Glycosylation of CD44 Is Implicated in CD44-mediated Cell Adhesion to Hyaluronan

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Abstract. CD44-mediated cell adhesion to hyaluronate is controlled by mechanisms which are poorly understood. In the present work we examine the role of N-linked glycosylation and Ser-Gly motifs in regulating CD44-hyaluronate interaction. Our results show that treatment of a panel of human cell lines which constitutively express CD44 with the inhibitor of N-linked glycosylation tunicamycin results in the loss of attachment of these cells to hyaluronate-coated substrate. In contrast, treatment of the same cells with deoxymannojirimycin, which inhibits the conversion of high mannose oligosaccharides to complex N-linked carbohydrates, results in either no change or an increase in CD44-mediated adhesion to hyaluronate, suggesting that complex N-linked oligosaccharides may not be required for and may even inhibit CD44-HA interaction. Using human melanoma cells stably transfected with CD44 N-linked glycosylation site-specific mutants, we show that integrity of five potential N-linked glycosylation sites within the hyaluronate recognition domain of CD44 is critical for hyaluronate binding. Mutation of any one of these potential N-linked glycosylation sites abrogates CD44-mediated melanoma cell attachment to hyaluronate-coated surfaces, suggesting that all five sites are necessary to maintain the HA-recognition domain in the appropriate conformation. We also demonstrate that mutation of serine residues which constitute the four Ser-Gly motifs in the membrane proximal domain, and provide potential sites for glycosaminoglycan side chain attachment, impairs hyaluronate binding. Taken together, these observations indicate that changes in glycosylation of CD44 can have profound effects on its interaction with hyaluronic acid and suggest that glycosylation may provide an important regulatory mechanism of CD44 function.

CD44 is a broadly distributed cell surface glycoprotein implicated in multiple physiologic cellular functions including cell–cell adhesion (Shimizu et al., 1989; St. John et al., 1990; Naujokas et al., 1993), lymphocyte activation (Huet et al., 1989; Haynes et al., 1989; Denning et al., 1990; Naujokas et al., 1993), and cell–substrate interaction (Carter and Wayner, 1988; Aruffo et al., 1990; Miyake et al., 1990; Stamenkovic et al., 1991; Thomas et al., 1992). Recent work has shown that CD44 can be expressed as several different isoforms, varying between 85 and >200 kD, each of which may display some degree of functional uniqueness (Stamenkovic et al., 1989, 1991; Brown et al., 1991; Gunthert et al., 1991; Jackson et al., 1992; Hoffman et al., 1991; Bartolazzi et al., 1994; Bennett et al., 1995). CD44 diversity is generated by differential usage of 10 exons encoding a portion of the extracellular domain (Screaton et al., 1992) and cell type–specific glycosylation (Brown et al., 1991; Jalkanen and Jalkanen, 1992).

The most widely expressed isoform is the 85–90-kD glycoprotein which represents the "standard" CD44 molecule, as it does not contain the products of differentially spliced exons (Stamenkovic et al., 1989; Goldstein et al., 1989; Screaton et al., 1992). It is commonly referred to as CD44H and has been shown to be a major cell surface receptor for hyaluronate (HA)[1] (Aruffo et al., 1990; Miyake et al., 1990). CD44H is the principal isoform found in hematopoietic cells, fibroblasts, melanomas, and some epithelial cells (Stamenkovic et al., 1989, 1991; Hoffmann et al., 1991; Quackenbush et al., 1990), and most of its known functions are attributed to its ability to recognize HA on the surface of adjacent cells or bound to extracellular matrix (ECM) proteins. Although all CD44 variants contain the HA-binding domain, their affinity for surface-bound hyaluronate is variable (Stamenkovic et al., 1991; Sy et al.,

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; CLP, cartilage link protein; GAG, glycosaminoglycan; HA, hyaluronate; NGS, N-linked glycosylation site.

1991), and at least some of the larger isoforms mediate only weak attachment of cells to hyaluronate-coated surfaces (Stamenkovic et al., 1991; Sy et al., 1991; Thomas et al., 1992). These observations suggest that structural features and/or posttranslational modification of variant exon products may partially impair hyaluronate recognition by the HA-binding domain of CD44.

The affinity for HA of CD44H itself varies according to the cell type in which it is expressed. Thus, normal lymphocytes, as well as certain lymphoid cell lines which express CD44H, do not bind soluble HA or adhere to HA-coated substrate (Lesley and Hyman, 1992; Hathcock et al., 1993; Liao et al., 1993; Murakami et al., 1993; Lesley et al., 1994). Similarly, several CD44H-positive nonlymphoid tumor cell lines fail to bind HA (Bartolazzi, A., and I. Stamenkovic, unpublished observations). However, a specific anti-CD44 mAb (Lesley and Hyman, 1992; Lesley et al., 1992), phorbolesters and certain cytokines (Liao et al., 1993; Hathcock et al., 1993; Murakami et al., 1993) can induce CD44-mediated adhesion to HA in murine lymphoma cells and normal murine and human lymphocytes. The observed augmentation of adhesiveness to HA is not the consequence of an increased number of CD44 molecules on the cell surface, but rather appears to be due to a change in conformation and/or aggregation of CD44 which may be induced by antibody, cytokine, or mitogen-triggered posttranslational modifications.

The extracellular region of CD44H is divided into two distinct domains. The NH_2-terminal 135 residues which form the HA-binding domain display a high degree of homology to cartilage link protein (CLP) and other ECM HA receptors (Stamenkovic et al., 1989). This domain contains five potential N-linked glycosylation sites and one BX_7B motif, where B is an arginine or lysine residue and X_7 contains one basic but no acidic residues. Two additional overlapping BX_7B motifs are found in close proximity to the CLP homologous region, spanning residues 150-162 (Yang et al., 1994). BX_7B motifs are required for HA binding and are present in most known cell surface and ECM HA receptors (Yang et al., 1994). The membrane proximal domain contains four Ser-Gly motifs, which constitute the minimal sequence for chondroitin sulfate (Mann et al., 1990), and possibly other glycosaminoglycan (GAG) attachment, as well as a large number of serine and threonine residues which may provide sites for O-linked glycosylation. Because glycosylation may contribute to and be an integral part of the physiological activity of a glycoprotein, we performed site-directed mutagenesis to address the role of the potential N-linked glycosylation sites and the Ser-Gly motifs in CD44H recognition of HA. We also used inhibitors of N-linked glycosylation to assess the importance of N-linked glycans in CD44H-HA interaction. Our results indicate that N-linked glycosylation plays an important role in CD44H-mediated adhesion to HA-coated substrate, as does the integrity of the four ser-gly motifs.

Materials and Methods

Development of CD44 Mutants

Fourteen CD44 site-directed mutants were prepared by encoding the desired mutation in overlapping oligonucleotide primers (Ho et al., 1989) and generating the mutations by PCR using CD44H cDNA in the CDM8 expression vector (Thomas et al., 1992) as a template. The mutants were generated in two groups. The first group was composed of mutations of the six potential N-linked glycosylation sites (NGS), five of which are located within the CLP homology domain. One set of mutants contained substitution of the Asn residue only, whereas a second set of mutants contained substitution of the corresponding Ser/Thr residue only (Table I). Mutants are labeled according to the specific mutation, N25I, and according to the corresponding N-linked glycosylation site, which in this case would be NGS1. NGS are numbered 1–6 starting from the NH_2 terminus (Table I).

Oligonucleotide primers used to derive each of the single NGS mutations were used to develop constructs containing mutations in multiple NGS. Two mutants containing substitution of Asn by Ile at NGS2-5 and of Ser/Thr by Ala at NGS3-5 were thus generated.

The second group of mutants was composed of substitution of each of the four serine residues that constitute the Ser-Gly dipeptides located in the membrane proximal domain by alanine. Mutants were labeled according to the specific mutations, e.g., S180G, and the corresponding ser-gly dipeptide, e.g., Ser-Gly 1. Constructs were then made containing mutation of S180G/S190G (Ser-Gly 1-2), S180G/S190G/S231G (Ser-Gly 1-3), and S180G/S190G/S231G/S258G (Ser-Gly 1-4).

Standard forward and reverse primers corresponding to the 5′ and 3′ extremities of the coding sequence of CD44, containing, respectively, a HindIII and an XhoI restriction site, were used in all PCR reactions, in combination with primers containing specific mutations. PCR conditions consisted of 30 cycles of 1 min at 94°C, 2 min at 58°C, and 3 min at 72°C. 5′ and 3′ amplified fragments were subjected to HindIII/X and Xho I/X digestion, respectively, where X represents the restriction site created for mutant generation, and ligated into HindIII–XhoI-cut CDM8 vector. In each case the creation of a new restriction site was performed by codon substitution that resulted in conservation of the wild-type residues or by generation of a silent mutation. All of the mutants were verified by dideoxy sequencing. PCR-generated errors were not observed.

Cell Lines

Development of stable CD44 transfectants in the human lymphoma Namalwa (Stamenkovic et al., 1991) and the human melanoma MC (Thomas et al., 1992) have been described previously. Transfectants expressing the NGS CD44 mutants were labeled according to the specific mutant, e.g., MC44N25I, or according to the position of the mutated NGS, e.g., MC44NGS1. Transfectants expressing composite mutants were labeled according to the mutated NGSs, including MC44NGS2-5 and MC44-NGS3-5. Similarly, transfectants expressing ser-gly dipeptide mutants were labeled according to the specific mutations within the transfected cDNA, e.g., MC44S180A/S190A or according to the position of the corresponding mutated Ser-Gly motifs, e.g., MC44Ser-Gly 1-2. MC44H and MC-T, respectively, express wild-type CD44H, and pSV2neo, the selection plasmid containing the neomycin resistance gene. Transfections were performed by electroporation (Gene Pulser, BioRad Labs, Hercules, CA) using the same conditions as those previously described (Thomas et al., 1992; Bartolazzi et al., 1994). Clones were selected for G418 resistance in DMEM (Irvine Scientific, Santa Ana, CA) supplemented with 2 mM glutamine (GIBCO BRL, Gaithersburg, MD), 10% FBS (Irvine), gentamycin and 1 mg/ml G418. All transfectants were periodically tested for expression of CD44 isoforms by indirect immunofluorescence.

T24 bladder carcinoma cells were purchased from Amer. Type Culture Collection (Rockville, MD). The renal carcinoma cell line KJ29, the melanomas CT1 and FV1, and the breast carcinoma cells VR1 and MN were derived from primary tumors.

Monoclonal Antibodies and Immunofluorescence

The anti–human CD44 BRIC series of mAb, including BRIC 214, 222, 235, and KZ1 (Antsee et al., 1991) were used in this study. Development of these antibodies has been described elsewhere (Antsee et al., 1991). Cells were detached from plates with EDTA, washed, resuspended in PBS, and incubated with monoclonal antibodies (10–50 μg/ml) for 45 min at 4°C. Cells were then washed in PBS, incubated with fluorescein-labeled $F(ab)_2$ fractions of goat anti–mouse affinity-purified antibody (Cappel, Malvern, PA) for 30 min at 4°C, washed, resuspended in PBS, and analyzed on a fluorescence-activated analyzer (Becton-Dickinson, Mountainview, CA).

Table I.

Mutant	Glycosylation site	Antibody binding of corresponding MC melanoma transfectants					
		BRIC 214		BRIC 235		BRIC 222	
		IF	IP	IF	IP	IF	IP
CD44H	wt	++	+	++	+	++	+
N25I	NGS1	++	+	++	−	+	−
T27A	NGS1	++	+	++	−	+	−
N57I	NGS2	++	+	++	−	++	−
N100I/N110I	NGS3-4	++	+	++	−	+	−
T102A/S112A	NGS3-4	++	+	++	−	+	−
N120I	NGS5	++	+	++	−	+	−
S122A	NGS5	++	+	++	ND	+	ND
N255I	NGS6	++	+	++	+	++	ND
N57I/N100I/N110I/N120I	NGS2-5	++	+	+	−	−	−
T102A/S112A/S122A	NGS3-5	++	+	+	−	−	−
S180G/S190G	Ser-Gly 1-2	++	+	++	+	++	ND
S231G	Ser-Gly 3	++	+	++	+	++	ND
S180G/S190G/S231G	Ser-Gly 1-3	++	+	++	+	++	ND
S258G	Ser-Gly 4	++	+	++	+	++	ND
S180G/S190G/S231G/S258G	Ser-Gly 1-4	++	+	++	+	++	ND
N255I/S258G	SerGly4:NGS6	++	+	++	+	++	ND

CD44H N-linked glycosylation site and Ser-Gly dipeptide motif mutants. Each specific mutation and the corresponding glycosylation site are indicated.
wt, wild type; *IF*, immunofluorescence; ND, not done; ++, high antibody reactivity, (MFI:80-150); +, low antibody reactivity (MFI:20-40); −, absence of detectable antibody binding by flow cytometry (MFI<10); IP, immunoprecipitation; +, effective immunoprecipitation by indicated mAb; −, loss of ability to immunoprecipitate CD44 mutants.

Adhesion Assays

96-well tissue culture plates were coated with 5 mg/ml hyaluronate, chondroitin sulfate, or 10 μg/ml fibronectin, collagen type I or collagen type VI or heat denatured BSA overnight at 4°C, and nonspecific sites were blocked with 1 mg/ml BSA. The wells were washed with PBS and seeded with ^{51}Cr-radiolabeled MC transfectant cells (10^5 cells/well) in PBS. Adhesion was allowed to proceed at 4°C for 30 min. The wells were then washed three times with PBS, the adherent cells lysed with 1% SDS, and lysate radiolabel determined in a γ-counter.

Development of CD44 Receptorglobulins

The development of CD44-Ig fusion proteins (Receptorglobulins, Rg) has been reported earlier (Aruffo et al., 1990). Receptorglobulins were prepared in COS cells and used to stain frozen rat kidney tissue sections before and following treatment with N-glycanase as described (Aruffo et al., 1990).

Radiolabeling and Immunoprecipitation

MC melanoma transfectants were washed and cultured in methionine-free medium (GIBCO BRL, Giathersburg, MD) supplemented with 10% dialyzed FBS for 2 h. Cells were then pulsed with 250 μCi/ml of [^{35}S]methionine (Amersham) for 12 h, washed in PBS, detached and lysed in a buffer containing 1% Triton X-100 (Sigma Chem. Co. St. Louis, MO), 10 μg/ml leupeptin (Sigma), 100 U/ml aprotinin (Sigma), and 10 μM PMSF (Bethesda Research Laboratories Inc., Bethesda, MD). After 1 h of lysis at 4°C, nuclei were removed by centrifugation and lysates were precleared with protein A-Sepharose CL4B (Pharmacia, Uppsala, Sweden) coated with affinity-purified rabbit anti-mouse IgG (Sigma). After preclearing, lysates were incubated with protein A-Sepharose CL4B coated with anti-CD44 mAb for 1 h at 4°C, the protein A-Sepharose beads washed, and the precipitates eluted by boiling. Immunoprecipitates were analyzed by SDS/7.5%PAGE. The gels were fixed, dried, and subjected to autoradiography.

For CD44Rg radiolabeling, COS cells transfected with CD44-Ig cDNA were incubated in methionine-free medium, as described above, 24 h after transfection, and pulsed with 250 μCi/ml of [^{35}S]methionine for a duration of 18 h. Supernatants were then harvested and incubated with protein A-Sepharose beads for 2 h at 4°C. The beads were then washed and receptorglobulins eluted as described (Bartolazzi et al., 1994) and analyzed by SDS-PAGE under reducing conditions.

To determine CD44 shedding, equal numbers of transfectants were radiolabeled with [^{35}S]methionine as above. 72 h after radiolabeling, culture supernatants were harvested, precleared with protein A-Sepharose and shed CD44 immunoprecipitated as above.

Inhibition of N-linked Glycosylation and Treatment with N-glycanase

Tunicamycin and 1-deoxymannojirimycin were purchased from Sigma Chem. Co. To inhibit N-linked glycosylation, 3–10 μg/ml of tunicamycin were added to the cell culture medium at the time of radiolabeling. Cells were incubated with tunicamycin for 12–18 h. To inhibit processing of N-linked glycans to complex carbohydrate forms, cells were incubated with 1-deoxymannojirimycin according to the protocol recommended by the vendor. To remove N-linked glycans from mature CD44 receptors, protein A-Sepharose-bound CD44 immunoprecipitates were treated with 10–20U of N-glycanase/sample (Boehringer Mannheim Corp., Indianapolis, IN) at 37°C at a pH of 7.2 overnight, according to the protocol recommended by the vendor. The immunoprecipitates were then eluted and subjected to SDS-PAGE as described above. Treatment of CD44Rg with N-glycanase was performed in a 100 μl vol using 30 μg of CD44 Rg and 1 U of N-glycanase. 30 μg of N-glycanase–treated or –untreated CD44Rg were then used to stain frozen tissue sections. Streptomyces hyaluronidase was used to pretreat frozen tissue sections as described previously (Aruffo et al., 1990).

Results

Development of CD44H N-linked Glycosylation Site Mutants

To determine the role of N-linked glycosylation in CD44-mediated adhesion to hyaluronate, ten individual potential N-linked glycosylation site (NGS)-specific mutants were developed (Fig. 1; Table I). The asparagine residue in each of the six potential NGS was substituted by an isoleucine (Table I). In addition, to ensure that any observed functional alteration associated with the mutations is due to a defect in glycosylation and not to the substitution of the asparagine residue per se, the serine/threonine residues of a subset of the potential NGS, at position 1, 3/4, and 5, were substituted by an alanine (Table I and Fig. 1). The mutants are labeled either according to the specific amino

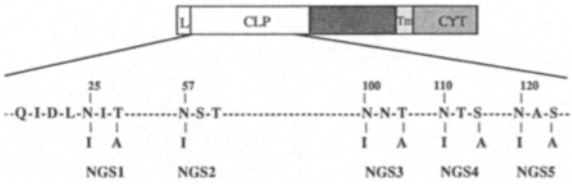


Figure 1. Schematic representation of NGS mutants in CD44H. *L*, leader peptide; *CLP*, cartilage link protein homology domain; *Tm*, transmembrane domain; *cyt*, cytoplasmic domain. Amino acids are denoted by their single letter code and numbered from the beginning of the leader peptide. Potential N-linked glycosylation sites (*NGS*) are numbered from the NH_2 terminus.

acid substitution, e.g., N25I, or according to the corresponding N-linked glycosylation site, numbered 1–6 starting from the NH_2 terminus, e.g., NGS1 (Table I). The effect of deficient glycosylation on multiple NGS was assessed by developing mutants containing substitution of Asn by Ile on NGS2-5 and of Ser/Thr by Ala on NGS3-5. All of the mutants were tested for expression in COS cells by flow cytometry, using the anti-CD44 mAb BRIC 214. COS cells transfected with each of the mutants were recognized by BRIC214 mAb (data not shown), indicating that none of the mutations had resulted in the abrogation of CD44 transport to the cell surface. Each of the mutants was then stably expressed in the MC melanoma (Thomas et al., 1992; Bartolazzi et al., 1994). Stable mutant CD44 transfectants are denoted by the prefix MC followed by the identification number of the mutated glycosylation site, e.g., MC44NGS1.

Selection-medium resistant MC44NGS mutant transfectants were tested for CD44 expression by flow cytometry using anti-CD44 BRIC214 mAb, and transfectants expressing comparable levels of CD44NGS mutants selected for further study. Immunoprecipitation of the CD44NGS mutants from lysates of radiolabeled transfectants revealed unequal glycan content of the six potential N-linked glycosylation sites (Fig. 2). Mutation of the Asn residue in NGS 1, 2, and 6 resulted in a minimal shift in the molecular mass (M_r) of CD44 (Fig. 2 and data not shown). Simultaneous mutation of Asn in NGS 3 and 4 resulted in a significant decrease in molecular mass, of ~5–8 kD, while immunoprecipitation of CD44 containing Asn substitution by Ile in NGS5 revealed a greater M_r reduction, of ~15 kD (Fig. 2). A combination of Asn mutations in NGS2-5 (Fig. 2) and of Ser/Thr mutations in NGS3-5 (data not shown) resulted in the expression of a 60–62-kD CD44 polypeptide, indicating that most of the N-linked glycosylation of CD44H expressed in MC melanoma cells requires the integrity of NGS 3/4-5.

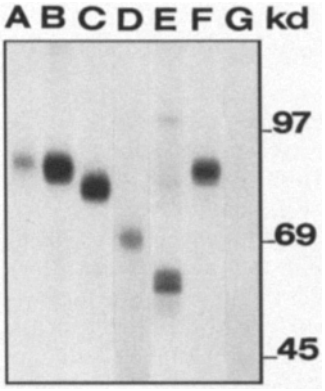


Figure 2. Immunoprecipitation of CD44HNGS mutants from ^{35}S-radiolabeled MC transfectant lysates. Immunoprecipitations were performed with the BRIC214 mAb from radiolabeled lysates of transfectants expressing mutants containing Asn substitutions at potential NGS. Lysates were from lane *A*, MC44HNGS1; lane *B*, MC44HNGS2; lane *C*, MC44HNGS3-4; lane *D*, MC44HNGS5; lane *E*, MC44HNGS2-5; lane *F*, MC44H; lane *G*, MC44H, immunoprecipitated with unrelated isotype-matched mAb. Molecular mass(kD) is indicated.

Substitution of serine/threonine residues of NGS 1, 3/4, and 5 by alanine (Table I) resulted in a shift in molecular weight comparable to that observed upon mutation of the corresponding Asn residues (data not shown).

NGS Mutations Induce Alterations in Antibody-binding Epitopes of CD44

All of the NGS mutants expressed in MC cells were tested for recognition by four anti-CD44 mAb, BRIC214, BRIC-222, BRIC235, and KZ1 (Antsee et al., 1991). BRIC214, BRIC222, and KZI are noncross-blocking antibodies (Antsee et al., 1991) and have been proposed to define three separate epitope groups. BRIC214 and BRIC235 cross-block each other, and belong to the same antibody group although they may recognize distinct epitopes (Antsee et al., 1991). All of the individual as well as the composite NGS mutants expressed in MC melanoma cells were recognized by the BRIC 214 and KZ1 mAbs (Table I and data not shown). Moreover, single NGS mutant and wild-type CD44H transfectants displayed comparable staining intensity with the two mAb in flow cytometry assays (Table I and data not shown), and all of the NGS mutants could be immunoprecipitated with BRIC214 (Fig. 1 and Table I). The BRIC 222 mAb bound MC transfectants expressing individual NGS mutants at positions 1 to 5 less strongly than MC44H cells and failed to stain MC transfectants expressing NGS2-5 and NGS3-5 mutants (Table I). The anti-CD44 mAb BRIC 235, which immunoprecipitates CD44H and blocks CD44-HA interaction (this study), was observed to bind MC44H and all of the individual NGS mutant transfectants comparably in flow cytometry assays. However, BRIC235 failed to immunoprecipitate CD44NGS mutants, with the exception of NGS6, from corresponding lysates (Table I).

NGS Mutations within the HA-binding Domain Abrogate CD44-mediated Cell Attachment to HA

To address the possible functional effects of NGS mutations, MC44NGS mutant transfectants were tested for adhesion to HA-coated plates. Although, CD44H expression promoted MC transfectant attachment to HA, none of the CD44NGS mutant transfectants, with the exception of MC44NGS 6, displayed significant CD44H-mediated adhesion to HA-coated substrate (Fig. 3 *A*). Binding of NGS mutant transfectants to fibronectin, collagen I, laminin, collagen VI, and chondroitin and heparan sulfate was comparable to that of wild-type CD44H transfectants (Fig. 3 *B* and data not shown).

Different Types of Inhibitors of N-linked Glycosylation Can Abrogate, Maintain, or Enhance CD44-mediated Adhesion to HA

Treatment of MC44H transfectants with tunicamycin ab-

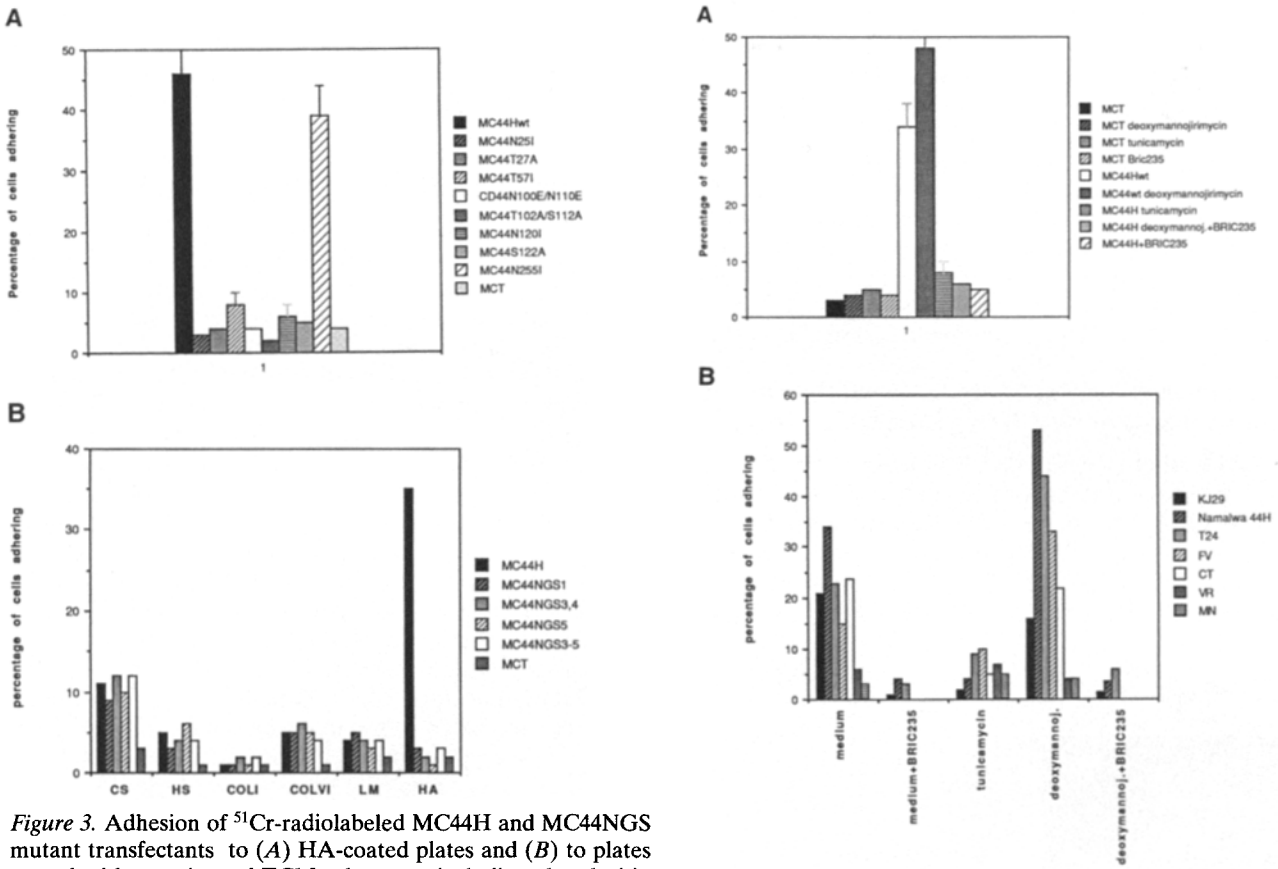


Figure 3. Adhesion of ^{51}Cr-radiolabeled MC44H and MC44NGS mutant transfectants to (*A*) HA-coated plates and (*B*) to plates coated with a variety of ECM substrates, including chondroitin sulfate (*CS*), heparan sulfate (*HS*), collagen type I (*ColI*), collagen type VI (*ColVI*), laminin (*LM*), and hyaluronate (*HA*). NGS mutants are indicated. MCT are MC cells transfected with pSV2Neo only. All experiments were performed in sextuplicate and adhesion is expressed as the percentage of cells which adhered to each substrate following the 30-min incubation period at 4°C.

rogated their adhesion to HA (Fig. 4 *A*) without significantly affecting the level of CD44 cell surface expression (data not shown). In addition, tunicamycin treatment of the human lymphoma Namalwa CD44H transfectants as well as several tumor cell lines which constitutively express CD44H and bind HA in a CD44-dependent manner, resulted in decreased adhesion to HA-coated plates (Fig. 4 *B*). To determine whether complex N-linked glycosyl chains may participate in CD44-mediated MC melanoma adhesion to HA, MC44H transfectants were treated with 1-deoxymannojirimycin which specifically inhibits mammalian Golgi α-mannosidase I activity and the conversion of high mannose to complex oligosaccharides (Roth, 1987). As a result, cell surface and secreted glycoproteins are synthesized which contain $Man_9GlcNAc_2$, but typically lack N-linked galactose, fucose, and sialic acid residues. Deoxy-

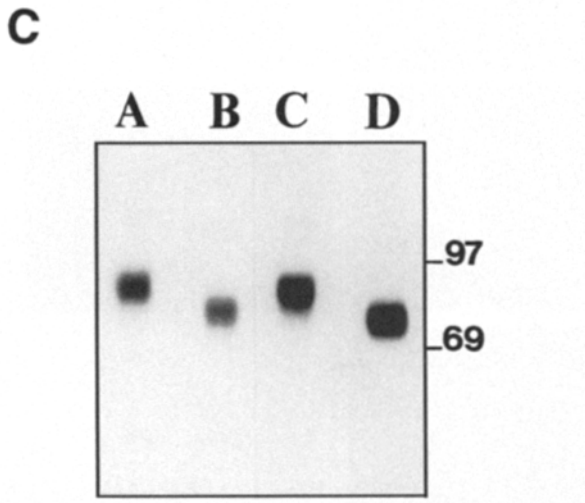


Figure 4. Effect of tunicamycin and 1-deoxymannojirimycin on CD44H-mediated cell attachment to HA-coated substrate. (*A*) Adhesion of ^{51}Cr-radiolabeled native, tunicamycin, and deoxymannojirimycin-treated MC-T (MC cells transfected with pSV2Neo only) and MC44H cells to HA in the presence or absence of the blocking BRIC235 mAb. The percentage of cells adhering to HA-coated plates after a 30-min incubation at 4°C is indicated. (*B*) Adhesion to HA-coated plates of a panel of ^{51}Cr-radiolabeled native, tunicamycin, and deoxymannojirimycin-treated cells which express CD44H. The cell lines used were KJ29, human renal carcinoma; Namalwa 44H, human lymphoma Namalwa, stably transfected with CD44H; T24, human bladder carcinoma; FV and CT, human melanomas derived from two different patients; VR and MN, human breast carcinomas derived from two different patients. Adhesion assays were performed as in *A*. All adhesion assays were done in sextuplicate. (*C*) Immunoprecipitation of CD44, using BRIC214 mAb, from lysates of ^{35}S-radiolabeled native and deoxymannojirimycin-treated MC44H and T24 cells. Lysates were from lane *A*, untreated T24 cells; lane *B*, deoxymannojirimycin-treated T24 cells; lane *C*, untreated MC44H cells; lane *D*, deoxymannojirimycin-treated MC44H cells.

mannojirimycin treatment resulted in a significant reduction in the molecular mass of CD44H (Fig. 4 *C*) but was accompanied by an augmentation in CD44-mediated attachment of MC cells to HA-coated surfaces (Fig. 4 *A*). The observed increase in HA binding was blocked by anti-CD44 mAb BRIC235 (Fig. 4 *A*). Deoxymannojirimycin treatment of the other cell lines revealed either no significant change or an increase in attachment to HA (Fig. 4 *B*). It is noteworthy, however, that two breast carcinoma cell lines failed to bind HA despite expressing CD44H. Tunicamycin and deoxymannojirimycin treatment of these cells failed to induce CD44-mediated adhesion (Fig. 4 *B*).

Deglycosylation of Soluble CD44-Ig Fusion Protein Reduces Its Ability to Bind HA in Mammalian Tissues

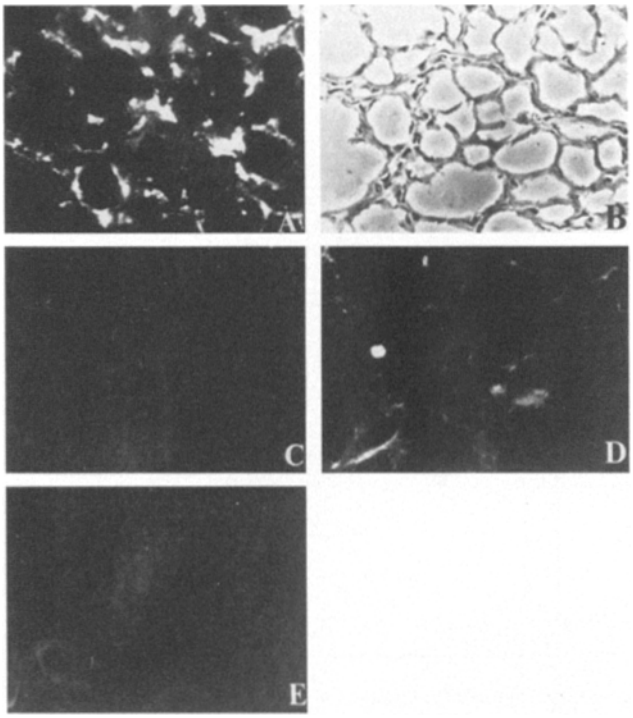
To provide further evidence that N-linked glycosylation of CD44 plays a role in regulating its ability to interact with HA, CD44-Ig fusion protein (CD44Rg) produced in COS cells was subjected to N-glycanase treatment and compared to untreated CD44Rg for staining rat kidney papilla which is rich in HA. Treatment of CD44Rg with N-glycanase abrogated its ability to bind tissue-associated HA (Fig. 5, *A–E*) and caused a significant reduction in its molecular mass (Fig. 5 *F*). Pretreatment of frozen tissue sections with hyaluronidase abolished CD44Rg binding (Fig. 5 *D*).

Development of CD44H Ser-Gly Dipeptide Mutants

CD44H contains four Ser-Gly dipeptides in the membrane proximal domain, which provide potential sites for glycosaminoglycan side chain attachment (Mann et al., 1990). To determine whether any of these sites is substituted in GAG side chains in MC cells, and to assess the potential role of these sites in regulating CD44H-HA interaction, the serine residue within each Ser-Gly motif was mutated to a glycine (Fig. 6). CD44 cDNA mutants encoding S180G/S190G (Ser-Gly1 2), S231G (Ser-Gly 3), S180G/S190G/S231G (Ser-Gly 1-3), S258G (Ser-Gly 4), and S180SG/S190G/S231G/S258G (Ser-Gly 1-4) were introduced into MC cells by electroporation and transfectants expressing comparable levels of CD44Ser-Gly mutants on the cell surface were selected for further analysis (Table I). Immunoprecipitation of each of the CD44Ser-Gly mutants from lysates of radiolabeled MC transfectants revealed that mutation of the serine residues had not resulted in a significant reduction of the molecular mass of CD44 (Fig. 7). This observation suggests that in MC melanoma cells these sites are not occupied by large GAG side chains, although it does not exclude the possibility that they may contain O-linked monosaccharides. Surprisingly, the molecular weight of Ser-Gly 4 and Ser-Gly 1-4 mutants was slightly increased with respect to that of wild-type CD44H (Fig. 7).

Ser-Gly Mutations Reduce CD44-mediated Adhesion to HA

CD44 Ser-Gly mutant transfectants were tested for adhesion to HA-coated plates. In comparison to CD44H transfectants, adhesion of CD44Ser-Gly mutant transfectants was found to be decreased by ~50% (Fig. 8). No significant difference in adhesiveness among the different CD44Ser-Gly mutant transfectants was observed, indicating that the effect of substituting one serine residue on CD44-mediated adhesion to HA was comparable to that of mutating all four Ser-Gly–associated serines. Similar to CD44NGS mutant transfectants, MC cells expressing Ser-Gly dipeptide mutants were not observed to display adhesiveness to collagens, laminin or chondroitin, and heparan sulfate (data not shown).



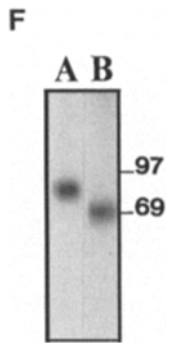


Figure 5. Immunofluorescence (*A*, *C–E*) and phase contrast (*B*) micrographs of rat renal papilla tissue sections. Untreated frozen sections were stained with (*A*) CD44Rg, (*C*) CD44Rg pretreated with N-glycanase and (*E*) CD8Rg control. Hyaluronidase-treated tissue stained with CD44Rg is shown in *D*. (*F*) Immunoprecipitation of untreated (lane *A*) and N-glycanase–treated (lane *B*) CD44Rg.

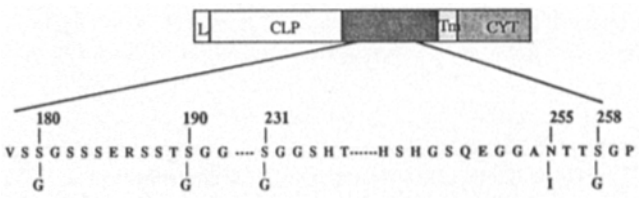


Figure 6. Schematic representation of Ser-Gly and NGS6 mutations. Amino acids are denoted by their single letter code and numbered from the beginning of the leader peptide (*L*).

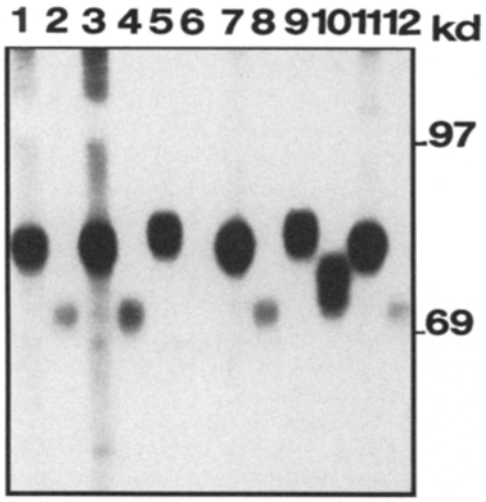


Figure 7. Immunoprecipitation of MC44Ser-Gly mutants. All mutants were immunoprecipitated from ^{35}S-radiolabeled transfectant lysates and corresponding 24 h cell culture supernatants with BRIC 214 mAb. Immunoprecipitates were, respectively, from lysates and supernatants of lanes *1* and *2*, MC44H; lanes *3* and *4*, MC44Ser-Gly 1-2; lanes *5* and *6*, MC44Ser-Gly 1-4; lanes *7* and *8*, MC44Ser-Gly 1-3; lanes *9* and *10*, MC44Ser-Gly 4; lanes *11* and *12*, MC44Ser-Gly 4/N258I. Molecular mass markers are indicated.

Mutation of 258S Induces Glycosylation of 255N (NGS6) and Enhances Spontaneous Shedding of CD44

In an effort to explain the increase in molecular mass after substitution of 258Ser by Gly, we addressed the possibility that the potential N-linked glycosylation site immediately upstream of 258Ser (NGS6) (Fig. 6) might become glycosylated as a result of the 258Ser mutation. Substitution of 255Asn by Ile in the Ser-Gly 4 mutant (N255I/S258G) resulted in the restoration of the molecular mass of CD44Ser-Gly 4 to the level of that of wild-type CD44H (Fig. 7, lane *11*). To provide further evidence that the increase in molecular mass of Ser-Gly 4 and Ser-Gly 1-4 mutants was due to N-linked glycosylation, CD44H, CD44Ser-Gly 4, and CD44Ser-Gly 1-4 immunoprecipitates were treated with N-glycanase and the corresponding molecular masses compared by SDS-PAGE. Migration of the different N-glycanase–treated immunoprecipitates was observed to be similar (Fig. 9), supporting the notion that a mutation of 258Ser resulted in glycosylation of NGS6.

CD44H as well as other CD44 isoforms are spontaneously shed from various cell types (Bartolazzi et al., 1994, 1995). Spontaneous shedding of CD44 was observed to be significantly increased in MC44S258G cells with respect to MC44H transfectants (Fig. 7, lane *10*). However, mutation of N255I in the Ser-Gly 4 mutant restored shedding to a level similar to that observed in MC44H cells (Fig. 7, lane *12*). Mutation of Ser-Gly 1-4 resulted in abrogation of detectable CD44 shedding from MC transfectants (Fig. 7, lane *6*).

Discussion

The structural features of CD44 required for HA binding and regulation of CD44-mediated cell adhesion to HA are still poorly understood. Recent work has shown that mutation of a single arginine residue (41Arg) within the cartilage link protein homology domain of CD44 abrogates binding to HA (Peach et al., 1993). A subsequent, more extensive study on HA-binding domains showed that all known cell surface and ECM HA receptors contain one or several BX_7B motifs which are required for HA binding (Yang et al., 1994). Although the discovery of these HA-binding motifs is a major step toward understanding the mechanism of interaction between HA and HA receptors, the observation that several cell types which express CD44H do not bind HA unless they are triggered by specific stimuli suggests that additional structural features of HA-binding proteins may play a role in regulating receptor affinity for HA. The results of the present study indicate that

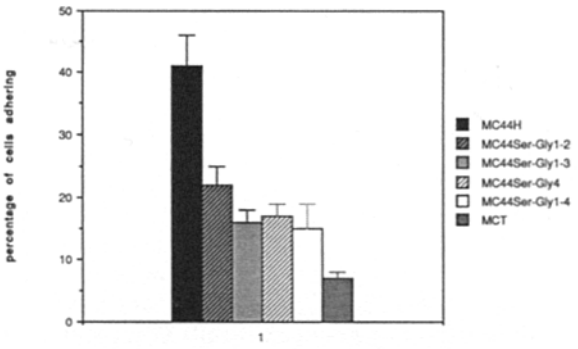


Figure 8. Adhesion of ^{51}Cr-radiolabeled MC44Ser-Gly transfectants to HA-coated plates. MC44Ser-Gly mutant transfectants are indicated. MCT represent MC cells transfected with pSV2Neo only. All experiments were done in sextuplicate and adhesion is expressed as the percentage of cells adhering to HA-coated plastic after a 30-min incubation at 4°C.

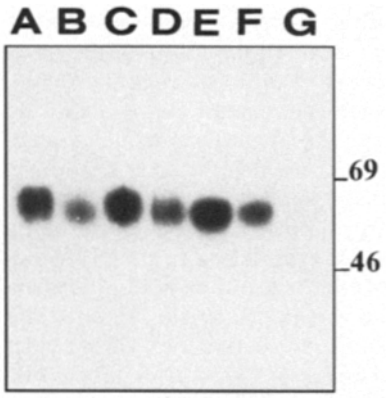


Figure 9. N-glycanase treatment of CD44H and CD44Ser-Gly mutants. Immunoprecipitation of CD44H and CD44Ser-Gly mutants from lysates of ^{35}S-radiolabeled MC transfectants was performed using the BRIC214 mAb. Immunoprecipitates bound to protein A–Sepharose beads were treated with N-glycanase before being eluted and subjected to SDS-PAGE. Lysates were from MC transfectants expressing: lane *A*, CD44H; lane *B*, CD44Ser-Gly 1-2; lane *C*, CD44Ser-Gly 1-3; lane *D*, CD44Ser-Gly 4; lane *E*, CD44Ser-Gly 1-4; lane *F*, CD44Ser-Gly 3; lane *G*, CD44H, immunoprecipitated with an isotype-matched mouse IgG.

CD44-HA interaction is highly sensitive to changes in CD44 N-linked glycosylation, and to a lesser extent to the integrity of Ser-Gly motifs.

Role of N-linked Glycosylation in CD44H-HA Interaction

N-linked oligosaccharides are thought to play an important role in the processing and physiologic function of glycoproteins (Olson and Lane, 1987; Rademacher et al., 1988; Yang et al., 1993), based on observations that inhibition of N-linked glycosylation can result in glycoprotein misfolding, abrogation of cell surface expression or loss of function. Treatment of MC transfectants as well as a number of cell lines which constitutively express CD44H with tunicamycin, resulted in abrogation of CD44-mediated attachment of the cells to HA-coated substrate which was not due to the loss of CD44 expression. Although these observations suggest that N-linked glycosylation of CD44 is important in CD44-mediated cell adhesion to HA, nonspecific glycosylation inhibitors, such as tunicamycin, block N-linked oligosaccharide addition to a multitude of cellular proteins. As a result, the abundance of misfolded proteins may obscure the normal fate and functional changes of any individual deglycosylated protein. We therefore used site-directed mutagenesis to develop single and multiple N-linked glycosylation site mutants in CD44H and address the effect of each mutant on the expression and HA-binding potential of CD44H in MC melanoma cells. A similar approach has shown that single NGS mutations alter expression, processing, cleavage as well as ligand-binding affinity of the transferrin receptor (Yang et al., 1993). Our results demonstrate that processing and transport of CD44H to the cell surface can occur in the absence of most N-linked glycosyl chains, since all of the mutants developed in this study could be expressed on the cell surface and were found to retain at least some epitopes recognized by the anti-CD44 mAb panel. However, changes in binding affinity of some of the antibodies, reflected by a reduction in staining or loss of the ability to immunoprecipitate CD44 mutants, suggest that mutation of each of the CLP-homology domain NGS may result in functionally significant conformational changes, sufficient to abrogate ligand binding. These results have at least three possible, nonmutually exclusive explanations. The first is that N-linked glycans themselves are involved in CD44-mediated adhesion, either by facilitating aggregation of CD44 molecules or by providing the appropriate conformation to the HA-binding domain. Such an explanation is not without precedent, since recent work has shown that a single N-linked oligosaccharide in the extracellular domain of CD2 provides the configuration necessary for CD2-CD58 interaction (Wyss et al., 1995), and that a single N-linked glycan regulates interactions between CD22 and its ligands (I. Stamenkovic, unpublished). The second possible explanation is that conformational alterations induced by mutation of asparagine and serine/threonine residues which constitute the potential N-linked glycan sites may participate in the loss of CD44-mediated cell adhesion to HA. It would imply that the capability of CD44 to mediate cell adhesion to HA is highly sensitive to the mutation of multiple residues within the CLP domain which are unrelated to the BX_7B motifs. Finally, conformational changes due to mutation of one N-linked glycosylation site may render other sites inaccessible to appropriate glycosyltransferases. Thus, it is possible that the 15-kD reduction in molecular mass after mutation of NGS5 is not due to the loss of glycans at NGS5 only but may reflect impaired glycosylation of other potential sites as well. Additional work will be necessary to resolve the mechanism by which each NGS mutation inhibits CD44-mediated cell adhesion to HA. However, the observation that treatment of CD44Rg with N-glycanase results in almost complete abrogation of its ability to bind HA in tissues provides support to the notion that N-linked glycosyl chains play an active role in the regulation of CD44-mediated adhesion to HA.

Treatment of MC44H cells with 1-deoxymannojirimycin, which inhibits the conversion of high mannose oligosaccharides to complex carbohydrates, enhanced adhesion to HA. Inhibition of complex N-linked oligosaccharide synthesis in Namalwa CD44H transfectants, T24 and FV1 cells all of which display CD44-mediated attachment to HA-coated plates, also resulted in a significant increase in CD44-dependent adhesion to HA-coated substrate. These observations suggest that high mannose N-linked glycans may help maintain the HA-binding domain in its appropriate conformation whereas complex N-linked oligosaccharides may inhibit CD44-HA interaction. It is conceivable that the structure and charge of complex galactose, fucose, and sialic acid–containing oligosaccharides synthesized by some cells may reduce CD44-HA–binding affinity.

Importantly, our results suggest that the mechanisms which regulate the adhesive properties of CD44H may not be the same in all cell types. In some cells, which bind HA, deoxymannojirimycin treatment failed to augment CD44-mediated adhesion. In two breast carcinoma cell lines, expression of CD44H failed to promote attachment to HA, and treatment of these cells with tunicamycin and deoxymannojirimycin did not induce adhesion. It therefore seems likely that glycosylation may promote different CD44-associated adhesive properties in different cell types, while in some cells factors other than glycosylation may be the primary regulators of CD44-mediated attachment to HA.

Recently, two independent studies (Katoh et al., 1995; Lesley et al., 1995) showed that glycosylation is involved in the regulation of CD44-HA interaction. Unlike the observations reported here, prolonged tunicamycin treatment (2–3 d) of some cell lines changed the endogenous CD44 HA-binding function from inactive to active (Lesley et al., 1995). The discrepancy between these observations and ours is most likely due to the different properties of the cell lines tested, and possibly to the different duration of cell exposure to tunicamycin which may have caused additional posttranslational changes. Thus, our observations do not necessarily contradict the results reported by Lesley et al. (1995) and Katoh et al. (1995) but rather underscore the complex nature of the regulation of CD44-dependent adhesion.

Effects of Ser-Gly Motif Mutation on the Structure and Function of CD44H

To determine the role of potential glycosylation sites distal to the HA-binding domain in the regulation of ligand rec-

ognition, we mutated the four Ser-Gly motifs in the membrane proximal domain of CD44H individually or in combination. Ser-Gly dipeptides are potential sites for GAG side chain attachment (Mann et al., 1990). Although high M_r GAG side chains have recently been shown to be associated with expression of CD44 variant exon 3 (Bennett et al., 1995; Bartolazzi et al., 1995), there is evidence that CD44H can behave as a facultative proteoglycan, bearing GAG chains in some cell types but not in others (Stamenkovic et al., 1989; Stamenkovic et al., 1991; Jalkanen and Jalkanen, 1992; Jackson et al., 1995). Mutation of all four Ser-Gly motifs failed to produce a significant reduction in M_r which would result from abrogation of GAG side-chain attachment, suggesting that in MC cells, CD44 does not contain Ser-Gly–associated high molecular weight GAGs. However, this observation does not exclude the possibility that these serine residues may be sites of attachment of O-linked glycosyl chains. Because O-linked carbohydrates typically consist of oligo- or even monosaccharides, removal of a few O-linked glycosylation sites would not be expected to detectably alter the M_r of an 85-kD glycoprotein on a standard SDS gel.

Ser-Gly mutations in CD44H highlighted the sensitivity of HA binding to substitution of amino acids distal to the HA-recognition domain. Each of the Ser-Gly mutations reduced CD44-mediated binding to HA of the corresponding transfectants by ~50% with respect to wild-type CD44H-expressing counterparts. The effect of a single Ser-Gly mutation, however, was the same as that of mutation of all four motifs. The reduction in HA binding is likely to be due to a conformational change of CD44, but whether such a change is due to the substitution of the serine residues themselves or to the loss of putative oligosaccharides which they may carry remains to be determined.

Two unexpected observations were made using Ser-Gly dipeptide mutants. The first was that mutation of the serine residue constituting the fourth Ser-Gly dipeptide, immediately downstream of NGS6 resulted in glycosylation of NGS6 reflected by an increase in M_r which could be restored to the wild-type level by mutation of the NGS6 asparagine residue. Migration of immunoprecipitates of N-glycanase–treated CD44 from MC44H and MC44SG4 lysates on SDS-PAGE was comparable, giving further support to the notion that mutation of Ser-Gly 4 results in glycosylation of NGS6. This finding suggests that 258Ser introduces a structural feature which blocks N-linked glycosylation on 255Asn.

The second unexpected event resulting from the mutation of Ser-Gly 4 was a significant increase in spontaneous shedding of CD44. The mechanisms which regulate CD44 shedding are unknown, although proteolytic cleavage of the extracellular domain appears likely (Bazil and Horeijsi, 1991; Bartolazzi et al., 1994, 1995). We have shown recently that spontaneous shedding of CD44 splice variants expressed in MC melanoma (Bartolazzi et al., 1994) and in Namalwa lymphoma cells (Bartolazzi et al., 1995) is significantly greater than that of CD44H. Mutation of 258Ser enhanced CD44 release whereas simultaneous mutation of 255Asn and 258Ser restored shedding to baseline levels. These observations suggest that glycosylation of NGS6 as a result of mutation of 258Ser altered local CD44 conformation, rendering the cleavage site more accessible to the putative protease. Analogous observations have been made on N-glycosylation–dependent proteolytic cleavage of the transferrin receptor (Hoe and Hunt, 1992). Interestingly, mutation of all four Ser-Gly dipeptides resulted in abrogation of detectable shedding suggesting that the structural change induced by the substitution of all four serine residues may conceal or alter the proteolytic cleavage site.

Taken together, our results show that CD44H-mediated cell adhesion to HA-coated surfaces and tissue-bound HA is highly sensitive to structural changes in both the HA-binding and the membrane-proximal domain. N-linked glycosylation is shown to be involved in the regulation of CD44-mediated adhesion to HA, but it is not the only regulator, since we have shown that some cell lines which express CD44H fail to adhere to HA irrespective of whether they are treated with various inhibitors of glycosylation or not. Furthermore, glycosylation-associated regulatory mechanisms of CD44-HA interaction appear to be both complex and cell type–specific. Elucidation of the composition of N-linked carbohydrates capable of inhibiting or enhancing CD44-HA interaction will be of interest and may help elucidate, in part, the selectivity with which CD44H mediates adhesion to HA.

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