Monoclonal Antibodies to CD44 and Their Influence on Hyaluronan Recognition

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Abstract. Antibodies to CD44 have been used to inhibit a variety of processes which include lymphohemopoiesis, lymphocyte migration, and tumor metastasis. Some, but not all, CD44-mediated functions derive from its ability to serve as a receptor for hyaluronan (HA). However, sites on CD44 that interact with either ligands or antibodies are poorly understood. Interspecies rat/mouse CD44 chimeras were used to analyze the specificity of 25 mAbs and to determine that they recognize at least seven epitopes. Amino acid substitutions that resulted in loss of antibody recognition were all located in the region of homology to other cartilage link family proteins. While at least five epitopes were eliminated by single amino acid replacements, multiple residues had to be changed to destroy binding by other antibodies. One antibody was sensitive to changes in any of three separate parts of the molecule and some antibodies to distinct epitopes cross-blocked each other. Certain antibodies had the ability to increase HA binding by lymphocytes but this did not correlate absolutely with antibody specificity and was only partially attributable to CD44 cross-linking. Antibodies that consistently blocked HA recognition were all sensitive to amino acid changes within a short stretch of CD44. Such blocking antibodies interacted with CD44 more strongly than ligand in competition experiments. One large group of antibodies blocked ligand binding, but only with a particular cell line. This detailed analysis adds to our understanding of functional domains within CD44 and requirements for antibodies to influence recognition of one ligand.

MONOCLONAL antibodies have provided powerful tools for identifying and characterizing cell-adhesion molecules. They usually reflect species differences in amino acid sequences between the source of the immunogen and the animal being immunized. Certain antibodies influence adhesive functions and others are informative about ligand-binding states. Particularly well studied in this regard are antibodies to integrins, which appear to undergo conformational changes in response to intracellular or extracellular signals (2, 38, 48). One antibody that binds only to an "activated" integrin may do so by mimicking the natural ligand (1). Some antibodies dramatically increase the affinity of integrins for ligand, perhaps by stabilizing a particular active conformation (9, 38). Still other reagents block or change the specificity for ligands and there are antibodies which recognize binding site occupancy (10, 36). Finally, antibodies have been described that recognize combinations of particular α and β integrin chains (3, 5). Interspecies chimeras have been successfully used to "map" the recognition specificity of antibodies to particular regions of integrins (6, 47, 52).

CD44 has attracted considerable interest because of its possible importance in lymphohemopoiesis, tumor metastasis, lymphocyte activation, and leukocyte extravasation. These functions may in part relate to the ability of CD44 to act as a receptor for soluble or cell-bound hyaluronan (HA)[1] (4, 25, 27, 32). Cells bearing CD44 can have different functional "states" with respect to ability to recognize this ligand (reviewed in 25). For example, resting B lymphocytes do not bind HA, and become able to do so only after prolonged incubation with activation stimuli such as IL-5. T lymphocytes represent a second state, where ligand binding is markedly up-regulated by a particular mAb, IRAWB14. Fully activated normal lymphocytes and comparable cell lines typify a third state, as they constitutively recognize HA with apparent high affinity.

Many antibodies have been prepared to multiple epitopes on CD44 of various species. However, with the ex-

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1. *Abbreviations used in this paper*: HA, hyaluronan; FL-HA, fluorescein labeled HA; MFI, median fluorescence intensity.

ception of some directed to exon products not commonly used by lymphocytes, most have not been well characterized with respect to specificity. The actual binding site for HA has not yet been identified and essentially nothing is known about how antibodies can influence HA recognition. We now characterize antibodies that recognize at least seven epitopes on CD44. All antibodies that uniformly blocked ligand recognition were dependent on residues in a single CD44 loop. Antibodies that were similarly sensitive to substitutions in another part of the molecule differed in their ability to enhance ligand binding. Still another portion of CD44 contained a single residue polymorphism which accounts for the specificity of antibodies that only block HA recognition by one cell type.

Materials and Methods

Cell Lines

The CD44-negative murine lymphoma AKR1 (15) was used to prepare stable transfectants with chimeric cDNAs. The AKR1.CD44WT and AKR1.CD44ΔNC clones are stable transfectants which have been described previously (13, 24). The human 293T renal cell carcinoma line (33) expresses extremely low levels of human CD44 and was used for transient transfection experiments. Other cell lines including BW5147 (a T lymphoma), WB (a derivative of SAKRTLS12.1, described in 16), and W279 (a B lymphoma) were maintained in Dulbecco's minimal essential medium with 10% FCS. The BM2 B cell hybridoma was grown in RPMI 1640 with 10% FCS. All cell lines have been used in our previous studies of CD44 function (16, 23, 24, 32).

Chimeric Plasmid Construction

Our previous constructs of the hemopoietic form of murine CD44.1 were prepared from cDNA generously donated by Dr. E. Butcher and have been described (13, 24). Nucleotide and amino acid numbering are as in those publications. Murine CD44.1 was cloned into the pBS+/− vector (Stratagene Inc., La Jolla, CA) to yield a plasmid designated mWTpBS. A 1.0-kb rat CD44 full-length cDNA in the EcoRI site of vector pT_7T_3-S was provided by Dr. U. Gunthert of the Basel Institute for Immunology (12). We designated it $rWTpT_7T_3S$.

Megaprimer PCR-directed mutagenesis was used to generate three rat/mouse CD44 chimeric expression plasmids which contain large segments (63, 85, and 107 residues) of rat CD44 at the 5′ end instead of murine sequence. These were designated R/MI, R/MI+II, R/MI+II+III, and are indicated in Fig. 1, which shows part of the extracellular domain of murine and rat CD44. Two previously described oligonucleotides were used throughout this study for PCR priming (13). The 29-bp upstream primer OM-6AR, corresponds to a 21-bp stretch overlapping the initiation codon ATG in the murine and rat CD44 cDNA clones mWTpBS and $rWTpT_7T_3S$, whereas the downstream primer OM-2AR is complementary to a 19-bp fragment around the stop codon TAG in the mWTpBS clone. Both oligonucleotides had an EcoRI site added to the 5′ end for use in cloning. Antisense primers of R/MI, R/MI+II, and R/MI+II+III were designed to amplify the three large fragments of rat cDNA. PCR reactions were cycled 25 times at 94°C for 1 min, 50°C for 2 min, and 72°C for 3 min. First-round PCR was primed by OM-6AR and the antisense primer of each chimera using the rat cDNA as template. The amplified 5′ end fragments of rat sequence were purified from agarose gels. We used mouse cDNA as template for second-round PCR, amplified rat 5′ end fragments, and OM-2AR as primers to obtain rat/mouse chimeric cDNA. This was then subcloned into an EcoRI site of the pBS+/− plasmid.

Construction of Chimeras Using Site-directed Mutagenesis

PCR-introduced site-directed mutagenesis was used to change single or a cluster of residues of the mouse CD44 sequence to rat. Sites that differed between mice and rats that were substituted are boxed and indicated by small letters in Fig. 1. For the first round of PCR, mWTpBS was used as template. OM-6AR and an antisense primer for each chimera were used as a set to amplify the 5′ end fragment of the mouse cDNA. In each case, the antisense primer contained the introduced rat sequence. A sense primer for each chimera containing the introduced rat sequence and complementary to the above antisense primer, was then used with OM-2AR to amplify the 3′ end fragment of the mouse cDNA. PCR conditions were as described above and products from both PCR reactions were then purified from agarose gels. For the second round PCR, we used the two purified PCR fragments as template, with OM-6AR and OM-2AR as primers to amplify the complete rat/mouse chimeric cDNA. The amplified products were then run on agarose gels, and 1.1-kb bands were purified using Geneclean (BIO 101, Inc., Vista, CA), digested with EcoRI, and subcloned into pBS+/−.

All of the chimeric clones contained full-length mouse CD44 cDNA. R/Ma contains a single Ile of rat sequence instead of Val-8. R/Mb contains a single residue change from Gln-36 to Glu. R/Mc contains a single residue change from Ser-40 to Thr. R/Md contains three residues changed (Asp-46 to Ala, Lys-49 to Glu, Ser-53 to Arg). R/Me contains a single residue changed from Asn-67 to His. R/Mf has a single residue changed from His-83 to Asn. RMg contains two residues changed (Val-90 to Leu, Thr-91 to Ala). R/Mh contains a single residue changed (Pro-108 to Leu).

Sequencing and Construction of Expression Plasmids

Chimeric cDNAs were cloned into pBS+/− vector and sequences determined using the dideoxy chain termination method with Sequenase (United States Biochemical Corp., Cleveland, OH) from double-stranded templates. The chimeric cDNA inserts of R/Md, R/Mg, and R/Mh were subcloned via blunt-end ligation into a HindIII site of the expression vector pRc/RSV (Invitrogen, San Diego, CA) which has an LTR promoter from Rous sarcoma virus, a polyadenylation signal from the bovine growth hormone and a *neo*^{r} gene for selection in G418 supplemented media. A BstXI/EcoRI adaptor (Stratagene Inc.) was added to other chimeric cDNA inserts before cloning into the BstXI site of the expression vector pEF-BOS (33). This vector has a human EF-1 promotor, the stuffer fragment from CDM8 vector, and a polyadenylation signal from human G/M-CSF cDNA.

Transfection of Cells

Mouse wild-type CD44 and the chimeric structures RMd, RMg, RMh in the pRc/RSV vector were transfected into AKRI cells by electroporation

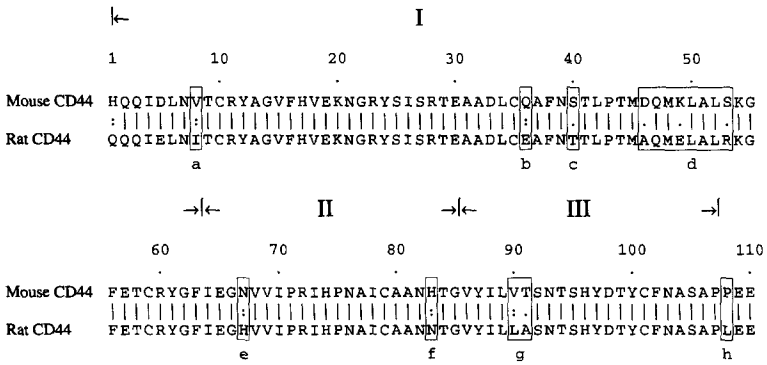

Figure 1. Comparison of amino acid sequences for part of the extracellular regions of murine and rat CD44. Numbering of residues starts at the beginning of the mature protein. Regions exchanged in large segment chimeras are indicated by roman numerals. Single or clustered residue substitutions are boxed and indicated by small letters.

using a gene pulser (BioRad Laboratories, Hercules, CA) with 550 V and 25 μF of capacitance as described previously (13). G418 was added at 1 mg/ml after 48 h of growth and drug-resistant colonies appeared within 2 wk. Mouse wild-type CD44 and all other chimeric CD44s in the pEF-BOS vector were transfected into 293 T cells with a standard calcium phosphate method. The transfectants were stained with CD44 mAbs and analyzed by flow cytometry with a FACScan® (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Antibodies and Flow Cytometry

Many of the rat anti-mouse CD44 mAbs have been previously described and include IM7.8.1 (IgG_{2b}) (55); I42/5 (IgG_{2a}) (55); RAMBM55.5 (IgM), RAMBM5.5.8 (IgM), RAGN185.6 (IgM), RAMBM17.4.3 (IgM), R7166.7 (IgM), REL72.1 (IgG_{2a}) (23); RAMBM44.5.2 (IgM) (21); IRAWB14.4 (IgG_{2a}) (24); IRAWB26.6 (IgG_{2a}) (26); RAW45.106.2 (IgM), IRAWB27.3 (IgG_1) (Lesley, J., R. Schulte, and R. Hyman, unpublished data); and KM201 (IgG_1), KM81 (IgG_{2b}), KM114 (IgG_1) (31). One group of previously unpublished antibodies, KM703 (IgG_{2a}) and KM03, arose from the same series (31). C71/26.4 (IgM) recognizes a polymorphism (CD44.2), and RAMBM44.5.2 (IgM) is specific for the CD44.1 allele (21). The rat D12 (IgG_{2b}) and the hamster anti-mouse 1.5.1 (IgG) CD44 antibodies were prepared by immunization of Sprague Dawley rats and Syrian hamsters, respectively (Farr, A., unpublished data). 1B6 is a recently prepared antibody which induces spreading of activated B lymphocytes (Santos-Argumedo, R. W. Kincade, and R. M. E. Parkhouse, manuscript submitted for publication) and methods used for its preparation have been described elsewhere (46). The previously unreported SK45.9 (IgM), SK47 (IgG_{2a}), and SK159 (IgG_{2a}) antibodies were made by fusion of Sp2/0 with spleen cells from Fisher rats immunized with affinity-purified CD44 from KLN205 tumor cells. OX49 (IgG_{2a}) and OX50 (IgG_1) are mouse anti-rat CD44 mAbs (39), and were generously donated by Dr. Neil Barclay (University of Oxford, Oxford, UK). Most of the flow cytometry experiments were conducted with hybridoma culture supernatants, but antibodies were purified by protein G chromatography (Pierce Chemical Co., Rockford, IL) and used in cross-blocking and other studies. The HPC4 antibody and its use with an epitope tag sequence have been described (43, 50).

Fluorescein-conjugated HA (FL-HA) was prepared and used at a final concentration of 36 μg/ml as described in our previous studies (13, 24). Transfected cells were first stained with ~1 μg of each anti-CD44 antibody, followed by FITC-labeled mouse anti-rat Ig in the case of rat antibodies, goat anti-mouse Ig for the two mouse anti-rat CD44 mAbs, or goat anti-hamster Ig for the 1.5.1 antibody. All the transfections and flow cytometry analyses were repeated at least three times. The 1.5.1 hamster mAbs efficiently recognized both rat and mouse CD44 and served as a standard for normalizing expression levels. The median fluorescence intensity of staining achieved with this antibody on wild-type murine CD44 transfectants was assigned a value of 1.0. Each of the other transfectants prepared on the same day were analyzed in the same way and the level of expression normalized to the wild-type result. When staining of a chimera (median fluorescence intensity) was reduced more than fivefold relative to that of wild-type CD44, it was indicated by − in Table I. All other results were consistently within twofold of wild-type values. In Table II, antibodies which gave <20%, 20–40%, or >40% of wild-type staining with chimeric CD44 were designated −, ±, or +, respectively.

Construction of Epitope-tagged CD44

The pEFBos expression vector was engineered to include the transmembrane segment of tissue factor (44) and 14 amino acids recognized by the HPC4 mAb (43, 50). A cDNA corresponding to the entire extracellular domain of the hemopoietic form of CD44 was then ligated into this vector. The resulting construct was then expressed in AKR1 cells and stable transfectants with high level expression were selected by cell sorting and cloning by limiting dilution. These cells were used to study the influence of antibody-mediated receptor oligomerization on HA binding and similar results were obtained with six different clones.

Results

Reactivity of mAbs to Rat/Mouse CD44 Chimeras

Preliminary experiments showed that none of 22 rat mAbs recognized rat CD44. However, all were positive on a previously prepared transfectant designated AKR.CD44ΔNC (13). These cells express a truncated murine CD44 molecule which contains only the NH_2-terminal 163 amino acids of the protein. We then exploited the extensive conservation between murine and rat CD44 sequences to localize residues encoding epitopes recognized by these antibodies. A hamster mAb (1.5.1) that recognized both rat and mouse CD44 served as a useful control for normalizing expression levels (see Materials and Methods).

Interspecies chimeras were constructed with increasingly large segments of rat CD44 cDNA sequence introduced into an otherwise mouse CD44 (Fig. 1). The resulting constructs were then used for transient transfection of human 293T cells, which constitutively express extremely low levels of CD44. Typical results from flow cytometry characterization are shown in Fig. 2 and the results are summarized in Table I. CD44 which included only the NH_2-terminal 63 amino acids of rat sequence (segment I) was still recognized by the majority of antibodies. However, residues in this most NH_2-terminal portion of the molecule were needed for recognition by a group of seven rat anti-mouse antibodies. Additionally, one mouse anti-rat CD44 antibody (OX50) was now positive. Introduction of a larger portion of rat CD44 sequence (segments I and II, the first 85 amino acids) resulted in loss of reactivity by an additional six rat anti-mouse antibodies, along with gain of reactivity by one mouse anti-rat CD44 antibody (OX49). Exactly the same pattern of reactivity was seen with transfectants prepared with an even larger portion (107 residues) of rat CD44 sequence (included segments I, II, and III). Therefore, residues required for recognition of CD44 by the remaining group of ten rat anti-mouse antibodies were localized to a region between amino acids 108

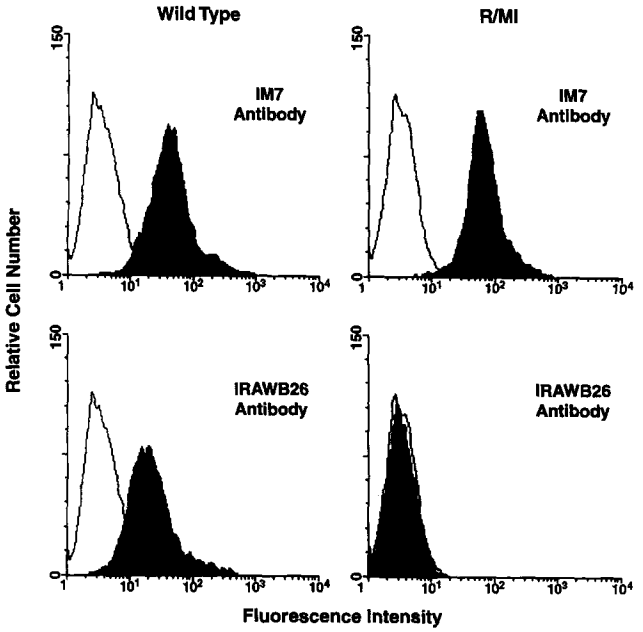

Figure 2. Example of epitope loss on chimeric CD44. These typical flow cytometry results were obtained by staining 293T cells transfected with CD44 cDNA. The IM78.1 antibody recognizes both wild-type and chimeric R/M I CD44, while the latter is not stained by the IRAWB26 antibody.

Table I. Recognition of Large Segment Rat/Mouse CD44 Chimeras by Antibodies

CD44 mAbs	Wild type	R/M I	R/M I+II	R/M I+II+III
IM7.8.1	+	+	+	+
RAMBM5.5.8	+	+	+	+
RAMBMI7.4.3	+	+	+	+
I42/5	+	+	+	+
R7 166.7	+	+	+	+
RAGN 185.6	+	+	+	+
IRAWB27.3	+	+	+	+
KM703	+	+	+	+
SK45.9	+	+	+	+
SK47	+	+	+	+
REL 72.1	+	−	−	−
RAWB45.106.2	+	−	−	−
IRAWB14.4	+	−	−	−
IRAWB26.6	+	−	−	−
RAMBM44.5.2	+	−	−	−
KM03	+	−	−	−
SK159	+	−	−	−
KM114	+	+	−	−
KM81	+	+	−	−
KM201	+	+	−	−
RAMBM55.5	+	+	−	−
D12	+	+	−	−
1B6	+	+	−	−
HAMSTER Ab 1.5.1	+	+	+	+
OX49 (MαR)	−	−	+	+
OX50 (MαR)	−	+	+	+

Transfectants were prepared and analyzed as detailed in Materials and Methods. Staining of rat/mouse chimeras was designated as − or + when it was reduced more than fivefold, or less than twofold of that obtained with wild-type CD44. R/M, rat/mouse.

and 163 (Table I). All of the constructs were found to confer hyaluronan-binding ability on transfected cells (data not shown). We conclude from this, and the reciprocal loss/gain of murine/rat epitopes, that the chimeric proteins retained structural integrity.

More extensive characterization of the antibodies was then done with chimeric constructs which had single, clustered, or combinations of rat amino acids placed into the murine sequence (see Fig. 1, *a–h*). The first three substitutions (*a–c*) had no effect on the binding of any of the rat anti–mouse antibodies (Table II). Chimeric murine CD44 which included three residues from the rat sequence (*d*) was no longer recognized by five of the antibodies. One of these (SK159) failed to recognize CD44 with substitution of just aspartic acid–46, while this had no effect on binding of IRAWB14 and three other antibodies in this group. Also, a large segment rat/mouse chimera which had the murine residue (serine) at position 53 was not recognized by any of these four antibodies (results not indicated in Table II). Thus, the rat/mouse difference at lysine-49 probably determines this antigen specificity.

Analysis of chimeric CD44 revealed that one set of two antibodies (KM114 and KM81) had indistinguishable specificity (Table II). Their reactivity was completely dependent on histidine-83 (*f*). These antibodies efficiently recognize hamster cells (data not shown) which are identical to mice at this position (4).

The specificity of a set of ten reagents, including the IM7.8.1 mAb, was dependent totally on a single proline-108 (*h*). Many of the antibodies in this group were previously known to cross-react with human CD44 (17, and

Table II. Recognition of Single/Multiple Residue Rat/Mouse CD44 Chimeras by Antibodies

CD44 mAbs	a or c	b	d	e	f	g	h	e + f	d + e + f	f + g	e + f + g
IM7.8.1	+	+	+	+	+	+	−	+	+	+	+
RAMBM5.5.8	+	+	+	+	+	+	−	+	+	+	+
RAMBMI7.4.3	+	+	+	+	+	+	−	+	+	+	+
I42/5	+	+	+	+	+	+	−	+	+	+	+
R7 166.7	+	+	+	+	+	+	−	+	+	+	+
RAGN 185.6	+	+	+	+	+	+	−	+	+	+	+
IRAWB27.3	+	+	+	+	+	+	−	+	+	+	+
KM703	+	+	+	+	+	+	−	+	+	+	+
SK45.9	+	+	+	+	+	+	−	+	+	+	+
SK47	+	+	+	+	+	+	−	+	+	+	+
RAMBM44.5.2	+	+	+	+	+	+	+	+	+	+	+
KM03	+	+	+	+	+	+	+	+	+	+	+
REL 72.1	+	+	−	+	+	+	+	+	−	+	+
RAWB45.106.2	+	+	−	+	+	+	+	+	−	+	+
IRAWB14.4	+	+	−	+	+	+	+	+	−	+	+
IRAWB26.6	+	+	−	+	+	+	+	+	−	+	+
SK159	+	+	−	−	+	−	+	−	−	−	−
KM114	+	+	+	+	−	+	+	−	−	−	−
KM81	+	+	+	+	−	+	+	−	−	−	−
KM201	+	+	+	+	±	+	+	±	−	−	−
RAMBM55.5	+	+	+	+	±	+	+	±	−	±	±
D12	+	+	+	+	±	±	+	±	±	−	−
1B6	+	+	+	±	+	+	+	±	±	+	−
HAMSTER Ab 1.5.1	+	+	+	+	+	+	+	+	+	+	+
OX49 (MαR)	−	−	−	+	−	−	−	−	−	−	−
OX50 (MαR)	−	+	−	−	−	−	−	−	−	−	−

Transfectants were prepared and analyzed as detailed in Materials and Methods (see also Fig. 1). The results are given as −, ±, or + when staining of chimeras was <20, 20–40, or >40%, respectively of that seen with wild-type CD44.

data not shown), and we note that human and murine CD44 share the same amino acid at this position.

A single residue (glutamine-36, *b*) determined the binding of the OX50 mouse anti–rat CD44 antibody, and replacing only asparagine-67 (*e*) with histidine allowed recognition by the OX49 mouse anti–rat antibody (Table II).

The SK159 antibody had unique and complex recognition specificity. Binding of this interesting reagent was abrogated by changing residues in any of three well-separated areas (*d*, *e*, and *g*). As noted above, it was only necessary to change aspartic acid-46 in the "d" cluster of residues for loss of this epitope (results not indicated in Table II).

Other antibodies were complex in a different way, and multiple, noncontiguous residues had to be changed to lose reactivity. For example, while substitution of the asparagine-67 (*e*) caused greatly reduced staining by the 1B6 antibody, it was completely lost only when it was changed together with other residues (*e*+*f*+*g*).

Staining of the "f" chimera (histidine-83 to asparagine) by the KM201 antibody was diminished ~70%. However, recognition was lost completely only when an additional two residues (valine-90 and threonine-91, *g*) were changed at the same time. While single changes at d and e alone had no effect, KM201 recognition was completely lost with the combination d,e,f. In another study, we found that tunicamycin treatment of murine lymphoma cells did not influence staining with KM201, but the same treatment abolished recognition of CHO cells (18). This indicates that species cross-reactivity of this reagent may be dependent partially on carbohydrate. Two other CD44 antibodies (KM114 and KM81) also recognized hamster cells and the staining was diminished, but not lost, by tunicamycin treatment.

The RAMBM55.5 antibody had a reactivity pattern which was similar, but not identical to KM201. The D12 antibody also had complex recognition specificity. While a single change at position 83 (*f*) greatly reduced recognition, an additional change six residues away (*g*) was needed to completely abolish staining.

The epitope(s) for two antibodies (RAMBM44.5.2 and KMO3) was not lost by any of the single or clustered amino acid substitutions but our analysis revealed that residues within the first 63 amino acids (segment I) are critical (see Table I). The first residue in the protein (histidine versus glutamine) represents the only difference between mice and rats in this region that we did not substitute and it presumably determines the RAMBM44.5.2 epitope. The same antibody is known to recognize a polymorphism of CD44 (CD44.1; BALB/c mice are positive, AKR mice are negative). The KMO3 antibody was indistinguishable from RAMBM44.5.2 in recognition of the chimeras, but was not investigated with respect to mouse strain polymorphism. BALB/c mice are known to differ from C3H mice at position 174 and to have two fewer amino acids at the signal peptide cleavage site (13, 37, 56, 59). Therefore, the presence or absence of two amino acids (histidine and proline) at the amino terminus could account for the CD44.1 versus CD44.2 polymorphism.

This analysis provided a detailed profile of differences between mouse and rat CD44 that are immunogenic. We then considered information about recognition specificities of the various antibodies together with their influence on CD44 functions.

Monoclonal Antibodies to Distinct CD44 Epitopes Cross-block Each Other

All of the antibodies shown to be members of the same group (Table II), also cross-blocked each other (data not shown). However, it was surprising to find that some antibodies from different groups cross-blocked, and a dramatic example is shown in Fig. 3. This pair of reagents is particularly interesting because IRAWB14 can enhance ligand binding in some circumstances whereas KM114 always blocks (see below). Pretreatment of cells with excess unlabeled IRAWB14 antibody completely prevented staining by FITC labeled KM114 (Fig. 3, *left panel*). Furthermore, the order of pretreatment with the unlabeled forms of these antibodies determined whether or not cells recognized fluorescent HA (*center* and *right panels*). Cells which first received IRAWB14 recognized this ligand and a substantial level of staining was observed by flow cytometry. However, essentially no binding was seen when the blocking KM114 antibody was used as the first step reagent. One can conclude that the epitopes recognized by these unrelated antibodies are in close proximity, or that binding of one reagent alters CD44 conformation so that the other epitope is lost.

Antibody Specificity and Influence on Ligand Binding

Most of the antibodies in this panel have been compared for their ability to block or enhance HA recognition by transfected AKR.CD44WT or similar cells, and a general summary is given in Table III. Two antibodies with similar specificity (KM114 and KM81), and antibodies with partially related specificity (KM201, RAMBM55.5, and D12), blocked FL-hyaluronan binding by many cell lines (for an example, see Fig. 7 below). The ligand recognized by the SK159 antibody is unique and sensitive to changes in any of three locations on CD44 (Table II). It also interfered with ligand binding by multiple cell types. A common feature of these reagents is their dependence on histidine-83,

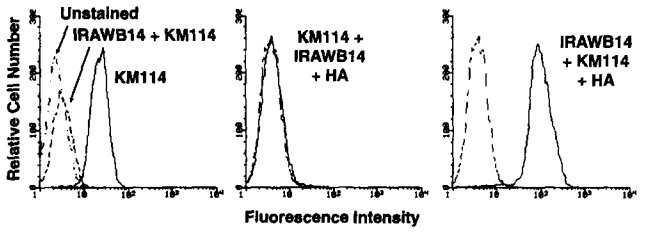

Figure 3. Antibodies to distinct epitopes cross-block each other. The left panel shows immunofluorescence staining of AKR1.CD-44WT cells treated with FITC-labeled KM114 antibody alone, or after pretreatment with an excess of unlabeled IRAWB14 antibody. Similar results were obtained with other antibodies from these two groups and when the antibodies were added in reverse sequence. The middle panel illustrates the inability of the same cells to bind flourescein-labeled hyaluronan (*HA*) after pretreatment with KM114, followed immediately by IRAWB14, followed by the labeled ligand. The right panel shows that fluorescent HA was bound if the order of pretreatment was reversed, i.e., IRAWB14, followed by KM114.

Table III. Antibodies with Similar Specificity Differ in Ability to Enhance Ligand Binding

Epitope group	Antibody	Isotype	Effect on HA binding
	IRAWB26	IgG_{2a}	↑
d	IRAWB14	IgG_{2a}	↑↑
	RAWB45	IgM	↓
	REL72	IgG_{2a}	↓
f	KM81	IgG_{2b}	↓
f	KM114	IgG_1	↓
f (complex)	KM201	IgG_1	↓
f (complex)	RAMBM55	IgM	↓
f (complex)	D12	IgG_{2b}	↓
d,e,g	SK159	IgG_{2a}	↓
	IM7	IgG_{2b}	~
	R7166.7	IgM	↑
	SK47	IgG_{2a}	~
	RAMBM5.5	IgM	~
h	RAGN185.6	IgM	~
	I42/5	IgG_{2a}	~
	KM703	IgG_{2a}	(↓)
	RAMBM17.4	IgM	~
	IRAWB27.3	IgG_1	~
	SK45.9	IgM	(↓)

This represents a general summary of the influence of antibodies on HA recognition. See Figs. 4 and 7 for specific examples.

or the nearby valine-90/threonine-91 for recognition of CD44.

Results obtained with a subclone of the WB lymphoma that does not constitutively bind HA are illustrated in Fig. 4. Four antibodies that were indistinguishable by interspecies chimera analysis (epitope d) had dissimilar influence on the ability of cells to bind HA. For example, the IRAWB14 and REL72 antibodies are both of the IgG_{2a} isotype, but only the former increased ligand binding. In fact, REL72 actually blocked ligand binding by other cells which constitutively had this function (not shown). Furthermore, one antibody in the h epitope group, R7166.7 (IgM), increased ligand binding ~10-fold. However, nine other antibodies in this category had no influence. The ability of antibodies to increase ligand binding may not be solely determined by epitope specificity.

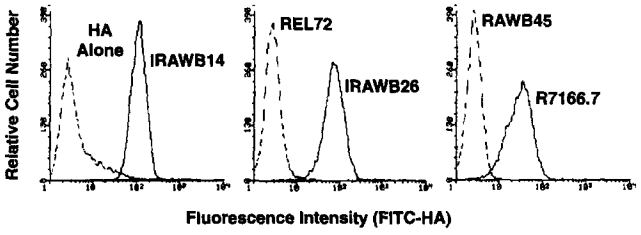

Figure 4. Antibodies with indistinguishable specificity differ in ability to enhance ligand binding. A non-HA-binding subclone of the WB lymphoma was first exposed to the indicated antibodies before addition of FL-HA. The extent of binding was then assessed by flow cytometry. All of the antibodies except R7166.7 (group h) were in the d group (Table II). Nine other antibodies of the h epitope group were tested in the same assay and found to have no influence on ligand binding.

Oligomerization of Cell Surface CD44 and its Influence on Ligand Binding

Monomeric Fab fragments of the IRAWB14 and IRAWB26 antibodies have reduced affinity for CD44 and only enhanced hyaluronan recognition by lymphoma cells when used with a second antibody (26). This indicated that enhancement might result in part from receptor cross-linkage. In agreement with that, dimerization of the receptor also lowered the threshold amount of CD44 required for detectable binding of HA (41). Although there are IgM and IgG antibodies which seem to have no effect on ligand recognition, these might in part interfere with the binding site because they recognize epitopes within the first 163 residues. We wondered if antibodies directed to a completely different part of CD44 might influence HA binding. A small HPC4 sequence tag was inserted into CD44 cDNA such that the epitope would be situated on the expressed protein near the cell membrane (see Fig. 8 below). A transmembrane segment from tissue factor was used to isolate the protein from intracellular events. The resulting stable transfectants were recognized specifically by multiple antibodies to CD44 as well as by the HPC4 antibody.

While the transfected cells did not constitutively bind hyaluronan, recognition of as little as 1 ng/ml of FL-HA was detectable when the cells were pretreated with the IRAWB14 antibody (Fig. 5). Simple exposure to the HPC4 antibody never increased ligand binding more than threefold over background (data not shown). CD44 which had been extensively cross-linked via the HPC4 epitope, followed by exposure to sheep anti-mouse IgG, could recognize above background amounts of this ligand. However, binding leveled off at a low level and contrasted to the extraordinary recognition ability of cells treated with just the IRAWB14 antibody alone. These results suggest

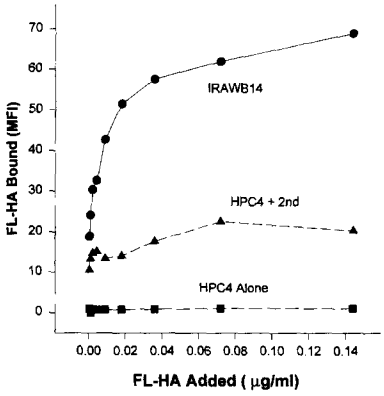

Figure 5. Cross-linkage of CD44 via an epitope tag slightly increases ligand-binding ability. Stable transfectants were prepared with AKR1 lymphoma cells and cDNA, which encodes the transmembrane domain of tissue factor, the HPC4 epitope (see Fig. 8 for placement), and the extracellular portion of the hemopoietic form of CD44. Flow cytometry was first done to establish that the transfected CD44 was recognized both by antibodies to CD44 and the HPC4 epitope tag (not shown). The cells were then exposed to FL-HA after pretreatment with the IRAWB14 or HPC4 antibodies alone, or HPC4 followed by a second antibody as described in Materials and Methods. The results are shown with background fluorescence obtained with untreated cells subtracted.

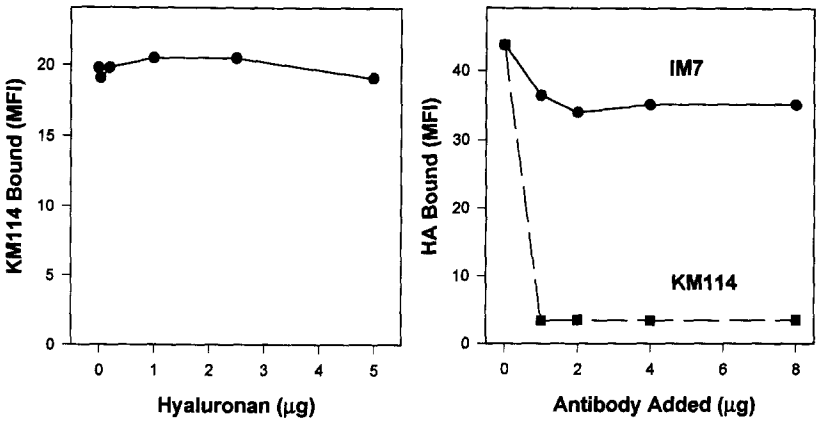

Figure 6. Competition between HA and antibody for CD44 binding. The left panel shows that even very high concentrations of unlabeled HA did not prevent staining of AKR1.CD44WT transfectants with FITC labeled KM114 antibody. The right panel illustrates that labeled HA was eluted from prestained cells with the same antibody, but not with IM7. The amount of label on cells was determined by flow cytometry and expressed as MFI.

that while simple oligomerization of CD44 improves its receptor function, the IRAWB14 antibody induces changes which have far greater consequences.

Competition between the Hyaluronan Ligand and a Blocking Antibody for CD44

A single amino acid (histidine-83) determines the epitope recognized by the hyaluronan blocking KM114 antibody; we performed simple competition experiments between this antibody and HA. CD44 positive AKR1.CD44WT lymphoma cells were placed in graded concentrations of unlabeled hyaluronan before addition of fluorescein-labeled KM114. The free ligand had no effect on antibody staining (Fig. 6, *left panel*). A reciprocal experiment was performed where the cells were first exposed to fluorescent hyaluronan, followed by unlabeled KM114 antibody (Fig. 6, *right panel*). In this situation, as little 1 μg of antibody completely reversed hyaluronan binding. The IM7 antibody, which fails to block ligand binding to these cells, also did not elute HA once it had attached. These experiments indicate that the ligand has a lower avidity and/or higher dissociation rate from CD44 than does the KM114 antibody. KM114 must either alter CD44 conformation so as to make hyaluronan recognition impossible or block the binding site.

The Ability of CD44 Antibodies to Block Ligand Binding Depends on Cell Type as well as Specificity

Previous studies revealed that although CD44 is broadly expressed on lymphocyte populations, only some cells are responsive to the enhancing IRAWB14 antibody (20, 24–27). We now show that the type of cell can influence whether certain antibodies interfere with hyaluronan recognition by cells that do so constitutively. 9 of the 10 antibodies in the h group efficiently blocked ligand binding, but only when examined with the WB lymphoma (Fig. 7 and Table IV). As noted above, all antibodies whose specificity was influenced by histidine-83 or valine-90/threonine-91 were uniform blockers, regardless of cell type. There was no correlation between the isotypes of the antibodies and their influence on CD44 function.

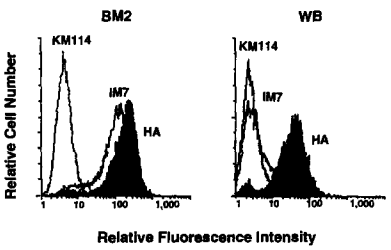

Figure 7. Some antibodies block HA recognition, but only on particular cell lines. Flow cytometry results are shown with a B cell hybridoma (*BM2*) and a T lymphoma (*WB*) which were exposed to unlabeled CD44 antibodies before FL-labeled HA. Binding of HA via CD44 was completely blocked by the KM114 antibody. However, pretreatment with the IM7 antibody only prevented ligand recognition by the WB lymphoma.

Discussion

While considerable information suggests that CD44 participates in tumor metastasis and other processes, its molecular structure has not been solved and little is known about its ligand-binding site(s). We performed a detailed analysis of a large panel of CD44 antibodies. This information can now be used to consider how the specificities of antibodies correspond to their abilities to influence ligand recognition functions. It also provides some basis for speculation about regions of the molecule that contribute to adhesive functions.

Species Polymorphisms in CD44 that Are Immunogenic

We selected the interspecies chimera approach because

Table IV. Some CD44 Antibodies Only Block Ligand Binding by Particular Cells

		Cell lines			
Eptiope group	Antibody	BM-2	W279	BW5147	WB
f	KM114 (IgG_1)	↓↓	↓↓	↓↓	↓↓
f (complex)	KM201 (IgG_1)	↓↓	↓↓	↓↓	↓↓
d, e, g	SK159 (IgG_{2a})	↓	↓↓	↓	↓
	IM7 (IgG_{2a})	~	~	~	↓↓
h	SK47 (IgG_{2a})	~	~	~	↓
	IRAWB27.3 (IgG_1)	~	~	~	↓
	KM703 (IgG_{2a})	↓	~	~	↓

Cells were incubated on ice with FITC-HA in the presence of the indicated CD44 antibodies for 15 min. Binding was then assessed by flow cytometry. MFI of staining in the presence of each antibody was normalized to the MFI of FITC-HA alone of each cell line.
Changes are indicated as: ~, 0-50%; ↓, 50-90%; ↓↓, >90% decreases in FITC-HA binding.

preliminary experiments with peptides were not promising, and many of the antibodies even failed to recognize reduced protein. All of the chimeric proteins retained ligand-binding ability and thus reflected species specific polymorphisms rather than mutations. Structural integrity of these molecules was also suggested by the reciprocal loss of mouse-specific epitopes and gain of reactivity by antibodies to rat CD44. Furthermore, some rat antibodies to murine CD44 recognized other species in a pattern that corresponds to shared amino acids. For example, the IM7 group of antibodies (epitope h) presumably recognize human CD44 because it shares a proline at position 108 with mouse. Similarly, the KM114 antibody recognizes hamster (shared histidine-83), but not human CD44 (different at this position). A number of our antibodies recognize single residue differences between species, which in one case occurs at the amino terminus of the protein. While this type of analysis does not precisely reveal epitopes on CD44 which the antibodies recognize, it allows grouping of antibodies according to similar specificity. We also assume that substitution of an amino acid subtly influences protein conformation within a limited area because rat and mouse CD44 are both functional receptors for HA.

Some antibodies determined to have different specificities by chimera analysis (groups *d* and *f*) were able to cross-block each other. This might advise caution in use of the term epitope mapping to describe results from simple antibody cross-blocking studies, because it implies that the precise locations of antibody binding sites are known. Although the h epitope is determined by a polymorphism at position 108 (proline), a fusion protein which stopped at threonine-113 was not recognized by IM7.8.1 (40). This stretch of six residues is interrupted by a postulated disulfide bond (Fig. 8). Structural integrity of the h epitope must therefore depend on residues in two neighboring regions of the molecule.

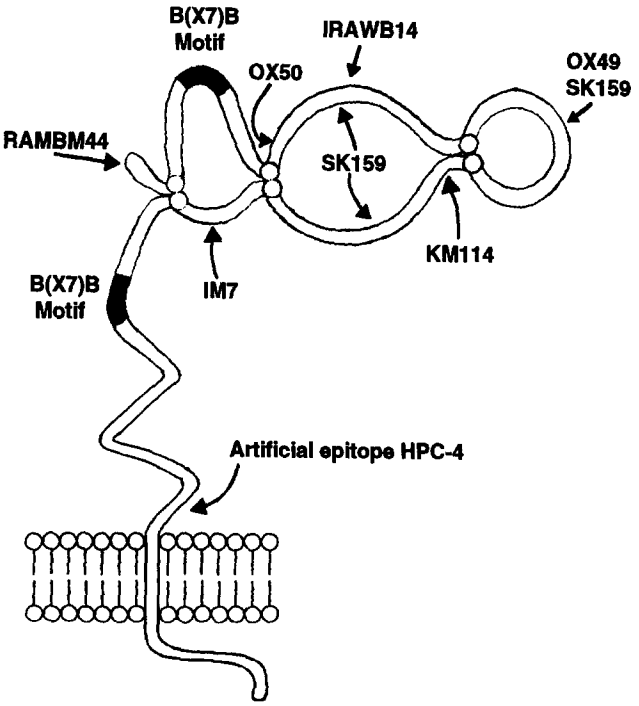

Figure 8. The secondary structure of CD44 has been extrapolated from that of the related cartilage link protein (11, 33). Positions of residues determining some of the murine and rat CD44 epitopes are indicated, along with the position of the HPC4 epitope tag used for one construct. Two shaded areas contain B(X7)B motifs which other studies indicate may be important for HA recognition (38, 56).

The KM201 antibody is partially related to KM114 in that its recognition was reduced, but not eliminated by substitution of histidine-83 and it did not give significant staining with human lymphocytes in our hands. However, the same reagent has been found to interfere with functions mediated by human CD44 (19, 53). This species cross-reactivity might not be due to peptide sequence because we recently found that tunicamycin-treated hamster, but not mouse, cells lose the KM201 epitope (18).

Relationships between Immunogenic Regions and Functional Domains

Large numbers of mAbs have been prepared to distinct human CD44 epitopes, and at least some of them inhibit hyaluronan recognition (29, 30, 34, 42). The epitopes for most of these have not been characterized. An exception is the HERMES 3 antibody, which does not interfere with HA recognition, but does block human lymphocyte adhesion to high endothelial venules in mucosal tissues (7). The HERMES 3 epitope is encoded by amino acids in the membrane proximal region (11), an area with discrete functions not necessarily related to hyaluronan. This domain is subject to great molecular diversity arising from alternative mRNA splicing and has attracted interest because of its possible function in cell migration and metastasis (12, 13, 45). Variation in this part of human CD44 can also influence HA recognition in certain cell types (8, 13, 49). This interesting region of the molecule was not detected by any of the anti-mouse CD44 antibodies used in our study. In fact, all of these reagents recognized the truncated CD44ΔNC protein, which contains only the first 163 residues (13).

Solution of the structure of CD44 will likely reveal close physical proximity between different loops. Antibodies have been prepared that recognize combinatorial epitopes involving separate integrin chains (3, 5). We now describe an antibody (SK159) whose epitope depends on three widely separated residues in the same protein. Available information indicates that multiple CD44 domains must function in concert for effective hyaluronan recognition and these antibodies may be helpful in understanding their spatial orientation.

CD44 can mediate recognition of ligands other than hyaluronan and some of these functions may involve the amino terminal domains. For example, it has been reported that hyaluronan and a proteoglycan might compete for the same site on CD44, in a manner inhibitable by the KM201 antibody (54). Also, the interaction between an erythroleukemia line and stromal cells seemed not to be hyaluronan dependent, but was partially blocked by our KM81 antibody (51). Precursors of thymocytes have no apparent affinity for hyaluronan, but their migration to the thymus is blocked by pretreatment with either the IM7 or KM201 antibodies (22, 57).

Several previous studies suggest that >145 amino acids are required for efficient CD44–hyaluronate interactions (13, 40). Furthermore, ligand-binding ability is probably dependent in part on critically spaced disulfide bonds (35). For these reasons, information is very limited on residues that might contribute to the actual binding site. One study concluded that two short stretches of amino acids corresponding to a B(X7)B motif (see Fig. 8) had affinity for HA when expressed as peptide–bacterial fusion proteins (58). In another study, mutation of a single arginine in one of these motifs completely abolished HA recognition and function was greatly reduced following alterations or truncations of the other motif (40). These motifs and the sequences which surround them are identical in mice and rats. Immunogenic residues corresponding to antibodies that influence ligand binding were not obviously localized to these portions of murine CD44 (Fig. 8). Rather, our findings indicate that some features of the molecule dictated by lysine 49 (group *d*, some enhancing antibodies) and the histidine 83 to valine 90 region (group *f*, blocking antibodies) could be important in regulating its adhesive function.

Antibodies that Block Ligand Recognition

High concentrations of unlabeled HA had no effect on attachment of the KM114 antibody to CD44 and KM114 eluted previously bound ligand from cells. This universal blocking antibody may have higher affinity for CD44 than does the ligand, and it either causes a conformational change or physically obstructs the binding site. Antibodies with indistinguishable, or partially related specificities (KM81 and KM201, RAMBM55.5, and D12), also blocked hyaluronan binding in every situation. SK159 was another consistent blocking antibody and one of the three residues on which its epitope depends is in the same loop of the molecule. While it is premature to speculate on the actual binding site for HA, this portion of CD44 may well contribute to it.

All but one of the antibodies in another group (*h*), blocked hyaluronan recognition by some cells, but not by others (Table IV). The basis for these cell-specific effects are not known. It might be speculated that WB cells recognize HA with relatively low affinity, allowing the binding to be inhibited by a broader spectrum of antibodies. Alternatively, it is possible that different cells use different portions of CD44 to achieve the same function. We show elsewhere that the ability of some cells to bind HA can be largely explained by differential glycosylation of CD44 (18, 28). Treatment with tunicamycin or deglycosylating enzymes converts nonbinding cells to HA-recognizing cells. It will be interesting to learn if there are similarities in how antibodies and naturally occurring carbohydrate make the CD44 binding site inaccessible for ligand.

Antibodies that Enhance Ligand Binding

It remains unclear why the IRAWB14 antibody increases hyaluronan recognition by some lymphocytes, but it could mimic some naturally occurring structure on the same or apposing cells. Antibodies to several integrins are also known to activate receptors for ligand recognition, and it has been speculated the antibodies cause and/or maintain a favorable conformational state in the receptors (2, 9, 38, 48). We anticipated that antibodies with related specificity would similarly increase hyaluronan recognition, but this was not the case and there was also no correlation with Ig isotype. A previous study localized residues encoding epitopes for both activating and inhibiting antibodies to a short segment of the β_1 integrin chain (52). This region included four amino acids that differed between species. We found that antibodies with indistinguishable specificity for polymorphism of a single residue had opposite influence on hyaluronan binding. Furthermore, only 1 out of another group of 10 antibodies (R7166.7, group h) had enhancing activity. One residue difference between mice and rats could result in more than one epitope and the blocking and enhancing antibodies within one group could differ with respect to fine specificity. Other properties of antibodies, such as affinity or isoelectric point, might also be important for determining how they influence cell adhesion molecules.

All of the antibodies in our panel recognize the portions of CD44 that are conserved among HA-recognizing proteins. We wondered if antibodies directed to a completely different part of the molecule could influence HA recognition and designed constructs with the HPC4 epitope tag near the cell membrane (Fig. 8). A transmembrane domain from tissue factor was used, as it is known to be compatible with dimerization (44). A small but measurable increase was seen when the receptor was extensively clustered via the epitope tag and a second antibody (Fig. 5). A polyclonal antibody prepared to a membrane proximal peptide of human CD44 also had a slight positive influence on hyaluronan binding (30). These findings are compatible with previous studies which demonstrated a requirement for multivalent binding of enhancing CD44 antibodies (26). Artificial dimers of CD44 have been shown to have improved ligand recognition (41). Local densities of integrins and their ligands have also been thought to be important for cell adhesion (14). However, the degree of ligand binding we achieved by receptor cross-linkage plateaued at a much lower level than that caused by IRAWB14. We show elsewhere that IRAWB14 substantially increased the ligand binding ability of a purified CD44-Ig fusion protein, even after it was immobilized to beads (18). Thus, while clustering of CD44 molecules on cell membranes can influence the amount of ligand bound, interaction with a special type of antibody (or ligands it might mimic) can have a much greater effect.

These observations add to previous studies by helping to ascribe functions to particular CD44 domains. There might be naturally occurring substances that modulate ligand recognition by this cell adhesion molecule. Our observations with enhancing and inhibiting antibodies indicate that such regulatory molecules could function through multiple sites and mechanisms. An understanding of these phenomena might suggest means to influence CD44 mediated tumor metastasis. Also, experience obtained with this group of reagents may influence the design and interpretation of many other studies in which antibodies are used to probe and manipulate functions of cell adhesion molecules.

The authors appreciate helpful comments on the manuscript by Dr. Linda Thompson. We thank Drs. Charles Esmon and James Morrissey for the

HPC4 epitope tag system and the transmembrane segment of tissue factor.

This work was supported by National Institutes of Health grants AI-33085, AI-24137, AG-04360, AI-31613, and CD14195.

Received for publication 1 March 1995 and in revised form 21 April 1995.

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