Identification of Hyaluronic Acid Binding Sites in the Extracellular Domain of CD44

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Abstract. CD44 is a polymorphic glycoprotein expressed on the surface of many tissues and cell lines which has been implicated in a number of cellular functions including lymphocyte homing to mucosal lymphoid tissue (Peyers patches), leukocyte activation, lymphopoiesis, and tumor metastasis. The predominant isoform found on human leukocytes, CD44H, is a receptor for hyaluronic acid. Because of the prominent role CD44 plays in diverse biological processes, we set out to identify the hyaluronic acid binding site(s) in the extracellular domain of CD44H. Using truncation and site-directed mutagenesis we identified two regions containing clusters of conserved basic residues which are important in hyaluronic acid binding. One of these regions is situated near the NH_2 terminus and is homologous to other hyaluronic acid binding proteins including cartilage link protein. The other more membrane proximal region lies outside the link protein homologous domain. Mutagenesis of basic residues within these regions established their role as determinants in hyaluronic acid binding. Mutation of Arg 41, a position where a basic residue is conserved in all hyaluronic acid binding proteins, completely abolished binding suggesting that this residue plays a critical role in hyaluronic acid binding.

CD44 is a broadly distributed polymorphic transmembrane glycoprotein that is also known as Pgp-I (Hughes et al., 1981; Lesley and Trowbridge, 1982), In (Lu)-related p80 (Telen et al., 1984), Hermes antigen (Jalkanen et al., 1986), ECMRIII (Carter and Wayner, 1988), HUTCH-1 (Gallatin et al., 1989) and p85 (Quackenbush et al., 1990). Studies using mAbs have implicated CD44 in a number of important cellular functions including lymphocyte homing to mucosal lymphoid tissue (Peyers patches; Jalkanen et al., 1987), leukocyte activation (Huet et al., 1989; Shimizu et al., 1989; Denning et al., 1990; Webb et al., 1990), lymphopoiesis (Miyake et al., 1990*a*), and as an extracellular matrix receptor (Carter and Wayner, 1988; Jalkanen and Jalkanen, 1992). It has become increasingly apparent that this diverse range of biological functions can be attributed to CD44 acting as a cell adhesion molecule. Recent reports also suggest that CD44 plays an important role in tumor metastasis (Sy et al., 1991; Günthert et al., 1991), with expression of CD44 transcripts being elevated in many human tumors (Stamenkovic et al., 1991) and repressed in others (Shtivelman and Bishop, 1991).

Isolation and sequencing of cDNAs encoding the predominant form of human CD44 expressed by cells of hemopoietic origin predicted a 37-kD protein with an NH_2-terminal domain homologous to the cartilage link protein family (Stamenkovic et al., 1989; Goldstein et al., 1989). Subsequent sequencing of cDNAs encoding mouse (Zhou et al., 1989; Nottenburgh et al., 1989), baboon (Idzerda et al., 1989), and bovine (Bosworth et al., 1991) CD44 have revealed >90% identity for the predicted NH_2-terminal 195 amino acids and considerable sequence disparity for the remainder of the extracellular domain (Bosworth et al., 1991). The NH_2-terminal 135-amino acid domain of CD44 not only has ~30% sequence homology to regions of cartilage link proteins (Dëak et al., 1986; Doege et al., 1986), but has since been shown to be homologous to regions of the proteoglycans versican and aggrecan (LeBaron et al., 1992), and to a recently described tumor necrosis factor-inducible protein TSG-6 (Lee et al., 1992). All of these proteins share the property of being able to bind the extracellular matrix glycosaminoglycan hyaluronic acid (HA).[1] In the case of cartilage link protein, the sites for interaction with HA have been determined (Goetinck et al., 1987). Using mAbs and synthetic peptides these sites were located in tandemly repeated sequences characterized by clusters of positively charged amino acids. These tandem repeats lay within the region homologous to the other HA-binding proteins including CD44.

There is considerable diversity in the size of CD44 molecules expressed on different cell types which has been attributed to variations in the degree of glycosylation and also to differential splicing of up to 10 exons in the membrane prox-

1. *Abbreviations used in this paper*: HA, hyaluronic acid; Rg, receptor globulins.

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imal region of the extracellular domain (Omary et al., 1988; Brown et al., 1991; Camp et al., 1991; Stamenkovic et al., 1991; Jackson et al., 1992; Screaton et al., 1992). In humans the predominant hemopoietic form (CD44H) has an actual molecular mass of 80–90 kD and is expressed by cells of both mesodermal and neuroectodermal origin. A splice variant called CD44E is a 150-kD protein found mainly on cells of epithelial origin, including carcinomas (Stamenkovic et al., 1991). This larger CD44E isoform, like all CD44 splice variants, has an identical NH_2-terminal region to CD44H but its cDNA codes for an additional 135–amino acid domain which is inserted after amino acid 220 in the 248 residue extracellular domain of CD44H (Stamenkovic et al., 1991).

CD44H has been shown to bind HA and other glycosaminoglycans (Aruffo et al., 1990; Miyake et al., 1990*b*; Culty et al., 1990), and to extracellular matrix proteins including types I and VI collagen and fibronectin (Carter and Wayner, 1988; Jalkanen and Jalkanen, 1992). Interestingly, CD44E, although containing the NH_2-terminal domain homologous to cartilage link and other HA-binding proteins, has considerably reduced affinity and/or avidity for HA (Stamenkovic et al., 1991). This is in contrast to the murine homologue of CD44E which, upon transfection into a CD44 negative T lymphoma cell line, mediated adhesion to HA-bearing cells (He et al., 1992). Many tissues also express CD44H but fail to bind HA (Hyman et al., 1991). Requirements for HA recognition by CD44 are not well defined although it is known that the cytoplasmic domain of CD44 is necessary for binding HA from solution and that the mAb IRAWB 14, can induce HA binding by some CD44+ cell lines that do not constitutively bind the ligand (Lesley et al., 1992).

Because of the prominent role CD44 plays in a number of diverse biological processes including leukocyte adhesion, we set out to identify the HA binding site(s) in the extracellular domain of CD44. Using truncation and site-directed mutagenesis of soluble forms of CD44 we have identified two clusters of basic residues that are associated with HA-binding. Mutagenesis of specific amino acids in these regions allowed us to identify critical residues involved in the CD44/HA interaction.

Materials and Methods

mAbs

The following anti CD44 mAbs were used in COS cell transfection immunofluorescence studies: A3D8 (Sigma Immunochemicals, St. Louis, MO); BU52 (Binding Site, Inc., San Diego, CA); IM7 (PharMingen, San Diego, CA), A1G3 (Oncogene Science, Uniondale, NY), and F10.44.2 (R&D Systems, Minneapolis, MN).

Construction of Truncated CD44 Rg Expression Plasmids

Mutant CD44 receptor globulins (Rg) plasmids encoding truncations of the membrane proximal extracellular domain were prepared by the PCR using a CD44H Rg expression plasmid previously described (Aruffo et al., 1990). An oligonucleotide primer was synthesized encoding a complementary sequence upstream of the MluI restriction site in the CMV promotor region of the πH3M expression vector (Aruffo and Seed, 1987). This primer (FP3) had the following sequence:

5′-GTA CGG GCC AGA TAT ACG CGT TGA CAT TGA TTA-3′

Reverse primers containing a BglII restriction site were synthesized with the following sequences:

F1-B 5′-CGC GAG ATC TGA TGT ACA ATC TTC TTC AGG-3′
F1-C 5′-CGC GAG ATC TAT GGT AAT TGG TCC ATC AAA-3′
F2 5′-CGC GAG ATC TTC ACT GGA GGA GCC GCT GCT-3′
F3 5′-CGC GAG ATC TGG ACT GTC TTC GTC TTC GTC-3′

Each primer encoded complementary sequences of regions in the membrane proximal extracellular domain of CD44H. PCR products therefore encoded the initial 131 amino acids of CD44 (F1-B), 145 residues (F1-C), 186 residues (F2), and 210 residues (F3) (see Fig. 1 *A*). Amino acids are numbered from the beginning of the signal peptide. PCR conditions consisted of 30 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C, using Taq polymerase and reagents recommended by the vendor (Perkin Elmer Cetus, Emeryville, CA).

PCR products were digested with HindIII and BglII and ligated to HindIII-BamHI–cut CDM7 vector containing genomic sequence encoding the hinge, CH2, and CH3 domains of a human IgG1 (Aruffo et al., 1992). Constructs encoding each of the mutants as well as wild-type CD44H Rg and CD44E Rg (Stamenkovic et al., 1991) were transfected into COS cells and the resulting fusion proteins recovered from culture supernatants using protein A-Sepharose as previously described (Aruffo et al., 1990). Purified proteins were visualized on 7.5% SDS-PAGE under reducing and nonreducing conditions. Protein concentrations were determined using a protein estimation kit (BioRad Labs, Hercules, CA).

Construction of CD44 Rg Site-directed Mutant Expression Plasmids

Nine CD44 Rg site-directed mutants were prepared by encoding the desired mutation in overlapping oligonucleotide primers and generating the mutants by PCR (Ho et al., 1989), using the CD44H Rg plasmid construct as a template. Mutants were prepared in two groups. The first group involved mutations of basic residues in a membrane proximal region. Four of these mutants were prepared encoding one of the following mutations: R150A, R154A, K158A, and R162A. A fifth construct was prepared using PCR primers encoding the K158A and R162A substitutions while a sixth construct was made with PCR primers encoding all four amino acid substitutions. Each PCR primer used for this group of mutants also encoded a silent point mutation which introduced a KpnI restriction site so that the constructs could be digested and assessed for correct frame maintenance.

A second group of three mutants giving rise to amino acid substitutions near the NH_2-terminus of CD44 were also prepared in a similar manner and encoded the following mutations: R29A, K38A, and R41A. Each PCR primer used for this group of mutants also encoded a silent point mutation which introduced a BsaHI restriction enzyme site used for diagnostic digestion purposes.

Primers that were required for PCR reactions but not for introducing mutations included the forward primer FP3 described above while the reverse primer (ScaB) was synthesized encoding a complementary sequence in the 5′ untranslated region of IgG1 genomic DNA. This primer contained a ScaI restriction site and had the following sequence:

5′-GGG ATG CGT CCA GGC AGG-3′

PCR conditions consisted of 6 min at 94°C, 3 min at 72°C, 2 min at 55°C followed by 25 cycles of 1 min at 94°C, 2 min at 55°C and 3 min at 72°C. Pfu polymerase and reaction conditions were used as suggested by the vendor (Stratagene Corp., La Jolla, CA). PCR products were digested with MluI and ScaI and ligated to MluI-ScaI–cut CDM7 vector containing genomic sequence encoding the hinge and constant domains of a human IgG1 (Aruffo et al. 1992). To confirm that the desired mutations had been inserted and to verify the absence of secondary mutations, all CD44Rg site-directed mutants were sequenced by the dideoxy chain termination/extension reaction with Sequenase reagents used according to the manufacturer's recommendations (United States Biochemical Corp., Cleveland, OH). Constructs were transfected into COS cells and the resulting fusion proteins recovered from culture supernatants as previously described.

Hyaluronic Acid Binding Assay

Binding of purified CD44 Rg's to HA was assessed using an ELISA assay. Wells of polystyrene ELISA plates (Cappel, Durham, NC) were coated with 50 μl of a stock 5 mg/ml solution of HA (Sigma Immunochemicals) diluted to 5 μg/ml in 50 mM sodium carbonate, pH 9.6, for 16 h at 22°C. Remaining binding sites were blocked by the addition of 250 μl of 10× Specimen Diluent Concentrate (Genetic Systems, Seattle, WA) diluted 1:10 with ddH_2O, for 1 h at 22°C. After washing with PBS containing 0.05% Tween-20 (PBS-Tw), wells were incubated with 50 μl of various concentrations of

CD44 Rg's diluted in PBS, for 1 h at 37°C. After several washes with PBS-Tw, wells were incubated with 50 μl of goat anti-human $F(ab')_2$ HRP-conjugate (Tago Inc., Burlingame, CA) diluted 1:5,000 in the blocking solution used above, for 1 h at 22°C. Wells were then washed with PBS-Tw and incubated with 100 μl of EIA Chromogen Reagent (Genetic Systems) for 15 min at 22°C. The reaction was stopped by the addition of 100 μl of 1N H_2SO_4 (Genetic Systems) and the optical density measured on an ELISA reader at dual wavelengths 450, 630 nm.

mAb Binding to COS Cell Transfectants

Plasmids encoding CD44 Rg's were transfected into COS cells using a DEAE-dextran procedure (Aruffo et al., 1990). 24 h after transfection, cells were trypsinized, seeded onto 15 × 35-mm culture dishes and allowed to grow another 24 h. Cells were then washed with PBS, fixed with 2% formaldehyde (30 min) and permeabilized with 0.1% Triton X-100 (30 min). Cells were then incubated for 30 min with anti CD44 mAbs (diluted 1:200 to 1:300 in PBS). After washing with PBS, cells were incubated with the appropriate anti species IgG-FITC-conjugated antibody (Tago Inc.) at a 1:500 dilution, for 30 min. Cells were then washed twice with PBS and examined by fluorescence microscopy.

Table I. Binding of Anti-CD44 mAbs to COS Cells Transfected with Plasmids Encoding CD44 Rg Mutants

	mAb				
Mutant	IM7	BU52	A3D8	A1G3	F10.44.2
F1-B	–	+	+	ND	ND
F1-C	+	+	+	ND	ND
F2	+	+	+	ND	ND
F3	+	+	+	ND	ND
29 R/A	+	+	+	+	+
38 K/A	+	+	+	+	+
41 R/A	+	+	+	+	+
150 R/A	+	+	+	+	+
154 R/A	+	+	+	+	+
158K/A	+	+	+	+	+
162 R/A	+	+	+	+	+
Double	+	+	+	+	+
Quadruple	–	+	+	+	+
CD44H	+	+	+	+	+
CD44E	+	+	+	+	+
MOCK	–	–	–	–	–

2 d after transfection, cells were fixed, permeabilized, and labeled with the antibodies listed. *Double* refers to the construct encoding mutations at amino acid positions 158 and 162. *Quadruple* refers to the construct encoding mutations at amino acid positions 150, 154, 158, and 162. *Mock* refers to control COS cells transfected with CDM8 plasmid only. +, positive labeling; –, no labeling.

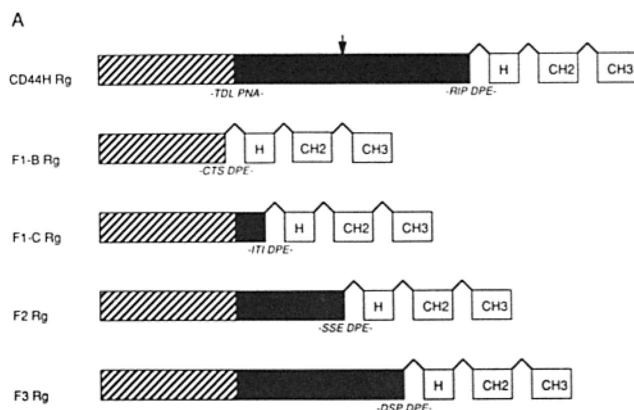
Results

Preparation of Soluble CD44 Rg Truncation Mutants

To define the limits of the extracellular domain of CD44H involved in HA binding, soluble chimeric proteins were prepared containing different-sized truncations of the membrane proximal and NH_2-terminal domains of CD44H fused to the hinge, CH2 and CH3 regions of a human IgG1 antibody. Four membrane proximal truncation mutants were prepared (Fig. 1 *A*). F1-B encoded the initial 131 amino acids of CD44 that make up the domain homologous to other HA-binding proteins, and included all conserved cysteine residues that may be involved in disulphide bonding. F1-C and F2 constructs (amino terminal 145 and 186 amino acids, respectively) encoded successively larger proteins, with F2 marking the end of the region of CD44 that is highly conserved among species. The F3 truncation mutant (amino terminal 210 amino acids) encoded a further region of 24 nonconserved residues.

Plasmids encoding these truncation mutant fusion proteins were prepared and transfected into COS cells as described in Materials and Methods. The resulting CD44 Rg proteins were purified using protein A-Sepharose columns and gave an average yield of 6 mg/L (Fig. 1 *B*).

The structural integrity of each CD44 Rg truncation mutant fusion protein was examined by assessing their ability to bind three anti-CD44 mAbs. All mutants bound the antibodies IM7, A3D8 and BU52 with the exception of F1-B which failed to bind IM7 (Table I). IM7 partially inhibits a B cell hybridoma from adhering to a bone marrow-derived



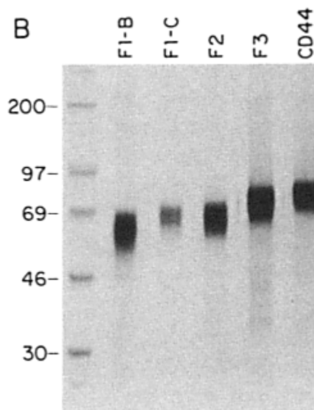


Figure 1. CD44 Rg truncation mutant fusion genes and purified proteins. (*A*) Fusion genes were constructed with truncation of cDNA coding for the membrane proximal region of the extracellular domain of CD44H. (▨) The NH_2-terminal 135-amino acid region homologous to other HA-binding proteins; (■) the remainder of the extracellular domain up to the site where alternatively spliced exons are inserted (amino acid 200; Stamenkovic et al., 1991). Arrow indicates the site where NH_2-terminal interspecies CD44 sequence homology ends (amino acid 195). H, CH2, and CH3 denote human IgG1 hinge and constant region exons, respectively. Introns are represented by connecting lines. The amino acid sequences predicted at the sites of fusion of the truncated extracellular domain with IgG1 are shown below each individual diagram. (*B*) ^{35}S-labeled fusion proteins purified from transfected COS cell culture supernatant by adsorption to protein A-Sepharose columns. Electrophoresis was performed in a 7.5% SDS-polyacrylamide gel under reducing conditions. Relative molecular mass of protein standards are shown on the left.

stromal line (Miyake et al., 1990*a*), and also partially blocks adhesion of lymphocytes to HA-coated culture wells (Miyake et al., 1990*b*). A3D8 recognizes an epitope on CD44 that modifies the ability of erythrocyte LFA-3 to interact with human T cell CD2 molecules (Haynes et al., 1989). The BU52 antibody has no known biological effect. The differential effect of these anti-CD44 mAbs in various biological assays suggests that they recognize distinct CD44 epitopes.

Four NH_2-terminal truncation constructs were also prepared, corresponding to deletions of the initial 55, 103, 131, and 165 amino acids of CD44H, respectively. Each of these constructs yielded several protein products when purified from culture supernatant by adsorption to and elution from protein A affinity columns. None of the anti-CD44 antibodies listed above bound to these truncation mutants. Because of this apparent lack of structural integrity of the NH_2-terminal deletion mutants, they were not used in further binding studies.

Binding of CD44 Rg Truncation Mutants to Hyaluronic Acid

To study the binding interaction between CD44 Rg membrane proximal truncation mutants and HA, an ELISA assay was developed in which soluble fusion proteins were incubated with HA immobilized on polystyrene wells. Bound CD44 Rg was detected by adding HRP-labeled anti-human IgG antibodies which recognized the human IgG portion of the fusion protein. Increasing concentrations of CD44H Rg gave a linear increase in the amount of fusion protein bound to HA (Fig. 2). When truncation mutants were incubated with immobilized HA, the two constructs having the smaller deletions, F2 and F3, showed enhanced binding with respect to CD44H. Despite retaining the cartilage link protein homologous domain, the two constructs that had larger deletions, Fl-B and Fl-C, exhibited a marked reduction in their ability to bind HA. As controls we used a soluble form of the splice variant CD44E which had been shown not to bind to HA when expressed on the surface of transfected tumor cells, and a CD7 Rg chimera. Both controls showed no appreciable binding to HA.

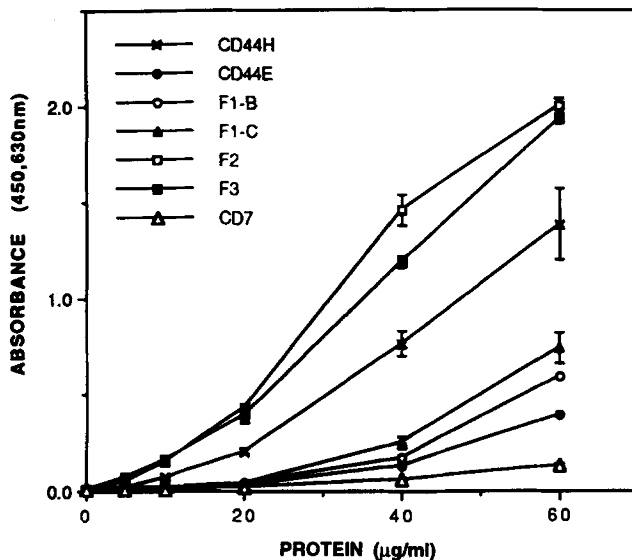


Figure 2. Binding of CD44 Rg truncation mutants to HA. Increasing concentrations of CD44 Rg truncation mutants were incubated with HA immobilized on ELISA plates. Bound fusion protein was detected using peroxidase-conjugated goat anti-human F(ab')$_2$ antibody. CD7 Rg was used as a human IgG fusion protein control. Data is expressed as the ±SD of duplicate wells and is representative of at least three experiments.

Preparation of CD44 Rg Site-directed Mutants

Hyaluronate binding assays established that the F2 construct was the smallest fully functional membrane proximal deletion mutant. The extracellular fragment of CD44 contained in this construct had two clusters of positively charged amino acids. One of these clusters was near the NH_2 terminus, within the link protein homologous domain (Fig. 3, shaded region *B*). The other cluster was located outside the link protein homologous region in a more membrane proximal position (Fig. 3, shaded region *A*) which had been deleted in the Fl-B and Fl-C constructs. Because glycosaminoglycans are known to interact via basic amino acids with their respective protein receptors (Jackson et al., 1991), and because both clusters of positively charged residues are conserved among species, we prepared site-directed mutants by individually substituting basic residues in each cluster with alanine (Fig. 3). Two additional mutants were prepared from region A in which the last two (double), and all four positively charged residues were replaced with alanine.

Site-directed mutants of soluble CD44 Rg were constructed using PCR oligonucleotide primer-directed mutagenesis. PCR products encoding the desired mutations were genetically fused to IgG heavy chain hinge and constant domains in the same manner as the soluble CD44 Rg truncation mutants described above. Subcloning, COS cell transfection and Rg purification were also performed as described above. An average protein yield of ~7 mg/L was obtained for the mutant proteins.

All constructs were sequenced to confirm that the desired mutations had been introduced and to ensure that no spurious mutations had been introduced by the PCR methodology. A Tyr to Ser substitution at position 109 was found in all of the constructs. However, independent CD44 clones also revealed a 109 Ser which is conserved in mouse (Zhou et al., 1989, Nottenburgh et al., 1989), bovine (Bosworth et

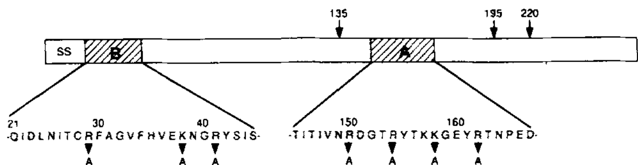


Figure 3. Schematic diagram of CD44H extracellular domain and the basic amino acid-rich regions where site-directed mutagenesis occurred. Basic amino acids mutated to alanine are indicated in the membrane proximal domain (A), and the NH_2-terminal domain (B). Amino acids are denoted by their single letter code and numbered from the beginning of the signal sequence (SS). Arrows at amino acid positions 135, 195, and 220 indicate the end of the NH_2-terminal domain homologous to other HA-binding proteins, the end of interspecies CD44 sequence homology, and the site where alternatively spliced exons are inserted, respectively.

al., 1991), and baboon CD44 (Idzerda et al., 1989), suggesting that this difference might have been due to a mistake in the original sequencing of the CD44H cDNA (Stamenkovic et al., 1989).

Binding of CD44 mAbs to Transfected COS Cells Expressing Site-directed Mutants

To examine whether site-directed mutagenesis had affected the structural integrity of mutant fusion proteins, the reactivity of each protein with five anti-CD44 antibodies was tested. All the constructs reacted with the anti-CD44 mAbs with the exception of the antibody IM7 which did not bind to the construct containing four mutations (quadruple) in the membrane proximal region (Table I). This was an indication that in this construct the mutations had either altered the epitope recognized by IM7 and/or the structure of the mutant protein had been perturbed. A1G3 has an epitope distinct from A3G8 (Denning et al., 1990), while F10.44.2 has no reported effect on the biological function of CD44 (Dalchau et al., 1980).

Binding of CD44 Site-directed Mutants to Hyaluronic Acid

To assess the ability of CD44 Rg site-directed mutants to bind HA we used the same ELISA assay used to study the truncation mutants. Results showed that all four membrane proximal mutants encoding a single substitution of a basic amino acid with alanine had reduced binding to HA (Fig. 4) with the mutant R154A having the least ability to bind HA. The construct which had two amino acid substitutions (double; K158A/ R162A), showed considerably reduced binding to HA compared with wild-type CD44H Rg. The construct with all four amino acid substitutions had an almost identical binding curve to the double mutation construct (data not shown).

Interestingly, in ELISAs where HA was substituted with other immobilized glycosaminoglycans, it was found that CD44H Rg also bound to chondroitin-4-sulfate and very weakly to chondroitin-6-sulfate, but not to dermatan sulfate or heparin sulfate (data not shown). However ~100 times more immobilized chondroitin-4-sulfate (500 μg/ml) was required to achieve similar binding levels of CD44H Rg as that seen with HA (5 μg/ml). It should be noted that neither HA nor chondroitin-4-sulfate can bind to plastic unless they are coupled in some way to protein which is present as a contaminant in the commercial glycosaminoglycan preparations. Therefore the amount of glycosaminoglycan bound to the plate may or may not reflect the concentration that was initially added. The double mutation construct also bound to a chondroitin-4-sulfate coated surface but to a much lower degree than to HA, even with an ~100-fold greater concentration of chondroitin-4-sulphate over HA immobilized to the plates (data not shown).

CD44 Rg NH_2-terminal site-directed mutants were also tested for their ability to bind HA by ELISA. All three mutants encoding single amino acid substitutions showed reduced binding to immobilized HA with the mutation R41A causing an almost complete loss of binding (Fig. 5). The mutant K38A bound HA somewhat less effectively than CD44H while the R29A mutant had HA binding capacity intermediate to the other two constructs.

Discussion

Truncation mutagenesis and site-directed mutagenesis of a

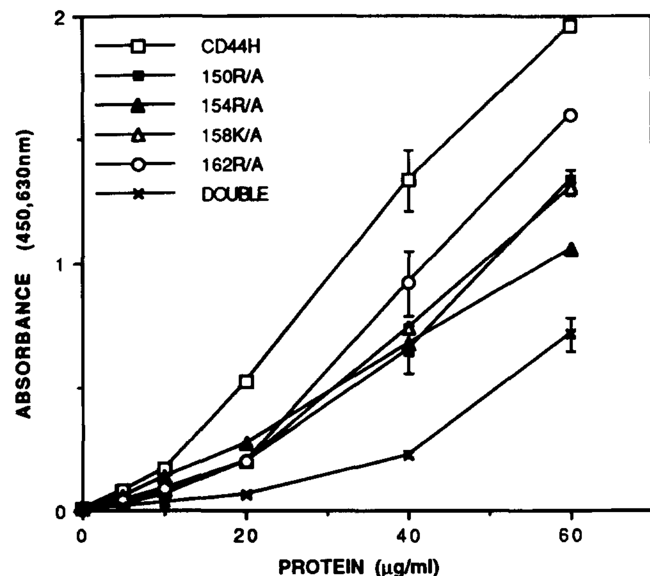


Figure 4. Binding of CD44 Rg membrane proximal site-directed mutants to HA. Increasing concentrations of CD44 Rg's encoding site directed mutations in the membrane proximal region were incubated with HA immobilized on ELISA plates. Bound fusion protein was detected as for Fig. 2. DOUBLE refers to the fusion construct containing both the K158A and R162A mutations. Data is expressed as the ±SD of duplicate wells and is representative of at least three experiments.

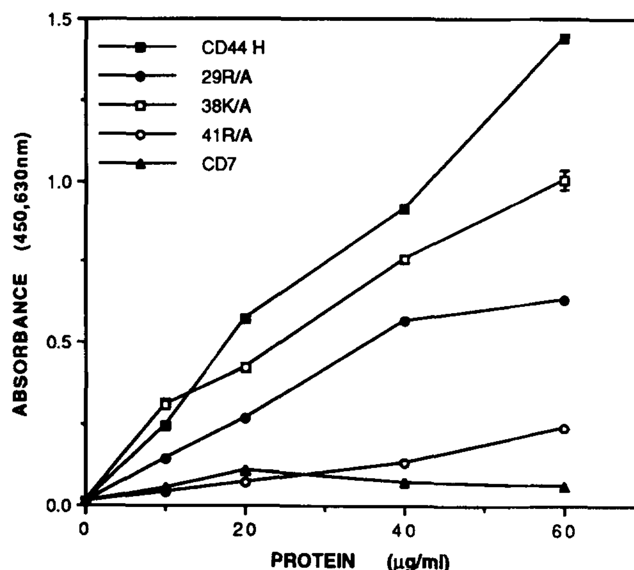


Figure 5. Binding of CD44 Rg NH_2-terminal site-directed mutants to HA. Increasing concentrations of CD44 Rg's encoding site-directed mutations in the NH_2-terminal region were incubated with HA immobilized on ELISA plates. Bound fusion protein was detected as for Fig. 2. CD7 Rg was used as a human IgG fusion protein control. Data is expressed as the ±SD of duplicate wells and is representative of at least three experiments.

soluble fusion protein of CD44H has allowed the identification of two clusters of basic residues and specific amino acids within these clusters as important determinants in CD44/HA binding. Truncation mutagenesis established that HA binding by wild-type CD44 requires more than just the cartilage link protein homologous domain. A cluster of positively charged amino acids situated membrane proximal to this homologous domain plays an important role in HA binding. Site-directed mutagenesis of basic residues both within this cluster and within a cluster in the NH_2-terminal link protein homologous domain confirmed that arginine and lysine residues within these regions were intimately associated with CD44/HA binding. It appears that HA binding is cooperative between the two basic regions in the extracellular domain of CD44. Mutation of any individual basic amino acid in either cluster affected the protein's ability to bind its ligand. The binding data clearly establishes Arg 41 as a critical residue in this interaction, yet the double membrane proximal mutant (K158A/R162A), can also significantly affect HA binding even with an intact Arg 41.

In contrast to the F2 and F3 truncation mutants, the Fl-B and Fl-C constructs had considerably reduced ability to bind HA. This was somewhat of a surprise since the cartilage link protein homologous region was still present in the NH_2-terminal region of these deletion mutants. These results suggested that this reduction in binding may have been due to deletion of amino acids not previously thought to interact with and bind to HA. Alternatively, because of the large number of deleted amino acids, Fl-B and Fl-C fusion proteins may have had an altered secondary structure such that they were unable to bind to HA. To address this latter possibility the ability of the truncation mutants to bind to anti-CD44 mAbs was examined. All deletion mutants, when expressed in COS cells, bound the antibodies IM7, A3D8, and BU52 with the exception of Fl-B which failed to bind IM7 (Table I). We can not completely rule out the possibility that these deletions had perturbed the CD44 molecule in such a way as to diminish HA binding. However our results suggest that the native configuration of the epitopes recognized by these antibodies and by extension the CD44 protein fragments, had been retained and that the loss of binding to HA by the Fl-B and Fl-C truncation mutants was most likely due to deletion of amino acids involved in the HA interaction. The inability of IM7 to bind to Fl-B may have been due to removal of part or all of the epitope recognized by this mAb.

Recent studies had shown that a membrane-bound truncated mutant of murine CD44H was still capable of binding to soluble HA (He et al., 1992). This construct had an 83-amino acid deletion immediately proximal to the transmembrane domain, the region least conserved among species. The F2 deletion mutant of human CD44 described here is similar to the murine truncation mutant and was also able to bind HA (Fig. 1 *A*). Taken together, this report and the results of He et al. (1992) suggest that the region immediately proximal to the transmembrane region is not involved in HA binding. The F2 deletion mutant did not display diminished ability to bind HA while the Fl-C mutant showed a marked reduction in HA binding. The region encoded in F2 but deleted from Fl-C contained a cluster of four positively charged amino acids situated within a 13 residue stretch (position 154 to 162). Site-directed mutagenesis of each of the four basic residues confirmed the observation that this region, and more specifically the basic residues within, played an integral role in HA binding. Each individual mutation caused a reduction in HA binding while a double mutant (K158A/R162A) showed a reduction in binding indicative of an additive effect of the two single mutants. This region is highly conserved among other species but lies outside the domain homologous to other HA-binding proteins and therefore identifies a unique domain involved in HA binding.

The only other cluster of basic residues that is conserved in CD44 lies close to the NH_2 terminus and is situated within a 135-amino acid region that has 30% sequence homology to other known HA binding proteins. Three basic residues in this cluster, all of which are conserved among species, were mutated to alanine. Of these the arginine 41 substitution had the greatest impact, eliminating HA binding almost completely. This result is of some significance to the group of proteins that share this sequence homology and are known to bind HA. All members of this group, CD44, link protein, versican, aggrecan, and TSG-6 protein, have a basic amino acid in the same relative position (Lee et al., 1992). This conservation of structure suggests a conservation of function leading us to predict that this residue is also of fundamental importance to the other members of the family in their ability to bind HA. It is likely that either an arginine or lysine in this position can fulfill the same role in HA binding as TSG-6 has a lysine residue while the other family members have an arginine. A basic residue at position 38 is less well conserved with arginine present in TSG-6 and link protein, and a lysine in CD44. Mutation of lysine 38 in CD44 did reduce HA binding but only to a level slightly less than normal suggesting that this residue was not as critical for binding HA.

Binding of selected mutant CD44 Rg's to tissue sections containing an abundance of HA confirmed the results of HA binding studies by ELISA. Whereas wild-type CD44H Rg bound extensively to HA present in tissue sections, binding by the double mutant K158A/R162A was weaker and binding by the R41A mutant was completely abolished (data not shown). Similar binding properties were observed in ELISA assays suggesting that these mutant proteins do not discriminate in their ability to bind HA either immobilized to plastic or immobilized in tissue sections.

Recent studies of glycosaminoglycan-protein interactions have revealed that binding occurs between negatively charged carboxyl and sulfate groups of the glycosaminoglycan and domains of clustered positively charged basic amino acids of the protein (Jackson et al., 1991). Indeed chemical modification of proteoglycan core protein (Hardingham et al., 1976), or cartilage link protein (Lyon, 1986), have implicated the basic amino acids arginine and lysine in the binding of these macromolecules to HA. Further, HA was shown to bind cartilage link protein within domains containing clusters of basic amino acids (Goetinck et al., 1987). Clusters of basic amino acids are found in the heparin binding domains of several proteins including antithrombin III and heparin cofactor II, both of which require heparin binding to perform their functions as inhibitors of serine proteases (Jackson et al., 1991). The two heparin binding domains of antithrombin III are somewhat analogous to the two domains in CD44 described here and the tandem repeat domains of cartilage link protein (Goetinck et al., 1987) that are important in HA binding. A portion of the heparin cofac-

tor II heparin binding domain can also bind to dermatan sulfate. We have shown that CD44 can bind to both HA and chondroitin-4-sulfate using a common domain although it is unclear whether both glycosaminoglycans use the same binding site. Underhill et al. (1983) previously showed that the same binding site of CD44 could bind both HA and chondroitin sulfate. It is clear that the sulfated chondroitin does not bind to CD44 with the same affinity/avidity as nonsulfated HA and that the position of the sulfate group in chondroitin is also important as chondroitin-6-sulfate binds very weakly to CD44.

He et al. (1992) have recently shown that a number of murine CD44 splice variants when expressed on T lymphoma cells, were capable of mediating adhesion to cells bearing HA. CD44E is a human homologue of one of these murine HA-binding variants and we have confirmed a previous report that CD44E has a very limited ability to bind HA whether expressed on transfected tumor cells (Stamenkovic et al., 1991), or as a soluble protein (present study). The explanation for this discrepancy is unclear. Whether the three amino acids deleted (one of which is an arginine) immediately upstream of the membrane proximal splice site play a role in HA binding remains unknown. CD44E is ∼60% homologous to the mouse and rat isoforms so amino acid differences in the alternatively spliced domain may also be responsible for the observed differences in HA binding. This will be an important issue to resolve as it would appear that there is an association between the ability of tumor cells to metastasize and the ability of CD44 isoforms expressed on tumor cells to bind HA (Sy et al., 1991; Thomas et al., 1992; Günthert et al., 1991).

By genetically manipulating the ability of CD44 to bind HA we can now study the biological role of a number of splice variants and determine whether these variants are capable of binding to as yet unidentified ligands and thus provide a means to explain the many biological functions attributed to CD44. The role CD44 plays in leukocyte trafficking (Jalkanen et al., 1987; Culty et al., 1990) can also be studied using HA-binding mutants to search for possible ligands expressed on high endothelial venules that appear to interact with CD44 via a functional domain distinct from the HA-binding domain.

We thank M. West for help with the preparation of this manuscript.

This work was supported by The Bristol-Myers Squibb Pharmaceutical Research Institute.

Received for publication 12 February 1993 and in revised form 30 March 1993.

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