Variant Exons v6 and v7 Together Expand the Repertoire of Glycosaminoglycans Bound by CD44*

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Alternative splicing and/or post-translational modification generate multiple CD44 isoforms (reviewed in Ref. 1). CD44 isoforms have been implicated in a wide variety of adhesion-dependent cellular processes, such as T-cell signaling and activation, lymphocyte recirculation, cell-cell and cell-matrix interactions, and cell migration and metastasis (2–4). The ligand binding activities of CD44 which mediate these adhesive processes, and functional differences between the different isoforms are both poorly understood.

The majority of CD44 isoform diversity is generated by the incorporation of amino acid stretches encoded by 10 alternatively spliced exons into a membrane proximal position of the extracellular portion. Transcripts in which these variant exons are spliced out encode the most common and widely expressed 85-kDa isoform (CD44s). The expression of CD44 isoforms containing sequences encoded by the variant exons (CD44v), however, is tightly regulated. Constitutive expression of these isoforms is restricted to a limited selection of epithelia and leukocytes, while transient and regulated isoform expression is observed during several physiological processes (5, 6). The expression of CD44v isoforms is up-regulated in many tumors during tumor progression (7).

It is well documented that CD44 isoforms bind to the extracellular matrix glycosaminoglycan (GAG) hyaluronate (HA), and HA binding motifs in the extracellular portion of the protein have been identified (e.g. Refs. 3, 8, and 9). However, other ligand binding activities must exist. For example, CD44s-mediated lymphocyte binding to mucosal high endothelial venules is independent of HA binding (10). Moreover, an antibody specific for CD44 variant isoforms containing an exon v6-encoded epitope blocks tumor growth, lymphocyte activation, and limb bud outgrowth, but does not block CD44-mediated HA binding (5, 6, 9). Although no specific ligands have been identified for CD44v isoforms, a plethora of ligands have been ascribed to CD44s, including mucosal addressin (11), collagen type I (12), fibronectin (13), MIP-1β (14), the chondroitin-sulfated form of invariant chain (15), serglycin (16), and osteopontin (17). While certain of these interactions are mediated by binding of the ligand to sugar moieties on the CD44s protein, the nature of the interaction with the other ligands is unclear. At least for serglycin, binding by CD44s is dependent on the presence of chondroitin sulfate (CS) modifications on the serglycin protein (16). In this case, however, the CD44s protein could not bind directly to CS, a finding also reported by others (18). Work with purified CD44s proteins, on the other hand, suggests that CD44s has a weak affinity for CS (19–21), a conclusion supported by CD44 transfection experiments in a B cell lymphoma line (22).

The role of CD44 splice variants in certain aggressive tumors arising from cells which constitutively express only CD44s and their restricted expression compared with CD44s in normal tissues indicate that these isoforms possess molecular properties in addition to those exhibited by CD44s. The variant portion of CD44v proteins may either mediate binding to new ligands, or modulate the function of domains expressed on all CD44 proteins, such as the HA-binding domain. Here we show that CD44 variants containing sequences encoded by exons v6 and v7 bind directly and avidly to multiple GAGs, both as soluble proteins and when expressed on the cell surface. Binding requires the N-terminal HA binding motif, suggesting that...
variant exon expression extends the spectrum of GAG binding via this motif. These findings suggest that alternative splicing of CD44 serves to regulate and define the range of ligands to which the protein can bind.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Antibodies**—The hybridomas expressing the monoclonal antibodies 1.1A8ML (23) and 5G8 (9), and the pancreatic tumor cell BSp73AS clone 10AS-7 (24) were maintained in RPMI 1640 medium containing 10% FCS. The CD44v4-v7-transfected 10AS cell line ASpSV14 and ASpSV15 (25) and other 10AS cell lines transfected with CD44 constructs were maintained in RPMI 1640 medium containing 10% FCS, supplemented with 300 \( \mu \)g/ml G418. BDX2 cells (9) were maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS, supplemented with 300 \( \mu \)g/ml G418. Clones were picked and checked for CD44 protein expression using the 1.1A8ML and 5G8 antibodies.

**Cetylpyridinium Chloride (CPC) Precipitations**—Confluent 10-cm plates of cells were washed three times in PBS and harvested with 5 mM EDTA/PBS. The cells were lysed in 1 ml of 0.5% Triton X-100, PBS, 1 mM phenylmethylsulfonyl fluoride and incubated on ice for 15 min. The lysate was then centrifuged for 5 min to remove insoluble material. Aliquots of lysate (100 \( \mu \)l) were added to \( 1 \) mg/ml aqueous solutions of GAGs (Sigma, Deisenhofen; catalogue numbers: CSA, C-0914; CSB, C-4259; CSC, C-4384; HA, H-5388; H, H-3393; HS, H-7641; KS, K-3001), or 50 \( \mu \)g/ml G418. Clones were picked and checked for CD44 protein expression using the 1.1A8ML and 5G8 antibodies.

**SDS-PAGE and Western Blotting**—Proteins were resolved by size and Western blotted from 10AS and BDX2 cells to create stable lines—Constructs were cotransfected with pRSVneo (28) into 10AS and BDX2 cells using DOTAP (Boehringer Mannheim) according to the manufacturer’s instructions. Transfectants were selected with 700 \( \mu \)g/ml G418. Plasmids were picked and checked for CD44 protein expression using the 1.1A8ML and 5G8 antibodies.

**Construction of CD44v4-v7**—Construction of CD44v4-v7 expression plasmids (obtained from Dr. Martin Hofmann) which encodes rat CD44s was digested with PmlI and SphI, blunt-ended and self-ligated to create the pSVNadp plasmid (see Fig. 1). Oligonucleotide primer pairs were used to generate PCR fragments encoding exons v5 (sv5For and sv5Rev primers), v7 (sv7For and sv7Rev primers), and v6-v7 (sv6For and sv7Rev primers) using the pSVmeta2 plasmid (27) as a template. PCR was performed using 30 cycles of 94 °C, 1 min, 55 °C, 1 min, and 72 °C, 1 min. The sequence of the primers was as follows: 5′-CACCCCTggCCACCACTCtg-3′ (sv5For); 5′-gggTCCATgATgTCTCgATCgTTTgCTgCTTgACATgACATg-3′ (sv6Rev); 5′-CACCCCTggCCACCACTCtgCCACCAACCAACCA-3′ (sv7For); 5′-gggTCCATgATgTCTCgATCgTTTgCTgCTTgACATgACATg-3′ (sv7Rev). The PCR products were digested with NcoI and BalI and ligated into similarly digested pSVNadp plasmid. The resulting plasmids containing the PCR-derived sequences were cut with Accl and BglII and in each case the fragment containing the PCR-derived sequence was ligated into Accl- and BglII-digested pSVCD44N plasmid. Positive clones were selected by restriction analysis and sequencing. The PCR-derived sequences in these were verified by dideoxy sequencing.

**Construction of HA Mutant Expression Plasmids**—To create the R44L point mutation in the most N-terminal HA-binding domain of CD44, a DNA fragment encoding this domain was generated by PCR in which one of the oligonucleotide primers (3-HA) contained the desired base changes. The primers had the following sequence: 5′-CgACTCAC-TATgTAACgCTgCTTgACCAACCAgAACAC-ACAT-3′ (HA5-BC); 5′-gATgTCTTgTgTgTgTACgATACTgTgTACATgATCgCCg- CCTgCTgTgTgTACgATACTgTgTACATgATCgCCg-3′ (HA3-BC). When annealed together, these oligonucleotides have overlapping 5′ and 3′ cohesive ends with BalI and ClaI cleavage sites. The CD44v4-v7 encoding plasmid was digested with BanI to give 2610- and 2456-base pair fragments. The wild type HA-binding domain was cut out by digesting the 2456-base pair fragment with ClaI to give a 2407-base pair fragment. The 2407- and 2610-base pair fragments were then ligated together with the annealed oligonucleotides, with the result that the annealed oligonucleotides were incorporated into the BanI (position 580) and ClaI (position 620) sites of the pSVmeta1 vector. The sequence of the newly inserted PCR fragments or oligonucleotides in these clones was verified by dideoxy sequencing.

**Plasmid Transfection into 10AS and BDX2 Cells to Create Stable Lines**—Plasmids were cotransfected with pRSVneo (28) into 10AS and BDX2 cells using DOTAP (Boehringer Mannheim) according to the manufacturer’s instructions. Transfectants were selected with 700 \( \mu \)g/ml G418. Clones were picked and checked for CD44 protein expression using the 1.1A8ML and 5G8 antibodies.

**SDS-PAGE and Western Blotting**—Proteins were resolved by size (30) using a resolving gel containing 7% polyacrylamide. Proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (Schleicher and Schuell, Dassel) by the method of Towbin et al. (31). Filters were washed in TBS (25 mM Tris-HCl, pH 8.1, 150 mM NaCl) for 10 min, then used or stored frozen at −20 °C. Blots were probed with antibody using the ECL detection system (Amersham, Braunschweig) according to the manufacturer’s instructions.
RESULTS

CD44v4-v7 but Not CD44s Binds Directly to Multiple GAGs—To understand the differential function of CD44 isoforms in their many physiological roles, the range of their ligand binding activities needs to be defined. We have previously demonstrated that CD44v4-v7 protein confers strong HA binding properties on the BSp73 rat pancreatic carcinoma 10AS cell line, while CD44s confers only weak binding (9, 33). Given that GAGs are structurally related sugars, we set out to determine whether the enhanced HA binding properties exhibited by CD44v4-v7 on these cells is extended to CS or other GAGs. To investigate this directly, we first employed CPC precipitation (29). In this procedure, GAGs mixed with cell lysates are precipitated by CPC. Proteins which interact with GAGs coprecipitate, while non-interacting proteins remain in solution. In this way, GAG-binding proteins can be isolated and identified by Western blot.

Fig. 2 shows Western blot of CPC precipitates using lysates from rat BSp73 rat pancreatic carcinoma cells expressing only the CD44s isoform (10AS) or from CD44v4-v7 transfectants of 10AS (A5SpSV14; for a summary of the cell lines used in this paper and their properties, see Table 1). CD44s was detected using the 5G8 antibody which binds to an epitope encoded by exon 15 (9), and CD44v4-v7 was detected with the v6-specific antibody 1.1ASML to allow detection of the CD44v4-v7 protein, while the blot from 10AS cells was probed with 5G8 to allow detection of CD44s. The negative control precipitate (Cont) contained water instead of GAG. The positive control (Lysate) is total protein from the same cell lysate as used for the CPC precipitations.

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Variant Exons v6 and v7 Are Together Required for Multiple GAG Binding—To ascertain the minimum exon requirement for the extended GAG binding exhibited by CD44v4-v7, constructs encoding CD44v6-v7, CD44v6, and CD44v7 were separately transfected into 10AS cells and the ability of the transfected protein to bind to GAGs was tested in each case by CPC precipitation. CD44 exon 15 was included in these constructs, permitting the detection of both CD44s and CD44v proteins with the 5G8 antibody. Since exon 15 is included in naturally occurring CD44s but spliced out of the metastasis-associated CD44v4-v7 (25) used in the CPC experiments of the previous section, exon 15 was also incorporated into a CD44v4-v7 expression construct and transfected into 10AS cells. CD44v4-v7
proteins with and without exon 15-encoded sequences bound GAGs equally well in CPC precipitations (data not shown), demonstrating that the presence or absence of exon 15 does not influence GAG binding.

While CD44v6-v7 efficiently binds to the same range of GAGs bound by CD44v4-v7 in addition to HA, neither CD44v6 nor CD44v7 bound to these GAGs (Fig. 3). Like CD44s, CD44v6 bound to HA, but interestingly CD44v7 did not even bind to HA. This suggests that exon v7 alone may alter the conformation of the corresponding CD44v protein such that it cannot longer bind HA. These results demonstrate that exons v6 and v7 are together required to extend the range of GAGs bound by CD44.

**Purified CD44v4-v7 Protein Also Binds to Multiple GAGs**—To ensure that the binding of CD44v4-v7 to GAGs we observed with the CPC precipitations was not due to bridging molecule in the cell lysates which permitted the CD44 variants to bind indirectly to the GAGs, we purified CD44v4-v7 from ASpSV14 cells over a 1.1ASML antibody affinity column. This procedure resulted in the purification of the CD44v4-v7 protein to homogeneity (Fig. 4A). The purified CD44v4-v7 protein was incubated with GAGs which in turn were precipitated with CPC as before. Fig. 4B demonstrates that the purified CD44v4-v7 protein binds to exactly the same GAGs as when ASpSV14 cell lysates were used, showing that the interaction between CD44 variants and GAGs is direct.

**CD44 Variant Isoforms Containing Variant Exons v6 and v7 Also Confer Multiple GAG Binding Properties When Expressed on the Cell Surface**—To determine if the extended GAG binding properties of v6- and v7-containing CD44 isoforms that we observed using solubilized CD44 proteins has physiological relevance, we investigated the GAG binding activity of CD44 variants on the cell surface. We biotinylated CS-A, heparin, HS, and HA, and used flow cytometry to look at the ability of these GAGs to be bound by cells expressing different CD44 isoforms.

Fig. 5 shows the binding of CS-A by cells expressing CD44s alone, or in addition CD44v4-v7, CD44v6-v7, CD44v6, or CD44v7. As expected from the CPC precipitations, CS-A was bound efficiently by cell lines expressing CD44 isoforms containing exons v6 and v7 together. The binding was relatively heterogeneous, while CD44v6 staining was uniform (data not shown), suggesting that determinants in addition to CD44v6-v7 expression may also regulate GAG binding. Cell lines expressing CD44 isoforms containing exons v6 or v7 separately, or only CD44s did not bind CS-A. Furthermore, ASpSV15 cells which express around twice as much CD44v4-v7 protein on their surface as AspSV14 cells (9), also bound more CS-A, both in terms of the percentage of cells binding and the amount bound per cell, demonstrating a dose dependence of the amount of CS-A bound on the amount of CD44v4-v7 on the cell surface. The observed binding of CD44v4-v7 and CD44v6-v7 to CS-A was not due to molecules in FCS able to bridge the CD44 proteins to CS-A, as equivalent staining was observed whether FCS was used during the FACS staining or not (data not shown). This was also true for HA staining. Incubation of HA or CS-A stained cells with hyaluronidase or chondroitinase, respectively, completely destroyed the FACS signal, further demonstrating the specificity of the staining.

To further demonstrate that CS binding is specifically conferred on 10AS cells by v6-v7-containing CD44 isoforms, we performed CPC precipitations with radiolabeled ASpSV14 cell lysates to determine how many proteins in the lysate could bind to CS. In the absence of CS, CPC did not significantly precipitate any proteins (Fig. 6). However, in the presence of CS, a number of proteins weakly coprecipitated, but one protein which migrated with the same molecular mass as immunoprecipitated CD44v4-v7 was clearly the major CS-binding protein in these lysates (see arrow, Fig. 6). To prove that this protein was indeed CD44v4-v7, we preleared the radiolabeled lysates using isotype-matched anti-CD44 antibodies before performing the CPC precipitation. Preclearing with the 5G8 antibody, which does not bind the CD44v4-v7 from ASpSV14 cells as the exon 15-encoded sequence is absent, did not affect the quantity of the major protein which coprecipitates with CS. However, preclearing with the CD44v6-specific antibody 1.1ASML specifically removed this major CS-binding protein.
This experiment thus suggests that CD44v4-v7 protein is responsible for the CS binding observed in ASpSV14 cells. Non-transfected 10AS cells showed a high endogenous level of binding to biotinylated H and HS in flow cytometry, although transfection with CD44 isoforms containing both exons v6 and v7 further increased this binding (data not shown). None of the cell lines used in this study bound to KS (data not shown), as would be expected from the CPC precipitation experiments. Together, these experiments demonstrate that the expanded GAG-binding repertoire of CD44 isoforms containing sequences encoded by both exons v6 and v7 is active both in solution and on the cell surface.

CD44 Exon v6-v7-mediated CS Binding Activity Is Not Limited to 10AS Cells—To demonstrate the generality of the finding that CD44 isoforms containing sequences encoded by exons v6 and v7 further increased this binding (data not shown), none of the cell lines used in this study bound to KS (data not shown), as would be expected from the CPC precipitation experiments. Together, these experiments demonstrate that the expanded GAG-binding repertoire of CD44 isoforms containing sequences encoded by both exons v6 and v7 is active both in solution and on the cell surface.

Like 10AS, the fibrosarcoma cell line BDX2 expresses CD44v6-v7 endogenously (9). Constructs encoding CD44v6-v7, CD44v6, and CD44v7 were separately transfected into BDX2 cells to make stable cell lines and the ability of the expressed protein to bind to GAGs was tested in each case by CPC precipitation. CD44v6-v7 expressed in BDX2 cells efficiently bound to the same range of GAGs bound by CD44v6-v7 expressed in 10AS cells, whereas CD44s, CD44v6, and CD44v7 bound significantly only to HA (Fig. 7). Unlike the situation in 10AS cells, CD44v7 bound to HA, suggesting that v7-induced changes in HA binding may also be modified by cell-specific factors such as post-translational modification. Furthermore, CD44v6-v7 expressed in BDX2 cells also bound moderately to KS, although...
this is probably accounted for at least in part by the low level of precipitation of the protein in the absence of GAG. These data show that the GAG binding activity mediated by CD44 isoforms containing amino acid sequences encoded by both v6 and v7 is a property of cells of vastly different origins.

The N-terminal HA Binding Motif of CD44v4-v7 Mediates Cell Surface Binding of Both HA and CS—GAGs are structurally related molecules, which suggested to us that HA and CS may be binding to common domains on the CD44 protein, and prompted us to investigate the role of the CD44 HA binding motifs in CS binding by mutational analysis. Two HA binding motifs are found in the extracellular portion common to all CD44 molecules, and both can individually bind to HA as isolated peptides (8). Basic amino acids in each motif are the critical residues for this HA binding activity. Point mutation of only one of these basic residues in the most N-terminal of these motifs efficiently abrogated HA binding by a CD44-immunoglobulin fusion protein, while mutation of two of the basic residues in the membrane proximal motif only partially reduced HA binding (21). However, this latter mutation ablates HA binding activity in other fusion proteins (8). We made similar point mutations in CD44v4-v7, and investigated their effect on the ability of the mutated CD44v4-v7 protein to confer HA and CS binding properties on 10AS cells.

Stable 10AS transfectants were made with CD44v4-v7 constructs containing mutations in the HA binding motifs. The cell line AS-R44 is a stable 10AS transfectant expressing CD44v4-v7 with a single point mutation of one of the basic amino acids in the N-terminal HA binding motif (R44L). This cell line did not bind to HA-coated surfaces (Fig. 8A), despite high levels of expression of the mutated CD44v4-v7 protein in comparison with CD44v4-v7 expression on the control cell line ASpSV14, as demonstrated by 1.1ASML staining (Fig. 8B). In contrast, the stable 10AS transfectant AS-K162R166, which expresses CD44v4-v7 with mutations of two basic residues in the membrane proximal motif (K162A,R166A), bound to immobilized HA, although less well than ASpSV14 (Fig. 8A). The AS-R44 cell line also failed to bind to both soluble HA and AS-A, showing that mutation of only one of the basic amino acids in the N-terminal HA binding motif (R44L) efficiently abrogates the ability of CD44v4-v7 to confer both HA and CS binding properties (Fig. 8B). Surprisingly, the AS-K162R166 cell line showed slightly enhanced soluble HA and CS binding compared with ASpSV14 cells (data not shown); while it is unclear why binding to solid phase HA should be reduced while solution phase binding remains unaffected by the K162A,R166A mutation, this result nevertheless further shows that GAG binding by CD44v4-v7 occurs with a single functional N-terminal HA binding motif. Together, these data demonstrate that the N-terminal HA binding motif is required for both the HA and CS binding activity of CD44v4-v7 protein expressed on the cell surface, while the membrane proximal motif plays at most a subsidiary role in this activity.
Glycosaminoglycan Binding by CD44 Variants

DISCUSSION

The elucidation of ligands bound by CD44 is of paramount importance if the biological role of this molecule is to be understood. Furthermore, differences in ligand binding properties between CD44s and CD44v isoforms are likely to be pivotal for their differential function in physiological and pathological processes. The data in this paper reveal new GAG binding properties of CD44, which moreover are only present in variant isoforms containing both exons v6 and v7. Like HA binding, these GAG binding properties require the most N-terminal HA binding motif.

The focus of this paper is to document the existence and structural requirements of CD44 binding to a wide range of GAGs. These GAG binding properties potentially extend the ability of CD44 to bind to many proteinaceous ligands, for while HA exists as a free carbohydrate within the extracellular matrix, other GAGs are always found as carbohydrate modifications on proteins. Many biological signaling molecules, cell surface molecules, and components of the extracellular matrix have been reported to be modified by CS, H, or HS (reviewed in Ref. 34). The extended GAG binding properties of CD44 isoforms bearing both exon v6- and v7-encoded sequences may permit these isoforms to interact with such molecules. An attractive model would be one in which the variant portion of CD44 not only permits interaction with GAG modifications on these molecules, but also itself interacts with the protein portion of the molecule to confer avidity and specificity on the binding interaction. We have tested the ability of CD44v4-v7 to bind to the CS-modified forms of the cytokine macrophage colony-stimulating factor (35), but were unable to detect binding (data not shown). This would be compatible with the notion that two discrete binding activities on CD44 may be required, each of which is insufficient to allow a fruitful CD44-ligand interaction. Such a model could suggest a mechanism by which the anti-CD44v6 antibody 1.1ASML blocks lymphocyte activation (5), limb bud outgrowth (6), and metastatic tumor growth (9, 36) while not inhibiting HA binding (9), or other CD44-GAG interactions (data not shown). Additionally, the GAG binding properties of CD44v6- and v7-containing isoforms may also increase their affinity for the CS-modified ligands ascribed to CD44s, a possibility which we are exploring.

CD44 interactions with GAG-bearing proteins may have several functional consequences. First, they may promote the binding of the CD44 to other cell surface or extracellular matrix components, resulting in enhanced cell-cell or cell-matrix interactions. Second, the interaction may trigger signal transduction via the CD44 molecule. Signal transduction activity through CD44 expressed on T lymphocytes has been reported (37). Third, the CD44 may bind to biological signaling molecules and present them to their receptors, permitting signal transduction through these receptors by facilitating high affinity interactions. In this case, CD44 would act analogously to cell surface proteoglycans such as the syndecan family. These sequester cytokines and growth factors such as members of the fibroblast growth factor family, which bind to GAG modifications on the cell surface proteoglycan and are subsequently presented to their receptor (for a recent review, see Ref. 38). Indeed, it has already been shown that GAG-modified CD44 isoforms can present growth factors in this way (39). However, if a CD44 isoform were to act as a growth factor presentation molecule by means of its GAG binding properties, it would instead bind to GAG modifications on the growth factor and then present the factor to its receptor.

It has been suggested that the chondroitin sulfate-modified invariant chain functions as an accessory molecule during T cell responses through its interaction with CD44 (15). In this regard, it is interesting to note that CD44 variants are transiently up-regulated during lymphocyte activation, and that this up-regulation is necessary for an immune response to develop (5, 40). Clearly, one possibility would be that CD44 isoforms bearing exon v6- and v7-encoded peptides may confer on activated T cells the ability to interact more avidly with the chondroitin sulfate-modified invariant chain on cellular targets during the development of the immune response due to their enhanced GAG binding properties.

The mere expression of CD44 on the cell surface has been amply documented to be insufficient to confer constitutive HA binding properties on cells (3). There is mounting evidence to suggest that the binding to HA by CD44 is regulated at multiple levels, and to date these data point toward a crucial role for the structural conformation of the protein in this regulation. Thus, although the extracellular portion of CD44 possesses two functional HA binding motifs (8), we show here that the most N-terminal motif provides the vast majority of GAG binding activity by CD44 on the cell surface. This is in agreement with fusion protein studies (21), and suggests that the tertiary structure of CD44 masks the HA binding activity of the second, membrane proximal HA binding motif. Furthermore, we demonstrate that incorporation of the peptide sequence encoded by exon v7 into CD44 in some instances abrogates HA binding. This has also been observed for other exons (41), and further suggests that alterations in the tertiary structure of the protein regulate HA binding. In this regard, it is interesting to note that glycosylation can have a positive or negative effect on HA binding (33, 41–44). Here we demonstrate a novel twist in this structural story, namely that the incorporation of the amino acid sequences encoded by variant exons 6 and 7 into the CD44 protein permits the N-terminal HA binding motif to bind to several different types of GAG in addition to HA.

There is obviously specificity in the extension of CD44 GAG binding properties mediated by v6- and v7-mediated structural changes, as we detected no or only low KS binding by CD44 in these experiments. On the basis of the data presented here, it is not possible to determine whether the exon v6- and v7-encoded epitopes only indirectly alter the structure of the protein to expose latent GAG binding properties, or whether they actively collaborate with the HA binding motif to extend the types of GAG bound by CD44. The weak CS binding capacity of CD44s suggests that the former possibility is more likely. Very long exposures of the Western blots in Figs. 3 and 7 revealed a weak interaction of CD44s and CD44v6 with GAGs other than HA (data not shown). Clearly, the incorporation of variant exon-encoded sequences dramatically up-regulates the GAG binding capacity of CD44, but other structural parameters may also regulate this on a cell line-specific basis.

A number of CD44 isoforms have been described which are GAG-modified (38, 45). The ability of isoforms of CD44 containing sequences encoded by exons v6 and v7 to bind to CS, heparin, and HS raises the possibility that these isoforms may be able to bind to other, GAG-modified CD44 proteins. This cannot, however, be the explanation for the CD44 multimerization we have previously reported (33), as the isoforms involved are not GAG-modified (data not shown). Furthermore, it is possible that other variant exon combinations not analyzed in this present study may also be able to promote GAG binding by CD44. In this regard, it is interesting that Droll et al. (46) reported adhesive interactions between alternatively spliced CD44 isoforms.

In conclusion, our results show that changes in CD44 splicing alter the specificity of ligand binding by the CD44 protein. The implications of this are wide ranging as CD44 variant isoforms have been shown to play a functional role in many
aspects of immunology, embryology, and tumor biology. Further understanding of the ligand binding activities of CD44 will reveal how CD44 functions in these varied processes.

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