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A Novel Ligand for CD44 Is Serglycin, a Hematopoietic Cell Lineage-specific Proteoglycan

POSSIBLE INVOLVEMENT IN LYMPHOID CELL ADHERENCE AND ACTIVATION*

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The lymphocyte adhesion molecule CD44 recognizes a non-hyaluronate proteoglycan, gp600, secreted by mouse T cell line CTLL2. We now demonstrate that gp600 is identical to serglycin, a member of the small proteoglycan family stored in intracellular secretory granules of lymphoid, myeloid, and some tumor cells. Purified gp600 has the ability to bind specifically to CD44, and the binding is dependent on activation of CD44. The CD44-binding elements on gp600 or serglycin are glycosaminoglycans consisting of chondroitin 4-sulfate. Serglycin is readily exocytosed, and its interaction with active form CD44 augments the CD3-dependent degranulation of CD44 positive CTL clones. We conclude that the serglycin secreted from secretory granules of hematopoietic cells is a novel ligand for CD44, and could regulate lymphoid cell adherence and activation.

CD44, which exhibits significant sequence homology to the phylogenically conserved amino-terminal domain of cartilage link proteins, is an important cell surface adhesion molecule expressed on lymphoid cells, myeloid cells, fibroblasts, epithelial cells, and endothelial cells (Jalkanen *et al.*, 1986; Stamenkovic *et al.*, 1989; Goldstein *et al.*, 1989; for reviews, see Haynes *et al.* (1989) and Lesley *et al.* (1993a)). Recent studies reveal that this molecule has many isoforms with various inserts in the membrane proximal portion (for review, see Herrlich *et al.* (1993)). Ligands for CD44 have been shown to be extracellular matrix components such as hyaluronic acid (Aruffo *et al.*, 1990), fibronectin (Jalkanen and Jalkanen, 1992), and collagen types I and VI (Wayner *et al.*, 1987; Carter and Wayner, 1988). In addition, the chondroitin sulfate-modified invariant chain has recently been suggested to be a ligand for CD44 (Naujokas *et al.*, 1993). The CD44 molecule is thought to participate in various adhesive events including lymphocyte recirculation (Jalkanen *et al.*, 1987), lymphohemopoiesis (Miyake *et al.*, 1990), and tumor cell invasiveness (Gunthert *et al.*, 1991). Monoclonal antibodies directed against the CD44 molecule enhance the proliferation of T cells (Shimizu *et al.*, 1989; Denning *et al.*, 1990). In addition, some anti-CD44 monoclonal antibodies can trigger effector functions of murine and human T cell clones (Seth *et al.*, 1991; Galandrini *et al.*, 1993). Therefore, one of the important functions of CD44 in the immune system seems to be the activation of lymphocytes, although the natural ligand interacting with CD44 remains to be determined.

An anti-CD44 monoclonal antibody, Hermes-3, that does not interfere with hyaluronate binding (Culty *et al.*, 1990), inhibits the binding of human lymphocytes to high endothelial venules on frozen lymph node sections, indicating the involvement of CD44 in lymphocyte homing (Jalkanen *et al.*, 1987). It was also reported that the binding of murine lymphocytes to high endothelial venules is resistant to hyaluronidase treatment (Culty *et al.*, 1990), indicating that hyaluronate is not a ligand for CD44 in lymphocyte-high endothelial venule interaction. In the search for a novel ligand for CD44, we identified a sulfated macromolecule, gp600, in the culture supernatant of a murine T cell line, and reported that gp600 is a proteoglycan consisting of a small core protein (18–22 kDa) and chondroitin sulfate-like glycosaminoglycans (Toyama-Sorimachi and Miyasaka, 1994).

Proteoglycans found ubiquitously in tissues are composed of a protein core and glycosaminoglycan side chains (for reviews, see Ruoslahti (1989) and Kolset and Gallagher (1990)). In hematopoietic cells, a member of the proteoglycan family termed serglycin is found in the secretory granules (for review, see Stevens *et al.* (1988)). Serglycins can be classified into 2 groups on the basis of the nature of the glycosaminoglycans, heparan sulfate serglycins and chondroitin sulfate serglycins. Various types of serglycin have been characterized, *e.g.* a heparin sulfate type in serosal mast cells, a chondroitin 4,6-sulfate type in mouse bone marrow-derived mast cells, a chondroitin 4-sulfate type in natural killer cells, eosinophils, and HL-60 leukemic promyelocytes, and a chondroitin 6-sulfate type in megakaryocytes and platelets (Stevens *et al.*, 1988a; Kolset and Gallagher, 1990). Serglycin is distinct from all other cell surface- and matrix-localized proteoglycans both in its high degree of sulfation and its resistance to proteolysis. Although the molecular masses of these proteoglycans are heterogenous (60–750 kDa) due to differences in their glycosaminoglycan side chains, the gene responsible for the peptide core, which is composed primarily of tandem serine-glycine repeats, is a single gene (Tantravahi *et al.*, 1986). The serglycin peptide core is estimated to be M_r 16,000–18,000 (Bourdon *et al.*, 1985; Stevens *et al.*, 1988b; Avraham *et al.*, 1989), similar to that of gp600 (Toyama-Sorimachi and Miyasaka, 1994). The expression of serglycin seems to be restricted to the yolk sac, hematopoietic cells, and some tumor cells. Serglycin has been suggested to participate in the packaging of basically charged serine pro-

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teases in secretory granules and the regulation of their enzymatic activity (Stevens *et al.*, 1988a). It has also been postulated that serglycin plays a role in cell-mediated cytotoxicity, since these proteoglycans are exocytosed when an effector cell kills tumor target cells (MacDermott *et al.*, 1985). However, the functions of serglycin have not been fully defined.

In the present study, since the various characteristics of gp600 so far identified remarkably resemble those of chondroitin sulfate serglycin, we isolated and biochemically characterized gp600 in detail to determine whether gp600 is indeed serglycin. We show that the amino acid sequence of the core protein of purified gp600 is identical to that of serglycin and that chondroitin 4-sulfate, a major glycosaminoglycan of gp600, is essential for CD44 binding. Furthermore, we indicate that CD44-serglycin interaction is involved in lymphoid cells adhesiveness and activation. This study provides further understanding not only of the physiological functions of CD44 but also those of serglycin.

EXPERIMENTAL PROCEDURES

Antibodies and Cells—KM201 is directed against mouse CD44 and inhibits CD44 binding to hyaluronate (Miyake *et al.*, 1990). Anti-CD44 monoclonal antibody IRAWB14 (Lesley *et al.* 1992), which induces hyaluronate binding to CD44, was kindly provided by Dr. J. Lesley (Department of Cancer Biology, The Salk Institute). Human IgG was purchased from Cappel. $F(ab')_2$ fraction of biotin-conjugated goat anti-human IgG was purchased from Zymed Laboratories Inc.

CTLL2, CTLL2 transfectants (Toyama-Sorimachi and Miyasaka, 1994) of mouse CD44, and mouse thymoma cell line BW5147 were grown in RPMI 1640 supplemented with 10% fetal calf serum (Iansa), 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 10^{-4} M 2-mercaptoethanol, 1% (v/v) 100 × nonessential amino acids (Flow Laboratories), 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). For the culture of CTLL2 and its transfectants, 1 nM recombinant mouse interleukin-2 was added to the complete medium (Karasuyama *et al.*, 1989). In the case of large scale culture of CTLL2, serum-free medium EX-cell 300™ (JRH Bioscience) was used. The culture of bone marrow-derived mast cells was performed as described previously (Razin *et al.*, 1984).

Purification of Gp600—Gp600 was monitored by the presence of uronic acid, and also by its ability to bind to a soluble fusion protein of CD44 and IgG (CD44-IgG) (Aruffo *et al.*, 1990). Conditioned medium from CTLL2 cells was centrifuged for 20 min at 5000 × *g*, and supernatant was recovered. The sample was concentrated in a Millipore concentrator (Minitan™ system, M_r 30,000 cut-off). The initial volume of 16 liters was concentrated to 450 ml, diluted with a buffer containing 4 M urea, 20 mM Tris (pH 8.0), and 0.2 M NaCl (TSKG-DEAE equilibration buffer), and concentrated again to 450 ml. The concentrated sample was loaded onto a TSKG-DEAE (Toso) at a flow rate of 1 ml/min, and eluted with a 60-ml linear NaCl gradient (0.2–1 M) in the same buffer. Gp600 was observed at approximately 0.5 M NaCl. Fractions containing gp600 were pooled, concentrated using a Centriprep-30 concentrator (Amicon), and dialyzed against distilled H_2O. The dialyzed sample was adjusted to 6 M guanidine, 20 mM Tris (pH 8.0), and 0.1% CHAPS,[1] and loaded at a flow rate of 0.5 ml/min onto a TSKG-3000 column (Toso) previously equilibrated with 6 M guanidine, 20 mM Tris (pH 8.0), and 0.1% CHAPS. In this step, gp600 was recovered in the void fraction. The fractions containing gp600 were pooled, concentrated, and dialyzed against distilled H_2O. The dialyzed sample was treated with DNase I to remove contaminating nucleic acid. The resulting sample was diluted with the same volume of buffer containing 6 M guanidine, 20 mM Tris (pH 8.0), and 0.1% CHAPS, and applied onto TSKG-3000 at a flow rate of 0.5 ml/min. Gp600 was recovered in the void fraction. Fractions containing gp600 were concentrated, dialyzed against 10 mM sodium phosphate buffer, and subjected to hydroxylapatite chromatography (HA-1000). By this hydroxylapatite column chromatography, the gp600 activities were clearly resolved into two fractions, the flow-through fraction and a fraction eluting at approximately 150 mM phosphate buffer. Since the first fraction was purer than the second, we focused on the flow-through fraction. The fraction was dialyzed against distilled H_2O, adjusted to 4 M urea, 20 mM Tris (pH 8.0), and 0.2 M NaCl, and loaded onto a TSKG-DEAE column. Gp600 was eluted with a linear gradient of NaCl (0.2–1 M) in the same buffer. The final preparation was dialyzed against distilled H_2O and lyophilized. The purity of the preparation was assessed by electrophoresis after radiolabeling the sample with $Na^{125}I$. Uronic acid was determined according to the method of Bitter and Muir (1962).

Preparation of CD44-IgG Fusion Protein—The CD44-IgG expression plasmid (Aruffo *et al.*, 1990) was kindly provided by Dr. B. Seed (Department of Molecular Biology, Massachusetts General Hospital). CD44-IgG fusion protein was generated as described previously (Aruffo *et al.*, 1990). Purification was performed using Protein G-Sepharose column chromatography.

ELISA—Purified gp600 and defined glycosaminoglycans were coated onto 96-well microtiter plates overnight at 4 °C. Nonspecific sites were blocked with 1% BSA in PBS at room temperature for 2 h. CD44-IgG fusion protein or control human IgG (20 μg/ml final concentration) was added and the plates were incubated for 1 h at 4 °C. The plates were washed and incubated with biotinylated goat anti-human IgG for 30 min at room temperature, followed by incubation with avidin-peroxidase for 30 min. The plates were developed after the addition of the *o*-phenylendiamine substrate and read with a microplate reader. A background value defied as the signal in the absence of CD44-IgG was subtracted from the experimental values to yield the specific signal.

Cell Adhesion Assay—The cell adhesion assay was performed using BCECF-labeled cells as described previously (Toyama-Sorimachi *et al.*, 1993). Gp600 (100 μg/ml) was coated onto 96-well microtiter plates (Sumitomo H-plate). After incubation overnight at 4 °C, the wells were filled with PBS containing 1% BSA and incubated for 2 h at room temperature to block nonspecific protein absorption. Cell lines were washed and resuspended in serum-free RPMI containing 5 μM BCECF-AM. After incubation for 45 min at 37 °C, the cells were washed with RPMI containing 10% fetal calf serum, and then resuspended in Ca^{2+} and Mg^{2+}-free PBS. BCECF-labeled cells (2×10^5 cells/well) were then added and the plates were incubated in the presence or absence of anti-CD44 antibody or control antibodies. Each experiment was run in duplicate or triplicate. Nonadherent cells were removed by inverting the plate. Adherent lymphocytes were solubilized with 0.1% Nonidet P-40 in PBS, and the fluorescence intensity of each well was measured with a Fluoroscan II (Flow Laboratories). The background level, defined as the binding to BSA, was subtracted from all values to yield the specific signal.

Enzyme Treatment—Enzymes used for proteoglycan digestion were as follows; chondroitinase ABC (from *Proteus vulgaris*), chondroitinase ACII (from *Arthrobacter aurescens*), hyaluronidase (from *Streptomyces hyalurolytics*), heparinase (from *Flavobacterium heparinum*), heparitinase (from *Flavobacterium heparinum*), and α-L-fucosidase (from *Charonia lampus*). All these enzymes were purchased from Seikagaku Kogyo Co. Treatment of immobilized gp600 with chondroitinase ABC or hyaluronidase was carried out as described previously (Toyama-Sorimachi and Miyasaka, 1994). Chondroitinase ABC and ACII were used at 20 μg/ml and 100 milliunits/ml, respectively, and hyaluronidase was used at 20 μg/ml (40 turbidity reducing units/ml). Heparinase, heparitinase, and fucosidase were used at 10 milliunits/ml.

Amino Acid Sequence Analysis—Amino acid sequence analysis of the purified gp600 core protein was performed with an Applied Biosystems model 475A gas-phase sequencer. The analysis was kindly performed by Dr. S. Tsubuki, Department of Molecular Biology, the Tokyo Metropolitan Institute of Medical Science.

Flow Cytometry Analysis—Flow cytometry analysis was performed as described previously (Toyama-Sorimachi and Miyasaka, 1994). In a competition assay (Table I), cells were preincubated with various concentrations of proteoglycans, and then incubated with 5 μg/ml fluorescein isothiocyanate-conjugated hyaluronic acid (Seikagaku Kogyo). Chondroitin sulfates E and K were kindly provided by Dr. N. Seno, Department of Molecular Biology, the Tokyo Metropolitan Institute of Medical Science. At least 10,000 cells/sample were analyzed on an EPICS-CS flow cytofluorometer (Coulter).

Disaccharide Analysis—After treatment of the prepared gp600 with chondroitinase ABC, unsaturated disaccharide analysis was carried out by high-performance liquid chromatography (HPLC) as reported previously (Sugawara *et al.*, 1989, 1992). HPLC analysis was performed with a Waters model 600E (Millipore Corp.) on an amino silica gel column (NH_2-125-N, Senshu Scientific, Tokyo; 4.6-mm inner diameter × 150 mm), with a programmed gradient elution from 16 to 500 mM NaH_2PO_4.

Immunofluorescence Staining of Secretory Granules—Cells were

[1] The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; CTL, cytotoxic T cell; BCECF, 2′,7′-bis(2-carboxyethyl)-5(and -6)-carboxyfluorescein.

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TABLE I
Isolation of gp600 from mouse CTLL2 conditioned medium

Purification of gp600 from conditioned medium (16 liters) of mouse cytotoxic T cell line CTLL2 was performed as described under "Experimental Procedures."

Step	Protein	Proteoglycan	Total unit[a]	Specific activity[b]	-Fold
	mg		*units*	*units/mg protein*	
Conditioned medium (CM)	4,610.00				
Concentrated CM	604.00	36.00	15,600	26	1
TSKG-DEAE	15.30	7.98	22,900	1,500	58
TSKG-3000	13.50	7.73	60,000	4,440	172
DNase, TSKG-3000	2.70	4.59	35,700	13,200	511
HA-1000	0.96	3.45	52,900	55,100	2,130
TSKG-DEAE	0.09	1.21	22,100	246,000	9,520

[a] One unit of activity was defined as the amount of hyaluronate that represented 50% of maximal binding of CD44-IgG in ELISA.
[b] Specific activity was calculated as follows: specific activity (units/mg protein) = total unit (units)/total protein (mg).

mounted onto slide glass by cytospin and air dried. After fixation with 3% formalin, cells were incubated with 10 μg/ml purified CD44-IgG at room temperature for 60 min. After washing with PBS containing 0.1% of BSA, the samples were incubated with affinity purified biotin-conjugated anti-human IgG at 1:200 dilution. After washing, the samples were incubated with fluorescein isothiocyanate-labeled avidin at 1:1000 dilution. Samples were examined by fluorescence microscopy.

Granzyme Release Assay—Mouse CTL clones 557 and 560 were kindly provided by Dr. S. Aizawa (Department of Physiology and Pathology, National Institute of Radiological Science). Release of granzyme A from CTL cytoplasmic granules was induced by plastic immobilized anti-CD3. ELISA plates were first coated overnight at 37 °C with 50 μl of various concentrations of anti-CD3 (2C11) in PBS. Wells were washed with PBS, and triplicate aliquots of 2×10^5 cytolytic clonal T cells were dispensed into the wells in 100 μl of RPMI 1640 containing 10 μg/ml IRAWB14 and 1% BSA in the presence or absence of 50 μg/ml purified gp600. After centrifugation at 1000 × *g* for 1 min, the plates were incubated for 3 h at 37 °C. Control cells were incubated in the absence of anti-CD3. Granzyme A activity was tested by adding 180 μl of the substrate (0.2 M Tris-HCl, pH 8.0, 2×10^{-4} M N^{α}-CBZ-L-lysine thiobenzyl ester (BLT), 2.2×10^{-4} M dithiobis(nitrobenzoic acid)) to 20 μl of cell supernatant or to cell lysates solubilized with 0.1% Nonidet P-40 (Pasternack and Eisen, 1985). The absorbance at 405 nm was determined using an ELISA reader after a 1-h incubation at room temperature. The amount of granzyme secreted over the spontaneous release was plotted as the percent of the total enzyme content of the effector cells.

RESULTS

Purification of Gp600—As a ligand for CD44, we recently identified a chondroitinase-sensitive proteoglycan, gp600, found in the culture supernatant of the mouse CTL line, CTLL2 (Toyama-Sorimachi and Miyasaka, 1994). To further characterize gp600, we purified it from the conditioned medium of CTLL2. In the final purification step, gp600 eluted as a single peak from a TSKG-DEAE column (Fig. 1). Nucleic acid represented less than 1% of the material in this peak. Overall, 1.21 mg of the proteoglycan was obtained from 16 liters of CTLL2 conditioned medium with 3.4% recovery. The activity to bind to CD44 was concentrated by 9,500-fold (Table I).

Specific Binding of CD44 to Gp600—We first investigated the binding ability of purified gp600 to CD44 by ELISA. This was performed by examining the binding of soluble CD44-IgG to gp600 immobilized on a plastic plate. CD44-IgG bound to gp600 in a dose-dependent manner, while no significant binding was observed with control human-IgG (Fig. 2).

We then examined CD44 binding to gp600 by a cell binding assay. CD44 positive or negative cell lines were labeled with fluorescent dye, and the adherence of cells to immobilized gp600 was assessed by measuring fluorescence intensity. CD44 positive BW5147 cells adhered to both gp600 and hyaluronate in the absence of Ca^{2+} and Mg^{2+} cations (Fig. 3). In contrast, CD44 negative CTLL2 cells failed to adhere to either gp600 or hyaluronate, but transfection of CTLL2 cells with CD44 cDNA (Toyama-Sorimachi and Miyasaka, 1994) resulted in a marked

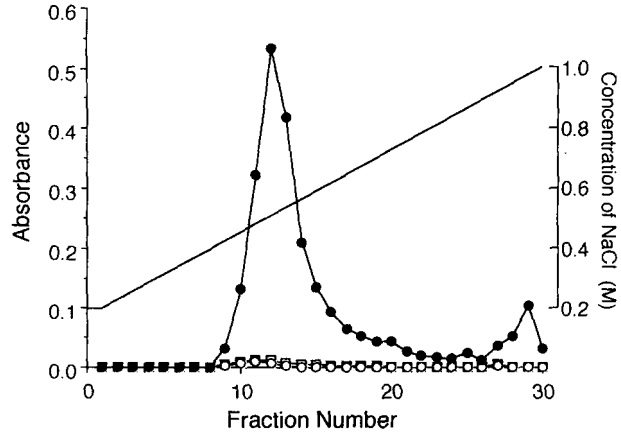

FIG. 1. **DEAE-ion exchange chromatography profile of gp600 obtained by hydroxylapatite chromatography.** The gp600 fraction from hydroxylapatite chromatography was applied to a TSKG-DEAE column. After washing, the column was eluted at 20 °C with a linear gradient of NaCl as indicated. Fractions were analyzed for uronic acid; A_{530} (●), A_{280} (○), and A_{260} (□).

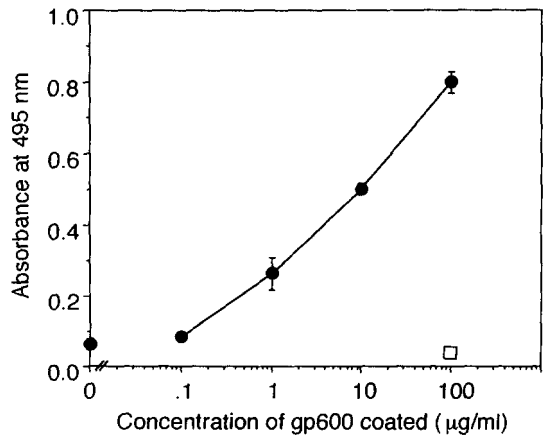

FIG. 2. **Binding of soluble CD44 to purified gp600.** Increasing amounts of purified gp600 were immobilized on an ELISA plate, and the binding of CD44-IgG (●) and control human IgG (□) to gp600 was assessed as described under "Experimental Procedures."

increase in adhesion. The binding of CD44 positive cells to both gp600 and hyaluronate was completely inhibited by an anti-CD44 monoclonal antibody KM201 (Fig. 3) but not by control rat IgG or isotype-matched monoclonal antibodies (data not shown), indicating that cell adhesion to gp600 is mediated by CD44. Hyaluronidase had no effect on the binding of CD44 positive cell lines to gp600, although the binding to hyaluronate was completely eliminated by enzyme treatment (Fig. 3, *right column*), clearly indicating that hyaluronate is not in-

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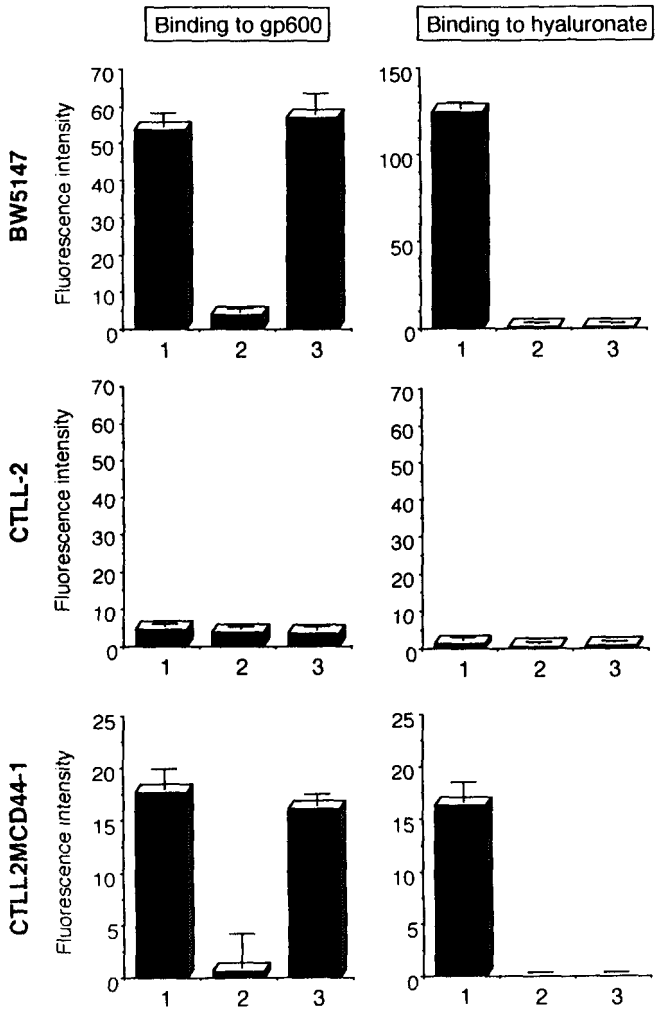

FIG. 3. **CD44-dependent cell adhesion to immobilized gp600.** CD44 positive (BW5147 and CTLL2MCD44–1) and negative (CTLL2) cell lines were labeled with fluorescent dye, and their binding to immobilized gp600 (*left column*) or hyaluronate (*right column*) was examined. *Lane 1*, control binding to immobilized materials in the absence of anti-CD44; *lane 2, lane 1* plus 50 μg/ml anti-CD44 (KM201); *lane 3*, hyaluronidase treatment of immobilized materials.

volved in the interaction between CD44 and gp600.

Gp600 Is Identical to Serglycin—To further characterize gp600, we prepared the core protein by treating gp600 with chondroitinase ABC (Fig. 4*A*; and Toyama-Sorimachi and Miyasaka (1994)), and determined its NH_2-terminal amino acid sequence. Ten amino acid residues were identified, identical to those found in the published sequence of mouse serglycin (Fig. 4*B*) (Avraham *et al.*, 1989). Furthermore, a remarkable sequence similarity to rat (Bourdon *et al.*, 1985) and human (Stevens *et al.*, 1988b) serglycin was observed. No other significant sequence homology was found in the homology search (EMBL protein data base, release 27.0). The determined sequence contained a portion of the tandem serine-glycine repeats highly conserved among human, mouse, and rat serglycin. The fact that the assumed serine residues were not detected by amino acid sequencing may be due to extensive glycosylation of the serine residues and/or a relatively lower recovery of serine derivatives. A minor sequence, YDDYG, found in mouse serglycin, was also identified. Corroborating these findings, the molecular size of the core protein of gp600 is 18–22 kDa, quite similar to that of serglycin (Toyama-Sorimachi and Miyasaka, 1994). Polymerase chain reaction analysis confirmed transcription of the serglycin gene in CTLL2 cells as well as its CD44 transfectants (data not shown). These results indicate that the core protein of gp600 is identical to that of serglycin.

Serglycin is known to localize in the secretory granules of granular leukocytes. Therefore, we examined immunohistologically whether soluble CD44 binds to intracellular secretory granules in CTLL2. It was revealed that CD44-IgG stained the cytoplasmic granules of CTLL2 distinctly while no significant fluorescence was observed with control human IgG (Fig. 5). A similar observation was obtained with interleukin-3-dependent mouse bone marrow-derived mast cells (Fig. 5), which produce chondroitin sulfate-type serglycin in their secretory granules (Razin *et al.*, 1982; Stevens *et al.*, 1985). These results support the notion that CD44 binds to serglycin.

Chondroitin Sulfate Glycosaminoglycans on Gp600 Are Necessary for CD44 Binding—Previous studies suggest that CD44 recognizes chondroitin sulfate (Aruffo *et al.*, 1990; Sy *et al.*, 1991; Naujokas *et al.*, 1993). To determine whether the chondroitinase-sensitive sugar chain on gp600 is necessary for CD44 binding, binding of CD44-IgG to gp600 was assayed by ELISA before and after the treatment of gp600 with various mucopolysaccharide degrading enzymes. The CD44-IgG reactivity was lost following digestion of gp600 with chondroitinase ABC or ACII but not with heparinase, heparitinase, fucosidase, or hyaluronidase (Fig. 6*A*). The effect of chondroitinase ABC was abolished in the presence of dermatan sulfate and Zn^{2+} which together inhibit specifically the enzymatic activity of chondroitinase ABC. These results indicate that the chondroitin sulfate sugar chains on gp600, which can be digested by chondroitinase ABC or ACII, are essential for recognition by CD44.

To investigate the kind of chondroitin sulfate involved in CD44 recognition, disaccharide analysis was performed. Gp600 was completely digested with chondroitinase ABC and the disaccharides obtained were subjected to HPLC analysis. The digest eluted at the position of 4-sulfated disaccharides, and neither non-sulfated nor disulfated disaccharides were detected (Fig. 7). The mass spectrum of the digest also supported this observation (data not shown). These results indicate that gp600 is a chondroitin 4-sulfate type serglycin, and that chondroitin 4-sulfate chains on gp600 are essential for CD44 binding.

We next tested the reactivity of CD44-IgG with various chondroitin sulfates by ELISA. Although the binding of CD44-IgG to gp600/serglycin and hyaluronic acid was readily detected, no significant binding to either chondroitin 4-sulfate or chondroitin 6-sulfate was observed (Fig. 6*B*). Similarly, these chondroitin sulfate preparations did not interfere with the binding of fluoresceinated hyaluronate to CD44-positive BW5147 cells, although gp600 strongly inhibited hyaluronate binding to CD44-positive cells as assessed by flow cytometry. Dose-dependent blockage of hyaluronate binding was observed with gp600, and almost complete blockage was obtained at 500 μg/ml (Table II). These results suggest that the gp600 binding domain on CD44 overlaps with or is close to the hyaluronate binding portion. Chondroitin sulfates A and E were slightly effective at 500 μg/ml, but other chondroitin sulfates were inactive. The finding that none of the defined chondroitin 4-sulfates tested so far was recognized by CD44 suggests that the association of chondroitin sulfates with the core protein is important for CD44 binding.

gp600 Binds to Active Form of CD44 and Induces CTL Activation—To evaluate the functional importance of CD44-serglycin interaction, we used two different experimental approaches. First, we examined whether normal lymphocytes bind to gp600/serglycin. CD44 expressed on peripheral lympho-

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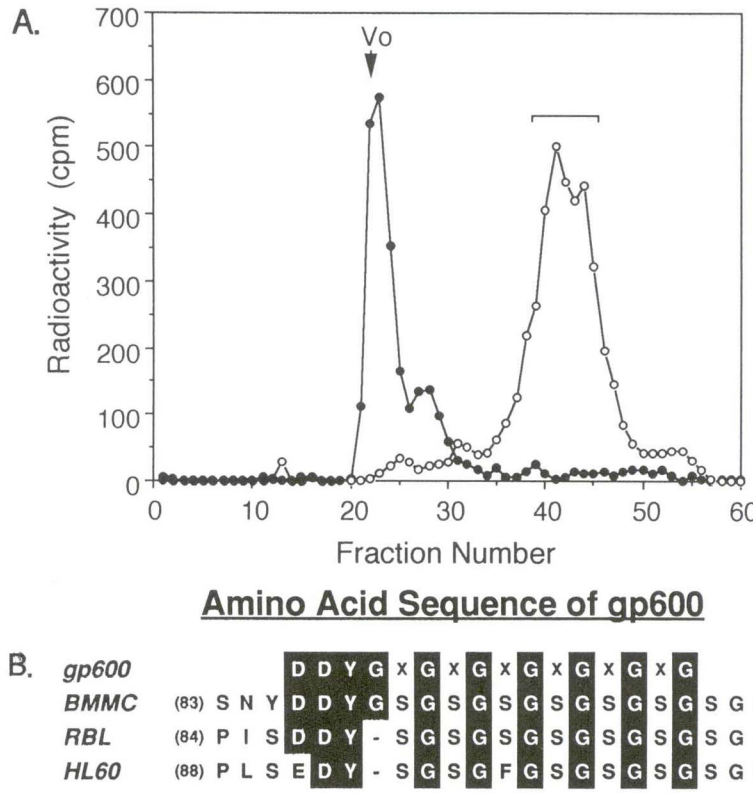

FIG. 4. **Identification of gp600 core protein as serglycin.** *A*, preparation of the gp600 core protein by degradation of the glycosaminoglycans with chondroitinase ABC. The core protein of gp600 was radiolabeled with $Na^{125}I$ by the lactoperoxidase method. A small amount of radiolabeled gp600 (10^4 cpm) was mixed with 1 mg of purified, unlabeled gp600 for detection of the core protein. After overnight treatment with chondroitinase ABC, the core protein was precipitated by adding acetone/ethanol. Gp600 (●) or gp600 treated with chondroitinase ABC (○) was applied to a gel filtration column. Fractions (No. 39–46) from gel filtration chromatography were pooled for NH_2-terminal amino acid sequence analysis. *B*, comparison of the amino acid sequences (single letter code) of the gp600 core protein with serglycin peptide cores expressed in mouse bone marrow-derived mast cells (*BMMC*), rat basophilic leukemia-1 (*RBL*) cells, and human HL-60 cells. *Numbers* indicate the residue numbers in the respective sequences.

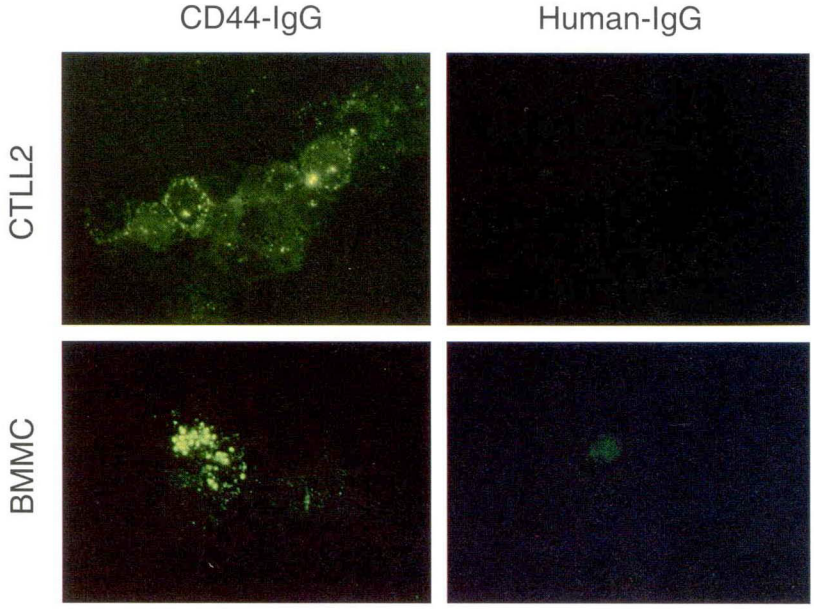

FIG. 5. **Immunofluorescence staining of intracellular granules of CTLL2 and mouse bone marrow-derived mast cells with CD44-IgG.** Cytospin samples of cells fixed with 3% formalin were incubated with 10 μg/ml CD44-IgG or control human IgG.

cytes is normally inactive and unable to recognize hyaluronate (Hyman *et al.*, 1991), unless the lymphocytes are activated by appropriate stimuli such as phorbol ester (Hyman *et al.*, 1991), interleukin-5 (Murakami *et al.*, 1990), or agonistic anti-CD44 monoclonal antibody IRAWB14 (Lesley *et al.*, 1992). As seen with binding to hyaluronate, peripheral lymphocytes did not bind to immobilized gp600 (Fig. 8*A*, *control*), unless treated with a CD44-stimulating antibody IRAWB14 (Fig. 8*A*), suggesting that the binding ability of CD44 to gp600 is regulated in a similar way as the binding to hyaluronate. The binding was observed in the absence of Ca^{2+} and Mg^{2+} cations and completely inhibited in the presence of anti-CD44 monoclonal antibody, KM201. These results suggest that gp600 binds to the active form of CD44 expressed on lymphocytes.

Second, the effect of gp600 on cytotoxic T cell (CTL) activation was examined, since the ligation of cell-surface CD44 has been shown to lead to T cell proliferation (Shimizu *et al.*, 1989; Denning *et al.*, 1990) and activation of CTL (Seth *et al.*, 1991; Galandrini *et al.*, 1993). Inasmuch as the CD44 expressed on CTL clones that we used was also inactive and did not bind to hyaluronate or gp600, we pretreated the clones with IRAWB14. After treatment, CD44 expressed on CTL clones could recognize hyaluronate and gp600 (data not shown). Under these conditions, anti-CD3 induced granzyme A release was examined in the presence or absence of gp600. As shown in Fig. 8, the addition of gp600 to CTL clones significantly enhanced anti-CD3 induced granzyme A release. Treatment with IRAWB14 alone had no effect on the CD3-dependent granzyme release (Fig. 8*B*, *closed square*). The enhancement of granzyme release was noticeable especially at suboptimal concentrations

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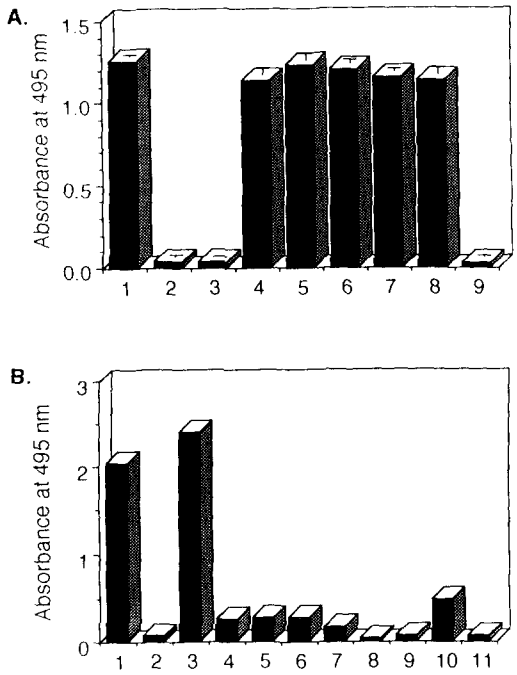

FIG. 6. **Quantitation of CD44-IgG binding to gp600 by ELISA after treatment with mucopolysaccharide degrading enzymes.** *A*, binding of CD44-IgG to immobilized gp600. Purified gp600 (100 μg/ml) was coated on plastic plates before (*lane 1*) or after treatment with chondroitinase ABC (*lane 2*), chondroitinase ACII (*lane 3*), chondroitinase ABC in the presence of 100 mM Zn^{2+} and 1 mg/ml dermatan sulfate (*lane 4*), hyaluronidase (*lane 5*), heparinase (*lane 6*), heparitinase (*lane 7*), or fucosidase (*lane 8*). In *lane 9*, BSA was used instead of gp600. CD44-IgG binding was examined as described under "Experimental Procedures." *B*, binding of CD44-IgG to various glycosaminoglycans. ELISA plates were coated with 1 mg/ml purified gp600 (*lane 1*), chondroitin (*lane 2*), hyaluronate (*lane 3*), chondroitin 4-sulfate (*lane 4*), chondroitin 6-sulfate (*lane 5*), dermatan sulfate (*lane 6*), heparan sulfate (*lane 7*), keratan sulfate (*lane 8*), keratan polysulfate (*lane 9*), heparin (*lane 10*), or BSA (*lane 11*) at 4 °C overnight.

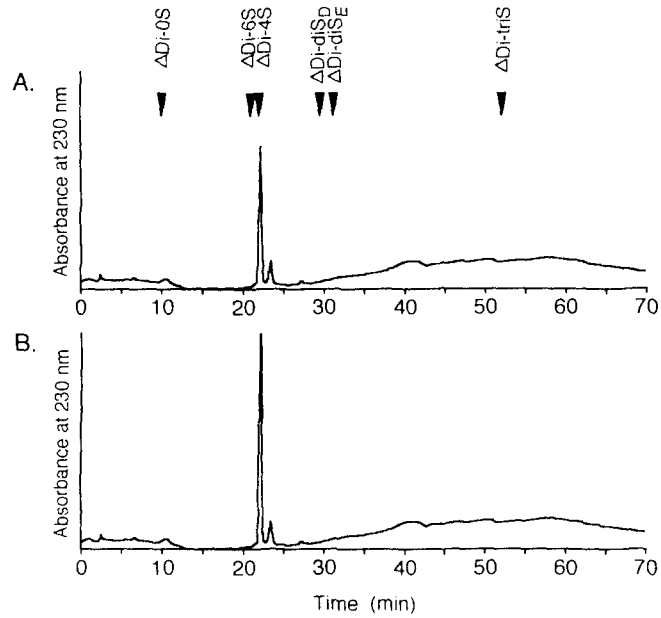

FIG. 7. **Disaccharide analysis of gp600 glycosaminoglycans by HPLC.** The oligosaccharide fraction prepared by chondroitinase ABC treatment of gp600 was chromatographed on an amino silica gel column. The elution positions of authentic unsaturated chondro-disaccharides are indicated. *A*, oligosaccharide obtained from gp600; *B*, *A* plus authentic ΔDi-4S. Disaccharide standard used are: ΔDi-0S, Δ4,5-GlcA(β1–3)GalNAc; ΔDi-6S, Δ4,5-GlcA(β1–3)GalNAc(6-*O*-sulfate); ΔDi-4S, Δ4,5-GlcA(β1–3)GalNAc(4-*O*-sulfate); ΔDi-diSD, Δ4,5-GlcA(2-*O*-sulfate)(β1–3)GalNAc(6-*O*-sulfate); ΔDi-diSE, Δ4,5-GlcA-(β1–3)GalNAc(4,6-*O*-sulfate); ΔDi-triS, Δ4,4-GlcA(2-*O*-sulfate)(β1–3) GalNAc(4,6-*O*-disulfate).

of anti-CD3 antibody, and was not observed in the absence of anti-CD3 (data not shown). In contrast to gp600/serglycin, hyaluronate did not enhance CD3-dependent granzyme release at any concentration examined (Fig. 8*B*). These results suggest that gp600/serglycin can activate CTL in a CD3-dependent manner, and could be important ligand for CD44.

DISCUSSION

In this report we show that a novel ligand for CD44, gp600, is a chondroitin 4-sulfate type serglycin stored in secretory granules of lymphoid and myeloid cells. Based on the following observations, we conclude that gp600, a novel ligand for CD44, is identical to serglycin. First, the NH_2-terminal amino acid sequence of the gp600 core protein coincides with that of the mouse serglycin core protein. Second, soluble CD44 recognizes intracytoplasmic secretory granules where serglycin is known to be present. Third, the molecular mass of gp600 treated with chondroitinase ABC or ACII is similar to that of serglycin (10–30 kDa) on SDS-PAGE (Stevens *et al.*, 1985; Toyama-Sorimachi and Miyasaka, 1994). Fourth, transcription of the serglycin gene was confirmed in CTLL2 cells by polymerase chain reaction analysis. In addition, polyclonal antibody raised against a synthetic serglycin core peptide recognized the gp600 core protein.[2] A specific interaction between CD44 and gp600 was verified by the following observations: 1) purified gp600 binds dose dependently to CD44-IgG but not to control human-IgG; 2) purified gp600 binds to CD44-positive cells but not to CD44-negative cells; 3) the binding is completely inhibited by anti-CD44 monoclonal antibody; 4) purified gp600 interferes with CD44 binding to hyaluronate.

The NH_2-terminal sequence of the gp600 core protein we evaluated corresponds to the sequence starting from the 84th residue of the predicted amino acid sequence of mouse serglycin (Avraham *et al.*, 1989). Similarly, the NH_2-terminal sequence of the rat yolk sac tumor serglycin corresponded to the sequence from the 75th residue of the predicted amino acid sequence of rat serglycin (Bourdon *et al.*, 1985). In the rat yolk sac tumor, the serglycin core protein is translated as a 19-kDa prepro-core protein, and subsequently processed to a 10-kDa core protein (Bourdon *et al.*, 1985). These results presumably indicate that the NH_2-terminal portion of mouse CTL serglycin is also removed during maturation similar to rat serglycin.

Glycosaminoglycans on gp600 synthesized by a mouse CTL line, CTLL2, were predominantly chondroitin 4-sulfate, consistent with the previous observation that glycosaminoglycans synthesized by T cells are mainly chondroitin 4-sulfate (Kolset and Gallagher, 1990). Degradation of the chondroitin sulfate moiety resulted in the loss of reactivity with CD44, suggesting that CD44 binds to chondroitin 4-sulfate on gp600/serglycin. It is unlikely that CD44 recognizes the serglycin core protein itself, since CD44 binding to gp600 was completely eliminated by chondroitinase treatment but resistant to protease (data not shown). It appears that CD44 also binds to chondroitin 4,6-sulfate on serglycin, since the soluble CD44 fusion protein binds to secretory granules of mouse bone marrow mast cells where chondroitin 4,6-sulfate type serglycin is known to accumulate (Razin *et al.* 1982; Stevens *et al.* 1985). However, binding of CD44 to purified chondroitin 4-sulfate or other chondroitin sulfates was not observed in the present study. In addition, the binding competition assay using fluoresceinated hyaluronate indicated that the affinity, if any, of purified chon-

[2] N. Toyama-Sorimachi, unpublished observation.

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TABLE II

Cell surface CD44 binds gp600 but not other chondroitin sulfates

Mouse thymoma cell line BW5147 (5×10^5 cells) was incubated at 4 °C for 30 min with anti-CD44 (KM201) or purified proteoglycans at various concentrations as indicated. After incubation, FITC-labeled hyaluronate (5 μg/ml) was added to the cell suspension, and fluorescence intensity was measured as described under "Experimental Procedures." Inhibition was calculated as follows: % inhibition = (mean fluorescence intensity (MFI) of each sample − MFI of control)/(MFI of cells stained with FITC-HA − MFI of control) × 100. The abbreviations used are: CS-A, chondroitin sulfate A; CS-B, chondroitin sulfate B; CS-C, chondroitin sulfate C; CS-D, chondroitin sulfate D; CS-E, chondroitin sulfate E; CS-K, chondroitin sulfate K.

	Concentration	Mean fluorescence intensity	% of Positive	% of Inhibition
	μg/ml			
Control		41.2	0.3	
FITC-HA	5	112.0	97.7	
KM201	50	39.1	0.2	100.0
Gp600	10	95.6	89.7	23.2
	100	59.7	4.6	73.9
	500	43.5	0.7	96.8
HA	10	79.2	50.8	43.6
	100	58.3	2.6	75.9
	500	47.8	0.7	90.7
CS-A	100	104.5	94.0	10.6
	500	103.7	95.1	11.7
CS-B	100	110.4	98.2	2.3
	500	112.1	98.5	0.0
CS-C	100	114.6	98.9	0.0
	500	108.9	97.5	4.5
CS-D	100	116.1	99.0	0.0
	500	113.2	98.7	0.0
CS-E	100	108.2	97.3	5.4
	500	103.9	96.5	11.5
CS-K	100	112.0	98.7	0.8
	500	111.3	98.5	1.1

droitin sulfates for CD44 is very low when compared to gp600 or hyaluronate. This observation is in agreement with reports by Miyake *et al.* (1990) and Murakami *et al.* (1990) but incompatible with three previous reports (Underhill *et al.*, 1983; Aruffo *et al.*, 1990; Sy *et al.*, 1991). Miyake *et al.* (1990) and Murakami *et al.* (1990) reported that CD44 binds to hyaluronate but not to chondroitin sulfates. In contrast, it has been reported that CD44 binds to conventional chondroitin sulfates (Underhill *et al.*, 1983; Aruffo *et al.*, 1990; Sy *et al.*, 1991). While it is uncertain whether these discrepancies are due to differences in the CD44 isoforms examined or the cell types or chondroitin sulfates used, we are currently inclined to think that CD44 may recognize a particular conformation of chondroitin 4-sulfate which might be formed by covalent bonding to the serglycin core protein. It may be that certain clusters of chondroitin sulfate chains on the gp600/serglycin core protein are important for high affinity binding of CD44. It is of note that CD44 has been reported to bind to chondroitin sulfate on the class II invariant chain (Naujokas *et al.* 1993). Although only a single chondroitin sulfate side chain associates with the core protein of class II invariant chain (Sant *et al.* 1985a, 1985b; Miller *et al.* 1988), it has been speculated that the lateral association of class II molecules may form clusters of chondroitin sulfate side chains (Brown *et al.* 1993).

Cell adhesion experiments suggest that the active form of CD44 can bind gp600/serglycin. The active form of CD44 is observed on the surface of cytotoxic splenic T cells and capable of binding ligands during an *in vivo* allogeneic response (Lesley *et al.*, 1994), while *in vitro* stimulated splenocytes is a rich source of gp600/serglycin and secrete it extracellularly.[3] The CD3-dependent granzyme A release by CTL clones of which CD44 had been activated by IRAWB14 antibody was demonstrated to be significantly augmented by the addition of serglycin. While it remains to be determined whether IRAWB14-activated CD44 and physiologically activated CD44 transduce the same intracellular signal, our results suggest that gp600/serglycin is involved in CTL activation, and that the binding of gp600/serglycin can induce a stimulatory signal in T cells. This is consistent with previous observations that anti-CD44 antibodies can induce cytolytic activity, granzyme release of CTL clones, and transduction of a co-stimulatory signal in T cells (Seth *et al.*, 1991; Galandrini *et al.*, 1993; Shimizu *et al.*, 1989). It is notable in this regard that only gp600/serglycin enhanced granzyme release and that hyaluronate had no effect. This may indicate that gp600/serglycin acts as a principal ligand for CD44 expressed on cytotoxic T cells.

The results obtained in antibody inhibition and flow cytometry analyses indicate that the gp600 binding domain is located close to the hyaluronate binding domain. However, the binding domains of these two ligands may be different. Alternatively, gp600/serglycin may have a higher affinity for CD44 than hyaluronate, although the binding affinities of gp600 and hyaluronate for CD44 have not been properly determined. Further study is required to understand how CD44 allows differential binding of diverse ligands in various adhesion events.

Due to its acidic nature, serglycin is implicated in packaging and stabilizing basically charged proteases, cytolytic proteins such as perforin, or cytokines within the secretory granules, and also in transporting them outside cells (Masson *et al.* 1990; Stevens *et al.* 1988a; Levitt and Olmstead 1986). Since serglycin can bind to cell surface CD44, it may prevent these molecules from random diffusion in the extracellular milieu when they are exocytosed, and help them to target and concentrate on the surface of CD44 positive cells, which would allow efficient delivery and presentation of effector molecules to target cells.

Since CD44 has been thought to play an important role in lymphocyte-high endothelial cell interaction (Jalkanen *et al.* 1987), it is of interest to investigate whether serglycin is also localized on the surface of endothelial cells. While immunohistological studies using antibody against serglycin have not yet been carried out, the observation that lymphocyte binding to the high endothelial venule is not affected by the anti-CD44 antibody KM201 (Culty *et al.* 1990), which blocks the binding of CD44 to serglycin, suggests that serglycin may not be directly involved in the recognition of the high endothelial venule by resting lymphocytes. However, in the case of inflamed tissues where the accumulation of serglycin-bearing leukocytes takes place, it is conceivable that serglycin secreted from cells would accumulate around endothelial cells or on the cell surface, thus allowing blood-borne CD44-positive cells to be recruited *in situ*. Detailed histopathological analysis will be required to resolve this issue.

Although CD44 is implicated in various types of immune responses, not only as an adhesion molecule but also as a signal transducer (Shimizu *et al.* 1989; Denning *et al.*, 1990; Lesley *et al.*, 1993a), the ligand(s) for CD44 that plays a major role in these responses remains to be elucidated. The ubiquitous expression of known ligands such as hyaluronate, collagen, and fibronectin is apparently incompatible with the cell-type specific function of CD44. In contrast, serglycin is a cell lineage-specific proteoglycan expressed in lymphoid and myeloid cells (Stevens *et al.* 1988a), and, as shown in our study, secreted extracellularly to bind to cell surface CD44. Such characteristics make serglycin an interesting candidate as a principal ligand for CD44 in immune responses, although the final conclusion awaits experimental verification.

[3] N. Toyama-Sorimachi, unpublished observation.

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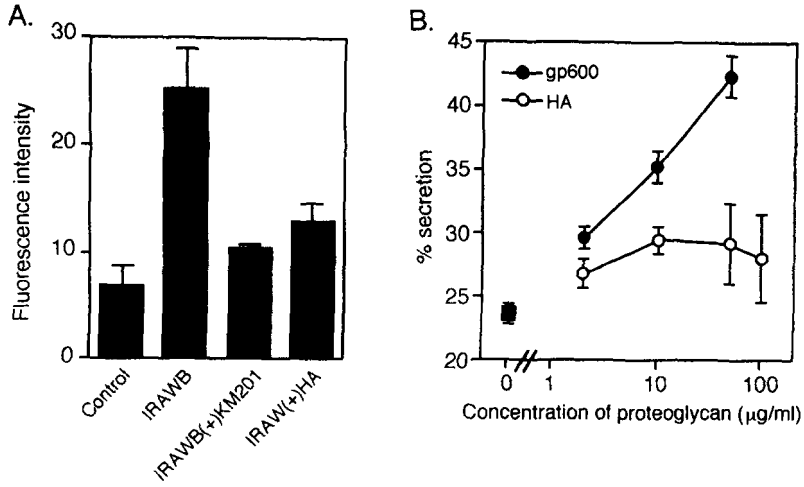

FIG. 8. **CD44-gp600 interaction is involved in lymphoid cell adherence and activation.** Cells were pretreated with IRAWB14 antibody at a concentration of 10 μg/ml to induce ligand binding activity of CD44. *A*, CD44-dependent binding of peripheral lymphocytes to gp600. This was examined in the presence or absence of blocking anti-CD44 antibody, KM201(20 μg/ml), or hyaluronate (1 mg/ml). *B*, granzyme A release of CTL clone. Anti-CD3 monoclonal antibody (2C11) was immobilized on a plastic plate at a concentration of 0.2 μg/ml, and the granzyme A release assay was performed using mouse CTL clone 5–57 in the presence of gp600 (●) or hyaluronate (○). Augmentation of granzyme release was not observed in the absence of gp600 (■) or IRAWB14 (□).

In conclusion, we demonstrate that chondroitin 4-sulfate type serglycin is a novel ligand for CD44. Although the results obtained in the present study indicate that CD44-gp600/serglycin interaction is involved in leukocyte adherence and CTL activation, different cells produce different CD44 isoforms and different types of serglycins, and the significance of the CD44-serglycin interaction may vary in different cell types. The *in vivo* significance of the CD44-serglycin interaction merits further investigation.

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