Hyaluronan Binding by Cell Surface CD44*

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CD44 is the primary cell surface receptor for the extracellular matrix glycosaminoglycan hyaluronan. Here we determined the relative avidities of unlabeled hyaluronan preparations for cell surface CD44 by their ability to block the binding of fluorescein-conjugated hyaluronan to a variety of cells. We show that hyaluronan binding at the cell surface is a complex interplay of multivalent binding events affected by the size of the multivalent hyaluronan ligand, the quantity and density of cell surface CD44, and the activation state of CD44 as determined by cell-specific factors and/or treatment with CD44-specific monoclonal antibody (mAbs). Using low $M_r$ hyaluronan oligomers of defined sizes, we observed monovalent binding between 6 and 18 sugars. At $\sim 20$ to $\sim 38$ sugars, there was an increase in avidity ($\sim 3\times$), suggesting that divalent binding was occurring. In the presence of the inducing mAb IRAWB14, monovalent binding avidity was similar to that of noninduced CD44, but beginning at $\sim 20$ residues, there was a dramatic and progressive increase in avidity with increasing oligomer size ($\sim 22 < 26 < 30 < 34 < 38$ sugars).

Kinetic studies of binding and dissociation of fluorescein-conjugated hyaluronan indicated that inducing mAb treatment had little effect on the binding kinetics, but dissociation from the cell surface was greatly delayed by inducing mAb.

Hyaluronan (HA)$^2$ is a linear polymer, composed of repeating disaccharides of glucuronic acid and N-acetyl-D-glucosamine. It can reach a molecular mass of several million daltons and is a ubiquitous component of extracellular matrices, where it is often associated with hyaluronan-binding proteoglycans and hyaluronan-binding proteins.

CD44, the best characterized cell surface receptor for HA, shares an $\sim 100$-amino acid region of homology with other HA-binding proteins referred to as the “link module” or the “proteoglycan tandem repeat” (see Ref. 1). The molecular basis of CD44 binding to HA is beginning to be unraveled by structural modeling and mutational analyses. NMR studies of the link module of TSG-6 (2) have provided a framework for modeling the homologous region in CD44 (3). HA binding studies of mutated CD44-Ig fusion protein chimeras have identified amino acid residues that are critical for HA binding (3, 4). Together, these studies give a consistent picture of the HA binding site of CD44, which has three-dimensional structural similarities to another family of carbohydrate-binding molecules, the C-type lectins (3).

However, CD44 differs in important ways from other HA-binding proteins. While the link modules of TSG-6 and link protein can bind HA on their own (2, 5, 6), HA binding by CD44 requires sequences outside the link module (4), is regulated by cell-specific factors (7, 8), and probably requires multiple CD44/HA engagements to achieve a functional avidity. Depending on the type and activation state of the cell in which it is expressed, CD44 may be inactive (unable to bind HA), inducible (able to bind HA upon treatment with certain CD44-specific mAbs or with inducers of cell activation such as phorbol ester), or constitutively active (able to bind HA without any treatment). The activation state seems to be determined, at least in part, by posttranslational modification (especially glycosylation) of the CD44 molecule itself, because CD44-Ig fusion proteins display the same activation phenotype as the endogenous cell surface CD44 of the cells in which they are made (9–12).

The most significant feature that distinguishes CD44 from other HA-binding proteins is that CD44 binding to HA takes place at the cell surface, where multiple, closely arrayed CD44 receptor molecules interact with the highly multivalent repeating disaccharide chain of HA. The affinity of a single CD44-HA binding domain for HA is likely to be very low. CD44-Ig fusion proteins (which are at least dimeric) were estimated to have $K_d$ ranging from 5 to $\sim 150$ $\mu$M by affinity capillary electrophoresis (11). Thus, binding of a CD44-positive cell to an HA substrate, or of a soluble HA molecule to the surface of a CD44-positive cell, must involve multiple weak receptor-ligand interactions. This is a feature common to cell surface adhesion receptor-ligand interactions (13) but is especially important in the case of CD44 because of the highly repetitive nature of its ligand HA.

In this study, we present a ligand blocking assay that provides a more quantitative assessment of HA binding by cell surface CD44 than other methods previously employed, such as cell adhesion and flow cytometry. We use cell lines with known CD44 receptor properties, CD44-specific mAbs that influence HA binding, and HA oligosaccharides of defined sizes to probe the interaction of receptor and ligand multivalency in binding of HA at the cell surface.

EXPERIMENTAL PROCEDURES

Cell Lines and Antibodies—Transfection of AKR1 (a CD44-negative mouse T lymphoma) with mutant and wild-type CD44.1 constructs has

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¶ The abbreviations used are: HA, hyaluronan; FKBP, human FK506-binding protein-12; FL-HA, fluorescein-conjugated hyaluronan; PMA, phorbol 12-myristate 13-acetate; mAb, monoclonal antibody.
been described previously (14–16). AKR1 transfectants used in this study all bind HA constitutively and express one of the following constructs: wild-type CD44 (designated as WT.CD44); “dimeric CD44” (14), a homodimer of CD44 in which the transmembrane domain is replaced with the transmembrane domain from the CD3ζ chain; cCD44-F3/CDD4 (15), a chimeric CD44 molecule in which the cytoplasmic domain of CD44 is replaced with three tandem repeats of the human FK506-binding protein 12 (FKBP12); and either M1, M2, M3, or M4, higher M, isoforms of CD44 that contain variable exons v9 and v10, v8–v10, v6–v10, and v4–v10, respectively (17). XJ(3) is a variant of the mouse pre-B lymphoma RAW264.7 that can be induced to bind HA by certain CD44-specific mAbs (18). CTLL/WT.CD44 is a transfectant of the CD44-negative cytotoxic T cell line CTLL that expresses wild type CD44 and binds HA constitutively (19). BW5147 is a CD44+ T lymphoma that binds HA constitutively. EL4 is a CD44+ T lymphoma that can be induced to bind HA by treatment with an inducing CD44-specific mAb or by overnight culture in phorbol ester (20). All cell lines were cultured in Dulbecco’s modified Eagle’s medium with 10% horse serum, except the CTLL-derived transfectant CTLL/WT.CD44, which was grown in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum and supplemented with supernatant from EL4 cells cultured in phorbol 12-myristate 13-acetate (PMA) as a source of interleukin-2. For phorbol ester induction of HA binding in EL4 cells, cells were cultured overnight (18 b) in 20 ng/ml PMA.

Rat mAbs specific for mouse CD44 (derived from rat × mouse hybridomas) included IM7 (21); the inducing mAbs IRAWB14 (22), IRAWB26, and R7166.7 (23, 24); and an inhibiting mAb KM81 (24). To “induce” HA binding with mAbs, mAbs were treated with hybridoma culture supernatants (1:10 dilution) for 30 min at room temperature and then washed once with excess HEPES buffer (HEPES-buffered Dulbecco’s modified Eagle’s medium containing 2% newborn calf serum). Fluorescein-conjugated IM7 (FL-IM7) was used for quantitation of cell surface CD44 by flow cytometric analysis on a FACScan® (Becton Dickinson, Mountain View, CA), as has been described (22).

Analysis of HA Binding—Hyaluronan from rooster comb (Sigma) was fluorescein-conjugated with fluoresceinamine (25). Cell surface binding of fluorescein-conjugated HA (FL-HA) was determined by flow cytometry on a FACScan®. Binding was mediated by cell surface CD44 as shown by 1) blocking of binding by CD44-specific blocking mAbs and 2) absence of binding in untransfected, CD44-negative cell lines (data not shown; see Refs. 16 and 20). Binding of unlabeled glycosaminoglycans was analyzed by inhibition of binding of FL-HA to cells as follows. Cells were dispensed into V-bottom 96-well plates at 10⁶ cells/well; serial dilutions of unlabeled glycosaminoglycans were added to the cells in 50 μl/well and incubated for 15–20 min at room temperature. Then 50 μl containing 1.0 μg/ml of FL-HA was added, and the plate was incubated at 4 °C for 25 min followed by two washes with 150 μl each of cold HEPES buffer. Finally, cells were analyzed for FL-HA binding on a FACScan®. Glycosaminoglycans used were high M, HA from bovine trachea (Sigma), chondroitin sulfate type A from bovine trachea (∼70% C-4-S and ∼30% C-6-S) (Sigma), heparin from porcine intestinal mucosa (Sigma), and HA oligosaccharides (see below).

Preparation of HA Oligosaccharides of Defined Sizes—Hyaluronan from umbilical cord (Sigma) was digested overnight in 0.1 M sodium acetate, 0.1 M NaCl, pH 6.0, with bovine testicular hyaluronidase (Calbiochem; ∼6000 units/ml) at 37 °C with 50,000 units/ml hyaluronan, and the resulting oligosaccharides were fractionated as described (26). Briefly, digest was filtered through a 10-kDa cut-off membrane and chromatographed on 95-cm columns of Superdex 30 (Amersham Pharmacia Biotech), eluted with 0.1 M NH₄HCO₃. The fractions corresponded to material collected under peak 0 and were as follows: HA 30, 26–34 monosaccharides (median 30); HA 34, 28–40 monosaccharides (median 34); and HA 36, 30–46 monosaccharides (median 38).

Oligomerization with AP1510—Cells expressing ΔCY-F3/CDD4 were incubated at 37 °C for 30 min with or without 100 nM of AP1510 (provided by ARIAD Pharmaceuticals Inc.). AP1510 is a synthetic, bivalent, membrane-permeable, small molecule that binds to and cross-links the FKBP sequences in ΔCY-F3/CDD4 (28).
Cells were incubated with the indicated concentrations of FL-HA for 30 min at room temperature, washed twice, and run on a Facscan® flow cytometer. Mean fluorescence intensity (MFI) indicates the mean fluorescence of the labeled cell population relative to background fluorescence of unlabeled cells (background normalized to 1.0). WT.CD44 cells bind HA constitutively (filled squares, non-, noninduced). XJ(3) cells are inducible; they do not bind FL-HA alone (filled triangles, non-, noninduced) but do bind when treated with the inducing mAb IRAWB14 (open triangles, ind., induced).

Rooster comb HA from Sigma is reported to contain HA chains ranging up to >600 kDa $M_r$ (29). Thus, each of the high $M_r$ HA molecules contains many fluorescein residues and potentially hundreds of binding sites for CD44. Detectable binding of FL-HA to cells is thought to depend on binding of cell surface CD44 to multiple sites along a HA chain, since binding of labeled low $M_r$ HA preparations cannot be detected by flow cytometry or microscopy. $^{2}$ The highly multivalent binding of the FL-HA makes it impossible to measure the affinity of a single CD44 molecule for a single HA binding site under these conditions.

An assay was developed to measure binding of unlabeled HA samples to cell surface CD44 by their ability to inhibit binding of FL-HA. In these experiments, FL-HA was used as a probe to detect HA binding sites unoccupied by the blocking reagent. For example, Fig. 3 shows the ability of unlabeled HA chains from bovine trachea (estimated $M_r$ between 80 and 600 kDa (29)), hereafter referred to as unlabeled high $M_r$ HA, to block the binding of a low concentration of FL-HA (0.5 $\mu$g/ml). The unlabeled HA was added 15–20 min prior to the addition of FL-HA and remained in the sample with the FL-HA (25 min). As seen in Fig. 3, much lower concentrations of unlabeled HA were required to block subsequent FL-HA binding to WT.CD44 cells when the cells had been pretreated with the inducing mAb IRAWB14 (open squares) compared with untreated cells (filled squares). This indicates a higher avidity of mAb-induced CD44 for the unlabeled HA.

Blocking curves, such as those shown in Fig. 3, can be used to compare relative avidities of cell surface CD44 for unlabeled HA preparations. Because the FL-HA and unlabeled competitors are not necessarily at equilibrium, these comparisons are only valid within a single experiment, where the same FL-HA dilution and labeling time are used for all of the samples. We have used the concentration of unlabeled HA giving 50% inhibition of FL-HA binding as the measure of relative avidity (e.g., arrows in Fig. 3). The lower the concentration needed to achieve 50% blocking, the higher the relative avidity of the unlabeled HA. The difference between noninduced (constitutive) and IRAWB14 mAb-induced HA binding avidity to CD44-transfected AKR1 cells ranged up to 100-fold (~40-fold in Fig. 3).

**Kinetics of Binding and Dissociation**—The requirement for multivalent interactions of each bound FL-HA molecule with the cell surface in order to achieve detectable binding makes it impossible to detect first order reactions and thus impossible to determine single binding site association and dissociation rates. Kinetic experiments, however, do allow an investigation of binding parameters and of the mechanism of mAb enhancement of HA binding avidity. In Fig. 4, binding of a standard concentration of FL-HA was measured on cells that were treated or not with mAb IRAWB14. The majority of binding occurred within 20–30 min for both cell treatments, but induced binding reached a higher level. After 20–30 min, binding curves tended to plateau, although induced binding continued to rise gradually (Fig. 4A). In repeated experiments, there was a slight lag in initial binding in the induced cells, as can be seen in the graph in Fig. 4B of the same data shown in 4A, here plotted as a percentage of binding at 45 min (when initial binding was at a plateau). The 25-min incubation with FL-HA used in the blocking assays was sufficient for stable and reproducible binding.

The dissociation of FL-HA was measured on pools of cells that had been preloaded with 1 $\mu$g/ml FL-HA in the presence or absence of mAb IRAWB14. Dissociation was begun by washing the cells in excess buffer. Then cells were resuspended in excess unlabeled HA (200 $\mu$g/ml) to prevent rebinding of FL-HA for the dissociation period. As shown in Fig. 5, mAb IRAWB14 treatment dramatically reduced the dissociation of FL-HA from the cell surface (open symbols, $t_{1/2} \sim 110$ min) compared with dissociation from untreated (noninduced) cells ($t_{1/2} \sim 25$ min).
The curve for non-induced cells leveled off at ~35% of maximum, perhaps due to rebinding of partially dissociated HA molecules. Longer incubation times would be needed to determine whether the induced curve continues to decline or levels off. The relatively rapid dissociation of FL-HA in the absence of IRAWB14, despite the highly multivalent association of the HA chains with the cell surface, implies that each single CD44/HA interaction must be extremely weak.

Specificity and Washing—Inhibition of FL-HA binding was used to determine the relative avidity of cell surface CD44 for other glycosaminoglycans, chondroitin sulfate and heparin (Fig. 6). 50- and 750-fold higher concentrations of chondroitin sulfate than unlabeled HA were required to block FL-HA binding to noninduced (Fig. 6A) and IRAWB14-induced (Fig. 6B) cells, respectively. Heparin did not interact with the HA binding site of CD44. Also shown are the effects of washing the cells (twice) between the blocking step with unlabeled HA and adding FL-HA. In some samples (open squares), cells incubated with the indicated concentrations of high M$_r$ HA were washed (twice) before adding FL-HA at 0.5 µg/ml for 25 min. All cells were washed 2 times after the FL-HA incubation.

Influence of CD44 Density on HA Binding—We have previously observed that differences between cell lines in the level of IRAWB14, despite the highly multivalent association of the HA chains with the cell surface, implies that each single CD44/HA interaction must be extremely weak.

In Fig. 5, above.
CD44 expression on the cell surface affected the binding of FL-HA, making it essential that comparisons of cell lines expressing mutant and wild-type CD44 constructs be done with cells expressing comparable CD44 levels (or, preferably, over a range of CD44 levels) (10, 14). The influence of CD44 expression level (and, thus, the overall density of CD44 on the cell surface) is seen in the blocking experiment shown in Fig. 7A, which is representative of several experiments done on many clones. Here, three clones of the AKR1 transfectant expressing wild-type CD44 were selected for differences in their CD44 expression levels (41-, 81-, and 106-fold over background). In B, CD44-positive transfectants expressing approximately equal levels of cell surface CD44 (see text) were assayed. Shown are WT.CD44 (open circles) or treated for 30 min at 37 °C with 100 nM of AP1510, a bifunctional agent that binds FKBP sequences, thereby cross-linking FKBP-containing molecules (filled circles).

FIG. 7. Influence of cell surface CD44 density on cellular avidity for high M₄ HA. Inhibition of FL-HA binding with high M₄ HA was carried out as described in the legend to Fig. 3. In A, three clones of WT.CD44, expressing differing levels of cell surface wild-type CD44 (as determined by binding of FL-IM7) were assayed: high CD44 (open squares, 106 times background); medium CD44 (filled squares, 81 times background); and low CD44 (open circles, 41 times background). In B, CD44-positive transfectants expressing approximately equal levels of cell surface CD44 (see text) were assayed. Shown are WT.CD44 (filled squares); dimeric CD44 with the disulfide-bonded transmembrane domain of CD3-ζ-chain (open squares); and ΔCY-F3/CD44, with the cytoplasmic domain of CD44 replaced by three FKBP repeats, mock-treated (open circles) or treated for 30 min at 37 °C with 100 nM of AP1510, a bifunctional agent that binds FKBP sequences, thereby cross-linking FKBP-containing molecules (filled circles).

FIG. 8. Inhibition of FL-HA binding by unlabeled low M₄ oligosaccharides of defined sizes. WT.CD44 cells were incubated with HA oligosaccharides at 2 times the indicated concentration (x axis) for 20 min at room temperature. Then an equal volume of FL-HA at 1.0 μg/ml was added for 25 min more. The percentage of maximum FL-HA binding (in the absence of any unlabeled HA) is shown on the y axis. Noninduced WT.CD44 cells are shown in A; IRAWB14 mAb-induced cells are shown in B.

Blocking with HA Oligomers of Defined Sizes—Unlabeled HA oligomers were prepared as described (see “Experimental Procedures”) and used to block binding of FL-HA to WT.CD44 cells. Examples of blocking curves for some of the low M₄ oligomers are shown in Fig. 8 (noninduced, Fig. 8A; induced with mAb IRAWB14, Fig. 8B). Table I summarizes the 50% blocking concentrations for a series of oligomers from 4 to 14 sugar residues. 4–5 times more of the HA₄ oligomer is required for 50% blocking than of the HA₈ and HA₉ residue oligomers, suggesting that HA₄ is the minimum oligomer size for efficiently occupying the HA binding site of CD44. The same minimal size specificity was observed for noninduced and mAb IRAWB14-induced cells.
For oligosaccharides HA$_4$–HA$_{10}$, the increase in avidity with each two-sugar increase in size is more pronounced than for oligomers between HA$_{10}$ and HA$_{18}$, with the biggest jump between HA$_4$ and HA$_6$ (Figs. 8 and 9). This suggests that while blocking begins with a six-sugar oligosaccharide (see Table I and Fig. 8), the HA binding site of CD44 is not optimally occupied until the oligomer size reaches HA$_{10}$. Monovalent binding appeared similar on induced cells and noninduced cells (see Table I and Fig. 9), suggesting that IRAWB14 induction does not enhance monovalent binding.

Subsequent experiments investigated blocking by HA oligomers of increasing size to determine when divalent binding began to occur. While the separation of the individual oligosaccharides was reasonably good up to HA$_{16}$–HA$_{18}$, oligosaccharides larger than HA$_{18}$ showed clusters of 3–8 main sizes (see Fig. 1). These oligomers are referred to as HA$_{22}$, HA$_{26}$, etc., where the nominal size is the median size of a cluster of oligomers (see “Experimental Procedures”). The range became broader with increasing size. Three experiments are summarized in Fig. 9, where 50% inhibition levels are plotted against oligosaccharide size. Between HA$_{10}$ and HA$_{18}$ in Fig. 9A (between HA$_{10}$ and HA$_{16}$ in Fig. 9B) there is little change in the micromolar concentration needed for 50% inhibition with increasing oligomer size, for both noninduced cells and IRAWB14-induced cells. For noninduced cells (filled symbols), there is a 2–4-fold drop in the 50% inhibition concentration with oligomers HA$_{22}$ and larger, compared with oligomers HA$_{10}$–HA$_{14}$. Since a single binding site accommodates 6–10 sugar residues, it is reasonable to expect that two adjacent binding sites will be available at some chain length around HA$_{20}$ and that CD44 binding to two sites on a chain would increase the avidity of binding by reducing the probability of dissociation. Therefore, we suggest that the increase in avidity above HA$_{18}$ represents divalent binding. For IRAWB14-induced cells (open symbols), the drop in micromolar concentration for 50% inhibition at HA$_{22}$ is more dramatic, and it continues to diverge from the noninduced curve with each increase in oligomer size above HA$_{22}$, reinforcing the idea that there is a change in HA binding above HA$_{18}$.

Despite the increase in avidity seen with oligomers above HA$_{18}$, we were not able to directly detect any binding of labeled HA$_{24}$ and HA$_{38}$. The avidity of these oligosaccharides is apparently still too low to detect by flow cytometry or microscopy, even when cells are induced with IRAWB14.

Several other CD44-specific mAbs induce HA binding in certain “inducible” cell lines that do not bind HA constitutively and also enhance HA binding in some constitutive lines (Fig. 10 and Ref. 24). This function did not correlate with antibody isotype or with the CD44 epitope recognized (24). Induction activities of IRAWB26, an IgG$_2a$, IRAWB14, an IgG$_2a$, and R7 166, an IgM, are shown in Fig. 10. All three mAbs increased the relative fluorescence intensity of WT CD44 cells for FL-HA and induced significant binding in EL4 cells, which do not bind HA constitutively. IRAWB14 was much more effective than the other mAbs at inducing EL4 cells to bind FL-HA. We did blocking experiments with monovalent (HA$_{16}$ and HA$_{14}$) and divalent (HA$_{22}$, HA$_{34}$, and HA$_{38}$) oligosaccharides to deter-

### Table I

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<thead>
<tr>
<th>Oligomer size (Ns. of sugars)</th>
<th>50% inhibition</th>
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<tr>
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<td>Experiment 2</td>
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<tr>
<td>Noninduced</td>
<td>IRAWB14-induced</td>
<td>Noninduced IRAWB14-induced</td>
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<tr>
<td>4</td>
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<td>14</td>
<td>150</td>
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**Fig. 9. Relationship between oligosaccharide size and inhibition of FL-HA binding.** The results of three experiments are plotted in terms of the micromolar concentration of oligosaccharide needed for 50% inhibition of FL-HA binding (y axis). Experiments were carried out as described in the legend to Fig. 8. The µg/ml concentration of oligosaccharide for 50% inhibition was determined from plots such as those shown in Fig. 8 and converted to an estimated micromolar concentration. A shows one experiment (expt#1) including HA oligosaccharides of 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 30, and 34 sugar residues (y indicates median size; see “Experimental Procedures”). WT.CD44 cells were noninduced (filled squares, non-, noninduced) or induced with IRAWB14 (open squares, ind-, induced). B shows two experiments using somewhat less complete sets of oligosaccharides than used in A. expt#2, noninduced cells (filled circles, non-, noninduced) and IRAWB14 induced (open circles, ind-, induced), expt#3, noninduced (filled triangles, dashed line) and IRAWB14-induced (open triangles, dashed line). For oligosaccharides 20 and above, the median oligosaccharide size of the population was used. The bars on the symbols of one of the curves in each panel apply to all of the oligomers of that size in the figure and indicate the range of oligosaccharide sizes for the population (see Fig. 1, B–D and “Experimental Procedures”).

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*J. Lesley, V. C. Hascall, M. Tammi, and R. Hyman, unpublished observations.*
D–F

A–C

ground for WT.CD44 cells (absence of inducing mAb: mean fluorescence intensity of 23.4
The MFI) of unlabeled cells equals 1.0. indicates relative fluorescence intensity, which has been normalized so

C

E

R7 166 (and

Flow cytometry histograms show FL-HA binding by constitutive HA binding cells (WT.CD44, A–C), and inducible cells (EL4, D–F) in the presence of inducing mAb IRAWB14 (A and D), IRAWB 26 (B and E), and R7 166 (C and F). The y axis indicates cell number. The x axis indicates relative fluorescence intensity, which has been normalized so that the mean fluorescence intensity (MFI) of unlabeled cells equals 1.0. The unshaded, dotted curve in each panel is FL-HA binding in the absence of inducing mAb: mean fluorescence intensity of 23.4 x background for WT.CD44 cells (A–C) and 1.3 x background for EL4 cells (D–F).

mine if IRAWB26 and R7 166 also enhanced divalent binding as did IRAWB14. Table II summarizes these results. All three inducing mAb increased the relative avidity of WT.CD44 cells for high Mr HA, although IRAWB26 and R7 166 were not as effective as IRAWB14 (relative avidities of 14, 43, and 86 times that of noninduced cells, respectively). R7 166 actually appeared to reduce monovalent avidity by about 2-fold, while IRAWB26 was neutral, and, in this experiment IRAWB14 appeared to enhance monovalent avidity by about 25%. Only IRAWB14 dramatically enhanced divalent binding to HA oligomers of ~22, ~34, and ~38 sugar residues (median sizes). Measurement of the dissociation of FL-HA from WT.CD44 cells induced with these mAbs showed that, like IRAWB14 in Fig. 5, mAbs IRAWB26 and R7 166 delayed dissociation. Dissociation from R7 166-treated cells was similar to that from IRAWB14-treated cells. Dissociation from mAb IRAWB26-treated cells was intermediate between noninduced and IRAWB14-induced dissociation (data not shown).

Another CD44-specific mAb, IM7, a rat IgG2b antibody with very high affinity, has neither a blocking nor an inducing effect on HA binding by mouse CD44. As shown in Fig. 11, IM7 pretreatment of WT.CD44 cells had no influence on the 50% dissociation level with high Mr HA, although it did change the slope of the blocking curve.

Binding of monovalent and divalent HA oligomers was not enhanced by intramembrane dimerization of CD44 (dimeric CD44) or by oligomerization of ΔCY-F3/CD44 mutant CD44 by treatment with AP1510 (Table III). If anything, it appears that the close apposition of CD44 receptors on the cell surface slightly reduced binding avidity for the HA oligomers, since higher concentrations of HA oligomers were required to block FL-HA binding by the clustered cell surface CD44 molecules.

HA Binding by Other Cell Lines and CD44 Isoforms—Blocking studies with defined HA oligomers were done on a set of AKR1 cells transfected with higher Mr mouse CD44 isoforms containing variable exons. M1 (v9 and v10), M2 (v8–v10), M3 (v6–v10), and M4 (v4–v10) all bind HA constitutively when CD44 expression levels are sufficiently high (17). In this study, CD44 expression was low on these transfectants, so only cells induced with IRAWB14 were analyzed, except in one case (M2) where noninduced binding could be assayed. No difference in the binding site size requirements was observed. The minimum blocking size was HA6, and divalent binding occurred at HA above (data not shown).

Several other constitutive and inducible cell lines were also analyzed to determine binding site size. BW5147 is a CD44 + T cell lymphoma that binds HA constitutively. CTLL/WT.CD44 is a transfectant of the CD44-negative cytotoxic T cell line CTLL-2 that expresses wild-type CD44 and binds HA constitutively. EL4 is CD44-positive but inducible. It only binds HA after induction by mAb (see Fig. 10) or by overnight culture in phorbol ester (20). XJ(3) is an inducible variant of the Abelson

| Table II
| Monoclonal antibody enhancement of HA binding
| WT.CD44 were untreated or pretreated with the indicated mAb. The concentration of each oligosaccharide needed for 50% inhibition of FL-HA binding was determined from curves such as those shown in Fig. 8. |
| Oligomer size (No. of sugars) | No mAb | IRAWB14 | IRAWB26 | R7166.7 |
| µg/ml HA | µg/ml HA | µg/ml HA | µg/ml HA |
| 10 | 120 | 90 | 130 | 240 |
| 14 | 130 | 100 | 160 | 240 |
| ~22 | 80 | 20 | 90 | 135 |
| ~34 | 90 | 7 | 96 | 85 |
| ~38 | 100 | 4 | 93 | 90 |
| High Mr HA | 4.30 | 0.05 | 0.30 | 0.10 |

* The ~ symbol indicates that the indicated oligomer size is the median size of a population of oligosaccharides (see “Experimental Procedures” and Fig. 1).
TABLE IV

| Cell line and treatment | μg/mL HA | MFI
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<tr>
<td></td>
<td>HA6</td>
<td>HA10</td>
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<tr>
<td>BWS147 (none)</td>
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<tr>
<td>CTLT/WT.CD44 (none)</td>
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<td>145</td>
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<td>EL4 + PMA (overnight)</td>
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<td>EL4 + IRAWB14</td>
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<td>40</td>
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<tr>
<td>XJ(3) + IRAWB14</td>
<td>30</td>
<td>35</td>
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a Mean fluorescence intensity, relative to background fluorescence of unlabeled cells = 1.0.

b The ~ symbol indicates that the indicated oligomer size is the median size of a population of oligosaccharides (see "Experimental Procedures" and Fig. 1).

Study of binding avidity of the HA oligomers showed little change in avidity with increasing size on noninduced cells, while as a group, their avidity was clearly higher than oligomers below HA−22. That a 20-sugar chain is the size where a transition to divalent binding occurs is reinforced by the dramatic change in IRAWB14-induced binding at this same oligomer size.

Virus transformed B lymphoma RAW 253 (18). Table IV summarizes binding avidities of these cells for HA oligomers, with results consistent with the binding site properties described above. Induction of EL4 by PMA, unlike that by IRAWB14 mAb, did not result in enhanced divalent binding of HA oligomer HA−34. Interestingly, PMA-induced EL4 cells bound HA significantly less well than cell lines that bound HA constitutively or than inducible cell lines treated with IRAWB14.

Discussion

These studies clearly demonstrate that cooperativity is the primary feature of HA binding by cell surface CD44. This cooperativity is the result of multiple binding sites on the repeating disaccharide ligand and multiple closely arrayed receptors on the cell surface. The ligand factor shown to be relevant here is the length of the carbohydrate chain, which determines the number of physically connected binding sites. The longer the sugar chain, the more linked binding sites are present, thus reducing the probability of the HA polymer dissociating from the cell surface. Studies using defined HA oligomers to block FL-HA binding indicate a minimal binding site size of six sugar residues, in agreement with several previous findings (30, 31). Oligomers of 10–18 sugars often appeared better at blocking FL-HA than six- and eight-residue oligomers (see Fig. 9 and Tables I, III, and IV), suggesting that monovalent binding was not quite optimal until HA10. However, all of the cell lines in this study showed significant binding to HA6. Tammi et al. (26) found that HA6 could not displace high M0 HA from cultured rat keratinocytes, while HA10 could. Keratinocytes express a similar range of HA isoforms, including the keratinocyte-specific isoform, epican (with variable exons v3-v10), as well as CD44H (31). Since we saw significant binding to HA6 by AKR1 transfectants expressing several high M0 isoforms, we believe that the difference in the keratinocyte results is probably not due to the isoform profile of the keratinocytes but more likely to the more stringent assay used, which required displacing previously bound high M0 HA from the keratinocyte cell surface with HA oligomers. Another possibility is that cell-specific factors, such as glycosylation, could influence the binding site size.

An increase in binding avidity became evident at HA−22 (ranging from 20 to 24 sugar residues; see Fig. 9, A and B). We propose that this represents divalent binding, with two hexamers engaging two CD44 binding sites and a minimum space of eight sugars. If this is indeed the configuration, the next step up in binding site engagement, to trivalent binding, would be expected at 34 sugar residues. However, we did not see evidence for a further increase in binding with oligomer preparations containing oligosaccharides above 30 residues (e.g. HA−30–HA−38) in the absence of IRAWB14 induction. While the oligosaccharides of sizes HA−22 and above that we have used consisted of a range of sizes, the observation that the binding of these oligomers showed little change in avidity with increasing size on noninduced cells, while as a group, their avidity was clearly higher than oligomers below HA−22, indicates that a transition occurs at HA−20. That a 20-sugar chain is the size where a transition to divalent binding occurs is reinforced by the dramatic change in IRAWB14-induced binding at this same oligomer size.

Since a single binding site accommodates 6–10 sugar residues, it is reasonable to expect that two adjacent binding sites will be available to bind two CD44 receptors at some chain length around HA20. However, an alternative possibility to divalent binding is that the structure of the HA oligosaccharide changes at the 20-sugar length, assuming a conformation that is more favorable for CD44 binding (a helical structure, for example). While we cannot rule out this possibility, the difference in the competition by oligomers larger than HA18 between noninduced and IRAWB14-induced cells argues against a structural change in the HA as the sole explanation for the observed increases in avidity and implicates some rearrangement of CD44 on the cell surface that is influenced by the IRAWB14 mAb.

We have shown here that a number of cell surface factors contribute to cooperative binding. First, there is the overall density of receptors on the cell surface. Any increase in receptor expression results in increased binding avidity (Fig. 7A), as we have observed in other studies (10, 14). Factors that cause receptor clustering from within the cell, such as intramembrane disulfide bond formation or cross-linking of cytoplasmic domains, can increase binding avidity without a change in total receptor quantity (Fig. 7B). Binding of certain CD44-specific "inducing" mAb enhances the cooperativity of binding, as shown by increased relative avidity for high M0 HA (Table II) and reduced dissociation rate (Fig. 5).

The activation state of cell surface CD44, determined by the cell in which it is expressed, is a defining factor that has been discussed at length in a number of papers and reviews (see Refs. 7 and 8). Cells with CD44 in an "inducible" activation state, such as the XJ(3) and EL4 cells used here and most normal resting T cells, do not bind HA unless induced by the binding of certain CD44-specific mAb or upon conversion of the cells to an active state by agonists such as phorbol esters or by signaling through antigen receptors (16, 20, 22, 33–35). The CD44 activation state has been shown in several examples to be influenced by cell-specific glycosylation of the CD44 receptor (9–12, 18). For example, a CD44-negative mutant of XJ(3) requires induction when transfected with wild-type CD44 but binds HA constitutively when transfected with mutant CD44 constructs lacking specific N-glycosylation sites (10).

Inducing mAbs enhance HA binding to cells which constitutively bind HA by increasing the efficiency of multivalent binding. In the case of IRAWB14 (Fig. 9), but not two other inducing mAbs (Table II), this can be seen at the level of divalent binding. It is important to note that, while multivalency of the inducing antibody is necessary (23), antibody-mediated cross-linking of receptors alone is not sufficient for induction. IM7 mAb, which is a divalent IgG2a and has a very high affinity for CD44, does not enhance HA binding or induce binding in cells that do not constitutively bind HA (Fig. 11 and Ref. 24). Also, cross-linking receptors from inside the cell or at the membrane, while increasing relative avidity 3–5-fold (Fig. 7 and Table III), did not
have as dramatic an effect as mAb induction (14–100-fold increases in relative avidity). Perhaps the inducing mAbs hold two or more CD44 molecules in a specific orientation that favors binding at adjacent sites along the HA polymer. The closer together two bound sites are, the more likely that the HA molecule will remain bound. IRAWB14 appears to be the extreme case of fixing two CD44 receptors so close together and so optimally oriented that each two-sugar increase in size above 20 residues results in an increase in binding avidity. This is consistent with an increase in the probability of binding two or more CD44 molecules in a specific orientation that have as dramatic an effect as mAb induction (14–100-fold increase in binding avidity and retard HA dissociation. This is important to study how HA of different sizes interacts with the cell surface. The assay used in this study provides a way of comparing the binding of different unlabeled HA reagents, including ones whose binding cannot be detected directly. The experiments also provide insight into the mechanism of enhancement of HA binding by certain CD44-specific mAb that are shown to increase binding avidity and retard HA dissociation.

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