed in both biotinylated HA-coated plates and heparin-coated plates. Fig. 9A shows that heparin did not significantly compete for HA binding to prQC43 and prQC44; it only reduced by about 15% the HA binding to prQC45. Whereas Fig. 9B shows that CSA was able to compete for heparin binding to both prQC43 and prQC44. With prQC45, HA was able to reduce about 60% of its binding to heparin. This suggests that HA-Spacrcan interaction is more efficient than heparin-Spacrcan interaction.

The relative binding between heparin and chondroitin sulfates can be compared by the competition power of the GAGs to influence the protein binding on biotinylated HA-coated plates. As shown in Fig. 9A, although preincubation of the proteins with heparin, CSA, or CSC led to a decrease of prQC45 binding to the HA plate to a similar extent (by ~12% (heparin), ~18% (CSA), and ~4% (CSC)), preincubation of the proteins with CSA or CSC led to greater reduction than with heparin of prQC43 (by ~10% (heparin), ~16% (CSA), and ~27% (CSC)) and of prQC44 (by ~11% (heparin), ~35% (CSA), and ~27% (CSC)) binding to the HA plate. This suggests that chondroitin sulfates tend to bind more efficiently to proteins in general when compared with heparin.

**DISCUSSION**

In this study, we have defined the molecular interaction taking place in the IPM between GAGs and a specific matrix protein, Spacrcan. Our results show that Spacrcan can bind to HA, chondroitin sulfates, and heparin. The binding of Spacrcan to HA is through the RHAMM-type HABMs, based on the elimination of HA binding following mutagenesis of these motifs. Binding of Spacrcan to chondroitin sulfates may also be mediated through the HABMs. However, the binding of the protein to heparin seems to be mediated more through specific spatial conformations rather than the basic amino acid residues in HABMs.

HABMs evaluated in this study were first identified in the HA receptor RHAMM (30). The motif consists of two basic amino acid residues, either Arg or Lys, flanking a seven amino acid stretch, BXX2B (30). The presence of acidic residues between the flanking basic residues reduces HA binding. While this motif appears in several HABPs, its presence is not sufficient to conclude that HA binding will occur (36); experimental
confirmation is necessary for each protein containing this motif. Here we show that like RHAMM, the presence of acidic residues within the motif dramatically decreases its HA binding because the multiple acidic residue-containing NF-HA binding motifs in Spacrcan prevented specific HA binding.

FIG. 7. Effects of HA binding motifs in heparin binding. The effects of HA binding motifs on heparin binding were analyzed by ELISA using plates precoated with biotinylated heparin. In the presence of various amounts of proteins, the colorimetric reaction was recorded at 450 nm. The absorbance reading corresponds to the affinity of proteins to heparin. Changes in heparin binding of the wild type and corresponding mutants are shown here.

FIG. 8. Effects of HA binding motifs on chondroitin sulfate binding. The effects of HA binding motifs on chondroitin sulfate binding were analyzed by competition experiments using chondroitin sulfate A or C against biotinylated HA precoated on ELISA plates. In the presence of various amounts of proteins, the colorimetric reaction was recorded at 450 nm. The absorbance reading corresponds to the level of protein binding to HA. Effects of chondroitin sulfates on HA binding were calculated as the relative absorbance to the control reaction without the presence of competitors.

Moreover, like RHAMM, binding of the IPM matrix protein Spacrcan to HA required Arg or Lys as the flanking residues. When the flanking basic residues are mutated from Arg to His (as in prQC43), the recombinant protein showed lower binding activity to HA. Although Arg, Lys, and His are each basic

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amino acids, they have substantial differences in their individual isoelectric points. The pIs are 11.15 for Arg, 9.59 for Lys, and 7.47 for His. The higher pIs for Arg and Lys will result in more ionized positive charges at neutral pH and thus may favor HA binding. This is consistent with the finding that clustering of basic amino acids in HABM results in higher HA binding affinity (30). Structurally the three basic amino acids also differ in their conformation. Lys is more like Arg in that its positive charge is due to its amine-containing linear side chain. However His is positively charged because of its aromatic nitrogen-nitrogen cyclicimidazole side chain. Therefore the difference in charge and conformation may explain the preference of Arg and Lys over His in the RHAMM-type HA binding motif.

The HA binding domain of RHAMM binds to HA and heparin (35) with approximately the same affinity. Based on this finding, an engineered protein has recently been developed as a detector for heparin (37) by linking three binding motifs of RHAMM in tandem. These results are consistent with what we have found in Spacrcan, revealing that RHAMM-type HA-binding proteins can interact with multiple GAGs to establish complex macromolecular networks in vivo.

Because of the binding difference between HA, CS, and heparin, plus the preference of Lys and Arg over His in the HA binding motif, ionic interaction does not seem to be the sole requirement of the flanking residues. Other interactions and possibly a unique conformation may be required to assist the binding. For example, the other group of HA-binding proteins, such as CD44, bind to HA through a link module (36). The specific tertiary structure of the link module brings the basic binding residues to the surface and in close proximity allowing HA binding. Therefore, proteins binding to HA through RHAMM-type HA binding motifs may also require a certain three-dimensional conformation to assist in this interaction.

To our knowledge, this is the first report indicating that RHAMM-type HA binding motifs are involved in chondroitin sulfate binding. Such dual binding ability to both HA and CS could provide a way to anchor and thus retain other CS proteoglycans to the HA scaffold in the IPM (38). If the anchored protein is a CS proteoglycan such as Spacrcan, then binding of the protein to other HA molecules would serve as a cross-linking bridge to stabilize the HA scaffold. Furthermore, the interaction of HA binding motifs with CS allows Spacrcan-Spacrcan interaction to increase the complexity of the matrix and further stabilize these interactions. Therefore the binding potential of HA binding motifs to CS may provide a way to stabilize the photoreceptor mosaic. In addition, HSPGs are prevalent in cell surfaces, extracellular matrix, and basement membranes. Interactions of the heparin-like GAG chain of the HSPGs with other proteins may promote cell adhesion, proliferation, and neurite growth in the retina, since HSPGs are known to mediate fibronectin interactions (39, 40). Moreover, HSPGs facilitate the binding of fibroblast growth factor and vascular endothelial growth factor to the cognate receptors on cell surfaces (24, 41–44). The work described here suggests that the presence of the RHAMM-type HA binding motifs in proteins may contribute to tissue development in retina

### Table II

Summary of competition results to HA with chondroitin sulfates

| Proteins   | HA binding motifs | Competed by CSA | Competed by CSC |
|------------|-------------------|-----------------|-----------------|
| prQC43     | RDPSSALYR         | Yes*            | Yes             |
| prQC49     | HXXXXXXXH         | No*             | No              |
| prQC55     | HXXXXXXXR         | No              | Yes             |
| prQC57     | HXXXXXXXH         | No              | No              |
| prQC44     | RVGSWNYWR         | Yes             | Yes             |
| prQC58     | QXXXXXXXR         | Yes             | No              |
| prQC59     | HXXXXXXXQ         | Yes             | No              |
| prQC45     | KMLQAQVRERQRPTSSR | Yes*           | Yes             |
| prQC56     | KXXXXXXXH         | No              | No              |
| prQC60     | KXXXXXXXH         | Yes             | No              |
| prQC61     | KXXXXXXXH         | No              | No              |

* Yes, chondroitin sulfate significantly competed binding to plates.
  
* No, competition was not significant.

**Fig. 9.** Relative GAG binding affinity of the three wild-type recombinant proteins. Wild-type recombinant proteins prQC43, prQC44, and prQC45 were analyzed for binding to biotinylated HA-precoated plates (A) or biotinylated heparin-precoated plates (B) in competition ELISA with various GAGs including HA, heparin, chondroitin sulfate A, and chondroitin sulfate C. The A450 was recorded and corrected with negative controls. Competition effects on binding to HA (A) and heparin (B) were calculated as the relative absorbance to the control reaction without the presence of competitors.
through these extensive binding interactions and the consequent biological functions.

It should be noted that the IPM also contains a Spacrcan-like HA binding molecule, Sparc. Each is a separate gene product but both of the molecules are highly homologous in their molecular features (4, 5, 29, 45). They both contain a signal peptide at the N terminus, a central mucin-like domain, several N-linked glycosylation sites, two EGF-like domains, and several RHAMM-type HA binding motifs. In rodents, both are chondroitin sulfate proteoglycans. One of the main differences between them is that only Sparc contains a hydrophobic stretch of amino acid residues close to the C terminus. If functional, this could anchor Sparc to the photoreceptor plasma membrane, in contrast to SPACR, which is secreted into the IPM but not attached to the plasma membrane. Though they both have been shown to bind to HA (4, 5), functional tests on the involvement of HA binding motifs in Sparc have not yet been performed. Since all the HA binding motifs in Sparc tested in this study show involvement in mediating HA binding, it is possible that the HA binding motifs in Sparc also function in such binding. This could allow a higher order of complexity in HA interactions with matrix proteins in which the secreted SPACR would be involved in stabilizing the matrix inside the IPM, whereas Sparc would play a role attaching the matrix to the photoreceptor plasma membrane.

It is currently unknown whether the Sparc intracellular C terminus interacts with other proteins. In this study we show that the Sparc intracellular fragment prQC45 has the potential to bind to HA through the HA binding motif. HA has been shown to be present in the granule cell cytoplasm of rat cerebellum (46). Consistently HA binding epitopes are also present in the granule cell cytoplasm of rat cerebellum (47). Though its biological significance is still unknown, one possibility is that the presence of cytoplasmic HA, either through internalization (48) or through other pathways, might serve as a master regulator in HA-binding protein complex formations. Alternatively, an HA binding domain, D5, of RHAMM has been shown to bind to other proteins intracellularly to regulate cell motility and cell cycle by intracellular signaling (49). It would be interesting to know whether prQC45 also binds to intracellular proteins, which in turn may have additional functional implications.

In addition to its binding to HA, prQC45 also binds to chondroitin sulfates and heparin. This is similar to the yeast cell cycle control protein Cdc37 and its vertebrate homologue (50). It is unlikely that this Sparc intracellular region is involved in cell cycle control because this molecule is not expressed until photoreceptors become postmitotic (29, 51).

In conclusion, we report here our studies characterizing the binding of Sparc to IPM GAGs. We show that Sparc binds to HA, heparin, and chondroitin sulfates. We also demonstrate that binding of Sparc to HA and chondroitin sulfates is through RHAMM-type HA binding motifs and that the flanking basic amino acid residues of the HA binding motif are involved in mediating HA and chondroitin sulfate binding. This study has extended our knowledge of RHAMM-type HA binding motifs in mediating chondroitin sulfate binding which was not addressed previously. We also show that RHAMM-type HA binding motifs may play an important functional role in maintaining this unique photoreceptor extracellular matrix.

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