

## Analysis of CD44 interactions with hyaluronan in murine L cell fibroblasts deficient in glycosaminoglycan synthesis: a role for chondroitin sulfate

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### SUMMARY

CD44 is a widely expressed cell adhesion molecule that binds the extracellular matrix component, hyaluronan, in a tightly regulated manner. Previous studies have shown that the CD44-hyaluronan interaction is affected by changes in the glycosylation state of CD44. In this study, we take advantage of several well-characterized murine L cell mutants defective in heparan sulfate synthesis (gro2C cells), heparan sulfate and chondroitin sulfate synthesis (sog9 cells), and glycosaminoglycan and oligosaccharide processing (sog8 cells) to assess the effects of these defects on the hyaluronan binding ability of CD44. In parental L cells and gro2C cells, CD44 was induced to bind hyaluronan after addition of the activating, anti-CD44 monoclonal antibody, IRAWB 14. By contrast, no inducible binding was observed in sog9 cells. Treatment of L cells

with sodium chlorate, an inhibitor of sulfation, also abolished inducible hyaluronan binding. However, inducible and some constitutive hyaluronan binding was observed in sog8 cells. This indicates that sulfation and, in particular, the addition of chondroitin sulfate are required for inducible hyaluronan binding by CD44 in L cells. However, in the absence of fully processed oligosaccharides, chondroitin sulfate is not essential for hyaluronan binding, indicating that the effect of chondroitin sulfate is dependent upon the glycosylation state of the cell. Thus, in addition to glycosylation, chondroitin sulfate biosynthesis is an important post-translational modification that can affect the hyaluronan binding ability of CD44.

Key words: CD44, Hyaluronan, Chondroitin sulfate, Glycosylation

### INTRODUCTION

CD44 is a widely expressed cell surface glycoprotein that has been implicated in a variety of cellular functions involving cell adhesion and migration, and in the metastasis of certain cancers (reviewed in Lesley et al., 1993a). Hyaluronan (HA) is a high molecular mass glycosaminoglycan (GAG), which has been identified as a major ligand for CD44 (Aruffo et al., 1990; Culty et al., 1990; Lesley et al., 1990; Miyake et al., 1990b). The interaction between HA and CD44 promotes cell-cell and cell-matrix interactions and CD44 has been implicated in bone marrow-stromal cell interactions (Miyake et al., 1990a,b), lymphocyte-endothelial cell interactions (Aruffo et al., 1990; Lesley et al., 1992; Camp et al., 1993; Zahalka et al., 1995; Clark et al., 1996; DeGrendele et al., 1996), angiogenesis (Trochon et al., 1996) and in arterial wall repair (Jain et al., 1996). As with other cell surface molecules involved in cell adhesion and cell migration, the adhesive property of CD44 is tightly regulated. Three different binding states have been identified for CD44 binding to HA: constitutive, inducible and non-binding (Lesley and Hyman, 1992). In general, cells expressing CD44 do not constitutively bind HA; however, some cells can be induced to bind HA after an in vivo allogeneic reaction (Lesley et al., 1994), an in vivo chronic graft-versus-host reaction (Murakami et al., 1991), an in vitro induction by

interleukin-5 in B cells (Murakami et al., 1990) and after arterial wall injury (Jain et al., 1996). Phorbol myristate acetate (PMA), which can mimic aspects of T cell activation, increases the expression of CD44 and induces the binding of CD44 to HA (Lesley et al., 1990; Hyman et al., 1991; Liao et al., 1993). The inducible binding state of CD44 can be identified using the anti-mouse CD44 specific monoclonal antibody (mAb), IRAWB 14, which can bind CD44 and induce HA binding (Lesley et al., 1992).

CD44 can exist in multiple isoforms generated by alternative splicing of the membrane proximal region of the molecule, which provides additional sites for glycosylation and GAG addition (Screaton et al., 1992). The standard form of CD44, CD44H, contains none of the alternatively spliced exons and exhibits different HA binding abilities, depending upon the cell type or the activation state of the cell (Lesley et al., 1990; and reviewed in Lesley et al., 1993a). Within a particular cell line or cell type, the alternatively spliced forms of CD44 can exhibit different HA binding abilities from the standard, CD44H form (Stamenkovic et al., 1991; Liao et al., 1993; Bennett et al., 1995; Jackson et al., 1995; van der Voort et al., 1995; Sleeman et al., 1996). Both N- and O-linked glycosylations have also been shown to influence the HA binding ability of CD44 in both a cell type- and CD44 isoform-specific manner. The inhibition of N-linked glycosylation in a variety of cell lines

can either inhibit (Bartolazzi et al., 1996; Sleeman et al., 1996) or enhance (Kato et al., 1995; Lesley et al., 1995) the HA binding ability of CD44. Inhibition of O-linked glycosylation either enhances (Bennett et al., 1995; Dasgupta et al., 1996) or has no effect (Lesley et al., 1995) on the HA binding ability of CD44. In some cell lines, modification with keratan sulfate (KS) and heparan sulfate (HS) has been reported, and KS modification of CD44 has been shown to have a negative effect on HA binding in a colon carcinoma cell line (Takahashi et al., 1996). Aggregation of CD44 has also been shown to correlate with its ability to bind HA, as demonstrated by induced dimerization of chimeric CD44 molecules and by aggregation of a particular CD44 isoform expressed in pancreatic carcinoma cells (Perschl et al., 1995; Sleeman et al., 1996). Thus the HA binding ability of CD44 can be affected by the CD44 isoform expressed, the glycosylation state of the cell and the aggregation state of CD44, which may differ depending on the activation state of the cell and cell type. It is clear that the HA binding ability of CD44 is tightly regulated, but which of these factors regulate HA binding in vivo in any one cell type is not known.

Although CD44 can be modified by the addition of GAGs, particularly chondroitin sulfate (CS), the effect of this GAG on HA binding ability has not been investigated. There are several approaches to assessing the effects of GAGs, such as enzymatic digestion and the use of biosynthetic inhibitors. However, these methods are problematic due to the difficulty in determining the precise effects of the treatment on the cell surface GAG moieties. To overcome this problem, we have taken advantage of a series of well-characterized L cell lines deficient in GAG synthesis (Gruenheid et al., 1993; Banfield et al., 1995), to determine the effect of HS and CS on CD44 binding to hyaluronan. These L cell lines were isolated on the basis of their resistance to herpes simplex virus-1 (HSV-1) infection (Tufaro et al., 1987). Gro2C cells are L cell variants resistant to HSV-1 infection and defective in the synthesis of HS (Gruenheid et al., 1993). Selection of these gro2C cells for further resistance to HSV-1 infection yielded two L cell lines, sog9 and sog8, which are defective in GAG synthesis (Banfield et al., 1995). Sog8 cells have an additional defect in oligosaccharide processing. By comparing the binding capacity in each cell line of CD44 for HA, we demonstrate that CS plays an important role in facilitating the functional interaction of HA with CD44 in these cell lines.

## MATERIALS AND METHODS

### Cell lines and antibodies

The parental L cell line (clone 1D line of LM(tk<sup>-</sup>) murine fibroblasts) and NIH 3T3 cells were from American Type Culture Collection (Rockville, MD). The isolation of mutant L cell lines was described previously (Tufaro et al., 1987). Mutant cell lines used were: gro2C (Gruenheid et al., 1993); sog9 (Banfield et al., 1995), and a ricin-resistant variant of sog9 (sog8) (B. W. Banfield and F. Tufaro, unpublished). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) (BRL Life Technologies, Burlington, Ontario) and 50 units/ml of penicillin and streptomycin at 37°C in 5% CO<sub>2</sub>. Tissue culture supernatants (TCS) containing rat mAbs against murine CD44: IM7.8.1 (Trowbridge et al., 1982), KM201 (Miyake et al., 1990a) or IRAWB 14 (Lesley et al., 1992) were used for flow cytometry and

immunoprecipitation, inhibition of HA binding, or flow cytometry and induction of HA binding, respectively, and were kindly given to us by J. Lesley and R. Hyman (IM7.8.1 and IRAWB 14) and P. Kincade (KM201). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rat antibodies were obtained from Kirkegaard and Perry Laboratories (Burlington, Ontario) and horseradish peroxidase (HRP)-conjugated goat anti-rat antibodies were from Southern Biotechnology Associates, Inc. (Birmingham, AL).

### Fluorescein conjugation of hyaluronic acid

Rooster comb HA was obtained as a sodium salt (Sigma, Mississauga, Ontario) and was conjugated to fluorescein according to the method of de Belder and Ove Wik (1975).

### Flow cytometry

Approximately 1-2×10<sup>5</sup> adherent cells were removed from dishes with 1 mM EDTA in phosphate-buffered saline (PBS) and labeled with mAbs for 20 minutes on ice. Cells were washed with PBS containing 2% FCS and 2 mM EDTA, pH 8.0. Cells were then incubated in a 1/100 dilution of FITC-conjugated goat anti-rat antibodies for 20 minutes on ice, washed, and resuspended in PBS/2% FCS/2 mM EDTA containing 5 µg/ml of propidium iodide (Sigma, Mississauga, Ontario). Alternatively, cells were incubated for 5 minutes with IRAWB 14, KM201 or with buffer alone, to either induce fluorescein-labeled hyaluronan (FL-HA) binding, prevent FL-HA binding, or as a control, respectively. The cells were then incubated with 15 µg/ml of FL-HA for 20 minutes on ice and washed. To block IRAWB 14-induced FL-HA binding, cells were first pretreated for 5 minutes with KM201, then washed prior to treatment with IRAWB 14 for 5 minutes followed by incubation for 20 minutes with FL-HA. All labeled cells were analyzed on a FACScan® flow cytometer (Becton Dickinson, Mississauga, Ontario) using Lysis II® software. Competition assays were done by incubating cells with 15 µg/ml of FL-HA in the presence of 3 mg/ml HA, 3 mg/ml chondroitin-4-sulfate (CSA) or 3 mg/ml chondroitin-6-sulfate (CSC) (Sigma, Mississauga, Ontario). For inhibition of sulfo-transferase activity, cells were incubated with 10 mM sodium chlorate (Sigma, Mississauga, Ontario) for 2 days in DMEM supplemented with 10% FCS at 37°C in 5% CO<sub>2</sub>.

### Detection of CD44 by western blot

5×10<sup>5</sup> cells were washed once in PBS and lysed in 60 mM *n*-octyl-β-D-glucopyranoside (Calbiochem, San Diego, CA), 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 200 µM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin (lysis buffer) and incubated on ice for 10 minutes. Lysates were clarified by centrifugation at 12,000 g at 4°C for 10 minutes, supernatants were resuspended in an equal volume of 2× non-reducing sample buffer, boiled, separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) Immobilon-P membranes (Millipore, Mississauga, Ontario). Dried membranes were incubated with 2 µg/ml purified, biotinylated IM7.8.1 in 5% skimmed milk powder, 0.1% Tween-20, 20 mM Tris, pH 7.5, 150 mM NaCl (TTBS) for 1 hour, washed, and then incubated with a 1/5000 dilution of HRP-conjugated streptavidin in 5%-skimmed milk-TTBS for 30 minutes. Membranes were washed thoroughly and developed using the enhanced chemiluminescence assay according to the manufacturer's instructions (ECL kit; Amersham, Oakville, Ontario). In all cases, prestained molecular mass markers (New England Biolabs, Mississauga, Ontario) were run on the SDS-polyacrylamide gels and transferred to the PVDF membrane.

### Sulfate labeling and treatment with chondroitin ABC lyase

Three sub-confluent 100 mm<sup>2</sup> plates of L cells were each incubated with 200 µCi of [<sup>35</sup>S]sulfate (specific activity approx. 43 Ci/mg, ICN Biomedicals, St Laurent, Québec) in 4 ml DMEM/2% FCS for 3 days. Cells were washed with 5 ml of PBS and lysed in 1% Triton X-100, 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA, 200 µM

phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin or in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 20 mM Tris, pH 7.5, 150 mM NaCl, 200 µM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin and 1 µg/ml pepstatin). Lysates were pooled, centrifuged at 12,000 g for 10 minutes, and then precleared three times with 30 µl of a 50% slurry of Sepharose CL-4B (Sigma, Mississauga, Ontario) for 1 hour at 4°C, rotating end-over-end. IM7.8.1 mAb (100 µl TCS) was precoupled to 20 µl of protein G Sepharose-4 Fast Flow (Pharmacia, Baie d'Urfe, Québec) for 2 hours, washed, and added to the precleared lysates for 2 hours at 4°C, rotating end-over-end. Immunoprecipitates were washed three times in lysis buffer and divided into two samples containing 40 µl of 0.01% BSA, 40 mM Tris, 40 mM sodium acetate, pH 8.0, one of which was treated with 15 milliunits of chondroitin ABC lyase (Boehringer Mannheim, Laval, Québec). Samples were incubated for 1 hour at 37°C before 3× reducing sample buffer was added, the samples boiled and loaded on a 7.5% SDS-polyacrylamide gel. Gels were treated with Amplify® (Amersham, Mississauga, Ontario), dried, and exposed at -70°C with Kodak BioMax MR film (InterScience, Markham, Ontario) with an intensifying screen for 7 days.

#### Pulse-chase of L cells and sog8 cells and treatment with endoglycosidase H

One confluent 60 mm<sup>2</sup> plate of L cells and sog8 cells per time point was washed with PBS and then starved of methionine and cysteine by incubation in 2 ml of DMEM methionine(-) and cysteine(-) medium (ICN, St Laurent, Québec) for 45 minutes at 37°C. The cells were then labeled for 20 minutes at 37°C with 250 µCi of [<sup>35</sup>S]Express (specific activity >3000 Ci/mmol, 70% methionine, 30% cysteine; NEN DuPont Canada, Markham, Ontario) in 2 ml of methionine- and cysteine-deficient DMEM supplemented with 1% Glutamax I (BRL Life Technologies, Mississauga, Ontario). Cells were then either harvested immediately or washed in 5 ml PBS and incubated in DMEM/10% FCS for 15, 30, 60 or 120 minutes. Cells were lysed as above. A small sample of each lysate was precipitated in 10% trichloroacetic acid to determine the amount of radiolabel incorporated into each cell line and lysates containing equivalent amounts of radioactivity were used for immunoprecipitation. Lysates were immunoprecipitated as above then washed twice in high salt buffer (1% Nonidet P-40, 10 mM Tris, pH 7.5, 500 mM NaCl, 2 mM EDTA), three times in low salt buffer (1% Nonidet P-40, 10 mM Tris, pH 7.5, 150 mM NaCl, 2 mM EDTA) and once in 10 mM Tris, pH 7.5, and divided in two. Half of the immunoprecipitate was digested by the addition of 12 µl 85 mM sodium citrate, pH 5.5, 2 µl (2 milliunits) Endoglycosidase H (endoH; Boehringer Mannheim, Laval, Québec) and 2 µl 100 mM PMSF, and incubated at 37°C overnight. The other half of the immunoprecipitated material was incubated at 37°C overnight in 85 mM sodium citrate, pH 5.5, and 2 µl 100 mM PMSF without endoH. All immunoprecipitates were boiled in an equal volume of 2× reducing sample buffer and separated on 7.5% polyacrylamide gels. Samples were treated as above except that gels were exposed for 2 days.

#### Treatment of cells with PNGase F, neuraminidase and O-glycosidase

1×10<sup>7</sup> cells were washed once in PBS and lysed as described above in 60 mM *n*-octyl-β-D-glucopyranoside. IM7.8.1 mAb (200 µl TCS) precoupled to protein G Sepharose (Pharmacia, Baie d'Urfe, Québec) for 2 hours and washed, was then added to precleared lysates for 1 hour at 4°C, rotating end-over-end. Alternatively, for immunoprecipitation of surface CD44, cells were incubated with IM7.8.1 mAb (200 µl TCS) for 30 minutes, washed, lysed and incubated with protein G Sepharose for 1 hour at 4°C, rotating end-over-end. Immunoprecipitates were washed twice with PBS and divided in two. Half of the immunoprecipitate was treated with 1000 units of peptide N-glycosidase F (PNGase F; New England Biolabs,

Mississauga, Ontario) at 37°C overnight in the buffer supplied by the manufacturer. The other half of the immunoprecipitate was treated in the same manner, but without PNGase F. To remove the sialic acid residues, CD44 immunoprecipitates were treated with 2 milliunits of neuraminidase from *Vibrio cholerae* (Boehringer Mannheim Canada, Laval, Québec) for 4 hours at 37°C in 40 µl of 50 mM sodium acetate, 4 mM CaCl<sub>2</sub>, pH 7.8. To remove O-linked oligosaccharides, neuraminidase-treated immunoprecipitates were washed three times with PBS and treated with 1 milliunit of BSA free O-glycosidase (Boehringer Mannheim, Laval, Québec) in 40 µl of 20 mM sodium cacodylate, 20 mM sodium phosphate, pH 6.5, overnight at 37°C. An equal volume of 2× non-reducing sample buffer was added to the immunoprecipitates, which were then boiled, separated on large 10% polyacrylamide gels to maximize separation and transferred to Immobilon-P membranes (Millipore, Mississauga, Ontario). Immunoblotting of the membranes was performed as described above.

## RESULTS

### CD44 expression and HA binding ability of different L cell lines

In this study, we used well-characterized L cell lines that are defective in the synthesis of GAGs (Gruenheid et al., 1993; Banfield et al., 1995) and in the addition of oligosaccharides to investigate the role of these carbohydrate modifications on CD44-HA binding. It has been demonstrated previously using highly sensitive anion exchange chromatography that gro2C cells, derived from parental L cell fibroblasts, are defective in HS synthesis. Sog9 cells, derived from gro2C cells, contain an additional defect in the GAG synthesis pathway such that they have lost the ability to synthesize any sulfated GAGs. Sog8 cells are similar to sog9 cells except that they contain an additional defect in the processing of N- and O-linked oligosaccharide moieties. The characteristics of these cell lines are summarized in Table 1.

To assess the role of glycosaminoglycan synthesis on CD44-HA binding, fluoresceinated HA (FL-HA) was used to quantify the relative affinity of HA for each of the cell lines (Fig. 1). It can be seen that the parental mouse L cells did not constitutively bind FL-HA, but could be induced to bind FL-HA by pre-treatment with the IRAWB 14 mAb. To determine whether HS or CS synthesis was required for inducible binding, the assays were repeated with gro2C cells and sog9 cells. IRAWB 14-inducible FL-HA binding was observed in the gro2C cells, which indicates that HS is not required for HA binding to these cells. By contrast, sog9 cells could not be induced to bind FL-HA, which suggests that CS plays a role in the CD44-HA

**Table 1. Characteristics of L cell variants**

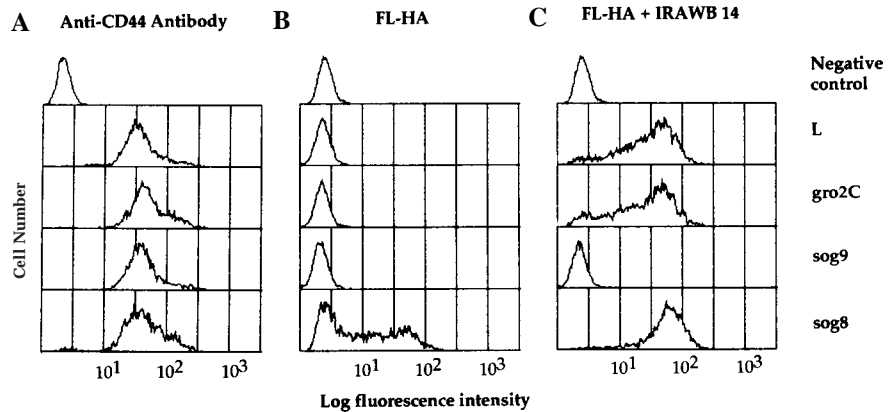
Cell line	Parental line	Glycosaminoglycan synthesis <sup>a</sup>			HA binding <sup>c</sup>
		HS	CS	Glycosylation <sup>b</sup>	
LM(tk <sup>-</sup> )	-	+	+	Normal	Inducible
gro 2C <sup>d</sup>	LM(tk <sup>-</sup> )	-	+	Normal	Inducible
sog 9 <sup>e</sup>	gro2C	-	-	Normal	None
sog 8 <sup>f</sup>	gro2C	-	-	Defective	Constitutive/ inducible

<sup>a</sup>Determined by HPLC; <sup>b</sup>glycosylation assessed by pulse chase labeling;

<sup>c</sup>determined by FACS analysis using FL-HA; <sup>d</sup>Gruenheid et al. (1993);

<sup>e</sup>Banfield et al. (1995); <sup>f</sup>Banfield and Tufaro, unpublished and this report.





**Fig. 1.** Surface expression of CD44 and HA binding ability of L cell lines (L, gro2C, sog9 and sog8) as determined by flow cytometry. Expression levels of CD44 were determined using the anti-CD44 mAb IRAWB 14 (A). (B) The constitutive FL-HA binding ability of these cells. (C) Inducible FL-HA binding ability after pre-treatment with IRAWB 14. Unlabeled L cells were the negative control.

interaction. We also showed that CD44 expression on all of the L cell lines was similar using either the anti-CD44 mAb, IRAWB 14 (Fig. 1) or IM7.8.1 (data not shown). Taken together, these results indicate that alterations in CS synthesis can have a profound effect on the interaction of HA with CD44. Interestingly, sog8 cells bound FL-HA constitutively and could be induced to bind additional FL-HA following incubation with IRAWB 14. These results were surprising, and suggested that CS was no longer required to induce HA binding when N and O-linked oligosaccharides were incompletely processed.

To verify that the observed inducible and constitutive FL-HA binding was mediated by CD44, L and sog8 cells were pre-incubated with the anti-CD44 antibody, KM201 (Fig. 2). KM201 was able to block the FL-HA binding on both IRAWB 14-induced L cells and sog8 cells, indicating that all FL-HA binding was mediated by CD44. In addition, FL-HA binding was inhibited by 200-fold excess unlabeled HA, indicating that FL-HA binding was mediated via HA (Fig. 2).

### Molecular characteristics of CD44 expressed on L cell lines

We next analyzed the forms of CD44 expressed in each cell line to determine what effect the GAG synthesis and glycoprotein processing defects had on CD44. CD44 in L cells has previously been shown to be a protein of  $M_r$   $85 \times 10^3$ ; this is the standard form of CD44, CD44H, which contains none of the alternatively spliced exons (Lesley et al., 1995). To characterize the forms of CD44 expressed in the control L, gro2C, sog9 and sog8 cells, cell lysates were immunoblotted with the anti-CD44 mAb IM7.8.1 (Fig. 3A). Using this method

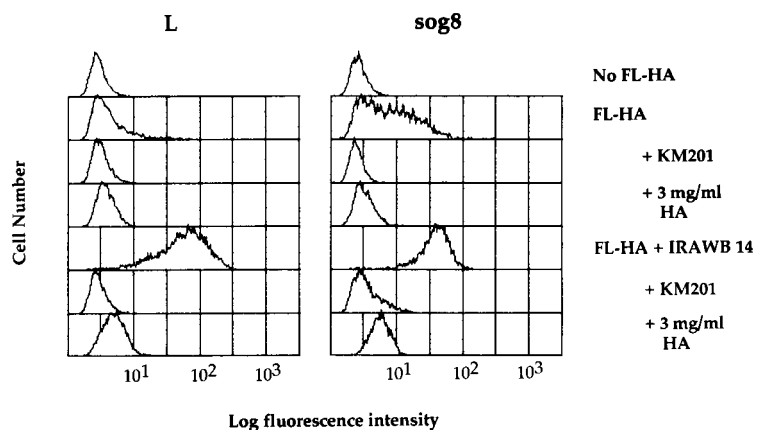
it was demonstrated that L, gro2C and sog9 cells expressed a major  $85 \times 10^3$   $M_r$  form of CD44, CD44H, with no higher  $M_r$  isoforms being detected. By contrast, the apparent  $M_r$  of CD44 expressed in sog8 cells was approximately  $70 \times 10^3$ . This reduction in apparent  $M_r$  was consistent with the presence of a defect in the glycosylation pathway in sog8 cells.

When CD44 was immunoprecipitated from L cells after radioactively labeling with [ $^{35}$ S]sulfate, the  $85 \times 10^3$   $M_r$  form of CD44, CD44H, and a higher  $M_r$  species of approximately  $175 \times 10^3$  was observed (Fig. 3B). This higher  $M_r$  species was immunoprecipitated using conditions that would eliminate co-precipitation of weakly associated CD44 proteins (lane 1), indicating that it was likely to be a sulfated higher  $M_r$  form of CD44. To further establish this, CD44 immunoprecipitates were boiled in SDS to separate all non-covalently associated proteins and then re-precipitated. Although less total protein was re-immunoprecipitated, approximately equal ratios of the higher and lower  $M_r$  forms were present (data not shown), indicating that this was a sulfated higher  $M_r$  form of CD44.

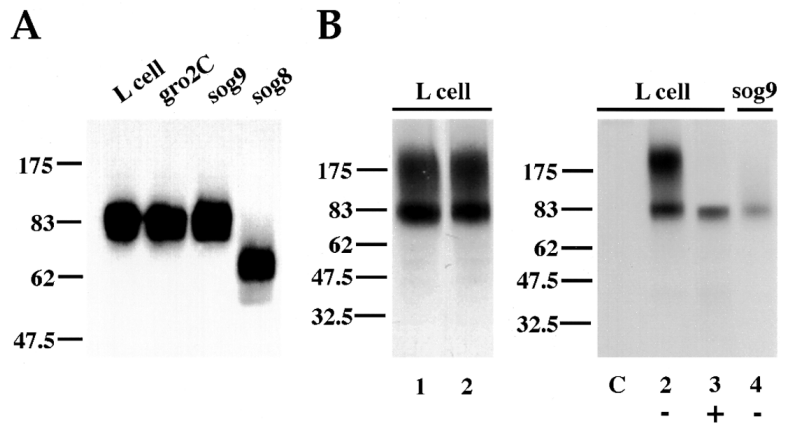
To identify the sulfated moiety on the larger form of CD44, the CD44 immunoprecipitate was also treated with chondroitin ABC lyase (lane 3). The larger band was sensitive to digestion with this enzyme, indicating that the higher  $M_r$  form of CD44 was modified by CS. As expected, immunoprecipitation of CD44 from sog9 cells, which are deficient in CS synthesis, produced only the lower  $M_r$  sulfated species (lane 4).

### Characterization of the role of chondroitin sulfate in IRAWB 14-inducible HA binding

The absence of GAG synthesis in sog9 cells did not affect the



**Fig. 2.** FL-HA binding in the presence of the anti-CD44 mAb, KM201, or excess unlabeled HA. L and sog8 cells were incubated in the presence and absence of the inducing mAb, IRAWB 14, and FL-HA binding was then assessed. KM201 was used to block CD44 dependent HA binding and excess HA was used to compete for FL-HA binding.



**Fig. 3.** Relative molecular mass of CD44 expressed in the L cell lines. (A) Western blot of CD44 from cell lysates of the L cell lines using IM7.8.1 (see Materials and methods for details). (B) Immunoprecipitation of equivalent amounts of CD44 from [ $^{35}\text{S}$ ]sulfate-labeled L cells before (–, lane 2) and after (+, lane 3) treatment with chondroitin ABC lyase and sog9 cells (lane 4) (see Materials and methods for details). Cells were lysed in RIPA lysis buffer (lane 1) or in 1% Triton X-100 lysis buffer (lanes 2–4). A control immunoprecipitate in the absence of the immunoprecipitating mAb IM7.8.1 is indicated in lane C. The positions of the prestained  $M_r$  markers are indicated on the left ( $\times 10^{-3}$ ).

ability of the IRAWB 14 mAb to bind to CD44, but it did affect its ability to induce FL-HA binding. To determine whether the addition of exogenous CS could restore IRAWB 14-induced FL-HA binding, CS was added to sog9 cells and FL-HA binding was assessed by flow cytometry (Fig. 4). The addition of CSA, dermatan sulfate (data not shown) or CSC up to a concentration of 3 mg/ml did not restore IRAWB 14-inducible binding, suggesting that CS has to be bound to a cell surface protein to mediate its effect. In control L cells, where inducible FL-HA binding was observed, the addition of substantial amounts of CSA (100-fold excess) resulted in a slight decrease in FL-HA binding.

We next investigated the role of sulfation in CD44-HA interactions by treatment with sodium chlorate. Sodium chlorate is a potent inhibitor of sulfation of proteins and GAGs (Bauerle and Huttner, 1986; Greve et al., 1988). Because chondroitin containing GAGs are subsequently modified by sulfate addition, it was important to determine the relative importance of these moieties. Time-course and dose-response studies were performed, which indicated that 10 mM sodium chlorate treatment for 2 days was the optimal concentration to induce an effect without causing a significant decrease in cell viability. L cells grown in the presence of 10 mM sodium chlorate lost IRAWB 14-induced FL-HA binding (Fig. 5), whereas sog8 cells grown in the same manner were relatively unaffected. Thus, it appears that sulfation is important for inducible CD44-HA interactions in L cells. The lack of effect of this sulfate inhibitor on sog8 and sog9 cells (data not shown)

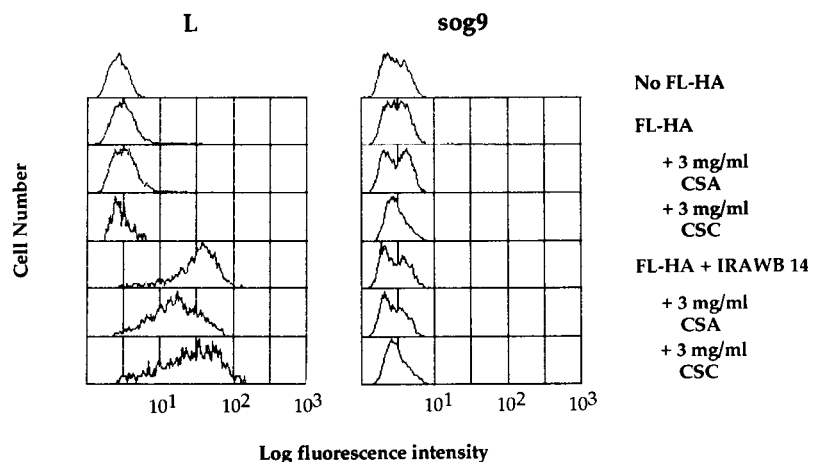
suggests that it is the sulfation of chondroitin that is required for inducible binding.

To determine whether sulfation affects inducible HA binding in other cells, similar studies were performed with sodium chlorate on NIH 3T3 cells (Fig. 5). It is clear from these data that IRAWB 14-inducible binding was inhibited in this cell line, which suggests that sulfation is an important post-translational modification in facilitating functional CD44-HA interactions in different cells.

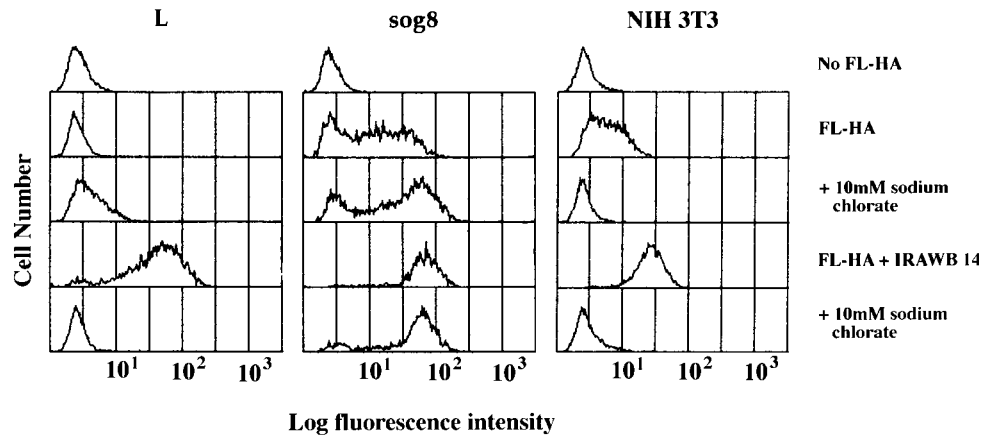
#### Characterization of CD44 in sog8 cells

Since the glycosylation defect in sog8 cells dramatically altered the binding profile of FL-HA to CD44, pulse-chase experiments with [ $^{35}\text{S}$ ]methionine and [ $^{35}\text{S}$ ]cysteine were performed to characterize CD44 in sog8 cells (Fig. 6). After a 20 minute pulse, newly synthesized CD44 had the same apparent  $M_r$  (approximately  $62 \times 10^3$ ) in both L cells and sog8 cells and was sensitive to digestion by endoH, indicating that protein translation and initial processing in these cells were normal. Although CD44 became endoH-resistant in both cell lines, the mature form present on sog8 cells was significantly smaller ( $70 \times 10^3$ ) compared to the normal form of  $85 \times 10^3$  present on L cells (Fig. 6). This indicates that initial processing of N-linked sugars occurs in sog8 cells, resulting in the addition of N-acetyl-glucosamine, but that there is a serious alteration in the processing of oligosaccharide moieties after they reach the medial Golgi.

To investigate whether both N- and O-linked oligosaccharide



**Fig. 4.** Effect of exogenous chondroitin sulfate on FL-HA binding ability of L and sog9 cells. FL-HA and IRAWB 14-induced FL-HA binding were assessed by flow cytometry in the presence or absence of 3 mg/ml of CSA or CSC.



**Fig. 5.** FL-HA binding after treatment of cells with sodium chlorate. L, sog8 and NIH 3T3 cells were incubated in the presence or absence of 10 mM sodium chlorate for 2 days and then tested for the ability to bind to FL-HA, either constitutively or after IRAWB 14 induction.

processing of CD44 was defective in sog8 cells, CD44 immunoprecipitates were treated with PNGase F and O-glycosidase. (Fig. 7). PNGase F treatment of CD44 immunoprecipitates from sog8 cells resulted in a smaller reduction in apparent  $M_r$  (approximately  $5 \times 10^3$  less) compared to the difference observed from CD44 isolated from L cells, indicating that sog8 cells possess incompletely processed N-linked sugars. O-glycosidase treatment of CD44 from sog8 cells indicated a severe defect in the addition of O-linked oligosaccharides.

To further characterize the specific glycosylation defect, CD44 was analyzed following treatment with neuraminidase. It can be seen in Fig. 8 that in sog8 cells, CD44 was relatively unaffected by neuraminidase treatment, which is consistent with a lack of terminal sialic acid residues in sog8 cells. Lectin intoxication studies also revealed that sog8 cells are 27-fold more resistant than L cells or sog9 cells to killing by the galactose-binding lectin RCA<sub>60</sub> (data not shown). This suggests that sog8 oligosaccharides display fewer galactose residues, which is consistent with the data that sog8 cell oligosaccharides are grossly underprocessed. We have also demonstrated that a similar pattern of underprocessing occurs for the VSV G protein expressed in sog8 cells, which possesses only N-linked oligosaccharides (data not shown). Taken together, these results show that N-linked processing and addition of O-linked oligosaccharides to CD44 were defective in the sog8 cells. This loss of fully processed oligosaccharides clearly altered the requirement for CS in CD44-mediated inducible HA binding in L cells. Thus we have demonstrated that although CS synthesis is required for IRAWB 14-inducible binding in L cells, it is not essential for binding when certain oligosaccharide moieties are absent.

## DISCUSSION

### Differential binding properties of L cell lines

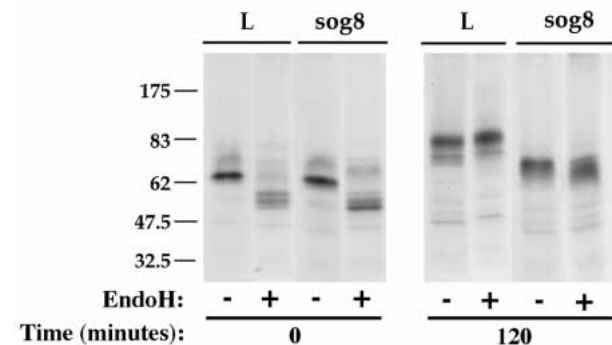
The presence of the three different HA binding phenotypes of CD44 in the L cell lines correlated with differences in CS synthesis and oligosaccharide processing. In the presence of both CS synthesis and complete N- and O-linked oligosaccharide processing, CD44 exhibits an inducible HA binding phenotype in L cells. With the loss of CS synthesis, CD44 has a non-inducible, non-binding phenotype. When both

CS synthesis and oligosaccharide processing are defective, inducible HA binding by CD44 is restored and some constitutive binding is observed.

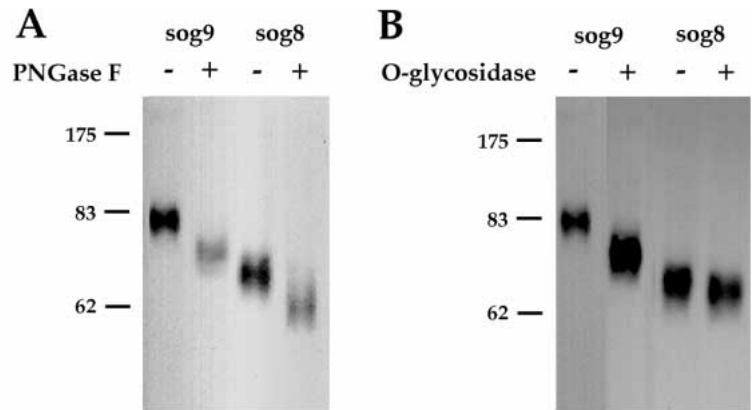
The cell lines used in this study are well characterized by a variety of assays, including anion exchange chromatography of labeled GAGs, and they have remained stable for over 5 years of observation (Gruenheid et al., 1993; Banfield et al., 1995). Studies on these cell lines thus provide an advantage over enzymatic and inhibitor studies where it is often difficult to determine their precise effects. For example, treatment of these cells with chondroitin ABC lyase from different commercial sources had different effects (data not shown). Likewise, treatment of cells with the GAG inhibitor, *p*-nitrophenyl  $\beta$ -D-xylopyranoside, produced different results to those obtained with chondroitin ABC lyase, and varied with the concentration used (data not shown).

### Effect of chondroitin sulfate and sulfation on IRAWB 14-inducible HA binding of CD44

Sulfation of CD44 has previously been reported and has been attributed to the presence of sulfated proteins or carbohydrates on CD44H, and to the addition of CS on the higher  $M_r$  forms of CD44 (Jalkanen et al., 1988). In this study both CS-modified and CD44H forms of CD44 were sulfated. However, the



**Fig. 6.** Pulse-chase analysis of [<sup>35</sup>S]methionine- and [<sup>35</sup>S]cysteine-labeled CD44 from L and sog8 cells. CD44 immunoprecipitates from L cells or sog8 cells were treated with (+) or without (-) endoH after a 20 minute pulse of radiolabel (time 0), and after 120 minutes chase time in unlabeled medium. The positions of prestained  $M_r$  markers are indicated on the left ( $\times 10^{-3}$ ).



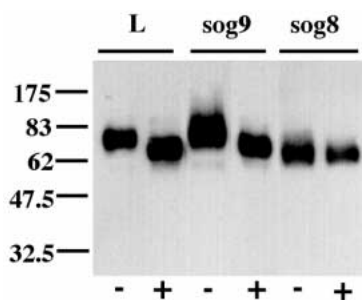
**Fig. 7.** Treatment of *sog9* and *sog8* cells with PNGase F and O-glycosidase. Anti-CD44 immunoprecipitates from cell lysates were prepared as described in Materials and methods. Following digestion with (+) or without (–) PNGase F (A) or O-glycosidase (B), samples were separated by SDS-PAGE under non-reducing conditions and immunoblotted with IM7.8.1. The positions of prestained  $M_r$  markers are indicated on the left ( $\times 10^{-3}$ ).

absence of the CS-modified CD44 form in *sog9* cells resulted in the loss of inducible HA binding. Exogenously added CSA or CSC did not restore inducible FL-HA binding in *sog9* cells, suggesting that CS had to be attached to a cell surface protein, possibly CD44, for it to affect IRAWB 14-induced HA binding (Fig. 4). The treatment of L cells, which contain CS-modified CD44, with sodium chlorate abolished IRAWB 14-inducible binding, whereas no inhibitory effect was observed in *sog8* cells where CS synthesis does not occur (Fig. 5). This is consistent with the possibility that sodium chlorate inhibits HA binding in L cells by preventing the sulfation of chondroitin.

The results in this study appear to conflict with those reported by Lesley et al. (1995), who found a negative regulatory role for both oligosaccharides and GAGs. Attempts to reconcile these differences by performing enzymatic and inhibitor studies generated data suggesting that these reagents have additional activities that lead to non-specific effects. It is possible that differences could arise from the following observations: the parental L cell clone used in each case was different and had different HA binding capabilities; the effect of CS can depend on the glycosylation state of the cell; and growing cells in different glucose concentrations can change their glycosylation state and HA binding ability (Zheng et al., 1997).

#### Characterization of the glycosylation defect present in *sog8* cells and its effect on HA binding

The core protein of CD44H is predicted to be  $37 \times 10^3 M_r$



**Fig. 8.** Treatment of L, *sog9* and *sog8* cells with neuraminidase. CD44 immunoprecipitated from the cell surface was prepared as described in Materials and methods. Following digestion with (+) or without (–) neuraminidase, samples were separated by SDS-PAGE under non-reducing conditions and immunoblotted with IM7.8.1. The position of prestained  $M_r$  markers are indicated on the left ( $\times 10^{-3}$ ).

(Goldstein et al., 1989; Nottenburg et al., 1989; Stamenkovic et al., 1989), yet the first synthesized CD44 protein detected after [ $^{35}$ S]methionine and [ $^{35}$ S]cysteine labeling had an apparent  $M_r$  of  $62 \times 10^3$  (Fig. 6). Pulse-chase analysis and treatment of CD44 immunoprecipitates with PNGase F and O-glycosidase indicated a defect in both N- and O-linked processing in *sog8* cells, but not a complete absence of glycosylation.

In *sog8* cells, the absence of the majority of O-linked oligosaccharides and of terminal sugar moieties on N-linked oligosaccharides enhanced the HA binding ability of CD44. Further analysis of *sog8* cells indicated the lack of terminal sialic acid residues (Fig. 8) and their relative resistance to ricin also suggested decreased expression of galactose residues. However, treatment of L and *sog9* cells with neuraminidase alone did not change their ability to bind FL-HA (data not shown), suggesting that sialic acids alone may be insufficient to impede HA binding or that enzymatic digestion did not go to completion. While an increase in binding of PNA lectin after neuraminidase treatment was observed (data not shown), we could not easily determine if all sialic acids had been removed. This result agrees with Lesley et al. (1995), who also found that treatment of L cells with neuraminidase did not affect the HA binding ability of CD44. However, it is interesting to note that neuraminidase treatment of soluble, purified CD44-Ig fusion protein did enhance its ability to bind HA (Katoh et al., 1995). It is possible that the factors that affect HA binding to purified CD44 may not be identical to the factors that affect HA binding to CD44 on a cell membrane.

#### Role of CS in the binding of HA to CD44

From these data we propose that the presence of CS has a positive effect on HA binding and that the presence of fully processed carbohydrates can have a negative effect. Data from the *sog8* cells indicate that CS is not essential for IRAWB 14-inducible HA binding, suggesting that one function of CS may be to overcome the negative effects of certain oligosaccharide moieties. One possible explanation is that the small percentage of CS-modified CD44 may act as a nucleus or scaffold upon which other CD44 molecules can bind to create aggregates, which then allow inducible HA binding. Indeed, CD44 has been shown to bind other CS modified ligands: serglycin (Toyama-Sorimachi et al., 1995) and the invariant chain of MHC class II (Naujokas et al., 1993) and aggregation of CD44 can enhance HA binding (Lesley et al., 1992, 1993b; Perschl

et al., 1995; Sleeman et al., 1996). Alternatively, perhaps only CS modified CD44 can bind HA in L cells, whereas the underglycosylated,  $70 \times 10^3 M_r$  form of CD44 binds HA in sog8 cells. Regardless of the precise mechanism of this regulation, the ability of CD44 to bind HA is clearly affected by CS addition. The presence of highly charged, rigid CS chains can provide an environment which allows CD44 to inducibly bind to HA. When CS is removed, the ionic milieu and the cell surface architecture are changed such that CD44 can no longer be induced to bind HA by the mAb, IRAWB 14. However, in the absence of both cell surface proteoglycans and certain oligosaccharide moieties (as occurs in sog8 cells), the binding of HA to CD44 becomes possible.

Inhibitors of glycosylation have previously been reported to affect the HA binding ability of CD44 (Hathcock et al., 1993; Bennett et al., 1995; Katoh et al., 1995; Lesley et al., 1995; Bartolazzi et al., 1996; Dasgupta et al., 1996). This study demonstrates that the presence of CS can also affect the HA-binding ability of CD44 in L cells and that this effect depends on the glycosylation state of the cell. Thus, the overall HA-binding state of CD44 in L cells appears to be determined by the combined effects of CS synthesis and glycoprotein processing. It is known that cells can vary oligosaccharide and GAG addition depending on their activation state, and changes in the glycosylation state of CD44 have been reported to occur in activated macrophages and B cells (Camp et al., 1991; Hathcock et al., 1993). It will thus be of interest to determine if changes in GAG and oligosaccharide addition occur under physiological conditions to regulate the HA-binding ability of CD44. Changes in these post-translational modifications would have to occur on newly synthesized CD44 molecules. Interestingly, CD44 levels are upregulated after PMA stimulation of T cells and this treatment results in the induction of HA binding (Lesley et al., 1990; Hyman et al., 1991; Liao et al., 1993).

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