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Molecular Isoforms of Murine CD44 and Evidence That the Membrane Proximal Domain Is Not Critical for Hyaluronate Recognition

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Abstract. We previously found that the CD44 glycoprotein on some lymphocytes can mediate adhesion to hyaluronate (HA) bearing cells. However, many questions remain about the molecular heterogeneity of CD44 and mechanisms which control its recognition of this ligand. In vitro mutagenesis and DNA sequencing have now been used to investigate the importance of the membrane proximal region of murine CD44 for recognition of soluble or cell surface HA. CD44 with an 83 amino acid deletion in this region mediated binding to soluble ligand and the apparent avidity increased markedly in the presence of a particular antibody to CD44, IRAWB14. The shortened CD44 was however inefficient in mediating adhesion of transfected cells to HA immobilized on cell surfaces. Four new murine isoforms of CD44 were isolated from a carcinoma line by use of the polymerase chain reaction. Only two of them correspond to ones recently discovered in rat and human cells. The longest variant nearly doubled the length of the extracellular portion of the molecule and introduced an additional 20 potential sites for glycosylation. When expressed on T lymphoma cells, all four of the new murine CD44 isoforms were capable of mediating adhesion to HA bearing cells. This result contrasts with a report that a related human CD44 isoform lacks this ability when expressed on B lineage lymphoma cells. The new murine isoforms also conferred the ability to recognize soluble HA and were very responsive to the IRAWB14 antibody. A brief survey of normal murine cell lines and tissues revealed that the hemopoietic isoform was the most abundant species. These findings indicate that the NH_2-terminal portion of CD44 is sufficient for HA recognition and that this function is not necessarily abrogated by variations which occur in the membrane proximal domain. They add to the known molecular diversity of CD44 and provide another experimental model in which isoform specific functions can be investigated.

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CD44 has been implicated in a variety of functions, which include hemopoiesis, tumor metastasis, and cell homing, as well as the activation of lymphocytes and monocytes (13–15, 30, 44). Some of these processes may depend on the ability of CD44 to recognize HA.[1] For example, CD44 bearing lymphoid cells can adhere to stromal cells and to certain endothelial cells by recognition of surface HA (27, 31). However, while virtually all normal blood cells express CD44, most do not have measurable affinity for HA (19, 27–29). The situation may be comparable to certain other cell adhesion molecules (CAM's) whose recognition function requires activation of the cells which express them (35). Indeed, activated B lymphocytes taken from mice with graft versus host disease or stimulated with IL-5 become adhesive for HA coated surfaces and some lymphomas are induced to recognize HA via CD44 after exposure to phorbol ester (19, 33, 34). While the molecular basis for regulation of ligand recognition by this molecule is unknown, possible mechanisms include structural diversity, conformational changes, focal clustering on the cell surface and interaction with other cell surface molecules. There is also recent evidence that association with cytoskeletal proteins may be cell type or activation dependent and possibly regulated by phosphorylation of intracellular serine residues (2, 4, 21, 22, 26). We previously found that the cytoplasmic domain is important for the adhesive function and that antibodies to particular epitopes on CD44 may dramatically increase ligand binding (27).

It has been known for some time that extensive posttranslational modifications of CD44, which involve O- and N-linked glycosylation, and in some cases, addition of chondroitin or heparan sulfates, account for some of the observed heterogeneity in molecules (3, 11, 23, 24, 36). More recently, multiple isoforms of rat and human CD44 have been described which may result from alternative exon utilization (3, 8, 14, 17, 42). This additional diversity has been associated with differences in growth rates in vivo and metastasis of tumors, as well as differences in HA recognition (14, 42, 43). These naturally occurring isoforms all display variations within a membrane proximal region of CD44.

We have investigated the importance of this domain for

1. *Abbreviations used in this paper*: HA, hyaluranate; PCR, polymerase chain reaction.

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HA binding by mutagenesis, as well as by isolation of new murine isoforms which differ in this region. The function of mutated and naturally occurring variants of CD44 were assessed after transfection into CD44 negative T lymphoma cells. We conclude that the NH_2-terminal 167 residues of CD44 are sufficient for recognition of HA when expressed on an appropriate cell type. Furthermore, this function is not necessarily dependent on sequence diversity involving the membrane proximal domain. Marked augmentation of HA binding by an enhancing monoclonal antibody was observed with all CD44 isoforms and variants.

Materials and Methods

Cells, Cell Cultures, and Antibodies

The murine T lymphoma AKR1 (18) was maintained in DME with 10% FCS and antibodies. The BM-2 B cell hybridoma, the 70Z/3 pre-B lymphoma, the W231 B lymphoma (30) and BMS-2 stromal cells (37) were cultured in RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME and antibiotics. The murine squamous cell carcinoma KLN205, Lewis lung carcinoma LL/2 and NIH 3T3 cells were obtained through the American Type Culture Collection (ATCC, Rockville, MD) and maintained in MEM with nonessential amino acids, 10% FCS and antibiotics (for KLN205), and in DME with 10% FCS and antibiotics (for LL/2 and NIH 3T3), respectively. The KM201, KM81, and IRAWB14 mAbs, recognizing epitopes on murine CD44 (27, 31), were used in purified form.

Isolation of Poly A^+ RNA

Poly A^+ RNA was isolated as described (1). Briefly, cultured cells were harvested, washed in HBSS and lysed with lysis buffer (200 mM NaCl, 200 mM Tris-HCl, pH 7.5, 1.5 mM $MgCl_2$, 2% SDS and 0.2 mg/ml Proteinase K). Oligo(dT) cellulose (Collaborative Research, Bedford, MA) was hydrated in elution buffer (10 mM Tris-HCl, pH 7.5), followed by equilibration in binding buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.5). The lysate and oligo(dT) cellulose were mixed and incubated for 30 min at room temperature with agitation. The oligo(dT) cellulose was then washed with binding buffer and packed onto a Poly Prep Chromatography Column (Bio-Rad Laboratories, Richmond, CA). The poly A^+ RNA was eluted with elution buffer.

Polymerase Chain Reaction

One microgram poly A^+ RNA was reverse transcribed with 14 U AMV reverse transcriptase (US Biochemical Corp., Cleveland, OH) in a 20-μl reaction primed with 0.7 μg of oligo $(dT)_{12-18}$ (10). The reaction was assembled by mixing poly A^+ RNA, oligo $(dT)_{12-18}$ and H_2O, followed by heating at 68°C for 4 min and quenching on ice. Then 4 μl of 5× first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$ and 50 mM dithiothretol) (US Biochemical Corp.), 1 μl of 10 mM dNTP, 10 U RNasin (Promega Corp., Madison, WI) and 14 U AMV reverse transcriptase were added. After 2 h at 39°C, the reaction was diluted with 1 ml TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and 10 μl was used as template for the subsequent polymerase chain reaction (PCR).

Two oligonucleotides were synthesized for PCR priming. The upstream primer OM-6AR, 5'-GGGAATTCGCCATGGACAAGTTTTGGTGG-3', corresponds to a 21-bp stretch overlapping the initiation codon ATG in the murine CD44 cDNA clone MHR6 (45), whereas the downstream primer OM-2AR, 5'-GGGAATTCAATGGCGTAGGGCACTACA-3' is complementary to a 19 bp fragment around the stop codon TAG in the same clone (45). Both oligonucleotides had an EcoRI site added to the 5' end for use in cloning.

The PCR cocktail was assembled in a 100 μl reaction by mixing 10 μl template, 10 μl of 10 × PCR buffer (Promega Corp.), 16 μl of 1.25 mM dNTP, 0.2 nmol each of OM-6AR and OM-2AR, and 5 U *Thermus aquaticus* (Taq) DNA polymerase (Promega Corp.). After 40 cycles at 94°C for 40 s, 55°C for 2 min and 72°C for 2.5 min, 5 μl of the reaction products were analyzed on a 1.2% agarose gel and the remainder purified using Geneclean (Bio 101 Inc., La Jolla, CA).

Construction of Mutant CD44ΔNC

A CD44 mutant, CD44ΔNC, lacking the region between Val161 and Arg244 (45) was made by loop out mutagenesis involving a two-round PCR. An oligonucleotide Blpl24 complementary to sequences before Val161 and after Arg244, 5'-CTGGAATCTGAGGTCTCCTATCGTCATCTATAATGTTTG-3', was synthesized. The first round was primed by OM-6AR and Blpl24 using the cDNA clone MHR6 as the template, resulting in a fragment containing all sequence up to that coding for Val161 and a stretch encoding Arg244 to Pro249. The product was purified, denatured by heating, and used as the upstream primer together with the downstream primer OM-2AR in a second round PCR with the same template, which created mutant CD44ΔNC. The final product was sequenced to confirm that the correct mutated sequenced had been obtained.

cDNA Cloning and Sequencing

The purified PCR products were digested with EcoRI and subcloned into plasmid pBS(+) (Stratagene Corp., La Jolla, CA). The nucleotide sequences of the inserts were determined using the dideoxy chain termination method with Sequenase (US Biochemical Corp.) from double-stranded templates. Sequence analysis software was from the Genetics Computer Group of the University of Wisconsin.

Construction of Plasmids for Expression

The cDNA inserts 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 gene for selection in G418 supplemented media.

Transfection by Electroporation

Cells were transfected by electroporation as described previously (16). Briefly, AKR1 cells were suspended at 1.0×10^7/ml in ice-cold PBS (Mg^{2+} and Ca^{2+} free). 0.5 ml of the cell suspension was transferred into a precooled electroporation cuvette and 100 μg of purified plasmid DNA was added. The cuvette was then pulsed using the Gene Pulser apparatus (Bio-Rad Laboratories) with 550 V and 25 μF of capacitance. G418 was added at 1 mg/ml after 48 h of growth and drug-resistant colonies appeared within 2 to 3 wk. The transfectants were analyzed by KM81 staining and bright populations were sorted on a FACStarPlus (Becton-Dickinson Immunocytometry Systems, San Jose, CA). In contrast to the experience of another group who used fibroblasts (40), none of our AKR1 transfectants displayed any marked tendency for spontaneous aggregation.

Northern and Southern Blotting

Northern blotting was performed as described (32). After electrophoresis on a 1.2% agarose gel, the PCR products were Southern blotted with various probes using procedures previously descried (38). The M4EX probe was created by PCR amplification using primers of M4UP, 5'-GCAACTACTCCACGGGTT-3', and IDON, 5'-CGTTGGAGTCAGTAGC-3', from domain A and D, respectively (Fig. 3 *a*), whereas probe M4AB is the PCR product of domain A and B using M4UP and M3DON (5'-GCTTTCTGTTTGATGACCTTG-3').

Cell Adhesion Assays

Cell adhesion to HA immobilized on stromal cell layers was assayed as described (31). Briefly, 2×10^6 cells were washed, suspended in complete medium and incubated with 10 μCi ^{51}Cr at 37°C for 1 h. The labeled cells were washed and added to 24-well plates (Corning Cell Well, Corning, NY) coated with a layer of the stroma cell line BMS-2 plated 24 h before the assay. Plates were incubated with labeled cells for 2 h at 4°C. Unbound cells were removed by three cycles of washing in complete medium with vigorous agitation on a minishaker (Dynatec Laboratories Inc., Chantilly, VA). Bound cells were lysed with 0.1 N NaOH/1% NP-40 and the ^{51}Cr counted with a gamma counter (Beckman Instruments Inc., Fullerton, CA). Percentages of bound cells were calculated as previously described (31). The adhesion of transfected lymphoma cells to BMS2 stromal cells is almost totally dependent on CD44 and HA (27). This was confirmed in these studies by use of HA and KM201 monoclonal antibody treatment (27).

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Soluble Ligand Binding Assays

Fluorescein-conjugated HA was prepared and used for soluble ligand binding experiments as described (27) except that cells were tested in serum free medium. The degree of ligand binding was determined by flow cytometry with a FACScan (Becton Dickinson Immunocytometry Systems). Numbers of molecules of bound fluorescein were determined by comparison of median channel numbers with values obtained with Immunobright bead standards (Coulter Corp., Hialeah, FL). The density of CD44 on transfected cells was similarly determined by flow cytometry after immunofluorescent staining with the KM 81 monoclonal antibody.

Cell Surface Biotinylation and Immunoprecipitation

Cells were surface labeled as described (32) and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM iodoacetamide, 0.1% NaN_3, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 10 μg/ml of soybean trypsin inhibitor, 1 μg/ml of leupeptin and 1 trypsin inhibitor unit/ml of aprotinin). The lysates were precleared with 100 μl of Affigel-10 (Bio-Rad Laboratories) conjugated with rat serum, followed by immunoprecipitation with 25 μl of Affigel-10 conjugated with either KM201 anti-CD44 antibody (2 mg/ml) or rat IgG as a negative control. After rotating 1 h at 4°C, the beads were washed twice with 50 mM Tris-HCl, pH 8.3, 0.6 M NaCl, 0.2% Triton X-100 and 0.1% NaN_3, and once with 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl and 0.1% NaN_3. Bound proteins were eluted by boiling 5 min in reducing sample buffer and run on a 7.5% SDS-PAGE gel, followed by transfer to a *trans* Blot membrane (Bio-Rad Laboratories). The membrane was blocked with 0.5% gelatin, 0.05% Tween-20 and 0.05% thimerosal in PBS for 1 h at room temperature, washed with 0.1% Tween-20 in PBS and incubated for 1 h with avidin HRP (Bio-Rad Laboratories) diluted 1:3,000 with 0.5% BSA and 0.05% Tween-20 in PBS. Biotinylated proteins on the membrane were visualized using the ECL Western blotting detection system (Amersham International, Arlington Heights, IL). Biotinylated molecular weight markers were used as standards.

Western Blotting

CD44 proteins were enriched by immunoprecipitating the unlabeled lysate with KM201 conjugated Affigel-10. The eluate was electrophoresed on a 7.5% SDS-PAGE gel, transferred onto a *trans* Blot membrane which was then blocked as described above. The membrane was first incubated for 1 h with 10 μg/ml KM201 in 1% BSA, 0.05% Tween-20 and 0.05% thimerosal, washed with 0.1% Tween-20 in PBS, and then incubated with 1:10,000 diluted HRP conjugated goat anti-rat IgG (Zymed Laboratories, Inc., San Francisco, CA). The protein bands were detected using the ECL system (Amersham Corp.). Prestained molecular weight markers were used as standards.

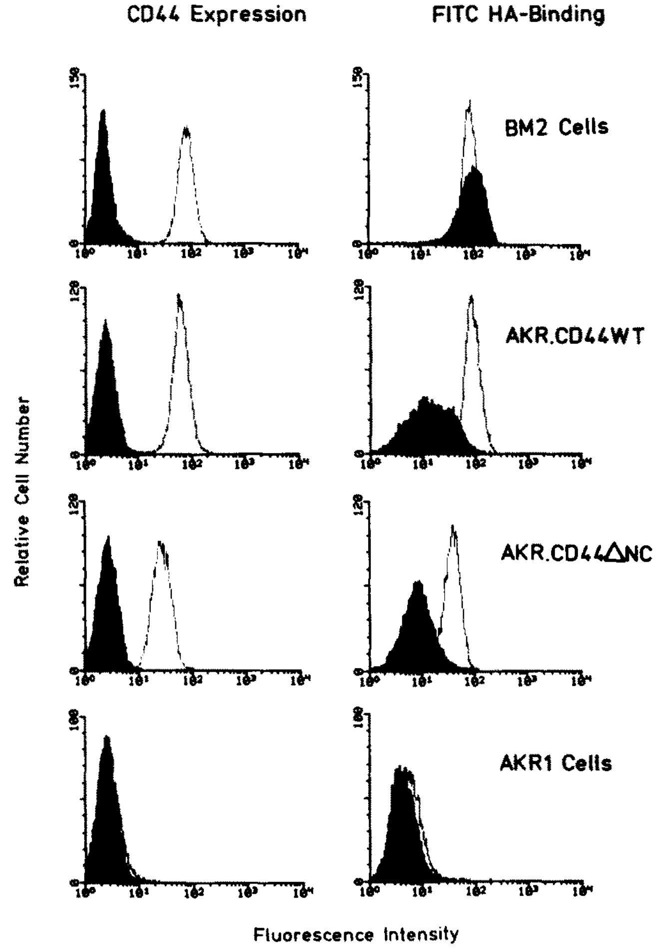

Figure 1. The membrane proximal domain of CD44 is not essential for recognition of soluble hyaluronate. Surface densities of CD44 (*left*) on an HA binding B lineage hybridoma (BM2) were determined by flow cytometry and compared with lymphoma cells transfected with either wild-type CD44 (CD44WT) or a mutant lacking the membrane proximal domain (CD44ΔNC). Shaded histograms depict unstained cell profiles and open histograms depict staining with FITC-labeled anti-CD44 antibody KM81. Flow cytometry results shown on the right depict binding of soluble fluorescein-labeled HA when tested alone (*shaded*), or when the enhancing IRAWB14 monoclonal antibody was added at the same time as the ligand (*open*).

Results

HA Binding Occurs in the Absence of the Membrane Proximal Domain

Recent reports have described various molecular isoforms of CD44 in humans and rats (3, 8, 14, 17, 42). These differ from the isoform expressed by most hemopoietic cells in having additional sequences inserted in the membrane proximal domain, which is the least well conserved part of the molecule. We investigated the importance of this region of the hemopoietic form of CD44 by mutagenesis and transfection experiments (Materials and Methods). A construct producing a molecule which lacks 83 amino acids of this domain was transfected into AKR1 T lymphoma cells (AKR1.CD44ΔNC). The sizes of the transcript and expressed protein were as predicted (1.7 kb and 58 kD, data not shown and Fig. 6 below). The epitope on CD44 which is recognized by the monoclonal antibody IRAWB14, as well as others defined by a panel of 22 antibodies, were all present in the mutated molecule (data not shown). We then evaluated the ability of the transfected lymphoma cells to recognize soluble fluorescein labeled HA (Fig. 1). The density of CD44 on this transfectant was ~36% of that on our previously described wild-type CD44 transfectant (AKR1.CD44WT). Nonetheless, there was constitutive recognition of the soluble HA ligand, which was markedly augmented in the presence of the inducing IRAWB14 monoclonal antibody. In contrast to the AKR1 lymphoma transfectant, maximum HA binding was constitutive with the BM2 B cell hybridoma and was usually unaffected by pretreatment with the IRAWB14 antibody. Note that BM2 cells appear to express the same "hemopoietic" isoform as other blood cells (see below). We conclude that the binding site for HA is in the amino terminal two thirds of CD44 and that the membrane proximal domain is not essen-

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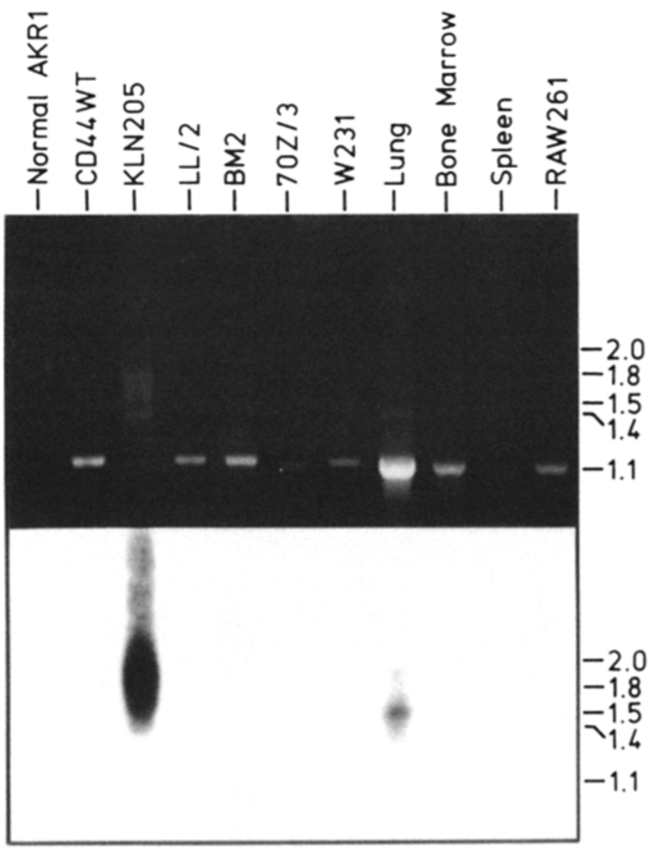

Figure 2. PCR amplification of CD44 from various cell lines, normal tissues and transfectants. Total or poly A selected RNA from various sources were reverse transcribed and PCR amplified using oligonucleotides OM-6AR and OM-2AR as detailed in Materials and Methods. The top illustrates the ethidium bromide stained products separated on a 1.2% agarose gel and visualized by UV illumination. The same PCR products were transblotted onto a nitrocellulose membrane and hybridized with cDNA probe M4EX as described in Materials and Methods. The resulting autoradiogram is shown in the bottom.

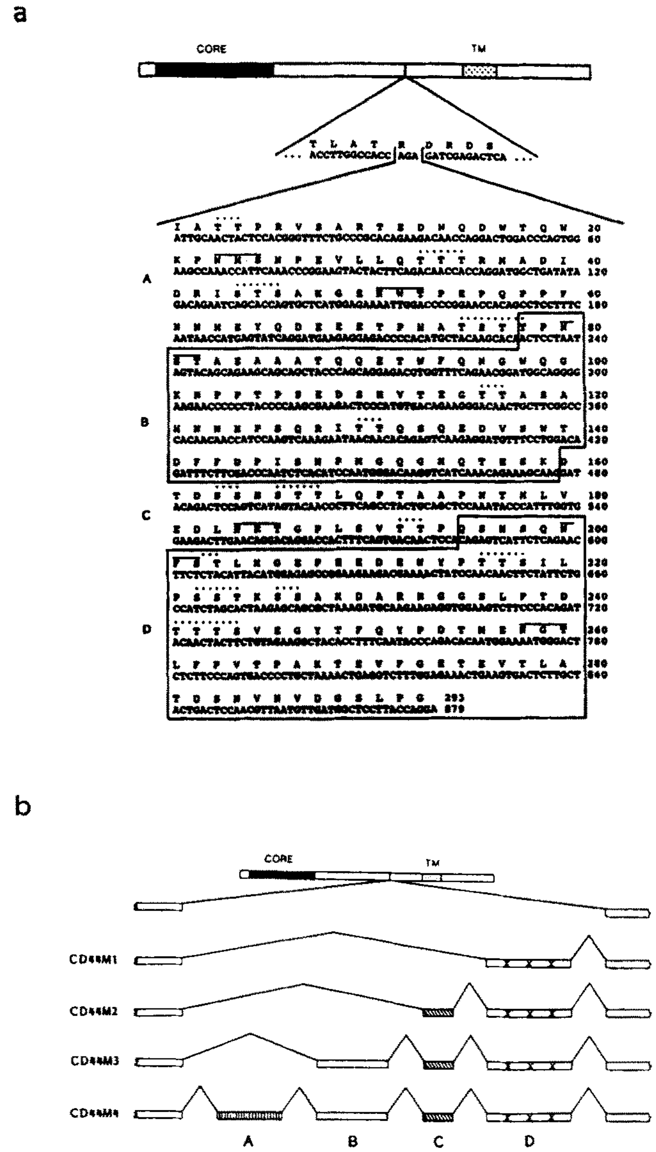

Figure 3. Complete cDNA sequences are shown for four new murine CD44 isoforms (*a*). The site of insertions in the previously described "hemopoietic" isoform is indicated relative to the proteoglycanlike "core" and transmembrane (TM) domains. Solid and dotted lines indicate potential N- and O-linked glycosylation sites, respectively. The blocks and letters on the left designate domains which are utilized in the various isoforms and the combinations found are depicted in *b*. These sequence data are available from EMBL/GenBank/DDBJ under accession numbers X66081-X66084.

tial for this function. Furthermore, the shortened molecule is fully responsive to the effects of the IRAWB14 antibody.

Molecular Isoforms of Murine CD44

Polymerase chain reactions were used to screen for murine cell lines which might synthesize high molecular weight isoforms of CD44. A squamous cell carcinoma (KLN205) was found to contain transcripts other than the expected 1.1 kb species when primers were used which span the coding sequence for the hemopoietic form of murine CD44 (Fig. 2). With this cell line, at least four discrete bands, corresponding to transcripts of 1.4, 1.5, 1.8, and 2.0 kb were resolved. Each band was purified by gel electrophoresis, digested with EcoRI, ligated into the pBS(+) plasmid and their nucleotide sequences determined. At least two independent clones were obtained from each, representing identical sequences. Each of these transcripts, which we designate M1–M4, encode CD44 isoforms which differ from the hemopoietic isoform by insertions after Thr 202 in the membrane proximal domain, extending the molecule by 99, 134, 216, and 293 amino acids, respectively (Fig. 3). The longest isoform (M4), has an additional six potential N-linked and 14 potential O-linked glycosylation sites. The only homology found for these new sequences by computer search was with recently described human and rat CD44 isoforms (17). Two of the four murine isoforms (M1 and M2) had exact homologues in other species and two (M3 and M4) represented unique combinations of sequences previously found in one rat isoform (see Discussion). With the exception of an NH_2-terminal stretch of 42 residues and two small gaps required for alignment of the murine and rat sequences, there was 88% identity between these two species (Fig. 4).

PCR amplification products obtained with RNA from a variety of cell lines and tissues were further assessed by

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Mouse   1 .........................................IATTPRVSARTEDNQDWTQWKPNHSNPEVLLQTTTRMADIDRISTSAHGENWTPEPQPP 59
                                                 |||||:|||:|.:||::|||.| ||||||||||||||.|||| ||||||||||.|||||
Rat     1 TESNTNPTGWKPNEENEDETDKYPNFSGSGIDDDEDFISSTIATTPWVSAHTKQNQERTQWNPIHSNPEVLLQTTTRMTDIDRNSTSAHGENWTQEPQPP 100

       60 FNNHEYQDEEETPHATSTT...PNSTAEAAATQQETWFQNGWQGKNPPTPSEDSHVTEGTTASAHNNNHPSQRITTQSQEDVSWTDFFDPISHPMGQGHQT 156
          |||||||||||||||||||   ||||.|.||||.|.||:|:||||||||||||||||||||||||||||||||:|||||||||||||||||||||||||||
      101 FNNHEYQDEEETPHATSTTWADPNSTTEEAATQKEKWFENEWQGKNPPTPSEDSHVTEGTTASAHNNNHPSQRMTTQSQEDVSWTDFFDPISHPMGQGHQT 200

      157 ESKDTDSSHSTTLQPTAAPNTHLVEDLNRTGPLSVTTPQSHSQNFSTLHGEPEEDENYPTTSILPSSTKSSAKDARRGGSLPTDTTTSVEGYTFQYPDTM 256
          |||||:||||||||||||||||||||||||||||||||||||||||||.|| ||:|:.||||:|||||||    :||||||| |||||:|||| ||||||
      201 ESKDTGSSHSTTLQPTAAPNTHLVEDLNRTGPLSVTTPQSHSQNFSTLPGELEEGEDHPTTSVLPSSTKS...GRRRGGSLPRDTTTSLEGYTPQYPDTM 297

      257 ENGTLFPVTPAKTEVFGETEVTLATDSNVNVDGSLPG 293
          ||||||||||||||||||||.|:|||||.|||||||
      298 ENGTLFPVTPAKTEVFGETEGTVATDSNFNVDGSLP. 333
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Figure 4. Protein sequence comparison of inserted residues in mouse and rat CD44 isoforms. The unique sequence from the new murine CD44M4 isoform (*top*) is compared with the longest CD44 isoform which was recently discovered in rats (17). Identical residues are aligned with vertical bars, whereas colons and dots between lines indicate conserved and less conserved replacements, respectively. Blanks represent nonconserved changes and gaps within the sequence were introduced for best alignment.

Southern blotting (Fig. 2) with a probe (M4EX) corresponding to the longest (M4) inserted sequence (Fig. 3 *a*). With this approach, lung was the only normal tissue examined which clearly had an alternative form of CD44. This transcript corresponded in size to the M2 isoform (1.5 kb) and did not hybridize with probe M4AB specific for M3 and M4 isoforms (data not shown). Furthermore, in Northern blot analysis using probe M4EX, an additional transcript (~5.3 kb in size) was only found in lung samples (Fig. 5 *b* below, and data not shown). All other samples had the three bands (~4.6, 3.3, and 1.8 kb) which have been repeatedly found by Northern blotting with murine hemopoietic cell RNA (27) and which presumably differ with respect to untranslated nucleotides.

Expression and Functional Assessment of New CD44 Isoforms

Each of the four new transcription products was ligated into an expression vector and transfected into the AKR1 lymphoma cell line. The resulting transcripts were evaluated by

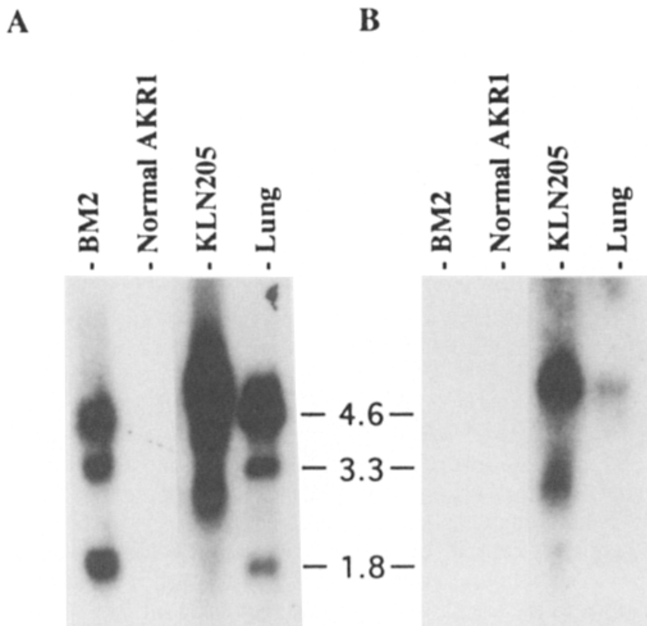

Figure 5. Northern blot analysis. RNA samples from the indicated cell lines and tissues were fractionated on a 1% agarose gel, transblotted onto a nitrocellulose membrane, and (*A*) hybridized with cDNA representing the full length "hemopoietic" CD44 isoform (CD44WT). The same blot was reprobed (*B*) with M4EX described in Materials and Methods. This probe corresponds to the unique inserted sequence found in our longest murine isoform (M4). The blot was deliberately overexposed to demonstrate the absence of variant CD44 isoforms in BM2 cells.

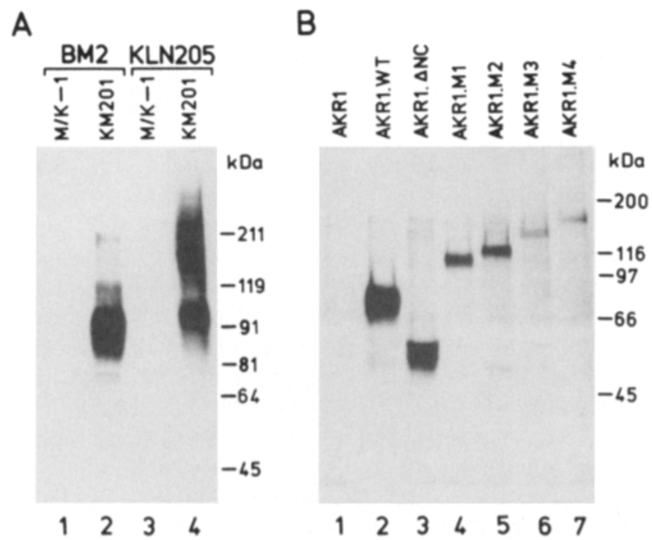

Figure 6. Expression of CD44 protein isoforms as revealed by Western blot (*A*) or cell surface labeling and immunoprecipitation (*B*). Cells were lysed and immunoprecipitated with either anti-CD44 mAb KM201 (IgG1) or anti-VCAM-1 mAb M/K-1 (IgG1). The precipitates were eluted by boiling 5 min in nonreducing sample buffer, run on a SDS-PAGE gel and transblotted onto a nitrocellulose membrane. CD44 proteins were then detected by mAb KM201 and HRP-conjugated goat anti-rat Ig using the ECL system (*A*). The mobilities of prestained molecular weight markers are indicated. cells were also surface biotinylated, solubilized and immunoprecipitated with mAb KM201. Immunoprecipitates were separated on a SDS-PAGE gel under reducing conditions, transferred onto a nitrocellulose membrane and detected with HRP-conjugated avidin using the ECL system (*B*). The mobilities of biotinylated molecular weight markers are indicated.

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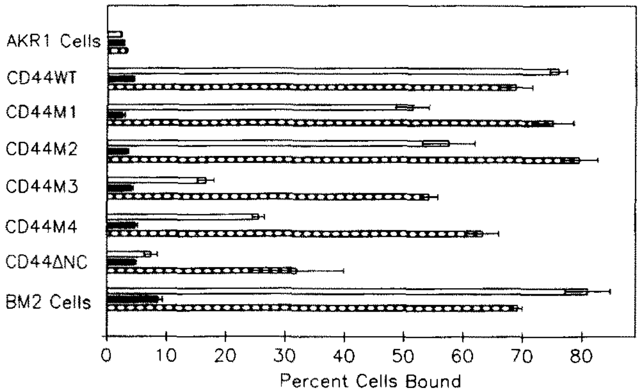

Figure 7. New murine CD44 isoforms can mediate adhesion of transfected cells to the BMS2 stromal cell clone. The levels of expression of CD44 in each of the transfectants was determined separately by flow cytometry as described in Materials and Methods. In three assays, these averaged 41% (*M1*), 32% (*M2*), 11% (*M3*), 28% (*M4*), 36% (*ΔNC*) and 238% (*BM2*) of the wild-type transfectant. No attempt was made to normalize the findings with respect to these differences. The extent of constitutive adherence of radiolabeled transfectants to monolayers of BMS2 cells is shown (*open bars*). The dependence of this adhesion on CD44 was determined by inclusion of the blocking KM 201 antibody (*closed bars*). Binding was also assessed in the presence of the enhancing IRAWB14 monoclonal antibody (*cross-hatched bars*). The results are typical of three similar experiments.

Northern blotting and prominent transcripts of the expected sizes (wild type = 1.9; M1 = 2.2 kb; M2 = 2.4 kb; M3 = 2.6 kb; M4 = 2.8 kb) were found (data not shown). Protein products made by hemopoietic and carcinoma cells were compared by Western blotting (Fig. 6 *A*), revealing a broad range of CD44 species in the latter cell type. Transfectants were surface labeled and immunoprecipitated to determine sizes of the expressed proteins. As in our previous study, the wild-type, hemopoietic isoform was ~80 kD when expressed in the transfected lymphoma cells (27). The mutated AKR1.CD44ΔNC construct produced an ~58-kD protein. Single protein species were obtained with each of the new isoforms corresponding to 110 kD (M1), 120 kD (M2), 140 kD (M3), and 160 kD (M4) under reducing conditions. Sizes predicted from the cDNA sequences of these molecules, without regard to glycosylation would be 48.1, 51.7, 60.7, and 69.5 kD, respectively. The ratio of predicted to actual sizes was constant (0.43) among all of the transfectants, indicating that posttranslational modifications were proportional to the lengths of the polypeptides.

All transfectants displayed measurable adhesion to an adherent stromal cell line, which was previously shown to bear HA (31) (Fig. 7). The CD44 dependence of this adhesion was confirmed by addition of the blocking KM 201 antibody. Pretreatment of the adherent layer with HA also reduced the binding by an average of 85% (data not shown). Adhesion was increased, and in some cases, dramatically, when the enhancing IRAWB14 antibody was present. Binding of the new isoform transfectants was less than with the wild-type hemopoietic isoform transfectant or BM2 hybridoma cells, which expressed substantially higher densities of CD44. For example, the M3 transfected cells had only 11% as much CD44 as wild-type transfected cells and displayed corresponding low constitutive binding to BMS2 monolayers. In an experiment done with a higher expressing subclone of M4, adhesion was nearly equivalent to that obtained with the wild-type CD44 transfectant (data not shown). In contrast, cells bearing the mutated CD44ΔNC molecule had surface densities comparable to the M2 and M4 transfectants, but an average of only 10% binding was observed in three independent experiments. In the same experiments, binding with M2, M4, and wild-type CD44 transfectants were 49, 35, and 77%, respectively. This inefficient binding was also increased when the IRAWB14 antibody was present. It should be noted that these assays were performed at 4°C to avoid potential activation of the cells by either ligand or antibodies. Studies done with LFA-3 demonstrated that differences relating to the overall length of that molecule are particularly noticeable at that temperature (5).

Recognition of soluble HA was assessed by flow cytometry, where densities of surface CD44 on each transfectant could be compared with their ligand binding abilities (Fig. 8). Constitutive HA binding was extremely low with some of these cell lines, but in every case, this increased when the IRAWB14 antibody was present. In that situation, the amount of HA bound tended to be proportional to the density of CD44 on the cell surface. No obvious pattern was observed where particular isoform sequences correlated with high or low HA binding.

These experiments demonstrate that all five known murine CD44 isoforms are able to confer HA recognition ability on transfected T lymphoma cells. The constitutive avidity for soluble ligand may be very low, but was sufficient to mediate adhesion to cells whose surfaces are coated with HA. All forms of CD44 in this T lymphoma model were responsive to the enhancing IRAWB14 monoclonal antibody, which dramatically increased binding to both soluble and cell immobilized ligand. This contrasts to the situation with B lineage hybridoma cells, which displayed very high HA recognition that was not consistently affected by IRAWB14 (Fig. 1). The enhancing antibody may mimic the interaction of some unknown ligand with CD44, when it is expressed on particular cells.

Discussion

These experiments address the importance of particular extracellular domains of CD44 for recognition of soluble or immobilized HA. We found that the amino terminal two thirds of the extracellular portion of the molecule bears epitopes recognized by a number of antibodies to murine CD44 and can mediate recognition of the ligand HA. Four new murine isoforms were characterized and circumstances found in which all mediated adhesion when expressed in transfected lymphoma cells. These findings increase our understanding of molecular polymorphisms of CD44 and indicate that variations in the membrane proximal region do not per se prevent HA recognition. They also extend recent observations of variable isoforms of rat and human CD44 to murine CD44.

Our results show that the amino terminal region of CD44, which bears homology to other HA binding proteins (41, 45), is clearly sufficient for HA recognition when expressed on T lymphoma cells. However, a number of recent findings have focused attention on the less well conserved membrane proximal portion of this molecule. First, this domain of hu-

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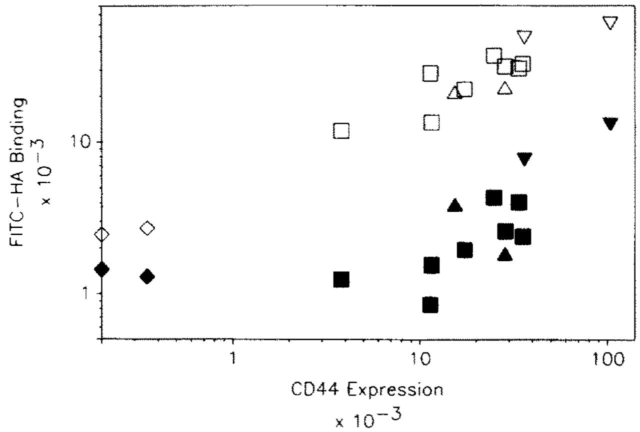

Figure 8. The density of CD44 on transfected cells influences binding of soluble ligand. Flow cytometry was used to determine the density of CD44 (numbers of FITC molecules bound by treatment with labeled anti-CD44 antibody) and binding of HA (number of FITC molecules bound after brief exposure to labeled HA). Closed symbols represent constitutive ligand binding ability and open symbols depict HA binding when the enhancing IRAWB14 antibody was present. The data are from two independent experiments where untransfected AKR1 lymphoma cells (♦,◇), wild-type transfectants (▼,▽), and cells expressing the CD44ΔNC mutant (▲,△) were compared to cells transfected with the new murine CD44 isoforms M1, M2, M3, M4 (■,□).

man CD44 bears the Hermes 3 epitope, which is thought to be functionally associated with lymphocyte homing and to interact with another ligand (13). This segment also contains several chondroitin sulfate addition sites and, when these are utilized, CD44 becomes capable of recognizing collagen and fibronectin, as additional ligands (9, 23). Furthermore, this high molecular weight form of chondroitin sulfated CD44 may confer a more aggressive phenotype on tumor cells which express it (9). Additional molecular heterogeneity of CD44 has been found to result from insertions of sequences of variable length in the membrane proximal domain (3, 6, 8, 12, 14, 17, 20, 39, 42).

A wide variety of cell types are now known to express some form of CD44. These include most blood cells, fibroblasts, bone marrow stromal cells and epithelial cells (15). In some cases, these have been shown to differ with respect to polypeptide length, as well as the degree and nature of post-synthetic modification by glycosylation (3). There is also reason to suspect that the same form of CD44 may function differently in different cell types. For example, HA binding is constitutively maximal in the BM2 hybridoma line and unaffected by the IRAWB14 antibody (Fig. 1). This may be comparable to B cells from mice undergoing graft versus host disease or exposed to interleukin 5, which had high intrinsic avidity for HA (33, 34). Similar sized CD44 molecules on T lymphocytes and lymphoma lines display moderate avidity for this ligand and HA binding is dramatically enhanced by IRAWB14. Still another pattern is found on resting splenic B lymphocytes and bone marrow cells, which bear the hemopoietic isoform of CD44, but do not adhere to HA regardless of whether IRAWB14 is present (27 and J. Lesley, unpublished observations). In contrast to our results with the murine M2 form of CD44 expressed in lymphoma cells, the homologous epithelial form of human CD44 failed to bind HA when expressed in a pre-B lymphoma (42, 43). These cell type specific differences might be attributable to associations of CD44 with other proteins or to differences in posttranslational modification of the CD44 molecule itself. We found that the cytoplasmic domain of the molecule is important for constitutive high recognition of HA (27). Others recently reported cell type specific differences in association with cytoskeletal elements, which may be related to the phosphorylation of cytoplasmic serine residues (2, 4, 21, 22, 26). This is an important area for further investigation and one that might be approached by transfection of multiple cell types with the same constructs.

The mechanism through which the IRAWB14 antibody enhances CD44 mediated binding of HA remains unclear. Enhancement occurs within seconds to minutes of adding antibody and occurs at 4°C. The antibody can enhance HA binding by CD44 molecules which lack a cytoplasmic domain and on cells which have been fixed by paraformaldehyde treatment (reference 27, and our unpublished observations). By analogy to antibodies which are known to increase ligand binding by an integrin (35), IRAWB14 may cause a conformational change in CD44. Other possibilities include antibody influences on association of CD44 with other proteins or on focal clustering on the cell surface. The epitope recognized by IRAWB14 was present on the AKR1.CD44ΔNC mutant and recognition of soluble HA was as markedly increased as it was with the new murine isoforms we characterized. We assume that shortening the molecule by mutation resulted in the relatively inefficient binding of transfected cells to HA coated stromal cells (Fig. 7). A minimal overall length may be required for interaction with immobilized ligand. Artificial lengthening of another adhesion molecule was reported to substantially increase function, particularly at cold temperature (5). Even this parameter was dramatically increased in our shortened CD44 transfectant by the enhancing IRAWB14 antibody.

At least 15 CD44 isoforms, which differ with respect to inserted or deleted sequences have now been found in transcripts expressed by rat, human, and murine cells. Included in that number are two unique murine isoforms (M3 and M4) characterized here. These represent "domains IV and V" or "domains II, III, IV, and V," respectively, as designated in a recent characterization of human and rat CD44 isoforms (17). Our M1 isoform is comparable to a human species designated "variant C" in a recent publication (20) and our M2 isoform is equivalent to the "epithelial" variant described by several laboratories (3, 17, 42). A short stretch of 31 amino acids is deleted in certain neuroblastomas which represents the NH_2-terminal boundary of the insertions (39). This incredible molecular diversity most likely results from alternative exon splicing (6), as is thought to be the case for generation of N-CAM isoforms (7). Inspection of all published cDNA sequences indicates that heterogeneity involving just the membrane proximal region of the molecule could result from differential utilization of at least nine exons.

Overall, the membrane proximal region of CD44 is the least well conserved portion of the molecule and is only 50% identical between humans and mice. However, there is considerable homology between the insertions that occur in this region with the various isoforms. For example, murine and rat insertions are 88% identical (Fig. 4). It is also noteworthy

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that Ala and Thr (position 201/202 in the mouse) have almost universally been found at this boundary in murine, rat and human CD44 sequences (3, 14, 17, 20). The longest insertion in the murine sequence (M4) nearly doubles the extracellular length of the molecule and introduces an additional 20 potential glycosylation sites. Although our experiments do not indicate that this region controls HA recognition, it may well be responsible for mediating other isotype specific CD44 functions. We isolated multiple isoforms of murine CD44 from a carcinoma line which was originally characterized for high metastasis to the lung and it is interesting that novel rat CD44 isoforms were discovered in a tumor which had the same property (14, 25). It remains to be determined if HA and/or some other ligand are responsible for selective migration of certain CD44 bearing tumors to that organ.

It has also been noted that the human insertions include a potential cleavage site (Arg Arg) for trypsin like proteases (8). The same dipeptide is present at the corresponding position of insertions in rodent CD44 isoforms, as well as near the transmembrane segment of the hemopoietic isoform. This would presumably increase the possibility of generating soluble CD44 by means of enzymatic cleavage and we have detected soluble CD44 in murine serum, as well as in the supernates of transfected cells (S. Katol and P. W. Kincade, unpublished observations).

The hemopoietic isoform appears to be the most common and abundant species of CD44 in the murine tissues we examined. Lung was the only untransformed source expressing any other isoform in our study and this species probably corresponds to the M2 isoform which we isolated from carcinoma cells. However, we consider it likely that multiple isoforms of CD44 are synthesized at some level by normal murine tissues. For example, a tailless isoform was originally cloned from human cells and subsequently shown to represent a very low abundance transcription product in several species (12). The sequence information we obtained may now make it possible to prepare isoform specific monoclonal antibodies and develop other approaches to understanding the distribution and functions of these molecules.

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