

Regulation of CD44 Binding to Hyaluronan by Glycosylation of Variably Spliced Exons

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Abstract. The hyaluronan (HA)-binding function (lectin function) of the leukocyte homing receptor, CD44, is tightly regulated. Herein we address possible mechanisms that regulate CD44 isoform-specific HA binding. Binding studies with melanoma transfectants expressing CD44H, CD44E, or with soluble immunoglobulin fusions of CD44H and CD44E (CD44H-Rg, CD44E-Rg) showed that although both CD44 isoforms can bind HA, CD44H binds HA more efficiently than CD44E. Using CD44-Rg fusion proteins we show that the variably spliced exons in CD44E, V8-V10, specifically reduce the lectin function of CD44, while replacement of V8-V10 by an ICAM-1 immunoglobulin domain restores binding to a level comparable to that of

CD44H. Conversely, CD44 bound HA very weakly when exons V8-V10 were replaced with a CD34 mucin domain, which is heavily modified by O-linked glycans. Production of CD44E-Rg or incubation of CD44E-expressing transfectants in the presence of an O-linked glycosylation inhibitor restored HA binding to CD44H-Rg and to cell surface CD44H levels, respectively. We conclude that differential splicing provides a regulatory mechanism for CD44 lectin function and that this effect is due in part to O-linked carbohydrate moieties which are added to the Ser/Thr rich regions encoded by the variably spliced CD44 exons. Alternative splicing resulting in changes in protein glycosylation provide a novel mechanism for the regulation of lectin activity.

CD44 represents a heterogeneous group of cell surface and secreted proteins generated by alternate splicing of a single gene (47). All CD44 isoforms contain at the amino terminus an HA-binding domain which is composed of two clusters of positively charged amino acids (44, 55). Protein heterogeneity arises predominantly from the variable splicing of exons encoding extracellular domains located between the invariant hyaluronan(HA)¹-binding domains and exons encoding the membrane proximal extracellular domain (13, 23, 33). Alternative splicing of exons encoding the cytoplasmic domain of CD44 has also been reported (15, 47). To date at least 18 different alternatively spliced CD44 isoforms have been reported. Cells can express more than one CD44 isoform at a time, however, in some cell types one CD44 isoform is preferentially expressed. Leukocytes predominantly express CD44H (50), an isoform whose extracellular domain contains no variably spliced extracellular domain exons, while epithe-

lial cells express many alternatively spliced isoforms (8, 17, 30, 51). The CD44 isoforms expressed by a given cell can also change in response to differentiation and/or activation (1, 28) suggesting that the contribution of CD44 to the function of a given cell is not always constant.

These observations suggest that these different CD44 molecules are endowed with distinct and possibly multiple functions. Indeed, in vitro evidence supporting this hypothesis has been reported. CD44 is the cell surface receptor for HA and there is evidence that the binding capacities of the various isoforms differ (22, 27, 44, 51). In addition, it has been shown that the CD44 isoforms whose extracellular domain contains variably spliced exon V3 can be modified with heparan sulfate and bind to a subset of heparin-binding growth factors (6, 22). Other CD44 functions have not been so clearly assigned to particular isoforms, nonetheless, CD44 is a widely distributed multifunctional glycoprotein. Among its many physiological functions, CD44 has been implicated in leukocyte homing (25), leukocyte activation (20, 43, 46), extracellular matrix binding (2, 9, 24, 54), and cell adhesion and migration (49, 53). Recent experiments have shown that abnormal expression of CD44 by tumor cells can enhance their ability to grow and/or metastasize in vivo suggesting that abnormal expres-

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1. *Abbreviation used in this paper:* HA, hyaluronan; MC, melanoma cells; RT-PCR, reverse transcriptase-polymerase chain reaction.

sion and/or regulation of CD44 function may play a role in some human cancers (5, 17, 51).

A number of different experimental results lead to the conclusion that CD44 functions as a lectin by binding HA. These include the demonstration that the binding of HA to cells expressing CD44 can be blocked by anti-CD44 mAbs (32, 35, 41), the finding that a soluble immunoglobulin fusion of CD44H can bind HA in a cell-free system (44), and the demonstration that a protein recognized by mAb K3 which binds to the hamster HA-receptor (52) is the hamster homologue of CD44H (2). These results were further supported by observations that anti-CD44 mAbs could precipitate [³H]HA-binding activity from murine and human cell lines, block the binding of labeled-HA to the receptor and inhibit the HA-dependent aggregation of CD44-expressing cells (9, 16).

In some cell lines the lectin function of CD44 is regulated (32, 40). Lesley et al. (32) showed that a number of cell lines which expressed high levels of CD44 bind HA only after activation with phorbol esters. This observation has been confirmed using additional cell lines (21, 31), peripheral blood lymphocytes (14), and transfected cell lines (35, 38), clearly establishing that in some cell types CD44 lectin function is tightly regulated. Additional studies indicate that high affinity binding of CD44 for soluble HA requires an intact cytoplasmic domain (35, 38), CD44 aggregation on the cell surface (34), protein synthesis (42), and cytoplasmic domain phosphorylation (45). Also, there is evidence that different CD44 isoforms have different HA-binding capacities suggesting that alternative splicing plays a role in the regulation of the lectin function of CD44 (22, 51).

Here we report on the results of experiments designed to examine the role of alternative splicing in the regulation of CD44 lectin function. Using CD44-negative cell lines which were transfected to express either CD44H or CD44E, an isoform with an extracellular domain containing variably spliced exons V8-V10, we found that cells expressing CD44H bound HA more effectively than cells expressing CD44E. This difference was also seen with soluble immunoglobulin fusion of these two CD44 isoforms. Using soluble forms of CD44 which contain peptide sequences encoded by one or more of the variably spliced exons found in CD44E or with a polypeptide fragment obtained from ICAM-1, we show that inclusion of the CD44 variably spliced exons specifically reduces the ability of CD44E to bind HA. We also present the results of experiments which suggest that the reduced HA-binding capacity of CD44E is due in part to the presence of O-linked carbohydrates which were added to the Ser/Thr rich regions found in the variably spliced exons of CD44E (38, 44, 51).

Materials and Methods

Cell Culture and Fusion Protein Expression

CD44 stable transfected human melanoma cell lines, melanoma cells (MC), have been previously described (53). MC44H, MC44E, and MC44E-trunc express wild-type CD44H, wild-type CD44E, and a CD44E deletion mutant which is missing exon E15 and E16 (the junction between V10 and E17 is NVNRSWLI), respectively. COS cells were purchased from Amer. Type Culture Collection (Rockville, MD), grown in DMEM/10% FBS, and used for transient expression of fusion proteins as previously

described (2). To generate O-linked deglycosylated CD44E-Rg, and CD44H-Rg, 1 mM phenyl- α -N-acetylgalactosaminide (phenyl- α -GalNAc) (Sigma Immunochemicals, St. Louis, MO) was added to DMEM during the protein production phase of the transfection, and to the melanoma transfectants for 16 h before testing HA binding.

FACS Analysis

The MC transfected cell lines were washed with PBS and added to staining media (RPMI/2% FBS/0.1% azide). The anti-CD44 mAb A3D8 and the IgG₁ isotype-matched control (Sigma Immunochemicals, St. Louis MO) were added at 10 μ g/ml, and the cells were incubated at 4°C for 30 min. Excess antibody was washed away with RPMI and an FITC-labeled secondary antibody (Tago, Carmarillo, CA) was added for 30 min at 4°C followed by washing the cells two more times. The cells were fixed in 2% formaldehyde/PBS and analyzed on a FACScan (Becton-Dickinson, Mountainview, CA).

Reverse Transcriptase-PCR

Total mRNA was obtained from MC, MC44H, MC44E, and MC44E-trunc by guanidine isothiocyanate/phenol extraction as previously described (6). A random primer method was used to prepare the cDNA for PCR. 10 μ g of total mRNA was incubated with 1 μ l of 0.1 M Hexamer (GIBCO BRL, Gaithersburg, MD) for 10 min at 65°C. Then 4 μ l of 5 \times first strand buffer (GIBCO BRL), 0.1 M DDT (GIBCO BRL), 2.5 M dNTP's (Boehringer Mannheim Corp., Indianapolis, IN), and 1 μ l superscript RT (GIBCO BRL) was added and incubated for 1 h at 37°C. 30 μ l of dH₂O was added, and then 3 μ l of the reaction volume was used for each PCR reaction. The PCR reactions were carried out in a total volume of 50 μ l with the following reagents added together: (a) 3 μ l of cDNA; (b) 3 μ l 1.25 mM dNTP's (Boehringer Mannheim Corp., Indianapolis, IN); (c) 2.5 μ l each oligonucleotide (10 mM); (d) 5 μ l 10 \times buffer, and 0.5 U Taq DNA polymerase (Boehringer Mannheim Corp.). The oligonucleotides used as PCR primers included: (a) CD44-E3-FP, GGGGTGTACATCCTCACATC; (b) CD44-E13-FP, GAAGGCTTGGGAAGAAGAT; (c) CD44-E17-RP CAAAGCCAAGGCCAAGAGG. The PCR reaction conditions were as follows: 94°C for 5 min; 35 cycles were carried out at 94°C for 30 s, 54°C at 1 min, 72°C at 1 min 45 s.

Construction of CD44-Rg Expression Vectors

The CD44Rg expression vector was generated by subcloning the extracellular domain coding region from the CD44V10 NarI/KasI cassette cloning vector (22) into pCDM8 Ig FC (6). CD44H Δ -Rg was made by digesting CD44V10Rg with BglII and SphI (Gibco BRL, Gaithersburg, MD), purifying the plasmid on 1% agarose, the ends of the DNA fragment were blunt ended by Klenow (Boehringer Mannheim Corp.) in the presence of 2 mM dNTPs (Boehringer Mannheim Corp.), and then the plasmid was ligated with T4 ligase (GIBCO BRL). PCR was used to clone in the different CD44 exons and domains from the ICAM-1 and CD34. The CD44 variable spliced exons were generated by PCR from CD44E-Rg and the oligonucleotide primers used were as follows: (a) CD44V9, V10-Rg insert was created with PCR primers CD44-E5-BglII, AGTGAAAGATCTAGCACTTCAGGAG, and CD44-E13-SphI, ACATGCATGCATGTGCTTGTATGTCAGAGTAGAAG; (b) CD44V9-Rg required oligonucleotide CD44-E13-SphI in conjunction with CD44-E13-BglII, GAAGATCTCAGAGTAAT-TCTCAGAGCTTCT. The PCR template used to clone the Ig-domain from ICAM-1 was ICAM-1-Rg (10) and the PCR primers were ICAM-1-950-FP, GAAGATCTTTTCCGGGGCCCAACGTG, and ICAM-1-1200-RP, ACATGCATGTCTCGGGGGCCATACAGGAC. CD44/CD34-Rg insert was generated from the template CD34-Rg (48) with oligonucleotides CD34-368-FP, GCCGCCAGATCTACTGCTACCCAGAGTTACCT, and CD34-764-RP, GCCGCCCATGCGTCACTTAGGATAGGAGAAGA.

Hyaluronic Acid-binding Assay

Transfectants were cultured in DME supplemented with 10% FBS and 1.5 mg/ml G418 (GIBCO BRL), and/or 2 mM phenyl- α -GalNAc for 16 h. Cells were then washed, detached with PBS/0.5 mM EDTA, washed and radiolabeled with 100 mCi ⁵¹Cr (New England Nuclear, Boston, MA) for 1 h at 37°C. After several washes in DME, 10⁴ radiolabeled cells were seeded per well onto 96-well plates previously coated with 5 mg/ml HA (Sigma), and allowed to attach for 30 min at 4°C. Nonadherent cells were removed by washing, adherent cells lysed with 1% SDS, and incorporated radioactivity determined in a β counter.

Binding of the purified fusion proteins to HA was assessed using an ELISA assay as previously described (44). The protein concentration was determined using the BioRad (Bradford) protein determination assay (Richmond, CA), and then each fusion protein was run on an SDS-PAGE gel and silver stained to confirm the protein concentrations. To demonstrate the specificity of the two assays, CD44-Rg interaction with HA were blocked by using an anti-CD44 specific mAb MEM-85 (DeDiCa, Carlsbad, CA) and/or Bric235 (provided by Frances Spring). The ELISAs were also done in the presence of isotype-matched control mAbs purchased from Sigma.

Glycosylation Determination

To verify that the O-linked glycosylation inhibitor, phenyl- α -GalNAc, was generating deglycosylated fusion proteins the CD44-Rg proteins (100 ng) were analyzed by SDS-PAGE, transferred onto Nitrocellulose, and then detected by using MAA (Maackia amurensis Agglutinin) from a DIG Glycan Differentiation Kit as recommended by the manufacturer (Boehringer Mannheim Corp.).

Results

Melanoma Transfectants Expressing CD44H Bind HA More Effectively Than the Same Cells Expressing CD44E

Previous reports suggested that CD44E, a CD44 isoform containing variably spliced exons V8-V10, had a reduced lectin function when compared with CD44H, the CD44 isoform containing no variably spliced exons. B cell lymphoma (Namalwa) (51) or T cell leukemia (Jurkat) (38) cell transfectants expressing one or the other CD44 isoforms were used to examine the ability of these two CD44 isoforms to bind HA. To examine if this difference in the HA-binding activity of membrane-bound CD44H and CD44E extended to other cell types we prepared melanoma cell transfectants expressing either CD44H or CD44E. This cell line was chosen since it does not express any CD44 isoforms (Fig. 1 A). In addition, we elected to use stable transfectants rather than transient transfectants since this would allow us to select CD44H and CD44E transfectants which express similar levels of CD44 and monitor the induction of endogenous CD44 transcripts, the expression of which has been shown to be stimulated in some cell types by the agents used to facilitate transient transfection (50). Anti-CD44 mAb binding assays were used to identify melanoma transfectants expressing approximately equal levels of CD44H and CD44E (Fig. 1, B and C). To ensure that the transfection procedure did not result in the expression of additional CD44 isoforms, we analyzed mRNA isolated from the melanoma transfectants and the parent cell line by reverse transcription-polymerase chain reaction (RT-PCR). As shown in Fig. 2 by RT-PCR with RNA isolated from stable transfectant melanoma cells encoding either CD44H, CD44E or melanoma cells transfected with vector only, express the expected CD44 transcript. The RT-PCR products run at the predicted size on the ethidium stained gel (Fig. 2 A), a random primed 32 P-CD44 probe did not detect any other mRNAs (Fig. 2 B), and a probe specific to exon V10 only detects the CD44E isoforms (Fig. 2 C).

Binding assays with the melanoma cells expressing CD44H and CD44E and the parent cell line showed that both transfectants were able to adhere to HA-coated plastic more effectively than the parent cell line, however,

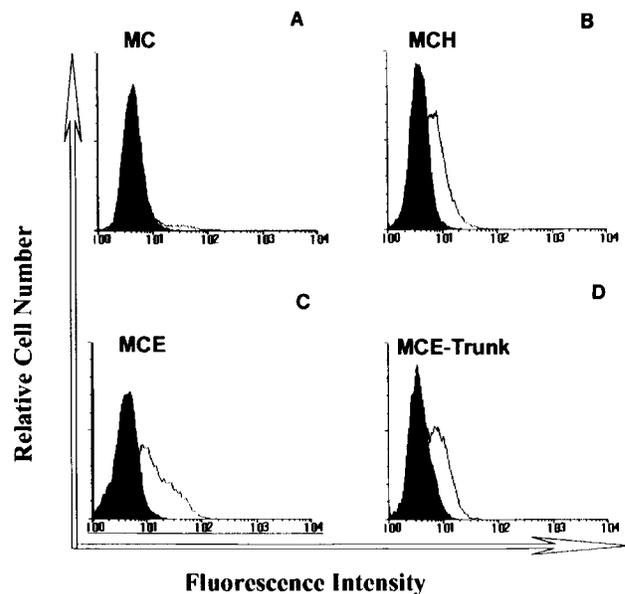


Figure 1. Line drawings representing flow cytometry profiles of stable transfected melanoma cells expressing different CD44 isoforms. (A–D) The cells were stained with either an isotype-matched control (shaded profile) or a mAb to the “constant” region of CD44. (A) MC is the parent cell line which does not express CD44. (B) MC44H, (C) MC44E, (D) and MC44E-Trunc, express CD44H, CD44E, and a CD44E deletion mutant missing exons E15 and E16, respectively.

the CD44H transfectants bound more efficiently than the CD44E transfectants (Fig. 3 A). The binding of both the CD44H and CD44E transfectants to the HA-coated plastic could be reduced to background levels by the anti-CD44 mAb indicating that the enhanced binding observed with the transfectants was due to the presence of CD44 (Fig. 3). These results are consistent with the data obtained with the Namalwa and Jurkat transfectants and shows that the different binding capacities of CD44H and CD44E can occur in both lymphoid and nonlymphoid cell types.

Receptor Shedding Does Not Account for the Difference in the HA-binding Capacity of Melanoma Cell Transfectants Expressing CD44H or CD44E

To examine the role that receptor shedding might play in the observed difference in HA binding between CD44H and CD44E transfected melanoma cells, we prepared melanoma transfectants expressing a truncated CD44E protein, CD44E-trunc. CD44E-trunc is lacking exons E15 and E16 which compose the membrane proximal extracellular domain. As reported by Bartolazzi et al. (4), CD44E-trunc, unlike CD44E and CD44H, is not shed from the transfected melanoma cells after HA binding. Melanoma transfectants expressing similar levels of CD44E-trunc to those of the CD44H and CD44E transfectants were selected for HA-binding studies (Fig. 1). RT-PCR analysis showed that the only CD44 transcripts expressed by these transfectants encoded CD44E-trunc (Fig. 2). HA-binding studies with melanoma cells expressing CD44-trunc showed that these cells adhere to plastic more efficiently than cells transfected with CD44E, however, they did not adhere to the HA-coated plastic as effectively as transfectants ex-

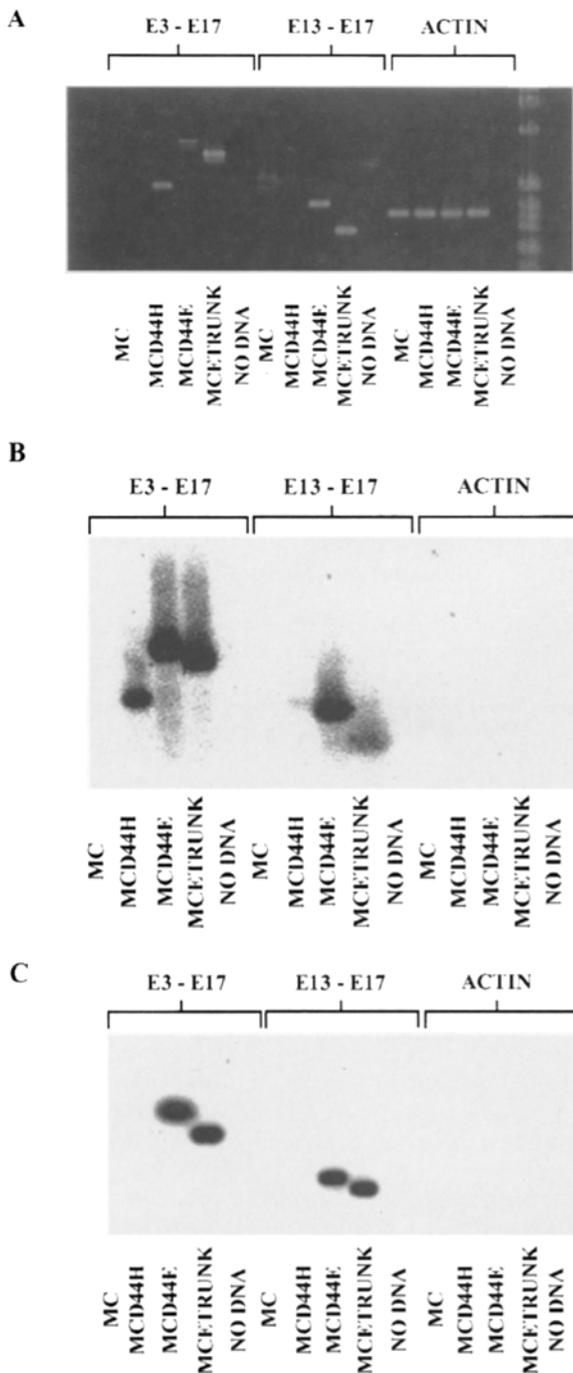


Figure 2. RT-PCR of the CD44-transfected melanoma cells. RT-PCR DNA products were generated with primers that span the alternatively spliced exons E3-E17, and with one oligonucleotide within the variably spliced exon, E13, expressed in CD44E, and with primers to Actin. The RT-PCR products were run on a 2% agarose gel and detected by (A) ethidium bromide, and then transferred onto a Hybond Nylon membrane and probed with a (B) 32 P-random primed probe to CD44E and (C) with a 32 P-end-labeled oligonucleotide to exon V10 which is expressed in CD44E but not CD44H.

pressing CD44H (Fig. 3). As with the other CD44 transfectants the adhesion of the CD44E-trunc transfectants to the HA-coated plastic could be prevented by pretreating the cells with a blocking anti-CD44 mAb (Fig. 3). These

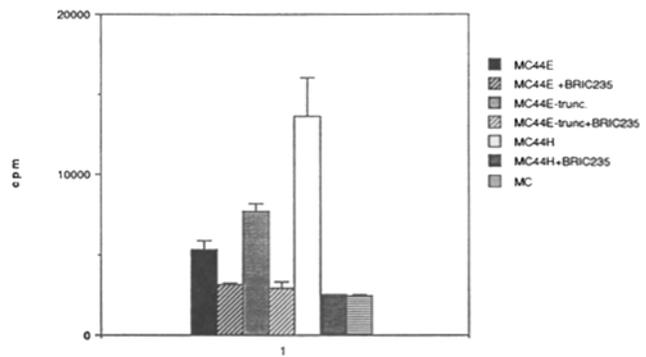


Figure 3. HA binding of melanoma cells transfected with CD44. A 51 Cr release assay was used to assess the binding to HA of stable MC transfected cells with CD44. MC, MC44H, MC44E, MC44E-trunc were radiolabeled with 51 Cr, and then seeded onto a plate with 5 mg/ml HA at 4°C for 30 min. Nonadherent cells were washed away, the remaining cells lysed, and the released 51 Cr was determined. Each data point represents triplicate wells and standard deviations are indicated. The binding of the cells to HA was blocked by preincubating the cells with an anti-CD44 mAb (Bric235) at 10 μ g/ml for 30 min at 4°C.

results suggest that the difference in the lectin capacity of the melanoma transfectants expressing CD44H or CD44E cannot be accounted for solely on the basis of CD44E shedding after ligand-mediated receptor cross-linking, but suggest instead a direct contribution by the alternatively spliced domain.

Reduced HA Binding by CD44E Is Specifically Mediated by the Inclusion of Variably Spliced Exons

We have previously reported the use of soluble immunoglobulin fusion proteins, containing the extracellular domain of CD44 (CD44-Rg), to study CD44-HA binding (44). CD44H-Rg, CD44E-Rg, and CD44-41R/A-Rg, a CD44H point mutant that lacks HA-binding activity (44) were used for this and the previous study (Fig. 4). For this study we used the modified chimeric gene encoding CD44V10-Rg which includes a pair of unique restriction sites between the sequences encoding the "common" CD44 exons and the human IgG (Fig. 4) (6, 22). These unique restriction sites allow for the convenient subcloning of one or more DNA fragments encoding variably spliced CD44 exons. Subcloning of the variably spliced exons (V9, V8V9) into the cassette results in either loss of amino acids from the common exon E5 and the loss of both common exons E15 and E16. To control for the loss of these sequences, a construct with both regions deleted was made and is referred to as CD44 Δ -Rg (Fig. 4). To examine the specificity and the role of O-linked carbohydrates of the CD44 alternatively spliced exons in decreasing the lectin activity of CD44, the fourth Ig domain of ICAM-1 and the mucin domain of CD34 were inserted into the cassette.

Comparison of the lectin activity of CD44H-Rg, CD44E-Rg, and CD44-41R/A-Rg (44), showed that CD44H-Rg bound HA in a concentration-dependent manner, whereas CD44E and CD44R/A-Rg have a greatly diminished HA-binding activity (Fig. 5 A). Antibody blocking studies showed that the binding of the CD44 Δ -Rg to HA-coated plastic could be specifically inhibited by a blocking anti-

CD44-Rgs

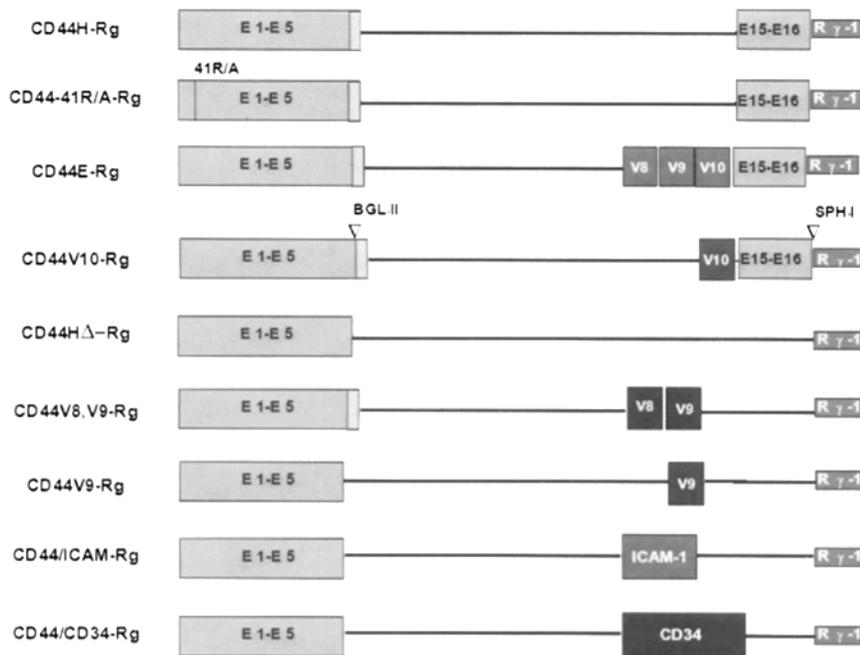


Figure 4. Line drawings of CD44-Rg constructs used to make soluble immunoglobulin fusion proteins. CD44H-Rg contains only the constant exons encoding the extracellular domain, and CD44E-Rg encodes for the constant exons plus three alternatively spliced exons V8, V9, and V10. CD44V10-Rg was engineered to contain a BglII and SphI site (6, 22) which were subsequently used to clone individual CD44 variable exons, ICAM-1, and CD34 domains.

CD44 mAb indicating that the lectin function of the CD44-Rgs is mediated by the CD44 moiety (Fig. 5 B). These experiments establish that HA-binding studies with CD44-Rgs can be used to study the effect of including variably spliced CD44 extracellular domains on CD44 lectin function.

The effect of including variably spliced exons V9 and V10, alone or the combination of V8 and V9, on CD44-HA binding was examined. Fig. 6 demonstrates that CD44HΔ-Rg has lectin-binding characteristics which are indistinguishable from those of the CD44H-Rg, and its binding to HA can be inhibited by a blocking anti-CD44 mAb (Fig. 5 B). The effect of including the variably spliced exons V9 or V10 individually on CD44 binding to HA was analyzed by testing the ability of the CD44V9-Rg and CD44V10-Rg fusion proteins to bind to plastic-immobilized HA. HA-binding studies with CD44V9-Rg and CD44V10-Rg showed that inclusion of variably spliced exons V9 or V10 significantly reduces the ability of CD44HΔ-Rg to bind HA, with exon V10 having a more pronounced effect than exon V9 (Fig. 6). Exon V10 has over twice the number of amino acids as exon V9, and since inclusion of either V9 or V10 did not reduce HA binding to the levels seen with CD44E-Rg suggesting that the effect of the variably spliced exons on CD44-HA binding is additive. To investigate this possibility we prepared a soluble CD44 fusion protein containing V8 and V9 (CD44V8,V9-Rg) and examined its lectin function. As shown in Fig. 6, CD44V8,V9-Rg binding to HA was intermediate to that seen with CD44HΔ-Rg or CD44E-Rg but lower than that seen with CD44V9-Rg indicating that the downregulation of CD44-HA-binding interaction by the inclusion of the variably spliced exons is additive.

To determine if the ability of the variably spliced CD44 extracellular domain exons to downregulate CD44 lectin function is specific or simply a spacing effect, we prepared a soluble CD44 fusion protein in which the cDNA frag-

ment encoding the variably spliced CD44 exons present in CD44E-Rg were replaced with a cDNA fragment encoding the fourth Ig-like domain of human ICAM-1 (Fig. 4). The fusion protein encoded by this chimeric gene, CD44/ICAM-1-Rg, bound to HA (Fig. 7 A) and its binding to HA could be blocked with an anti-CD44 mAb (Fig. 7 B). As an additional control we analyzed the ability of ICAM-1-Rg, a fusion between the extracellular domain of ICAM-1 and human immunoglobulin to bind HA (Fig. 7 A). Recently ICAM-1 has been purified from whole rat liver on an HA affinity column (39) but the extracellular domain has not been analyzed for HA binding. We found that ICAM-1-Rg did not bind plastic-immobilized HA in this assay, and previously we have shown that ICAM-1-Rg can provide costimulatory signals to CD4⁺ cells through interactions with LFA-1 (10). These results suggest that the down modulation of HA binding observed with CD44E is specifically dictated by the variably spliced CD44 exons and is not due to a spacing effect.

O-linked Carbohydrate Chains on the Variably Spliced Exons of CD44E Inhibit the Lectin Function

The most striking feature shared by the amino acid sequence of the variably spliced exons found in CD44E is the presence of multiple Ser and Thr residues which are potential sites of O-linked glycosylation, raising the possibility that O-linked carbohydrates may interfere with CD44-HA binding. Initial attempts to address the role of O-linked carbohydrate modifications on CD44-HA binding relied on the use of enzyme digestion to remove these moieties. Although these experiments seemed to indicate that O-linked carbohydrates play a role in regulating CD44-HA interactions, the results were variable (data not shown). We attributed this variability to incomplete digestion and designed alternative methods to investigate the

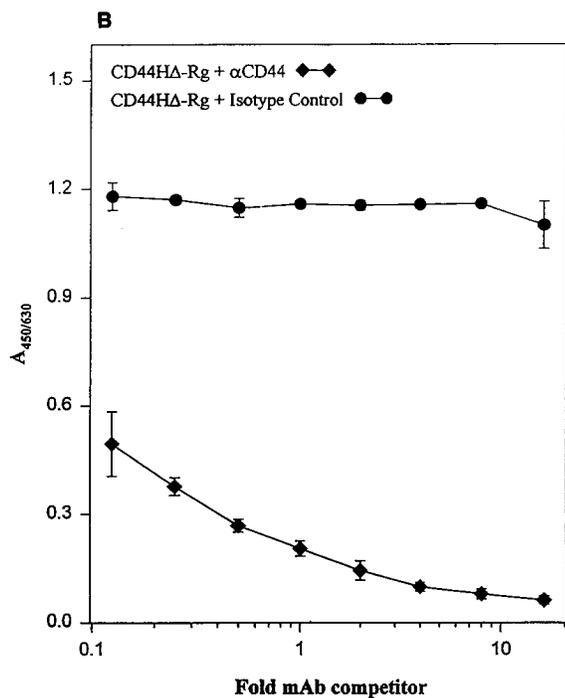
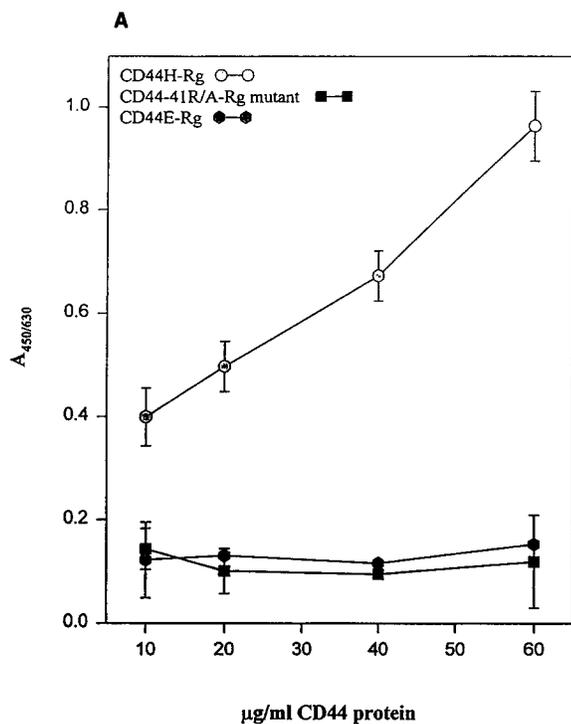


Figure 5. Binding of CD44 fusion proteins to immobilized HA was assessed by ELISA. (A) Increasing amounts of CD44 fusion proteins were incubated on ELISA plates with 5 µg/ml immobilized HA. Each data point represents the average and standard deviation of triplicate wells. (B) Specific blocking of 2 µg/ml of CD44HA-Rg to 5 µg/ml immobilized HA in the presence of increasing amounts of an anti-CD44 mAb but not an isotype-matched control IgG₁.

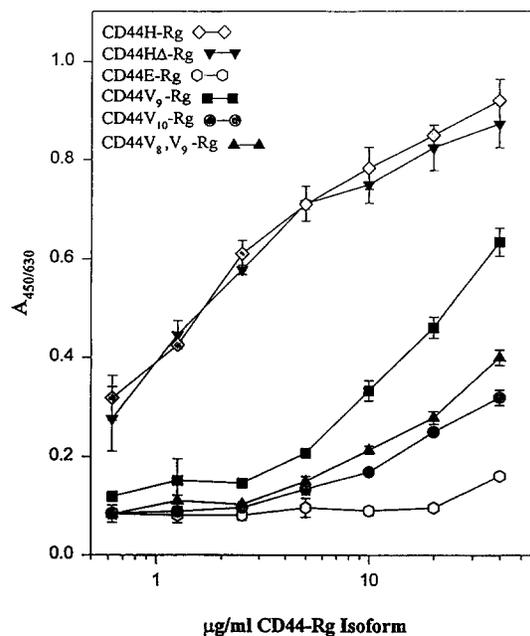


Figure 6. HA binding of CD44 fusion proteins with variably spliced exons. CD44H and CD44HA-Rg have very similar HA-binding profiles. CD44E-Rg has a very low binding capacity to HA at the concentration tested. The CD44 fusion proteins with the variable spliced exons V9, V10, and V8 plus V9 have an intermediate HA-binding capacity.

role of O-linked carbohydrate side chains on CD44-HA interaction. Two approaches were used, first, we prepared CD44E-Rg in the presence of a specific inhibitor of O-linked glycosylation and examined its ability to bind HA. Second, we replaced the variably spliced exons in CD44E with a Ser and Thr rich sequence from the mucin CD34, which is decorated with O-linked carbohydrate moieties, and examined the ability of this novel fusion protein (CD44/CD34-Rg) to bind to HA.

CD44E-Rg and CD44H-Rg were produced in COS cells in the presence of phenyl- α -GalNAc, a compound which has been shown to block the addition of O-linked carbohydrates to mucins without affecting cell growth, protein synthesis, N-linked glycosylation, or GAG synthesis ([29] and Wang, W.-C., personal communication). CD44H-Rg, CD44H-Rg O-glyc (-), CD44E-Rg, and CD44E-Rg O-glyc (-) made in the presence of phenyl- α -GalNAc were analyzed by running 2 µg of the proteins on SDS-PAGE. CD44H-Rg runs as a slightly lower molecular weight protein when made in the presence of phenyl- α -galNAc demonstrating the loss of O-linked carbohydrates (Fig. 8 A). CD44E-Rg is extensively modified by N-linked carbohydrates, O-linked carbohydrates, and glycosaminoglycans (8), and consequently runs as a diffuse band on SDS-PAGE when made in the presence or absence of phenyl- α -galNAc resulting in no strong detectable shift in molecular weight (Fig. 8 A). To ensure a reduction in O-linked glycosylation occurred during the production of CD44E-Rg, a lectin-blot was carried out. 100 ng of the proteins were separated on SDS-PAGE, transferred to nitrocellulose, and then blotted with MAA, a lectin that detects (2-3)-linked sialic acids in O-glycans (data not shown). The lectin-blot demonstrated that in the presence of the inhibitor that CD44E-

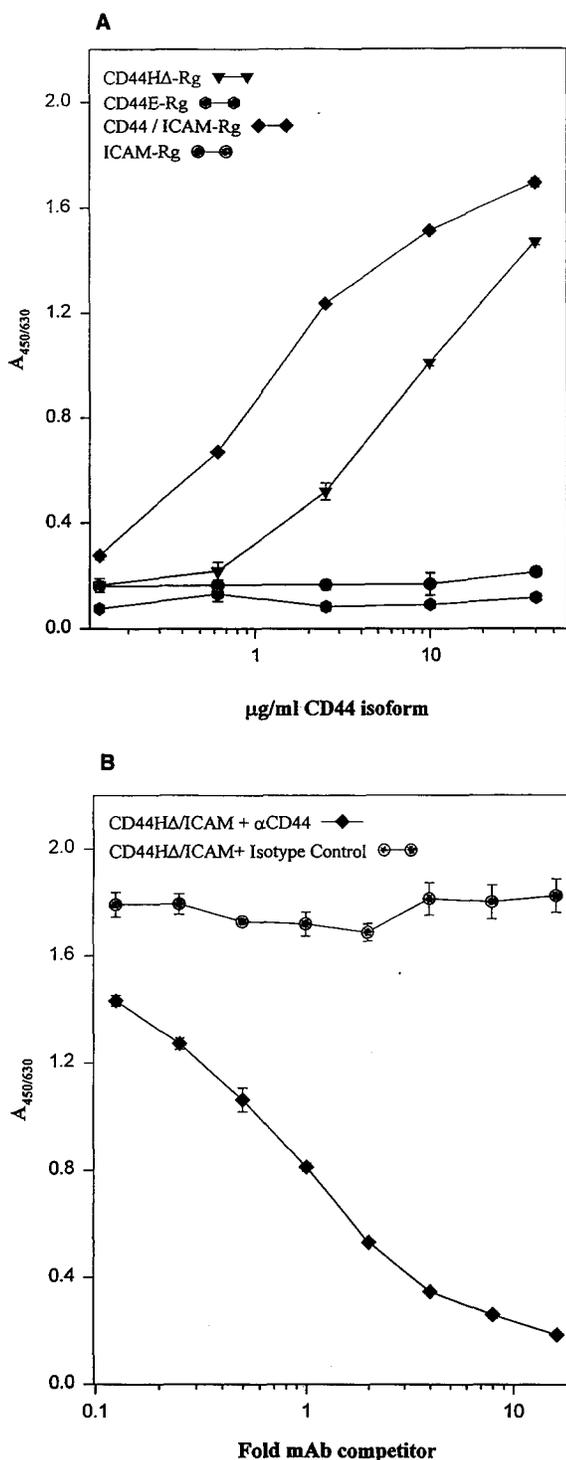


Figure 7. The HA-binding capacity of CD44 is not reduced by exon spacing. (A) The Ig domain from ICAM-1 (CD44/ICAM-1-Rg) did not reduce the HA-binding capacity of CD44. ICAM-1-Rg fusion protein does not bind to immobilized HA. (B) The binding of the CD44/ICAM-1-Rg chimeric protein (2 µg/ml) to HA was blocked in a concentration-dependent manner by an α-CD44 mAb but not by an isotype-matched control.

Rg O-glyc (-) contains substantially less O-linked carbohydrates. Binding studies with CD44E-Rg produced in the presence of phenyl-α-GalNac showed that this protein bound to plastic-immobilized HA as well as CD44H-Rg

(Fig. 8 B), while the presence of O-linked glycosylation did not influence the binding of CD44H-Rg to HA (Fig. 8 C). The ability of the deglycosylated CD44E-Rg to bind to HA was inhibited by a blocking anti-CD44 mAb showing that removal of the O-linked sugars unmasked the HA-binding site in CD44E (Fig. 8 D). These results suggest that O-linked carbohydrates play an important role in modulating the ability of CD44E to function as a lectin.

To independently examine the role of O-linked carbohydrates in the regulation of CD44-HA binding we prepared CD44/CD34-Rg (Fig. 4). The chimeric gene encoding this protein was prepared by replacing the DNA sequence encoding the variably spliced exons in CD44E-Rg with a DNA fragment encoding the mucin domain of CD34. This region of the protein contains 44% Ser/Thr residues and is known to be decorated with a large number of O-linked carbohydrate moieties. As shown in Fig. 9, inclusion of the CD34 mucin domain substantially reduced the ability of CD44H to bind to HA with the CD44/CD34-Rg fusion protein showing HA-binding properties which are comparable to those seen with CD44E-Rg. These results provide independent evidence that the O-linked carbohydrate moieties, which are added to the variably spliced exons found in CD44E, play a role in regulating the ability of this protein to function as a lectin.

To test the role of the O-linked glycosylation in vivo, the stable melanoma-transfected cell lines were grown in 2 mM phenyl-α-GalNac, and then assayed for HA binding (Fig. 10). A dramatic increase in HA binding of the melanoma transfectants expressing CD44E occurred while a slight increase in HA binding was observed with the CD44H transfectants. These data further support that alternatively splicing of CD44 provides a mechanism of regulating the lectin function by the addition of mucin-like domains.

Discussion

Using melanoma cell transfectants expressing either CD44H or CD44E, we show that although both forms of CD44 are capable of binding HA, CD44H-Rg binds HA significantly better than CD44E-Rg. This difference was also seen when the HA-binding activity of CD44H and CD44E, soluble immunoglobulin fusion proteins, was examined in a cell-free system using HA immobilized on plastic where CD44H binds HA at concentrations well below those required to detect CD44E interacting with HA. Experiments with CD44V9-Rg, CD44V10-Rg, CD44V8, V9-Rg, and CD44/ICAM-1-Rg showed that including the variably spliced exons found in CD44E, but not an ICAM-1 derived Ig domain, specifically inhibited CD44 lectin function. In addition, the effect of each exon was additive, suggesting that the level of HA binding retained by a given CD44 isoform can be finely regulated by varying the number of alternatively spliced exons present in that CD44 isoform.

Inspection of the amino acid sequence of variably spliced CD44 domains found in CD44E showed that they are rich in Ser and Thr residues and thus likely to be highly modified with O-linked carbohydrates in some cell types (Ser/Thr content in exons V8-V10 varies from 43% for exon V9 to 30% for exon V10). This led us to speculate that carbohydrate modifications of the variably spliced CD44 exons may play a role in regulating the HA-binding activity of

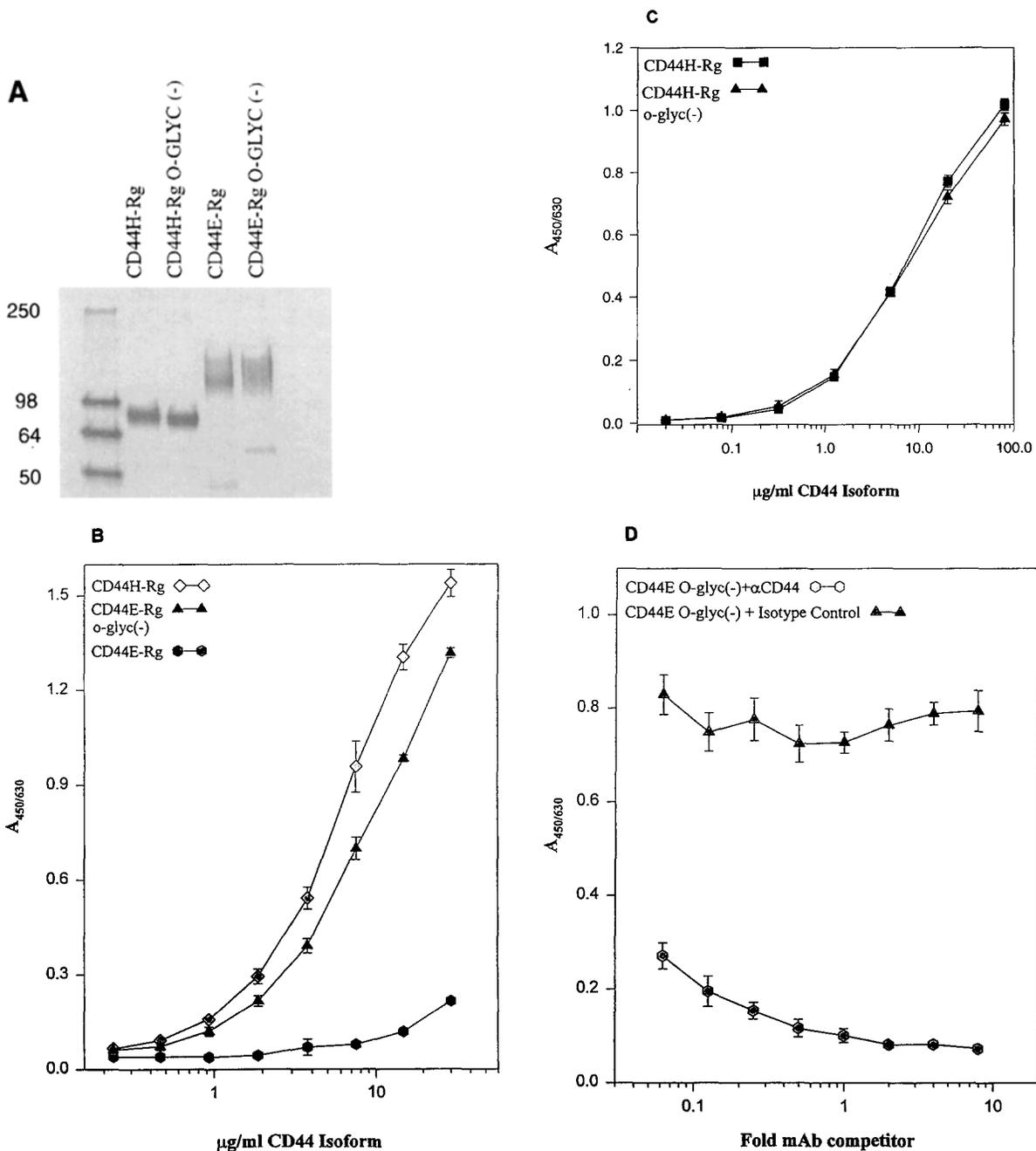


Figure 8. Production of CD44E-Rg in the presence of an O-linked glycosylation inhibitor generated a fusion protein with high capacity binding to HA. (A) A comparison of the molecular weights by SDS-PAGE of CD44H-Rg and CD44E-Rg made in the presence of (O-Glyc (-)) and absence of phenyl- α -galNac. (B) Increasing concentrations of CD44E-Rg produced in the presence of phenyl- α -GalNac bound to HA. (C) Increasing concentrations of CD44H-Rg produced in the presence and absence of phenyl- α -GalNac bound HA equivalently. (D) The binding to HA by CD44E-Rg (2 $\mu\text{g/ml}$) was blocked by an α -CD44 mAb but not by an isotype-matched control.

CD44. Indeed, such mechanisms have been observed to regulate the interaction between ICAM-1 and Mac-1 (11) and was proposed by Hodes and his colleagues to play a role in regulating CD44-HA binding (18). We investigated this possibility by digesting CD44E-Rg with a number of glycosidases and examining its ability to bind HA. These experiments were variable but suggested a role for O-linked glycosylation in the modulation of the HA-binding activity of CD44E. We attributed these variable results to the inability of the enzymes to completely remove the carbohy-

drates from the native protein. We circumvented this problem by producing CD44E-Rg in the presence of an inhibitor of O-linked carbohydrate addition. Binding studies with CD44E-Rg lacking O-linked carbohydrates showed that it was able to bind HA as efficiently as CD44H-Rg. This was in contrast to CD44H-Rg, where the O-linked carbohydrates had no effect on CD44-Rg/HA interaction. Furthermore, antibody blocking experiments demonstrated that the binding of the deglycosylated protein was specifically mediated by the CD44 HA-binding site. If O-linked

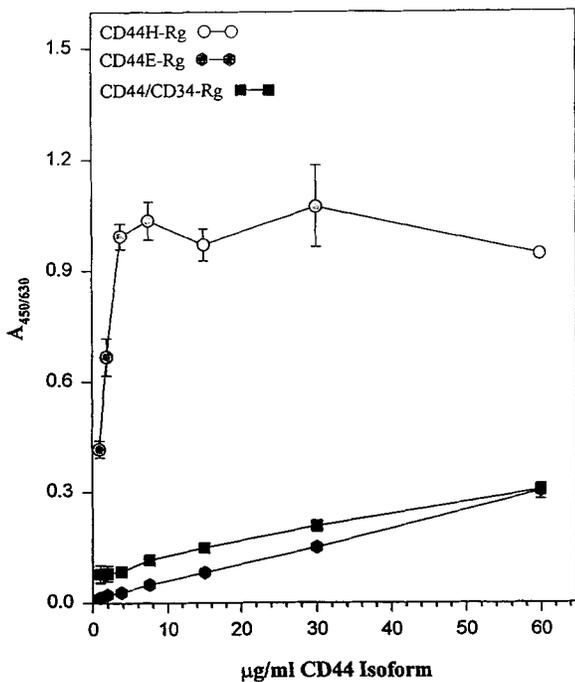


Figure 9. A mucin domain from CD34 reduced the HA-binding capacity of CD44. A CD44/CD34-Rg chimeric protein bound only weakly to immobilized HA.

carbohydrates play such an important role in modulating the lectin activity of CD44, we reasoned that it should be possible to mimic the effects of the variably spliced exons found in CD44E by including carboxyl-terminal to the HA-binding domain of CD44, a polypeptide fragment from a mucin which is heavily substituted with O-linked carbohydrates. For this reason we prepared CD44/CD34-Rg, a CD44 immunoglobulin fusion protein in which sequences corresponding to the variably spliced exons found in CD44E were replaced by a Ser/Thr rich polypeptide fragment derived from CD34 (Ser/Thr content in this region is 44%). Binding studies with CD44/CD34-Rg showed that the inclusion of this mucin fragment significantly inhibited the ability of CD44H to bind HA. Furthermore, the transfected melanoma cells expressing CD44E and CD44H bound equivalently HA when grown in the presence of 2 mM phenyl- α -GalNAc in a CD44-dependent manner. Taken together these results provide significant evidence that O-linked carbohydrate moieties added to the variably spliced exons of CD44 play a role in modulating the lectin activity of CD44. While O-linked glycosylation introduced by the variable spliced exons of CD44 modulate HA binding, other factors may also be involved in regulating CD44 isoform function. For example, two recent papers have shown that N-linked glycosylation reduces the ability of CD44 to bind HA (26, 31).

Although this study focused on the regulation of CD44-HA binding mediated by variably spliced exons V8-V10, inspection of the amino acid sequence of other extracellular domain variably spliced exons (V2-V7) showed that they are also rich in Ser and Thr residues (Ser/Thr content varies from 18% for exon V5 to 32% for exon V2) suggesting that the glycosylation of these exons might also play a role in modulating the lectin activity of the CD44 common

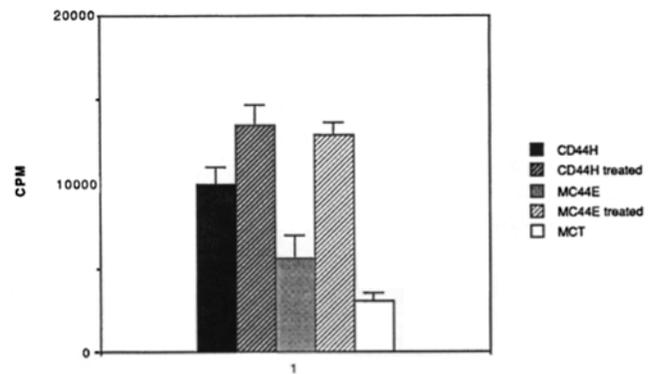


Figure 10. HA binding of melanoma transfectants grown in the presence of phenyl- α -GalNAc. A ^{51}Cr release assay was used to assess the binding to HA of the MC-transfected cells. Binding of MC44E cells to HA increased to a level equivalent to that of the MC44H cells when grown in the presence of an O-linked glycosylation inhibitor. All experiments were performed in sextuplicate.

exons in CD44 isoforms which contain them. The function of two other lectins have been reported to be regulated by glycosylation. Removal of sialic acid from the asialoglycoprotein receptor results in the exposure of galactose moieties which bind to the receptor blocking the binding activity of receptor preparations (3). Similarly, modification of CD22 with a 2,6-sialic acids blocks the ability of soluble receptor preparations to bind to lymphocytes, presumably due to CD22-CD22 interactions involving sialic acid (7). These results indicate that receptor glycosylation might be a common mechanism for regulating the function of a subset of lectins. The regulation of CD44-HA binding by glycosylation differs from that reported for the asialoglycoprotein receptor and CD22 in two important ways. First, in CD44 there are two levels at which glycosylation can be regulated; one is at the level of alternative splicing, the other is at the level of expression of the glycosyltransferases. Second, alternative splicing has the potential to give rise to CD44 isoforms with graded HA-binding capacities. These two mechanisms allow for the tight regulation of the HA-binding activity of CD44 isoforms containing variably spliced exons by glycosylation and suggest that a cell can rapidly regulate CD44/H A interaction by post-translational modification and/or alternative splicing.

The molecular mechanism whereby carbohydrate modification of the alternatively spliced CD44 exons modulates the binding activity of this protein remains to be determined. It is possible that glycosylation might alter the conformation of the molecule affecting its ability to function as a lectin. Alternatively, the negative charges on the O-linked carbohydrate chains might perturb and/or directly interfere with the two clusters of positively charged residues which form the HA-binding site located at the NH_2 terminus of all CD44 isoforms (55).

Experiments designed to address the role of alternative splicing in the regulation of CD44-HA binding have been carried out and yielded mixed results. CD44-negative B cell lines transfected with human CD44H but not CD44E or CD44 isoforms with additional alternatively spliced exons, were able to bind to hyaluronan-bearing cells (51). Likewise, CD44-negative T cells transfected with CD44H and CD44E showed different HA-binding activities after

activation (22, 38). CD44H binding to HA was inducible by PMA and a number of anti-CD44 antibodies, whereas, CD44E inducible HA binding was much more restricted. In addition, the CD44E transfectants induced HA binding at lower levels than that observed with the CD44H transfectants. Taken together these two reports suggest that although these two CD44 isoforms can bind HA, CD44H binds HA more effectively than CD44E. Furthermore, this work supports the notion that there are multiple mechanisms functioning to regulate HA binding by CD44. First, there appears to be an on/off switch that can regulate both isoforms, and second, the addition of alternatively spliced exons along with regulation of the level of glycosylation can result in the ability of fine tuning CD44/HA-binding interactions. These observations contrast with the results by Dougherty et al. (12) and in experiments showing that CD44-negative T cells transfected with murine CD44H or CD44E bound HA comparably (19). The results presented herein suggest that the reported differences in the ability of a given CD44 isoform to bind to HA when expressed in different cell types may arise from differences in the ability of the cells to appropriately and/or sufficiently modify CD44 isoforms containing variably spliced exons with O-linked carbohydrates.

The ability of CD44H to bind HA in a number of cell types is regulated. Cellular activation (14, 37), protein synthesis (42), and cytoplasmic domain phosphorylation (45) have been implicated in the regulation of the interaction of CD44H with HA. The finding that the interaction of CD44E with HA is regulated by the addition of O-linked carbohydrate moieties to the variably spliced exons adds an additional mechanism to the regulation of CD44-HA binding and suggests that in vivo the lectin activity of all CD44 isoforms is tightly regulated. Presently, it is not clear why it is physiologically important to regulate the HA-binding activity of this receptor, however, its widespread distribution and unique role in cell activation, adhesion, and migration suggests that its function as an HA-binding lectin requires precise regulation in vivo.

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