Site-specific De-N-glycosylation of CD44 Can Activate Hyaluronan Binding, and CD44 Activation States Show Distinct Threshold Densities for Hyaluronan Binding

Nicole M. English, Jay E. Lesley, and Robert Hyman
Cancer Biology Laboratory, The Salk Institute, San Diego, California 92186-5800

ABSTRACT

CD44 is a cell surface receptor for the glycosaminoglycan hyaluronan (HA). Not all CD44-positive cells bind HA, and binding ability is strictly regulated. Three different HA binding states have been defined: inactive, inducible (by certain CD44-specific monoclonal antibodies), and constitutively active. The observation that sets of genetically related cell lines representing different HA binding states showed correlated differences in N-glycosylation of CD44, and that inhibition of N-glycosylation enhanced HA binding (Lesley et al., J. Exp. Med., 182: 431-437, 1995) led us to examine directly whether specific N-glycosylation site modifications were involved in regulating the HA binding function. CD44-negative, -active, and inducible cell lines were stably transfected with mutant constructs in which each of the five N-glycosylation sites of murine CD44 had been separately inactivated. Ability to bind soluble HA was examined over a range of CD44 expression levels. For the active cell line, AKR1, transfectants for all N-glycosylation mutants bound HA as well as did transfectants for wild type CD44. No inhibitory effects of inactivating specific N-glycosylation sites were observed. HA binding was activated when two of the mutant constructs were transfected into a novel CD44-negative inducible cell line. Inactivation of N-glycosylation sites at residues 25 or 120 converted the inducible cell line to constitutively active, whereas inactivation of other sites had little or no effect.

Fusion proteins secreted from inactive, inducible, or active cell lines were purified, bound to beads, and assayed for HA binding activity by flow cytometric analysis. Fusion proteins derived from inactive, inducible, and constitutively active cells exhibited three distinguishable “threshold” densities required for HA binding ability. The results imply that the CD44 molecules produced in cells in these three activation states have intrinsic differences in HA binding function. Treatment of the fusion proteins with neuraminidase altered the HA binding state, and glycosylation mutations that affected the phenotype of the inducible cell line lowered the threshold required for HA binding of CD44-immunoglobulin fusion proteins derived from the inducible cell line. Thus, alterations of glycosylation of CD44 itself can affect HA binding ability as manifested by a change in HA binding state.

INTRODUCTION

Several studies have shown a direct correlation between aberrant glycosylation in tumor cells and their invasive and metastatic potential (1). Glycosylation may influence cell-cell interactions as well as cell-matrix interactions. This, in turn, can affect cell motility. Metastasis is a multistep process that includes de-adhesion, migration, adhesion, and invasion. CD44 and its specific interaction with HA (4, 5) has been implicated in many of these processes (2, 3, 5). Three activation states for CD44/HA binding ability have been defined: inactive, inducible (by particular CD44-specific mAbs), and constitutively active (2). Representatives of each of these activation states can be seen in both normal cells and in cell lines. Some cell types can only bind HA after activation by phorbol esters (6, 7), cytokines (8-10), or immobilized CD3-specific antibody (11). Also, HA binding can be affected by inhibitors of glycosylation (10, 12-16).

The standard form of murine CD44, CD44H, has a predicted polypeptide of 37 kDa but the observed molecular mass is ~80-90 kDa due to extensive posttranslational modification. In murine CD44H there are 5 potential sites for N-linked glycosylation and 10 potential sites for O-linked glycosylation (3). Three serine-glycine motifs in the membrane proximal domain of CD44 can be modified by the glycosaminoglycans heparan sulfate or chondroitin sulfate. These modifications may alter ligand specificity (3, 4), CD44 is acidic largely due to sialic acid (3). Differential use of 10 variable region exons results in CD44 isoforms, which have additional sites of N- and O-glycosylation as well as for glycosaminoglycan attachment, adding to the potential diversity of the molecule. CD44 has been found to be differentially glycosylated depending on cell type and cell activation state (9, 10, 12, 13, 17); this differential glycosylation is one possible mechanism for modulation of function.

Our laboratory has described variants isolated from a single cell line and exhibiting different HA binding activation states (12). These differences in HA binding correlated with differences in N-glycosylation. The inactive parent was more heavily N-glycosylated than the inducible variant, which was more heavily N-glycosylated than the active variant. Furthermore, cells that were inactive or inducible were converted to constitutively active HA binding cells upon treatment with tunicamycin, an inhibitor of N-glycan addition (12). Other investigators have also reported effects on CD44/HA binding function upon disruption of glycosylation (reviewed in Ref. 4 and 16). Here, we use site-directed mutagenesis to inactivate individual N-glycosylation sites of CD44, and analyze the effects of these mutations on constitutive and inducible HA binding. Also, we present data indicating that sialic acid may be a major regulator of the HA binding function, supporting observations by the laboratories of Kincaid (10, 13) and Stamenkovic (16).

MATERIALS AND METHODS

Cell Lines and Antibodies. AKR1, a CD4+, CD8+, CD44.2+ T lymphoma, is described by Hyman et al. (18). RAW264 is a CD44.1+ pre-B lymphoma (19). XI(3) is a variant of RAW264 that can be induced to bind HA by a CD44-specific mAb (12). All cell lines were cultured in DMEM with 10% heat-inactivated horse serum. The CD44-specific IM7.8.1 mAb was used for immunoblotting, immunoselection, and purification of CD44/immunoglobulin fusion proteins (20). Supernatants of the CD44-specific mAb IRAWB14 were

Received 12/22/97; accepted 6/10/98.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
1 Supported by National Institute of Allergy and Infectious Diseases Grant AI-31613 (to R. H.) and National Cancer Institute Core Grant CA-14195.
2 To whom requests for reprints should be addressed, at Cancer Biology Laboratory, The Salk Institute, P. O. Box 85800, San Diego, CA 92186-5800.
3 The abbreviations used are: HA, hyaluronan; Ig, immunoglobulin; mAb, monoclonal antibody; CD44H, hematopoietic form of CD44; FI, fluorescein; PNA, peanut agglutinin; WGA, wheat germ agglutinin; LPA, Limulus polyphemus agglutinin; DSL, Datura stramon- nium lectin; MAL II, Maackia amurensis agglutinin; MFI, median fluorescence intensity.
used for induction of HA binding (21). The Fl-conjugated antibody IM7 was used for quantitation of cell surface CD44 and quantitation of CD44/Ig fusion proteins on beads. Fl-conjugated antibodies RAMBMM44, specific for CD44.1, and CD71/26, specific for CD44.2, were used to determine CD44 alleles (22).

Antibodies used to assess possible conformational changes in CD44 due to glycosylation sites and mutations. The numbering for amino acids is as according to Stamenkovic et al. (25) for human CD44. N-glycosylation sites are numbered from the N-terminal portion of CD44 showing the N-glycosylation sites and mutations. The numbering for amino acids is as according to Stamenkovic et al. (25) for human CD44. N-glycosylation sites are numbered from the amino terminus as N1-N5. Regions of clustered basic amino acids shown to be critical for HA binding (26).

CD44/Ig Fusion Protein Purification. Transfected cell lines producing the CD44/Ig fusion protein were selected by assaying the supernatants of individual clones using a sandwich ELISA. Briefly, 96-well plates were coated with 1 µg/ml IM7 followed by the addition of undiluted supernatants, which were incubated with peroxidase conjugated Protein A (Cappel, West Chester, PA), and finally detected with 2,2'-azino-2-3-ethylbenz-thiazoline sulfonate (ABTS) (Boehringer Mannheim, Indianapolis, IN). Positive transfecteds were grown in DMEM with 15% heat-inactivated horse serum in a MiniPERM Bioreactor (Heraeus, South Plainfield, NJ) to concentrate the secreted fusion proteins. CD44 fusion proteins were purified on an IM7-Sepharose affinity column, dialyzed against PBS, and determined to be pure by silver staining.

**Isolation of a CD44-negative Inducible Cell Line.** XJ(3) cells were treated with 600 µg/ml ethylmethanesulfonate for 14 h. After recovery from mutagenesis, selection was carried out by incubating the cells for 30 min at 0°C in CD44-specific mAb IM7 ascites diluted in HEPES-buffered DMEM containing 1 mM dinitrophenol, followed by 45 min at 37°C in a mixture of rabbit complement and rabbit antiserum Ig, both diluted in HEPES/DMEM containing 1 mM dinitrophenol. These incubations were repeated, the cells were washed and resuspended in growth medium, and the surviving cells were allowed to grow out. This procedure was repeated for a total of seven cycles. The cells, which still appeared detectably CD44-positive by flow cytometry, were then sorted for the least fluorescent cells. Cell sorting was performed as described (see Ref. 6). Upon growth of these cells, a CD44-negative population could be detected. This population was sorted for the least fluorescent cells that were cloned by limit dilution. A CD44-negative clone, termed XJ(3)/CD44~, was still appeared detectably CD44-positive by flow cytometry, were then sorted for the least fluorescent cells. Cell sorting was performed as described (see Ref. 6). Upon growth of these cells, a CD44-negative population could be detected. This population was sorted for the least fluorescent cells that were cloned by limit dilution. A CD44-negative clone, termed XJ(3)/CD44~, was detected in such experiments. The XJ(3)/CD44~ clone was used in subsequent experiments.

**Generation of CD44 Mutants.** cDNA clones encoding the standard form of murine CD44.1 or CD44.2 in pBluescriptSK (pSK) were used to generate mutants by oligonucleotide site-directed mutagenesis (24). Oligonucleotides were generated that replaced Asn25, Asn57, Asnl00, Asnl10, and Asnl20 with Ser. The numbering of amino acids is as according to Stamenkovic et al. (25) for human CD44H. The N-glycosylation sites were mutated as N1, N2, N9, N4, and N5, respectively (see Fig. 1). Mutants were verified by sequencing, excised from pSK with NotI and Kpnl, subcloned into pSL301 (Invitrogen, San Diego, CA) to achieve Xbal ends, and finally were subcloned into the Xbal cloning site of the eukaryotic expression vector pEFBos (27). Dr. Paul Kincade of the Oklahoma Medical Research Foundation (Oklahoma City, OK) kindly provided us with the pEFBos vector; cDNA for constructs of CD44.1 with individual N-glycosylation mutations in which Thr27, Thr59, Thr102, Ser112, and Ser122 were replaced with Ala; and cDNA encoding a CD44/Ig fusion protein in pEFBos (13). Briefly, this soluble fusion protein contains the extracellular domain of the murine hematopoietic form of CD44.1 and the hinge, CH2, and CH3 regions of the human IgG1. The extracellular domain containing the N-glycosylation sites was excised from the CD44/Ig cDNA in pEFBos with NotI and HincII and replaced with the NotI-HincII fragments from the pSK mutants N1-N5. This resulted in five CD44/Ig fusion proteins with Asn25, Asn57, Asn100, Asn110, and Asn120 each mutated to Ser as described above.

**Transfections.** CD44~ cell lines were transfected with constructs encoding CD44 of the alternative allele to ensure that effects seen were due to the introduced CD44 and not to activation of endogenous CD44. Cells (10²) were cotransfected with 40 µg of construct DNA plus 4 µg of pSV2NEO (28) by electroporation as described (see Ref. 29). Selection for stable transfecteds using G418 (Genticin) was as described (see Ref. 30).

---

### Table 1

<table>
<thead>
<tr>
<th>CD44.1</th>
<th>CD44.2</th>
<th>Constitutive HA binding</th>
<th>Inducible HA binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>XJ(3), parent</td>
<td>XJ(3)/CD44</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>XJ(3)/CD44−</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Transfectant</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Fluorescence at least 20 times over background.*

*No binding above background staining.*
RESULTS

Isolation of a CD44-negative Inducible Cell Line. Our laboratory has demonstrated that there is a progressive reduction in the N-glycosylation of CD44 between inactive, inducible, and constitutively active cell lines. To unambiguously test for possible activating effects of specific changes in CD44 N-glycosylation, a CD44-negative cell line that retains the inactive or inducible phenotype is required as a host for transfection. Therefore, a CD44-negative mutant of the inducible cell line XJ(3) was isolated as described in “Materials and Methods.” This CD44-negative derivative, termed XJ(3)/CD44−, retained the cell surface marker profile of its parent and has remained stable (data not shown). After transfection with a wild type CD44 construct encoding the alternative CD44.2 allele, the XJ(3)/CD44− cell line demonstrated an inducible HA binding phenotype (Table 1). Therefore, this mutant CD44-negative cell line is an appropriate host to examine whether inactivating specific sites of N-glycosylation has a positive or negative effect on inducible HA binding.

AKR1 is a CD44-negative cell line that on transfection of wild type CD44 binds HA constitutively (21); this cell line is in the active state. The AKR1 cell line was used to determine whether inactivation of specific N-glycosylation sites affected constitutive binding.

Effect of Inactivating Sites of N-glycosylation on Constitutive and Inducible HA Binding. Each individual glycosylation site of mouse CD44.1 and CD44.2 was mutated by substituting the asparagines with serines. These mutants are termed N1, N2, N3, N4, and N5 (see Fig. 1). The CD44.1 mutants were stably transfected into CD44-negative AKR1 cells and the CD44.2 mutants into XJ(3)/CD44− cells by electroporation. For sites N1–N4, analysis by SDS-PAGE and immunoblotting revealed that each N-glycosylation site was used by the cell because each mutant ran approximately Mr 2000–5000 faster than the CD44 from cells transfected with wild type (see Fig. 2). The reductions in molecular mass for each mutant were similar in both sets with wild type. For the XJ(3) transfectant, however, a gain in function suggested that the site was altered (see below).

A panel of CD44-specific mAbs (see “Materials and Methods”) that bind to several different epitopes of CD44 was used to look for possible conformational changes caused by the mutations (23). Ten different CD44-specific mAbs used in a flow cytometric assay bound the mutants and wild type with the same efficiency, suggesting that there were no major structural changes in the CD44 protein expressed on the cell surface as a consequence of these mutations (data not shown).

As shown in Fig. 2A, all five sites of N-glycosylation seem to be used in AKR1. Over a range of expression levels, transfectants for mutants N1–N5 bound HA as efficiently as cells transfected with wild type (Fig. 3A). Thus, inactivation of any single N-glycosylation site did not inhibit constitutive HA binding in AKR1 cells. AKR1 cells were also transfected with constructs that had mutations in the serine or threonine residues of the five N-glycosylation consensus sequences. Transfectants with disrupted sites at N1, N2, N4, or N5 due to serine or threonine mutations exhibited constitutive binding (data not shown). The transfectant with threonine mutated near N3 did not bind HA constitutively, but could be induced to bind HA with IRAWB14 mAb (data not shown).

Constitutive cell lines have been shown to differ from inducible cell
To determine whether inactivating specific sites of N-glycosylation would alter the inducible HA binding phenotype, the XJ(3)/CD44- cell line was transfected with wild type CD44.2 and with the N-glycosylation mutants in CD44.2 and the HA binding properties of the transfectants were characterized. The transfectant for wild type CD44 does not bind HA but will bind HA after induction with mAb IRAWB14; this cell line, therefore, has the "inducible" phenotype (Table 1). When the XJ(3)/CD44- cell line was transfected with N-glycosylation mutants it was found that transfectants for mutants N1 or N5 bound HA constitutively (Fig. 3B). In contrast, transfectants for N3 or N4 showed only very low levels of constitutive HA binding whereas mutation of N2 conferred no constitutive HA binding ability at all, similar to wild type CD44 in the XJ(3) background (Fig. 3B). XJ(3)/CD44- cells were also transfected with the N-glycosylation mutants in which the serine or threonine of the N-glycosylation consensus sequence was changed to alanine. These transfectants behaved similarly, i.e., mutation of N1 and N5 conferred constitutive HA binding, whereas mutation of N2, N3, and N4 had little or no effect on the inducible phenotype (data not shown). Mutations at the N-glycosylation sites did not affect the ability of the XJ(3)/CD44- transfectants to be induced by mAb IRAWB14 (data not shown). It should be noted that if wild type CD44 or any CD44 mutant was expressed in very large amounts on the cell surface, HA binding did occur. This "threshold" effect is similar to that observed with mutants lacking the CD44 cytoplasmic domain (30). Consequently, to accurately assess HA binding state comparisons between wild type and any CD44 mutant must be carried out over a range of CD44 expression levels.

**HA Binding by CD44/Ig Fusion Proteins.** Fusion proteins containing the external domain of CD44 allow the HA binding function to be assessed outside of the cellular environment (34). In addition, fusion protein concentrations can be controlled, thus, allowing examination of greater concentration ranges than those that occur on the cell surface. Wild type CD44/Ig fusion proteins were transfected into AKR1 (active), CD44-positive XJ(3) (inducible), and RAW253 (inactive) cell lines. CD44/Ig fusion proteins were purified from the supernatants of these transfected cell lines. Serial dilutions of CD44/Ig fusion proteins were bound to plastic beads of uniform size, resulting in beads with varying densities of CD44/Ig that were used in flow cytometric assays to determine HA binding (see "Materials and Methods"). The HA binding phenotype of the CD44/Ig fusion proteins produced in these cell lines reflects the HA binding phenotype of the cell from which they originated (Fig. 4A). CD44/Ig fusion proteins from AKR1 bound HA constitutively even at very low levels of protein, whereas fusion proteins from XJ(3) require a much higher density of CD44/Ig on the beads to bind HA constitutively. Fusion proteins from RAW253 only bound HA constitutively at the highest levels tested (Fig. 4A). These binding curves show that each fusion protein has a minimum threshold of CD44 density necessary for constitutive HA binding. Even inactive (defined as not able to bind HA) fusion proteins capable of binding FI-HA at very high concentrations of fusion protein; but, the threshold is at a higher concentration for the inactive fusion protein than for the inducible or active fusion proteins. The threshold for binding by inducible fusion proteins is lower than for inactive fusion proteins, but higher than for active fusion proteins. These different threshold binding levels imply inherent differences in the CD44 molecules themselves, which must be due to differences in posttranslational modification of the polypeptide. Detectable differences in migration of the fusion protein from the three cell lines are observed, consistent with differences in glycosylation (see Fig. 4B, compare Lanes a, c, and e).

Treatment of CD44/Ig fusion proteins with neuraminidase, which cleaves terminal sialic acids, altered the HA binding state. On removal of sialic acids, fusion proteins from inactive cells became inducible and fusion proteins from inducible cells lines became active, as judged by a change in the threshold binding level (Fig. 4A). Active fusion proteins showed only a slight (<2-fold over a range of densities) increase in HA binding after neuraminidase treatment (data not shown). A reduction in apparent molecular mass of the CD44/Ig fusion proteins occurred after neuraminidase treatment (Fig. 4B). On further analysis, it was found that the change in threshold of CD44/Ig from inducible to active could be achieved with a neuraminidase from the Newcastle disease virus specific for α2,3-linked sialic acid. However, treatment with neuraminidase derived from Newcastle disease virus had no effect on the activity of CD44/Ig derived from inactive cells. A change in threshold was seen when CD44/Ig from inactive cells was treated with neuraminidase from V. cholerae, which has a broader specificity and also digests α2,6-linked sialic acid.

CD44/Ig mutants were constructed in which each of the five N-glycosylation sites were inactivated. These mutants were transfected into CD44-positive XJ(3) cells and the mutant fusion proteins were assessed for HA binding ability as described above. Analysis by flow cytometry revealed that the CD44/Ig fusion proteins from the XJ(3) cell line that had mutations in sites N1 or N5 bound HA with a threshold similar to that of the fusion proteins derived from the active AKR1 cell line (Fig. 5). Thus, inactivating sites N1 or N5 of the XJ(3) CD44/Ig fusion proteins converted the protein from an inducible state...
to an active state. Inactivation of sites N3 or N4 resulted in intermediate levels of HA binding, whereas inactivation of site N2 resulted in HA binding only at very high levels of fusion protein concentration, similar to wild type binding (Fig. 5). Therefore, the mutant fusion proteins behaved similarly to the respective N-glycosylation mutants in the CD44-negative XJ(3) cell line (compare Fig. 3B and Fig. 5). Taken together, these data suggest that inactivation of site N1 or N5 is sufficient to convert the XJ(3) inducible phenotype to a constitutively active phenotype.

To further demonstrate that mutation of individual N-glycosylation sites altered the glycosylation of CD44/Ig, fusion proteins coupled to beads were assayed for binding to a panel of lectins with differing carbohydrate specificities. As shown in Table 2, each mutant fusion protein differed from wild type and from other mutants in its lectin binding profile. All mutants showed increased PNA binding and reduced binding of MAL II and DSL. All but N2 were reduced in LPA binding. These results suggest a general reduction in terminal sialic acid and especially in α2,3-linked sialic acid for each mutation.

**DISCUSSION**

Transfection experiments have shown that the binding of HA by CD44 is dependent upon the cell in which CD44 is expressed. Wild type CD44H transfected into the T cell line AKR1 leads to constitutively active HA binding; wild type CD44H transfected into the T cell lines EL4 or SAKRTLS12 shows inducible binding, with HA binding occurring only in the presence of the CD44-specific inducing mAb IRAWB14; and CD44H transfected into the pre-B line, RAW253, or the fibroblast line, L(TK)−K, does not bind HA at all and is inactive (2). The observations that sets of genetically related cell lines which differ in the HA binding function show reductions in N-glycosylation of CD44 correlated with increased HA binding activity and that HA binding function could be altered by inhibiting N-glycosylation (12, 13) led us to look at the role of each particular N-glycosylation site of CD44 in influencing HA binding function.

Mutations that inactivated individual N-glycosylation sites had no effect on constitutive HA binding in the active cell line AKR1 (Fig. 3A). All of the N-glycosylation sites on CD44 seem to be used in AKR1 cells because a reduction in apparent molecular mass was seen on SDS-PAGE with each mutant (Fig. 2A). In contrast to these results, Bartolazzi et al. (15) showed that mutation of one of the five N-glycosylation sites in human CD44 abolished adhesion to immobilized HA in a melanoma tumor line. On the basis of changes in CD44-specific antibody binding, Bartolazzi et al. (15) postulated that an altered protein conformation may have caused loss of HA recognition. We, on the other hand, detected no conformational changes when individual sites of N-glycosylation were inactivated in AKR1 because a panel of CD44-specific antibodies bound mutants and wild type equally. Mutation of the threonine in the consensus sequence for the N3 site did abolish HA binding in AKR1 cells, however point mutation of the asparagine did not. This indicates that that the loss of HA binding ability in AKR1 was not due to loss of N-glycosylation at that site. The differences between our results and those of Bartolazzi et al. might be due to the functional assay used; soluble HA binding versus adhesion to surface-coated HA (21). Cell-specific or species-specific differences may also exist. Cell-specific differences are exemplified by conflicting reports on the effects of tunicamycin on HA binding (10, 12, 13, 15).

Constitutively active cell lines have been demonstrated to show reduced amounts of glycosylation compared with inducible cell lines, and tunicamycin treatment of inducible cells results in a constitutively active binding phenotype (12, 13). The isolation of a CD44-negative cell line with an inducible phenotype allowed us to test specifically whether inactivation of N-glycosylation would alter the HA binding phenotype from inducible to constitutively active. Inactivation of either site N1 or site N5 altered the inducible, nonbinding state to an active, binding state (Fig. 3B). However, deglycosylation of site N1 or N5 is not invariably required for the active phenotype, per se, because it is clear that sites N1 and N5 are used in the AKR1 cell line, which binds HA constitutively. These results indicate that cell-specific N-glycan modifications differentially affect the HA binding function.

Bajorath et al. (35) have recently published a molecular model of the proposed HA binding region of CD44 based on the structure determined by nuclear magnetic resonance of the homologous domain of TSG-6. Site-specific mutagenesis of the CD44/Ig fusion protein was used to identify residues important for ligand binding and to map the HA binding site (35). Glycosylation sites N1 and N5 are outside of the region modeled, and sites N2 and N4 are not predicted to be involved in the ligand binding domain. However, this study found that residues around and including N3 were important for HA binding in

---

**Table 2 Lectin binding properties of glycosylation mutants in CD44/Ig fusion proteins**

<table>
<thead>
<tr>
<th></th>
<th>PNA*</th>
<th>WGA*</th>
<th>LPA*</th>
<th>DSL*</th>
<th>MAL II*</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>N1</td>
<td>4</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>N2</td>
<td>3</td>
<td>2</td>
<td>6</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>N3</td>
<td>6</td>
<td>5</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>N4</td>
<td>3</td>
<td>1</td>
<td>4</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>N5</td>
<td>5</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Recognizes galactose β(1→3) N-acetylgalactosamine (sialic acid blocks PNA staining).
* Recognizes N-acetylgalactosamine oligomers.
* Recognizes sialic acid.
* Recognizes β(1→4) linked N-acetylgalactosamine oligomers.
* Recognizes α(2→3) linked sialic acid.

---

N. M. English et al., unpublished observations.
that their mutation significantly reduced binding of fusion proteins to immobilized HA in an ELISA. We did not find that mutation of asparagine 100 to serine affected HA binding in AKR1 cells. However, mutation of the nearby threonine 102 did result in loss of constitutive, although not inducible, binding. Although not directly tested in the Bajorath study (35), this residue seems to be in a region important for ligand binding. Our results suggest that its function in influencing ligand binding is independent of glycosylation. Amino acid residues at N1 and N5 are not expected to be directly involved in the ligand binding site, yet mutation of these residues does affect the ligand binding state in Xj(3) cells. Thus, it is possible that carbohydrate chains extending from these residues affect the interaction between the ligand and the binding domain.

To determine whether cell-specific differences in CD44 function were influenced by interactions on the cell surface or were properties of CD44 itself, wild type CD44/Ig fusion proteins were produced in different cellular environments and assayed on beads in the absence of other cell-surface molecules. Assaying the fusion proteins on beads allowed the CD44/Ig concentration to be controlled and a wide range of densities to be examined. With each fusion protein there was a CD44 density at which HA was bound and a lower density at which it was not bound. The fusion proteins from active cells bound HA at a significantly lower CD44 density than did fusion proteins from inducible cells which, in turn, bound HA at a lower density than fusion proteins from inactive cells. These differences in the threshold at which HA binding occurred paralleled the activity of the cell surface CD44 of the cells in which the fusion proteins were synthesized. Zheng et al. (10) have also shown that CD44/Ig fusion proteins have the same HA binding capabilities as the cell line from which they originated. These differences in function of CD44/Ig fusion proteins synthesized in different cellular environments must be the result of cell-specific posttranslational modifications. Indeed, the same molecular size differences were detected on SDS-PAGE between fusion proteins representing different activation states as was seen with their cellular counter-parts (present study and Ref. 10).

CD44/Ig fusion proteins, in which individual N-linked oligosaccharides had been inactivated, were purified from the inducible CD44-positive Xj(3) cell line and assessed for HA binding ability. In parallel to the results with intact cells, mutations in sites N1 or N5 caused significantly enhanced HA binding as compared with wild type binding. This result confirms that mutation of a single N-glycan in CD44 itself is sufficient to activate HA binding in CD44 from an inducible cell. Lectin-binding profiles of each of the five N-glycosylation mutants differed uniquely from wild type CD44/Ig made in the same inducible cell line, indicating that each mutation affected fusion protein glycosylation in a different manner. Although this observation indicates unique changes in glycosylation for each mutation, the lectin binding studies do not allow conclusions about the structure of the carbohydrate at individual sites. It is also possible that mutation of a particular N-glycosylation site might indirectly result in structural changes at other sites of N- or O-glycosylation.

Zheng et al. (10) and Katoh et al. (13) observed that CD44/Ig fusion proteins from 293 or CHO cells had increased HA binding after treatment with neuraminidase and that intact non-HA binding CHO cells were able to bind HA upon neuraminidase treatment. Furthermore, column chromatography analysis of [3H]GlcN-glycopeptides from CHO variants showed that CD44 from the non-HA binding parental CHO line was more heavily sialylated than CD44 from its HA binding sub-clone (10). Recently, Skelton et al. (16) reported that removal of sialic acid from CD44/Ig fusion proteins with an α2,3-specific neuraminidase increased HA binding affinity. We have also found that removal of sialic acids from CD44/Ig fusion proteins altered their HA binding abilities (Fig. 4A). For the cell lines used in this study, we did not observe this effect on intact cells (12). In agreement with Skelton et al. (16), we found that specific removal of α2,3-linked sialic acids resulted in activation of fusion proteins from inducible cells. However, neuraminidase of broader specificity was required to shift the activity threshold of fusion proteins from inactive cells, indicating that sialic acids with other linkages were involved in regulating the ability of this CD44/Ig to bind HA. Furthermore, the increase in activity of the inactive fusion protein by neuraminidase digestion was only partial, bringing it to a level equivalent to fusion protein from inducible cells. This indicates that factors in addition to sialic acid contribute to the function of this molecule. It is possible that the changes in the HA binding function associated with deletion of site N1 or N5 in the inducible state (or inducible cells) are due to deletion of sialic acids at those sites. Indeed, lectin binding studies indicate loss of α2,3-linked sialic acid (detected by MAL II) as a consequence of each mutation (Table 2). However, much more detailed structural information would be necessary to establish this point.

The mechanisms by which glycosylation affects the ability of CD44 to bind HA remain unclear. Perhaps in certain cell types, exemplified by Xj(3), the specific structure at sites N1 and/or N5 serve to stabilize the CD44 molecule in an inducible or nonbinding conformation. Other studies have shown that N-glycans have critical roles in maintaining protein integrity and conformation. Nuclear magnetic resonance structural studies suggested that a single N-linked oligosaccharide of CD2 was responsible for the configuration required for the CD2-CD58 interaction (36). Hebert et al. (37) reported that different mutations of the N-glycans of the influenza virus hemagglutinin had distinct consequences for calcinein/calreticulin binding thus resulting in inappropriate posttranslational folding of the hemagglutinin. Sgroi et al. (38) found that mutation of a single N-glycosylation site in CD33 was sufficient to "unmask" its sialic acid binding site (38). Finally, there is mounting evidence that sialic acids contribute to altered CD44 ligand recognition (present study and Ref. 10, 13, 16, 39). It has been speculated that sialic acids contribute to the overall charge of the glycoprotein, thereby interfering with CD44 oligomerization or altering the HA binding domain (13), but, as yet, there is no definitive evidence.

Recent studies have indicated a correlation between carbohydrate profile of CD44 and metastatic potential. Two clonal variants of a human colon carcinoma that show differences in keratan sulfate substitution on CD44 also show differences in CD44/HA binding function (40). The clone in which CD44 is more heavily substituted with keratan sulfate is highly metastatic and binds HA poorly, whereas the clone which is less keratan-sulfated on CD44 is poorly metastatic and binds HA well (40, 41). Goupille et al. (42) transfected the α(1-2) fucosyltransferase gene into a rat colon carcinoma cell line, causing changes in glycosylation of CD44 and subsequent enhanced tumor cell motility and tumorigenicity. Finally, remodeling of glycoconjugates on CD44, induced by transfection of β(1-4) N-acetylgalosaminyltransferase III into a murine melanoma cell line, resulted in enhanced adhesion to HA, local tumor growth, and metastasis, all of which were inhibited by CD44-specific mAbs (39). Aberrant glycosylation and malignancy have also been correlated for other adhesion molecules such as integrins, selectins and cadherins. In fact, reagents that modulate glycosylation are currently being studied as a means to block metastasis in human cancers (1). A better understanding of the mechanisms by which glycosylation influences the function of adhesion molecules will help guide this approach.

REFERENCES

Site-specific De-N-glycosylation of CD44 Can Activate Hyaluronan Binding, and CD44 Activation States Show Distinct Threshold Densities for Hyaluronan Binding

Nicole M. English, Jayne F. Lesley and Robert Hyman