

# Variant Cell Lines Selected for Alterations in the Function of the Hyaluronan Receptor CD44 Show Differences in Glycosylation

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## Summary

CD44 is a major cell surface receptor for the extracellular matrix glycosaminoglycan hyaluronan (HA). However, the ability of CD44 to bind ligand is strictly regulated. Three activation states of CD44 have been demonstrated: (a) inactive; (b) inducible (by certain CD44-specific mAb); and (c) constitutively active. Starting with two parental cell lines expressing CD44 in the inactive state, a pre-B cell (RAW 253) and a fibroblast (L cells), we used fluorescence-activated cell sorting with fluorescein-conjugated hyaluronan in the presence of inducing mAb to derive variant cell lines with CD44 in the inducible state. Constitutively active derivatives were isolated from the inducible variants by a further round of fluorescence-activated cell sorting in the absence of inducing antibody. However, constitutively active variants could not be isolated directly from parental cells expressing CD44 in the inactive state. These results suggest that two genetic events must occur to obtain an active CD44-HA receptor from an inactive receptor. Variant and parental cell-derived CD44 molecules exhibited differences in migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis that were partly attributable to differences in N-linked glycosylation. Furthermore, culture in tunicamycin for 2–3 d converted parental and inducible cell lines into cells showing constitutive CD44-mediated HA binding. Also, removal of cell surface glycosaminoglycan chains by culture of cells in *p*-nitrophenyl  $\beta$ -D-xylopyranoside or treatment with chondroitinase ABC resulted in conversion of cells with an inactive CD44 receptor to an inducible state. These results indicate that carbohydrate side chains of CD44 and/or other molecules on the cell surface that interact with CD44 are potentially involved in regulating the HA-binding function of CD44 on the cell surface.

CD44 is a cell surface receptor for hyaluronan (HA),<sup>1</sup> a glycosaminoglycan (GAG) that is abundantly distributed in extracellular spaces (1–3). CD44 is found on diverse cell types and is thought to be involved in a number of functions, including lymphocyte activation, recirculation and homing, tumor metastasis, hematopoiesis, and HA metabolism (1, 3, 4). The HA receptor function of CD44 appears to be strictly regulated, with many cells that express CD44 failing to exhibit receptor function (1, 5). The mechanisms of this regulation are unknown, but the importance of regulated receptor function may be reflected in the correlation of altered CD44 expression with malignancy, inflammation, and autoimmunity, as well as cell activation in a normal immunological response (1, 4).

<sup>1</sup> Abbreviations used in this paper:  $\beta$ -D-xyloside, *p*-nitrophenyl  $\beta$ -D-xylopyranoside; BZ $\alpha$ GalNAc, benzyl 2-acetamido-2-deoxy- $\alpha$ -D-galactopyranoside; Fl-HA, fluorescein-conjugated HA; GAG, glycosaminoglycan; HA, hyaluronan.

We have previously described three activation states of CD44 with respect to HA-binding function (1, 6–8). Here we show that a single cell line can express CD44 in each of the three activation states as the result of a limited number of mutation events. By comparison of variant cell lines differing in CD44-mediated HA-binding function, we define differences in glycosylation that are potentially responsible for differences in CD44-mediated ligand binding.

## Materials and Methods

**Cell Lines and Antibodies.** RAW 253 (a pre-B lymphoma, 9), L.TK<sup>-</sup>(K) strain of L cell fibroblasts (10), and the variant cell lines derived from them were cultured in DMEM with 10% horse serum. Tissue culture supernatants of the CD44-specific mAb IM7(11), IRAWB14 (7), and KM81 (12) were used for immunoprecipitation, induction of HA binding, and inhibition of HA binding, respectively. Fluorescein-conjugated IM7 (7) was used for quantitation of cell surface CD44 by flow cytometry.

**Cell Sorting and Flow Cytometry.** Cell sorting to derive mutant

cell lines with alterations in HA-receptor function of CD44 was performed as previously described (13). Briefly,  $2-3 \times 10^7$  cells were labeled with fluorescein-conjugated HA (Fl-HA) (14) in the presence or absence of inducing mAb IRAWB14. Rare positive events, with relative fluorescence greater than tenfold above the bulk of the population, were isolated by fluorescence-activated cell sorting on a FACStar+® (Becton Dickinson and Co., Cockeysville, MD). After three or four rounds of sorting, when a distinctly positive population was evident, the cells were cloned, and positive clones were selected after analysis by flow cytometry. Flow cytometric analysis was performed as previously described (15) on a FACScan® (Becton Dickinson and Co.) using Fl-IM7 to measure CD44 expression, Fl-HA to measure HA binding, and Fl-HA plus IRAWB14 to measure inducible HA binding.

**Reverse Transcription and PCR.** Total RNA was isolated from both sets of parental and variant cell lines and from control cell lines transfected with higher relative molecular mass isoforms of CD44 (8) using an RNA isolation kit (Stratagene, La Jolla, CA). Reverse transcription, PCR amplification, and Southern blotting were as previously described (16). Primers used for PCR were upstream and downstream of the site of insertion of alternatively spliced exons (16).

**Iodination and Immunoprecipitation.** Iodination and immunoprecipitation were as previously described (17), using IM7 mAb supernatant plus anti-rat Ig-coupled Sepharose (Pharmacia Biotech, Inc., Piscataway, NJ). Treatment of precipitates with *N*-glycosidase F (Boehringer Mannheim Corp., Indianapolis, IN) was performed as described by Camp et al. (18).

**Inhibition of Glycosylation.** Cells were cultured in DMEM with 10% horse serum for 2 or 3 d with the following inhibitors of glycosylation: 2 mM deoxymannojirimycin (Calbiochem-Novabiochem Corp., La Jolla, CA); 5  $\mu$ g/ml tunicamycin (Calbiochem-Novabiochem Corp.); 1 mM *p*-nitrophenyl  $\beta$ -D-xylopyranoside ( $\beta$ -D-xyloside; Sigma Chemical Co., St. Louis, MO); 2 mM benzyl 2-acetamido-2-deoxy- $\alpha$ -D-galactopyranoside (BZ $\alpha$ GalNAc; Sigma Chemical Co.). Treated and control cells were analyzed for CD44 expression and HA-binding function by flow cytometry. Aliquots of cells were also lysed in extraction buffer (10 mM Tris, 50 mM sodium acetate, 150 mM NaCl, 5 mM EDTA, pH 7.5) with 1% Triton X-100 and 1 mM PMSF, diluted in sample buffer, run on SDS-PAGE, and immunoblotted (as described in 19) to detect changes in the relative molecular mass of CD44.

**Immunoblotting.** Immunoblotting of cell lysates was performed as previously described (19) using IM7 mAb supernatant or an mAb specific for the transferrin receptor cytoplasmic domain, H68.4 (20), and horseradish peroxidase-coupled rabbit anti-rat Ig (Boehringer Mannheim Corp.). Blots were visualized using the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL).

## Results

**Derivation of Cell Lines with Alterations in CD44 Function.** Parental cell lines RAW 253 and LTK<sup>-</sup>(K) strain L cells express substantial levels of the standard 85–90-kD isoform of CD44, but they do not bind the CD44 ligand HA, even in the presence of the inducing CD44-specific mAb IRAWB14 (see Figs. 2 and 5, *top row*). The activation state of these cells and of the CD44 molecules expressed on their surface is termed “inactive.” Using fluorescence-activated cell sorting, we derived stable variant lines that differ from the parent lines in

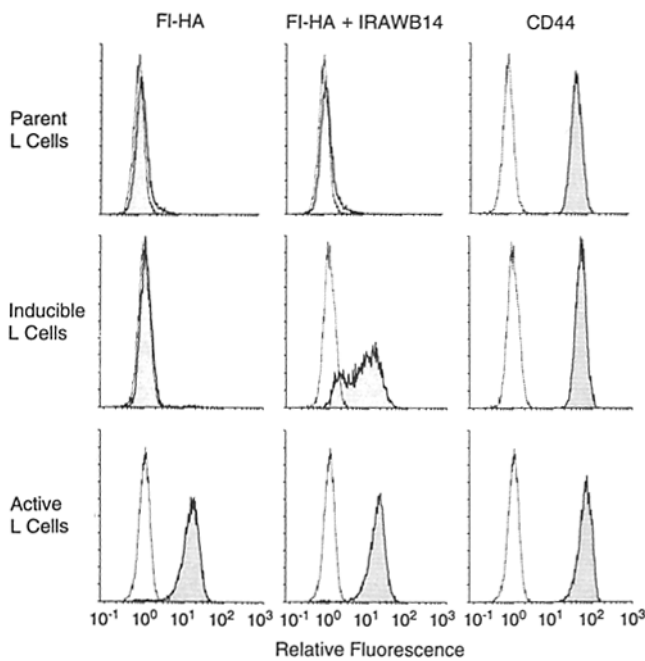
the HA-binding function of CD44. The derivation of these cell lines is outlined in Fig. 1, and the phenotype of the L cell series of variants is shown in Fig. 2. By enrichment sorting of the parental cell lines for rare HA-binding cells using Fl-HA plus the CD44-specific mAb IRAWB14, stable lines that bind Fl-HA in the presence of IRAWB14 mAb, but which do not bind Fl-HA in the absence of inducing antibody, were obtained. These cell lines are termed “inducible” (Figs. 1 and 2, *middle row*). Enrichment sorting of inducible cell lines for cells able to bind Fl-HA in the absence of IRAWB14 allowed isolation of cell lines with a constitutively active CD44 function (Figs. 1 and 2, *bottom row*). These latter cell lines are termed “active.” Cell surface CD44 levels of parental and inducible cell lines were not significantly different. Active variant cell lines showed a two- to threefold increase in cell surface CD44 (Fig. 2, *right column*). Though the increase in CD44 expression may contribute to the HA-binding activity of the constitutively “active” variant cell lines (17), it is unlikely that this increase alone accounts for the change in phenotype from inducible to active, since we have shown previously that inducible cell lines selected for high levels of CD44 expression retain their inducible phenotype (13). The L cell-derived variant lines and the inducible RAW 253 variant have been stable for >1 yr in culture. The active RAW 253 variant has been recloned several times to maintain the majority of cells in the active state.

We were not able to select a cell line with constitutively active CD44 function directly from an inactive parental line by sorting with Fl-HA in the absence of inducing antibody. Previous studies have estimated that the enrichment sorting method allows isolation of variants that are present at a frequency of  $10^{-6}$ – $10^{-5}$  (21). This is the approximate frequency of a mutation event in a single copy of a single gene (22). Thus, if the basis of the phenotypic changes observed is due to mutation, then each variant probably represents a single mutational event, with two mutations required to generate a constitutively active phenotype from the inactive parent.

**Isoform Expression and Cytoskeletal Association in Variant Cells.** One possible mechanism for activating the function of CD44 in variant cells would be a change in splicing of CD44 exons leading to expression of a novel CD44 isoform with an altered ability to bind HA. This possibility was examined by reverse transcription and PCR amplification of CD44 RNA from parental and variant cell lines. As shown

Activation State	CD44 Expression	HA Binding	HA Binding + IRAWB14
INACTIVE (Parent)	+	–	–
(Fl-HA + IRAWB14) (3–4 sorts)			
INDUCIBLE	+	–	+
(Fl-HA alone) (3–4 sorts)			
ACTIVE	+	+	+

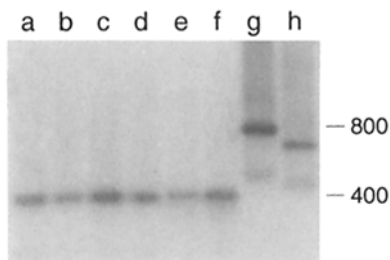
**Figure 1.** Derivation of variants in HA receptor function. Variant cell lines were derived from “inactive” parental cell lines as described in Materials and Methods. CD44 expression and HA-binding phenotype were determined by flow cytometry (see Fig. 2).



**Figure 2.** Flow cytometric analysis of the L cell [L.TK-(K)] series of lines. Cells were incubated with Fl-HA (left column), Fl-HA in the presence of inducing mAb IRAWB14 (center column), or fluorescein-conjugated IM7 specific for CD44 (right column) and analyzed by flow cytometry. Relative fluorescence was normalized such that unlabeled cells (indicated by the dotted insert in each panel) had a mean relative fluorescence intensity of 1.0.

in Fig. 3, all lines expressed the standard isoform of CD44. No novel isoforms were detected in the variant cell lines (Fig. 3, lanes a-f). Control cell lines expressing higher relative molecular mass isoforms of CD44 (8) had the expected larger reverse transcription-PCR products (Fig. 3, lanes g and h).

In L cells, a portion of the cell surface CD44 is insoluble

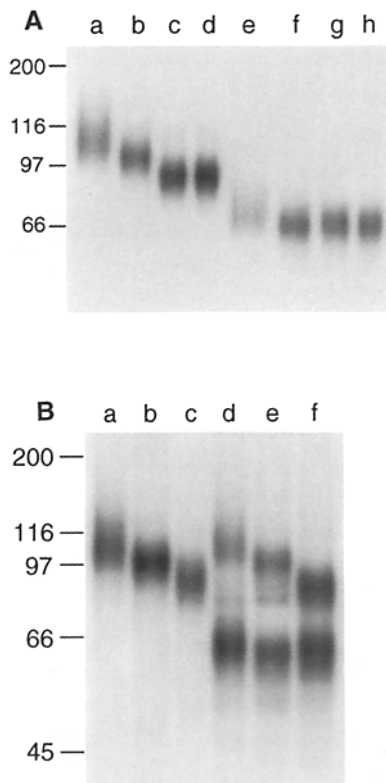


**Figure 3.** CD44 isoform expression in parental and variant cell lines. Reverse transcription, PCR amplification, and Southern blotting were performed as described in reference 16. Total RNA was isolated from cell lines as follows: parental L cells (lane a); inducible L cells (lane b); constitutively active L cells (lane c); parental RAW 253 (lane d); inducible RAW 253 (lane e); active RAW 253 (lane f); and CD44-negative AKR1 cells transfected with higher relative molecular mass CD44 isoforms, described in reference 8, containing variant exons v8-v10 (lane g) and v9-v10 (lane h). The legend indicates bp, and the expected size of standard CD44H is 400 bp.

in Triton X-100 (19). The proportion of Triton X-100-insoluble CD44 was not changed between parental L cells and variants for HA-binding function (data not shown). Also, RAW 253 cells and variants derived from them lack a Triton X-100-insoluble CD44 component. These results indicate that Triton X-100 insolubility of CD44 or lack of Triton X-100 insolubility does not correlate with HA-binding phenotype.

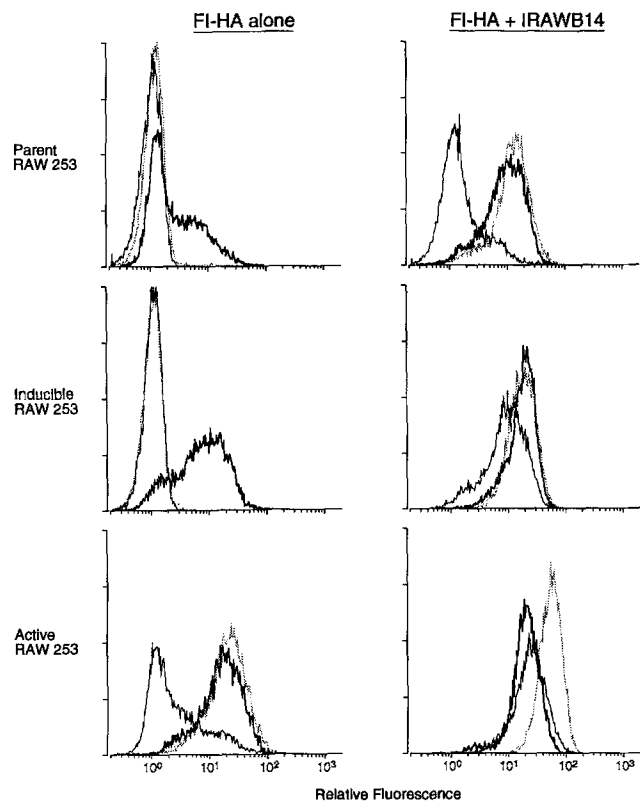
**Variant CD44 Molecules Show Differences in Glycosylation.** Slight differences in apparent relative molecular mass were seen when iodinated and immunoprecipitated CD44 molecules from parent and variant cell lines were analyzed on SDS-PAGE. These differences were seen only when immunoprecipitates were run on long 10% acrylamide gels, and/or run for longer times (after the marker dye had reached the bottom of the gel). CD44 from constitutively active variants migrated more rapidly than CD44 from inducible variants, which in turn migrated more rapidly than CD44 from parental cells. This suggested that inactive CD44 was more heavily glycosylated than the inducible form and that inducible CD44 was more heavily glycosylated than the active form. This result was found for both L cells and RAW 253 and is shown for RAW 253 and its variants in Fig. 4 (A, lanes a-d, and B, lanes a-c). Slight differences in migration were also seen when comparing migration of transferrin receptor from the three RAW 253-derived cell lines, indicating that these differences were not specific to CD44 but affected multiple glycoproteins. The differences in apparent relative molecular mass of CD44 could be partly attributed to N-linked glycosylation: As shown in lanes e-h of Fig. 4 A, most of the differences in mobility were eliminated by treatment of the CD44 immunoprecipitates with N-glycosidase F, though parental cell CD44 still migrated slightly more slowly than the two variant forms after N-glycosidase F treatment. When cells were iodinated and CD44 was immunoprecipitated after culture in 5  $\mu$ g/ml tunicamycin, the differences in migration of the deglycosylated lower migrating bands of CD44 from the three cell lines were again largely eliminated, but the deglycosylated band of the parental cells still migrated above those of the two variants (Fig. 4 B, lanes d-f). The lower relative molecular mass band in tunicamycin-treated cells always migrated faster than CD44 deglycosylated by treatment with N-glycosidase F, suggesting either that N-glycosidase F did not remove all of the N-linked sugars, or that tunicamycin affected other posttranslational modifications of CD44 besides N-glycosylation. A fraction of the immunoprecipitated CD44 from tunicamycin-treated cells migrated similarly to CD44 of untreated cells (Fig. 4 B, lanes d-f, upper bands). This observation suggests that CD44 does not turn over completely during the period of tunicamycin treatment.

**Inhibition of N-linked Glycosylation Alters the Activation State of CD44.** The HA-binding function of CD44 in tunicamycin-treated cells (after 2 d in the drug) is shown for the RAW 253 series of cell lines in Fig. 5 (bold solid lines). After tunicamycin treatment, parental RAW 253 cells (Fig. 5, upper row), were completely inducible by IRAWB14 mAb (upper right panel), and some cells bound Fl-HA constitutively, i.e., without induction by IRAWB14 (upper left panel). Inducible RAW 253 variants cultured in tunicamycin-bound HA con-



**Figure 4.** Migration of CD44 from RAW 253 parent and variant cells on SDS-PAGE. Surface iodinated cells were solubilized in 1% NP-40, and CD44 was immunoprecipitated. Samples were run on 10% acrylamide gels until ~60 min after the bromophenol blue marker dye ran off the bottom of the gel. (A) Lanes *a-d* are untreated CD44 immunoprecipitates. Lanes *e-h* are CD44 immunoprecipitates digested overnight with *N*-glycosidase F after denaturation in SDS and mercaptoethanol. Lanes *a* and *e* were from parental cells; lanes *b* and *f* were from inducible cells; and lanes *c*, *d*, *g*, and *h* were from two clones of constitutively active cells. (B) Cells were cultured for 28 h in the absence (lanes *a-c*) or presence (lanes *d-f*) of 5 µg/ml tunicamycin; parental cells (lanes *a* and *d*); inducible cells (lanes *b* and *e*); and constitutively active cells (lanes *c* and *f*). Migration of molecular mass standards in kilodaltons is indicated on the left.

stitutively (Fig. 5, *middle left panel*). The constitutively active HA-binding variant, which in this experiment bound HA only weakly in the absence of tunicamycin (Fig. 5, *thin solid line, lower left panel*), bound HA much more efficiently after culture in tunicamycin (Fig. 5, *bold solid line, lower left panel*). Thus, tunicamycin converted parental and inducible lines to the active state and enhanced the HA-binding function of active cells. 2 d (42–48 h) was required to effect the maximum change in HA-binding function shown in Fig. 5, and longer treatment resulted in toxicity to the cells. The same changes in HA-binding phenotype were obtained with the L cell series of lines, except that culture for 3 d in tunicamycin was tolerated and was required to convert the cells to an active phenotype. HA binding of treated cells was mediated by CD44 as shown by inhibition of FI-HA binding with the CD44-specific mAb KM81 (data not shown). In other experiments, tunicamycin treatment converted the in-



**Figure 5.** Flow cytometric analysis of the HA-binding function of RAW 253 parental and variant cell lines in the presence and absence of inhibitors of glycosylation. Cells were cultured for 42 h in the presence of no inhibitor (*thin solid line*), 1 mM  $\beta$ -D-xyloside (*dotted line*), or 5 µg/ml tunicamycin (*bold solid line*), and stained with FI-HA alone (*left column*) or FI-HA in the presence of the inducing mAb IRAWB14 (*right column*). Relative fluorescence was normalized such that mean fluorescence intensity of unstained cells was 1.0.

ducible T lymphoma SAKRTLS12 (described in 4 and 13) to a constitutively active state (data not shown). Treatment with tunicamycin either slightly reduced or did not significantly affect cell surface CD44 expression (data not shown).

Tunicamycin inhibits N-linked glycosylation of glycoproteins by preventing formation of the lipid-linked saccharide donor, which is transferred as a unit to the nascent polypeptide (23). Other inhibitors, such as deoxymannojirimycin, inhibit trimming of the mannose-rich immature carbohydrate structure linked to asparagine (23). When parental and variant cell lines were cultured for 3 d in 2 mM deoxymannojirimycin, the HA-binding phenotype of the cell lines did not change. Thus, inhibition of processing of N-linked sugars was not sufficient to alter the functional state of CD44. The only clear effect of deoxymannojirimycin treatment was that the active variant of RAW 253, which showed relatively weak constitutive HA binding, now bound FI-HA more efficiently, such that all cells showed detectable binding (data not shown). In other experiments, inhibition of O-linked glycosylation by culture for 3 d in 2 mM BZ $\alpha$ GalNAc (24) also did not influence the function of CD44 in any of the cell lines (data not shown). Western blotting to detect CD44 from deox-

ymannojirimycin and BZ $\alpha$ GalNAc-treated cells verified that there was a significant reduction in relative molecular mass as a result of these treatments. Inhibition of O-linked glycosylation reduced the difference in migration between parental cells and the two variants, possibly accounting for the component of the differential migration that was not eliminated by inhibition of N-linked glycosylation. Lectin-binding analysis confirmed that deoxymannojirimycin-treated cells expressed mannose-rich cell surface carbohydrates and lacked more terminal sugar structures. Neuraminidase treatment of parental RAW 253 cells did not change the HA-binding properties of these inactive cells. The effectiveness of the enzyme treatment was monitored by binding of lectins sensitive to the presence of terminal sialic acid residues (data not shown).

Culture of cells in  $\beta$ -D-xyloside prevents the addition of chondroitin sulfate GAG side chains to proteoglycan core proteins (25). We examined the effect of culture in 1 mM xyloside on CD44 function in RAW 253 cells (Fig. 5, *dotted lines*). Parental cells cultured in  $\beta$ -D-xyloside acquired inducible HA-binding activity, but still did not bind HA constitutively (*upper panels, right and left, respectively*). Inducible variant cells were not detectably affected. Constitutive HA-binding cells bound Fl-HA more efficiently after culture in  $\beta$ -D-xyloside (*lower left panel*). The same results were obtained with the L cell series of lines upon culture in  $\beta$ -D-xyloside. Parallel cultures in the presence of the inactive isomer,  $\alpha$ -D-xyloside, showed no effect on HA binding. These results were confirmed by treatment of intact RAW 253 cells with chondroitinase ABC, which digests chondroitin sulfate side chains (as well as HA). Again, parental inactive cells were converted to the inducible state, but no other changes in phenotype were found. It should be noted that tunicamycin has also been shown to selectively inhibit proteoglycan synthesis in some cell types (26). Thus, the effect of tunicamycin on HA-binding phenotype could represent combined influences on proteoglycan and N-linked carbohydrate synthesis. Hyaluronidase treatment, on the other hand, had no effect on HA binding in either the presence or absence of IRAWB14-inducing mAb, indicating that failure to bind Fl-HA was not due to endogenous synthesis of HA (data not shown). Western blotting of CD44 molecules from parental and variant RAW 253 cell lines did not show any detectable change in the migration in SDS-PAGE of CD44 from  $\beta$ -D-xyloside-treated cells compared with untreated cells, indicating that the bulk of CD44 molecules in these cells was not modified with chondroitin sulfate side chains.

**Maintenance of the Activation State of CD44 Does Not Require Cell Metabolism.** To determine whether active cell metabolism was required to maintain the functional state of CD44 in parental or variant cells, cells were fixed in 2% paraformaldehyde in a periodate lysine buffer designed to maintain carbohydrate structure (27) for 20 min at 4°C, washed in PBS, and held overnight at 4°C in PBS. After this treatment, cells were completely permeable to propidium iodide and trypan blue (15 and data not shown). When assayed for HA binding in the presence and absence of inducing mAb, the cells retained the same HA-binding properties that were found in living cells: Parental cells were inactive, inducible cells bound

HA only in the presence of IRAWB14 mAb, and active cells bound HA constitutively (data not shown).

## Discussion

Cellular regulation of the ligand-binding function of adhesion receptors controls the timing and specificity of cell adhesion in cell-cell and cell-substrate interactions. CD44 shares this property of regulated function with many of the integrins and with selectins (28). Though there is a great deal of heterogeneity in the CD44 family of cell surface molecules, due to alternative splicing and posttranslational modifications (1), it is not yet clear how these variations in CD44 molecular structure affect ligand-binding function. By comparing lines with different ligand-binding properties derived from a common parent, we sought to elucidate factors involved in regulating receptor function.

Here we have provided genetic evidence for three activation states of CD44 with respect to HA receptor function. The requirements for selecting variant lines suggest that two genetic events separate inactive and active states in the two series of cell lines that we have characterized here (see Fig. 1). However, the genes involved here are probably not the only ones that act to alter CD44 function. Any gene whose product influences CD44 function could give rise to a new activation state, and combinations of such gene products could give still more activation states. Additional assays might allow us to distinguish additional activation states.

Studies by Hathcock et al. (29) as well as those presented here noted that cells able to bind HA had less heavily glycosylated CD44 molecules than cells that failed to bind HA. We have now shown that modification of the carbohydrate composition of the cell surface can have dramatic effects on CD44 function. The general finding from these studies is that removal of cell surface carbohydrate results in a CD44-HA receptor with ability to bind HA either inducibly or constitutively. The treatments used to affect carbohydrate structure act on glycoproteins or proteoglycans throughout the cell. Thus, though we could demonstrate changes in CD44 glycosylation upon treatment with tunicamycin, we do not know whether the observed changes in function are due to deglycosylation of CD44 and/or deglycosylation of some other molecule(s) on the cell surface that influence(s) CD44 function. In preliminary experiments using HA-conjugated Sepharose beads (30) to assay the binding function of detergent-solubilized CD44 from the RAW 253-derived cell lines, only lysates from the constitutively active cells bound the beads. With lysates from tunicamycin-treated cells, the lower relative molecular mass deglycosylated band of all three cell lines bound, suggesting that the HA-binding properties of the cells are a consequence of posttranslational modification of CD44 itself.

In the case of  $\beta$ -D-xyloside and chondroitinase treatments, we could not detect any change in the mobility of CD44 on SDS-PAGE, suggesting that removal of GAG chains from some other molecule may have affected CD44 function in inactive cells. One possibility is that an endogenous ligand

for CD44 is expressed on the cell surface and blocks CD44 function by occupying ligand-binding sites. Specific binding of CD44 to two chondroitin sulfate-containing proteoglycans has been described (31, 32). Another possibility is that a chondroitin sulfate-containing molecule interacts with CD44 at some site distinct from the HA-binding region and alters CD44 conformation or distribution. It is also possible that a minor component of cell surface CD44 contains chondroitin sulfate side chains, or that chondroitin sulfate side chains are present on inactive CD44 but are too short to alter relative molecular mass detectably.

The parental and variant cell lines we describe here show differences in N-linked glycosylation, and elimination of N-glycosylation abolishes differences in CD44-HA receptor function. However, this does not prove that glycosylation differences are the basis of the differences in function in the variant cell lines. It is clear, however, that whatever the mechanism responsible for the differences in HA-binding cell phenotypes, it does not require ongoing cell metabolism, as HA-binding phenotypes are maintained even in fixed cells (15 and this paper). Thus, HA-binding phenotype appears to be a stable property of the cell surface environment that does not require active processes for its maintenance.

We have shown previously that activation of HA binding by inducing mAb requires multivalent antibody and cross-linking of CD44 (15), and that a mutant CD44 molecule expressed as a disulfide-bonded dimer on the cell surface binds HA more efficiently than wild-type CD44 (17). As a result of these studies, we have postulated that HA binding by CD44 is promoted by close association of CD44 molecules in a multivalent aggregate on the cell surface. This model has precedents in other experimental systems, as Dustin and Springer (28, 33) have shown that aggregated CD2 and LFA-3 had 10- and 200-fold higher avidity for substrate, respectively, than monomeric forms. Mobility within the membrane has been shown to be a significant factor in contributing to adhesion between the CD2 and LFA-3, presumably by allowing clustering of receptors at a point of contact between cells (34), and carbohydrate side chains have been shown to restrict lateral

diffusion of cell surface MHC class I molecules, as mutation of N-glycosylation sites resulted in increased mobility in fluorescence photo bleaching and recovery assays (35). Formation of multivalent CD44 receptor might be prevented by restriction of mobility due to glycosylation and/or by charge repulsion of carbohydrate side chains terminating in charged residues (28). Other possibilities include more direct inhibition of HA binding as a result of conformational or steric interference.

Several treatments that inhibit glycosylation also enhanced HA binding of constitutively active RAW 253 variant cells ( $\beta$ -D-xyloside, deoxymannojirimycin, and tunicamycin), indicating that, even in the active state, receptor function was restricted by carbohydrate. A recent study by Katoh et al. also found that HA binding by Chinese hamster ovary cells and several other cell lines was enhanced by deglycosylation (tunicamycin and neuraminidase treatment), again indicating restriction of receptor function by carbohydrate (see accompanying article, 36). In our studies, however, deoxymannojirimycin (or neuraminidase treatment of inactive RAW 253 parental cells), which eliminated most of the charged sugar residues from N-linked carbohydrate side chains, did not alter the function of CD44 in inactive and inducible cell lines. Therefore, the mechanism whereby N-linked glycosylation restricts CD44 function remains to be determined and may differ among different cell types.

Bartolazzi et al. (37) have shown that, in one model system, the ability of CD44 to bind HA is required for the enhancement of metastatic behavior when CD44 is transfected into a nonmetastasizing cell line. By selection of cells that vary in the HA-binding function of their cell surface CD44, we have shown that changes in the expression of only one or two genes are sufficient to convert a cell that does not bind HA to a constitutively active HA-binding phenotype. Mutations similar to those described here, which affect the regulation of CD44 ligand-binding function, might contribute to the function of CD44 in promoting tumor malignancy and metastasis.

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