The Hyaluronate Receptor is a Member of the CD44 (H-CAM) Family of Cell Surface Glycoproteins

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Abstract. The present study was undertaken to determine the relationship between the hyaluronate receptor and CD44 (H-CAM), cell-surface glycoproteins of similar molecular weights that have been implicated in cell adhesion. In initial experiments, a panel of monoclonal antibodies directed against CD44 were tested for their ability to cross react with the hyaluronate receptor. These antibodies immunoprecipitated [^{3}H]hyaluronate binding activity from detergent extracts of both mouse and human cells, indicating that the hyaluronate receptor is identical to CD44. In addition, one of these antibodies (KM-201 to mouse CD44) directly blocked the binding of labeled hyaluronate to the receptor and inhibited hyaluronate dependent aggregation of SV-3T3 cells. CD44 has also been implicated in lymphocyte binding to high endothelial venules during lymphocyte homing. Interestingly, the monoclonal antibody Hermes-3, which blocks lymphocyte binding to the high endothelial venules of mucosal lymphoid tissue, had no effect on the binding of labeled hyaluronate. Furthermore, the binding of lymphocytes to high endothelial cells of lymph nodes and mucosal lymphoid tissue was not significantly affected by treatment with agents that block the binding of hyaluronate (hyaluronidase, excess hyaluronate and specific antibodies). Thus, CD44 appears to have at least two distinct functional domains, one for binding hyaluronate and another involved in interactions with mucosal high endothelial venules.

THE hyaluronate receptor is a cell surface glycoprotein of 85 kD that is responsible for the adhesion of cells to hyaluronate and chondroitin sulfate in the extracellular matrix (36). This receptor is present on a wide variety of cells including most macrophages, epithelial cells, and some neurons (1, 10, 36). In the case of epithelia, it is preferentially expressed on actively proliferating cells as compared to their nonproliferating counterparts (1). In addition the hyaluronate receptor appears to be associated with actin filaments (20), a characteristic that may allow it to mediate transmembrane signaling between the extracellular matrix and the cytoskeleton.

Another membrane glycoprotein involved in cell adhesion has been described that is similar to the hyaluronate receptor with respect to molecular weight, tissue distribution (6, 28) and association with actin filaments (15, 19). This glycoprotein (or set of closely related glycoproteins) has been studied by a number of different laboratories, and has been referred to variously as Pgp-1 (13), Ly-24 (22), ECMRIII (4), gp90^{Hermes} (3), and H-CAM (28). However, subsequent studies have revealed that these proteins are in fact closely related or identical to each other (7, 22, 27, 29, 32), and represent CD44, one of the cluster of differentiation antigens in leukocytes (12). The CD44 protein has been shown to mediate the interaction between lymphocytes and high endothelial cells during lymphocyte homing (16, 17). It has also been implicated in a number of other lymphocyte adhesion events, including interactions of T-cells with erythrocytes and monocytes (12). And finally, CD44 appears to play a role in the interaction between the hemopoietic and stromal cells of the bone marrow, which may involve hyaluronate (24, 25).

The connection between the hyaluronate receptor and CD44 was initially suggested by the studies Goldstein et al. (9) and Stamenkovic et al. (30). These researchers independently reported that the extracellular domain of CD44 is homologous to the hyaluronate binding region of the link protein of cartilage. This finding suggested that CD44 may be able to bind hyaluronate, and thus closely resembles the receptor.

The present study was undertaken to define more clearly the relationship between the hyaluronate receptor and the CD44 family of molecules. For this, we first examined the hyaluronate receptor to determine whether it resembles CD44 with respect to both isoelectric point and presence of phosphate linkages. Secondly, we tested a variety of mAbs against CD44 to determine whether they cross react with the hyaluronate receptor. The results of this study indicate that the hyaluronate receptor is indeed identical to CD44.

Table I. Characteristics of the mAbs Used in this Study

mAb	Antigenic specificity	Biological effect
K-3	Hamster hyaluronate receptor	Blocks binding of hyaluronate to receptor (35).
Mel-14	Mouse lymph node homing receptor (Mel-14)	Blocks adhesion of lymphocytes to high endothelial cells of peripheral lymph node (2).
KM-201	Mouse CD44 (Pgp-1)	Blocks adhesion between lymphocytes and stromal cells (23).
Hermes-1	Human CD44 (H-CAM)	None known (26).
H2-7	Human CD44 (H-CAM)	None known (26).
H2-280	Human CD44 (H-CAM)	None known (26).
Hermes-3	Human CD44 (H-CAM)	Blocks adhesion of lymphocytes to endothelial cells of mucosal lymphoid tissue (16).
BU52	Human CD44	None known.
J173	Human CD44 (Pgp-1)	None known (29).

Materials and Methods

Cell Lines and Culture Conditions

The SV-3T3 (Swiss mouse) and the BHK cell lines were grown and cultured as previously described (37). The HCV-29T cell line (invasive human bladder carcinoma), which expresses the hyaluronate receptor (26), was kindly donated by Dr. Warren Knudson (Rush Medical Center, Chicago, IL). The cells were cultured in 10% Nu-Serum (Collaborative Research, Lexington, MA), 90% DME containing 100 U/ml penicillin, and 100 μg/ml of streptomycin in an atmosphere of 5% CO_2, 95% air. For the hyaluronate binding assay, the cells were cultured for 1 d in serum-free medium before harvesting to remove the last traces of serum.

Antibodies

A list of the various mAbs used in this study is shown in Table I. K-3, a mouse mAb against the hyaluronate receptor from hamster cells was isolated from ascites fluid by chromatography on a protein A-Sepharose column (11). The KM-201 mAb (rat IgG) against mouse CD44 was isolated and purified by ion-exchange chromatography (24). Mouse mAbs against the human CD44 molecule include the Hermes series (Hermes-1, H2-7, H2-280, and Hermes-3) (16, 17, 28), the BU52 (purchased in a purified form from the Binding Site, San Diego, CA), and J173 which was kindly donated by Dr. John M. Pesando (the Biomembrane Institute, Seattle, WA). This last mAb was used directly in the ascites form. Control antibodies consisted of IgG purified from nonimmune mouse serum (11), and the rat mAb Mel-14 (American Type Culture Collection, Rockville, MD) which is directed against a homing receptor of 90 kD distinct from CD44 (3).

The mAbs were coupled to biotin using the method of Updyke and Nicolson (41) except that sulfosuccinmidyl 6-(biotinamido) hexanoate (Pierce Chemical Co., Rockford, IL) was substituted for the sulfosuccinimodobiotin.

Electrophoresis and Western Blotting

For labeling proteins with $[^{32}P]PO_4^{-3}$, confluent cultures of BHK cells were grown in serum-free medium for 1 d, and then incubated for 1 h in a modified Krebs Ringer's solution without phosphate to deplete the intracellular pools of ATP (8). The medium was then changed to one containing 20 μCi $[^{32}P]PO_4^{-3}$/ml and incubated for an additional hour. The cells were washed, extracted, and dissolved in a urea sample buffer containing a 1:3 mixture of 3–10 and 5–7 ampholytes, (Bio-Rad Laboratories, Richmond, CA). The sample was processed for two-dimensional electrophoresis, using a 10% SDS polyacrylamide gel under nonreducing conditions for the second dimension. The resulting gel was electrophoretically blotted onto nitrocellulose, which was blocked in 5% nonfat milk, and then immunostained for the hyaluronate receptor with biotinylated K-3 mAb as previously described (11). The blot was subsequently processed for autoradiography using Kodak X Omat film.

Immunoprecipitation

The procedure for immunoprecipitation was based upon that of Updyke and Nicolson (41). Cultured cells (SV-3T3 for mouse, HCV-29T for human) were extracted in 0.1% Na deoxycholate, 0.5 M NaCl, 0.02 M Tris, pH 8.0 (DOC buffer),[1] and then homogenized with a polytron (Brinkmann Instruments, Westbury, NY). Nonspecifically binding material was removed by incubating 2-ml aliquots of the extract for 30 min with 50 μl of a 50% slurry of streptavidin-agarose (Pierce Chemical Co., Rockford, IL), followed by centrifugation at 9,000 *g* for 10 s in a Microfuge B (Beckman Instruments, Inc., Palo Alto, CA). Next, the various biotinylated mAbs were added to aliquots of the supernatant. After incubating for a minimum of 2 h on a rotating table, streptavidin-agarose (0.2 ml/ml extract) was added to each sample and further incubated for 1 h. The samples were then centrifuged (9,000 *g*, 10 s), and the supernatants were collected for further testing.

To determine the amount of biotinylated antibody removed by this procedure, samples of the extracts both before and after the addition of the immobilized streptavidin were serially diluted and blotted onto nitrocellulose using a dot blot apparatus (Bio-Rad Laboratories). The blot was then incubated with streptavidin coupled to horseradish peroxidase and followed by a substrate consisting of H_2O_2 and 3-amino-9 ethyl carbazole (11). The relative amounts of the biotinylated protein could be estimated from the intensity of the staining. In each case, at least 80% of the biotinylated antibody was removed by the procedure described above.

Hyaluronate Binding Assay

The hyaluronate binding assay was a miniaturized version of that described previously (38). In some experiments, the cell extracts were preincubated with the various mAbs at 22°C for 30 min before the assay. Samples of the cell extracts in DOC buffer (0.25 ml final volume) were mixed with 1 μg [^{3}H]hyaluronate (Batch 1, 7.7 × 10^4 cpm/μg; Batch 2, 9.8 × 10^4 cpm/μg) at room temperature. After 30 min, an equal volume of saturated $(NH_4)_2SO_4$ was added to each sample followed by 25 μl of nonfat milk that acts as a carrier. The samples were then centrifuged (9,000 *g*, 5 min), and the tubes containing the precipitates were washed 2 times with 50% saturated $(NH_4)_2SO_4$. The precipitates were resuspended in water and processed for scintillation counting. The background level of binding was determined by including 100 μg of nonlabeled hyaluronate in the assay. The results are expressed in terms of specific binding in which the background levels of binding have been subtracted. Since the specific binding depends upon the particular preparation of [^{3}H]hyaluronate and cell extract, the level of binding will vary from experiment to experiment.

Partial Purification of the Hyaluronate Receptor

The hyaluronate receptor was partially purified by hydroxylapatite and molecular sieve chromatography as described previously (39). HCV-29T cells were extracted in 0.5% Na deoxycholate, 0.01 M $NaHPO_4$, pH 8.0, and applied to an 8.5 × 1.5-cm column of hydroxylapatite, which was eluted with a linear gradient of 0.1% Na deoxycholate, 0.01 M $NaHPO_4$ to 0.3 M $NaHPO_4$, pH 8.0. The fractions containing [^{3}H]hyaluronate binding activity were pooled and concentrated on a pressure filter cell (YM-10 filter;

1. *Abbreviations used in this paper*: CMF-PBS, calcium- and magnesium-free PBS; DOC buffer, 0.1% Na deoxycholate, 0.5 M NaCl, 0.02 M Tris, pH 8.0.

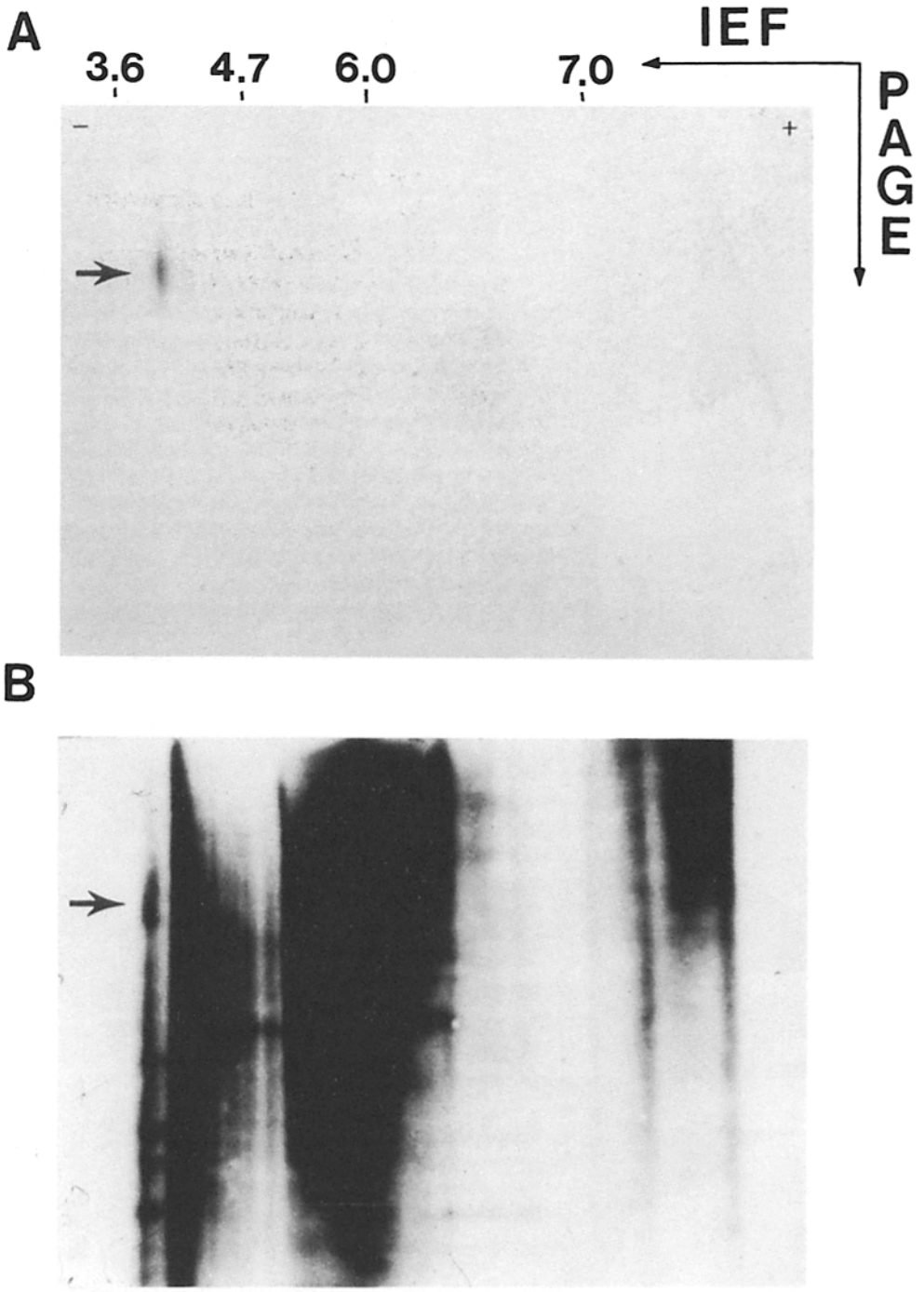

Figure 1. The isoelectric point and phosphorylation of the hyaluronate receptor. Confluent BHK cells were labeled with $[^{32}P]PO_4^{-3}$, processed by two-dimensional gel electrophoresis, and then transblotted onto a sheet of nitrocellulose. (*A*) The location of the hyaluronate receptor on the nitrocellulose (*arrow*) was determined by immunostaining with the K-3 mAb. The positions of various standards of different pI are indicated at the top of the figure. (*B*) The presence of ^{32}P was demonstrated by autoradiography of the nitrocellulose blot. The arrow indicates the presence of label in a location corresponding to the hyaluronate receptor. (Please note that the autoradiogram was deliberately overexposed to illustrate more clearly the protein of interest.)

Amicon Corp., Danvers, MA). This sample was mixed with $[^3H]H_2O$ (to locate the total volume) and applied to a 50 × 1.5-cm column of Sepharose CL-6B and eluted with DOC buffer. The eluted fractions were assayed for protein (BCA; Pierce Chemical Co.) and for [^{3}H]hyaluronate binding activity. For dot blot analysis, alternate fractions were diluted with 9 vol of water and 50 μl aliquots were applied to a sheet of nitrocellulose using a dot blot apparatus (Bio-Rad Laboratories). The sheet was blocked in 5% nonfat milk for 1 h and then cut into strips that were incubated individually with the various antibodies (4 μg/ml). The strips were incubated with the appropriate secondary probe (streptavidin coupled to peroxidase for biotinylated antibodies, and anti-mouse IgG coupled to peroxidase for non-biotinylated antibodies), followed by a substrate consisting of H_2O_2 and 3-amino-9 ethyl carbazole substrate (11). The reaction was stopped when an appropriate density level was reached.

Cell Aggregation

Subconfluent cultures of SV-3T3 cells were washed three times with calcium- and magnesium-free PBS (CMF-PBS) containing 0.02% EDTA. The cells were detached from the substratum with a stream of buffer, centrifuged (300 *g*, 5 min) and resuspended in a high salt buffer (0.5 M NaCl, 0.02 M Tris, pH 7.3), which optimizes hyaluronate dependent aggregation (35). 1-ml aliquots of this cell suspension were added to 20 ml plastic scintillation vials (Fisher Scientific Co., Columbia, MD) containing the different reagents, and placed on an orbital shaker at 100 rpm. To determine the number of single cells and small aggregates, 200 μl of the cell suspension was diluted with 20 ml of CMF-PBS and then analyzed with a model Z_F counter (Coulter Electronics Inc., Hialeah, FL) (1/amplitude = 2; 1/aperture = 4; window 5–100). Triplicate readings were taken at 0 and 30

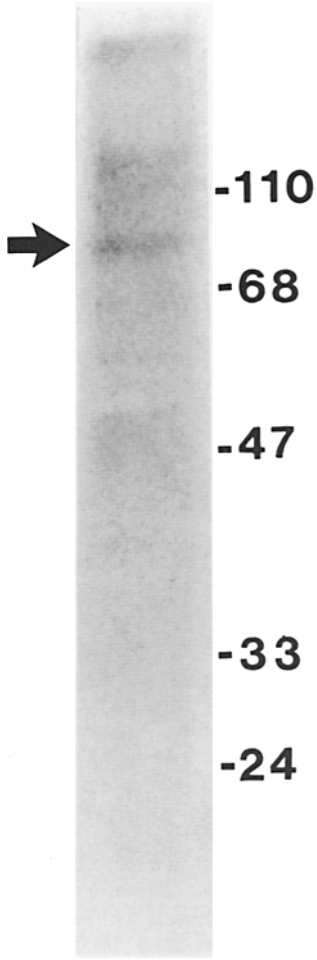

Figure 2. Phosphorylation of the hyaluronate receptor. BHK cells were labeled with [^{32}P]-PO_4^{-3}, extracted in DOC buffer, and then the hyaluronate receptor was immunoprecipitated with the K-3 mAb (41). The immunoprecipitate was then electrophoresed on a 10% Na dodecyl sulfate polyacrylamide gel, and blotted onto a sheet of nitrocellulose that was then exposed to x-ray film. The location of the hyaluronate receptor is indicated by the arrow on the left and the positions of various molecular weight standards are indicated on the right.

min. The results are expressed in terms of percent aggregation, which corresponds to the following:

$$\% \text{ Aggregation} = [1 - (\text{final/initial reading})] \times 100.$$

Testicular hyaluronidase (10 μg/ml; type VI-S; Sigma Chemical Co., St. Louis, MO) was added to one sample to block the hyaluronate dependent aggregation.

Lymphocyte Binding to High Endothelial Cells

The binding assay for lymphocytes to high endothelial venules in frozen sections of peripheral lymph nodes and Peyer's patches has been described previously (31, 42). Briefly, mesenteric node lymphocytes from BALB/c mice or Syrian hamsters were incubated in DME with 5% calf serum and 10 mM Hepes with mild rotation at 7°C for 30 min on unfixed frozen sections of BALB/c peripheral lymph node and Peyer's patches ($2.0 - 3.0 \times 10^6$ lymphocytes in 100 μl/section). The slides were fixed in cold PBS containing 2% glutaraldehyde. At least three sections containing multiple lymph nodes or Peyer's patches were counted for each condition or sample. The results are expressed as the number of bound lymphocytes per high endothelial venule. It should be noted that the binding specificity of the lymphocyte is conserved across species barriers (42), thus allowing the use of mouse frozen sections for assays with both mouse and hamster lymphocytes.

In the antibody blocking experiments, the mAbs KM-201 and K-3 were incubated with lymphocytes (20 μg antibody/2×10^6 cells in 100 μl) for 15 min at 4°C before the assay and left with the cells throughout the assay. In other experiments, lymphocytes were preincubated with varying concentrations of hyaluronate (from bovine vitreous humor; Sigma Chemical Co.) for 30 min on ice, and then this mixture was applied to frozen sections to assess binding. Controls were incubated with medium alone. In a separate experiment, frozen sections were preincubated with hyaluronidase (type VI-S; Sigma Chemical Co.) for 15 min at room temperature to remove endogenous hyaluronate. The sections were then washed with DME, and examined in the lymphocyte binding assays.

Results

Isoelectric Point and Phosphorylation of the Hyaluronate Receptor

In initial experiments, both the isoelectric point and phosphorylation of the hyaluronate receptor from hamster cells were examined. For this, BHK cells were grown in the presence of [^{32}P]PO_4^{-3} to label the phosphoproteins and then the cells were solubilized in detergent and analyzed by two-dimensional gel electrophoresis. The resulting gel was blotted onto nitrocellulose that was then stained for the hyaluronate receptor using the K-3 mAb. Fig. 1 *A* shows that the hyaluronate receptor migrated to a distinct spot in the acidic range of the gel. This region corresponded to an isoelectric point of approximately 4.0, which is consistent with that of the CD44 molecule (18).

The location of ^{32}P-labeled proteins was then determined by exposing the nitrocellulose blot to a sheet of x-ray film. As shown in Fig. 1 *B*, the region corresponding to the hyaluronate receptor contained a significant amount of label.

To further evaluate the presence of phosphate in this molecule, extracts of ^{32}P-labeled cells were directly immunoprecipitated with the K-3 mAb and analyzed by SDS-PAGE followed by blotting onto nitrocellulose. Autoradiography of the blot revealed a prominently labeled band corresponding to the hyaluronate receptor (see arrow, Fig. 2), confirming the presence of phosphate in this molecule. Thus, the hyaluronate receptor appears to be an acidic phosphoprotein, as is CD44 (14, 18).

Immunoprecipitation of Hyaluronate-binding Activity by mAbs against CD44

The relationship between the hyaluronate receptor and CD44 was examined using a panel of mAbs. In this approach, aliquots of a detergent extract of cultured cells containing the hyaluronate receptor were incubated with biotinylated mAbs against CD44 (see Table I for a description of each mAb). The mAbs and any specifically bound protein were then removed from the extract by using immobilized streptavidin, and the supernatant was assayed for [^{3}H]hyaluronate binding activity. The results of this experiment are shown in Table II.

In the case of the hyaluronate receptor from mouse cells, neither nonimmune mouse IgG nor the Mel-14 mAb (which is directed against a lymphocyte receptor distinct from CD44) were able to immunoprecipitate significant amounts of [^{3}H]hyaluronate binding activity. As a positive control, the K-3 mAb which cross reacts to some extent with the receptor from mouse cells (11, 40) was tested and found to remove a significant fraction of the hyaluronate binding activity. And finally, the KM-201 mAb against the mouse CD44, was also found to reduce significantly the level of hyaluronate binding activity. These results suggest that the KM-201 mAb can bind to the hyaluronate receptor from mouse cells.

When a similar approach was used with the receptor from human cells, the K-3 mAb had only a small effect on the level of hyaluronate binding activity, presumably reflecting a

Table II. Immunoprecipitation of Hyaluronate Binding Activity by mAbs to Mouse and Human CD44

Source of receptors	Experiment No.	Treatment	Amount	Specific binding	Control
			μg/ml	*cpm ± range*	%
Mouse	1	Control IgG	40	2,492 ± 121	100
		Mel-14	40	2,871 ± 104	115
		K-3	40	1,282 ± 45	51
		KM-201	40	614 ± 18	25
Human	1	Control IgG	40	5,735 ± 135	100
		K-3	40	5,084 ± 104	89
		H2-7	40	2,640 ± 301	46
		H2-280	40	1,928 ± 21	34
		Hermes-3	40	1,337 ± 44	23
		BU52	20	1,722 ± 473	30
		J173	40	3,437 ± 28	60
	2	Control IgG	40	37,195 ± 61	100
		K-3	40	34,791 ± 765	94
		Hermes-1	40	23,393 ± 63	63

The SV-3T3 and HCV-29T cell lines were used as sources of mouse and human cells, respectively. The cells were extracted in DOC buffer, preadsorbed with streptavidin–agarose, and then incubated with the various biotinylated antibodies. After immunoprecipitation with streptavidin–agarose, the supernatants were assayed for [^{3}H]hyaluronate binding activity. Batch 1 [^{3}H]hyaluronate was used in experiments 1 (mouse and human) and batch 2 was used in experiment 2 (human).

lower level of cross-reactivity between this antibody and human receptor as compared to mouse. However, the mAbs directed against human CD44 were able to immunoprecipitate significant amounts of the receptor as compared to control mouse IgG (Table II).

The hyaluronate binding activity remaining after immunoprecipitation could be due to either incomplete removal of a homogeneous species or the presence of a heterogenous population of receptors, some fraction of which is unable to react with the antibodies. To distinguish between these two possibilities, an extract was subjected to two rounds of immunoprecipitation with Hermes-3 mAb. Under these conditions, 90% of the [^{3}H]hyaluronate binding activity was removed, suggesting that the receptor is homogeneous with respect to its ability to bind to the Hermes-3 mAb.

The conclusion from this set of experiments is that mAbs directed against the CD44 molecule can immunoprecipitate [^{3}H]hyaluronate binding activity from both mouse and human cells, suggesting that the hyaluronate receptor and CD44 share similar epitopes and that they are closely related if not identical molecules.

Copurification of the Hyaluronate Receptor and CD44

To evaluate further the relationship between the hyaluronate receptor and CD44, the behaviors of these proteins on molecular sieve chromatography were compared. For this, the receptor was partially purified from the human HCV-29T cells by hydroxylapatite chromatography and then analyzed by molecular sieve chromatography on a column of Sepharose Cl-6B. Fig. 3 shows that the hyaluronate receptor elutes with a partition coefficient of 0.4–0.5, consistent with that previously reported for the receptor from the mouse SV-3T3 cells (39). Since the receptor has a highly elliptical shape, it behaves as an anomalously large molecular on molecular sieve chromatography (39). When the fractions were analyzed by dot blot, each of the antibodies to human CD44 revealed a peak of immunoreactive material eluting with a partition coefficient of 0.4–0.5 (Fig. 3; fractions 13 and 15), which corresponds to the position of [^{3}H]hyaluronate binding activity. Thus, CD44 coelutes from the molecular sieve column with the hyaluronate receptor, confirming that these proteins are either highly related or identical.

Unfortunately, a similar approach was not possible with the hyaluronate receptor from mouse SV-3T3 cells, since the KM-201 mAb did not react with nitrocellulose blots of these cells. Presumably, the epitope recognized by the KM-201 is not stable to blotting.

Effect of Antibodies on the Binding of Hyaluronate

The various mAbs were also examined for their ability to directly block [^{3}H]hyaluronate binding activity shown (Table III). In the case of the receptor from mouse cells, high concentrations of Mel-14 (control mAb) had only a modest effect on the binding activity, while relatively small amounts of the K-3 mAb blocked most of the binding, as had been demonstrated previously (11). Likewise, the KM-201 mAb, which immunoprecipitated the hyaluronate receptor, also inhibited [^{3}H]hyaluronate binding. These results suggest that the KM-201 mAb recognizes an epitope very close to the hyaluronate binding domain on the CD44 molecule.

In the case of the receptor from human cells, the K-3 mAb as well as the Hermes-1 mAb blocked [^{3}H]hyaluronate binding activity, but only at relatively high concentrations. However, none of the other mAbs directed against human CD44 had a significant effect on the binding of [^{3}H]hyaluronate to the receptor. These results suggest that with the possible exception of the Hermes-1 mAb, the mAbs against human CD44 appear to be directed to epitopes distinct from the hyaluronate binding region of the CD44 molecule.

Inhibition of Aggregation by KM-201

The results of the previous section indicated that the KM-201 mAb directly blocked the binding of [^{3}H]hyaluronate to the

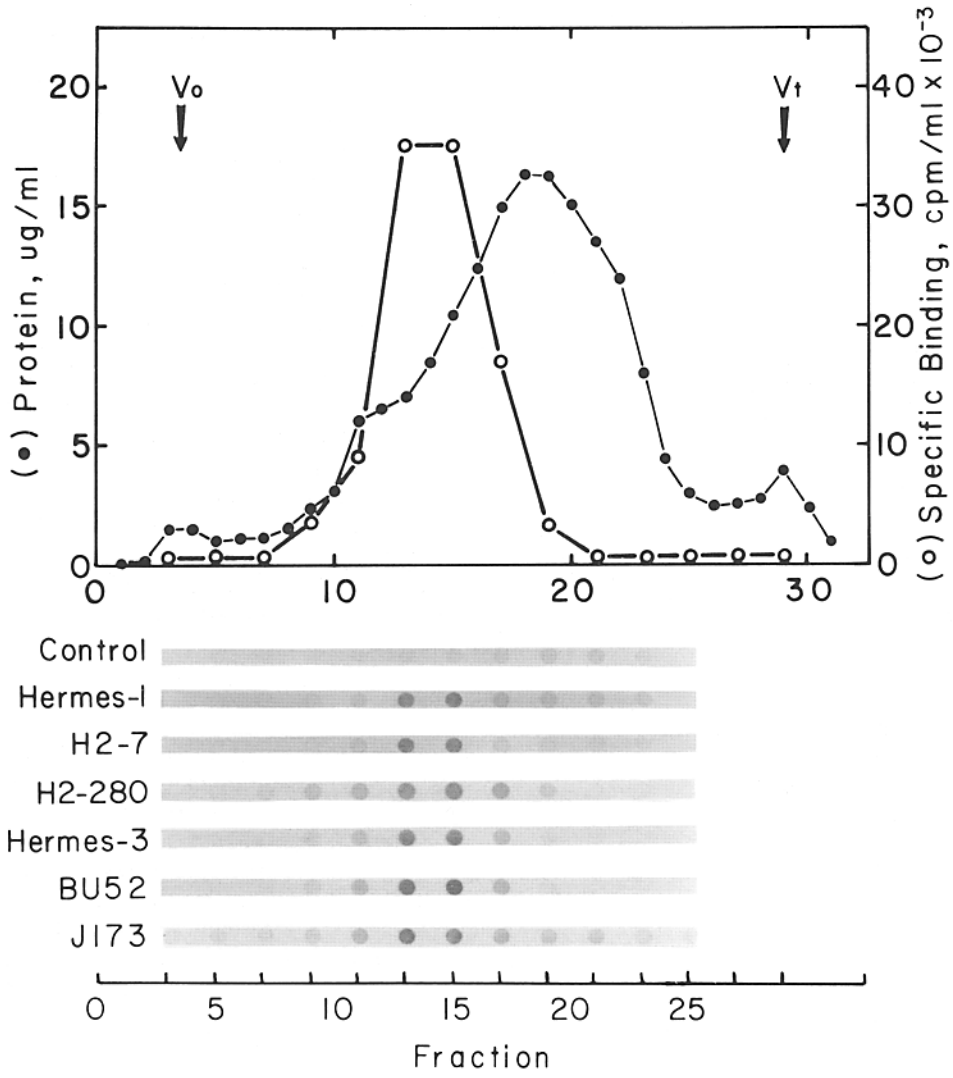

Figure 3. Coelution of [^{3}H] hyaluronate binding activity and CD44 immunoreactivity by molecular sieve chromatography. The hyaluronate receptor from cultured HCV-29T cells was partially purified by hydroxylapatite chromatography and then applied to a column of Sepharose Cl-6B that was eluted with DOC buffer. The positions of the void volume (*Vo*) and the total volume (*Vt*) are indicated. Each fraction was assayed for protein and alternate fractions were assayed for [^{3}H]hyaluronate (Batch 2) binding activity. Alternate fractions were also blotted onto nitrocellulose and assayed for immunoreactive material with the indicated mAbs to CD44. Fractions 13 and 15 contain the peak of [^{3}H]hyaluronate binding activity as well as the maximum amount of immunoreactive material.

Table III. Direct Inhibition of Hyaluronate Binding Activity by mAbs to CD44

Source of receptors	Experiment No.	Treatment	Amount	Specific binding	Control
			μg/ml	*cpm ± range*	%
Mouse	1	None (control)	0	5,371 ± 647	100
		Mel-14	60	4,453 ± 156	83
		K-3	1	2,274 ± 129	42
			2	714 ± 108	13
		KM-201	1	3,152 ± 519	59
			2	829 ± 35	15
Human	1	None (control)	0	10,181 ± 207	100
		K-3	20	6,585 ± 529	65
			40	3,926 ± 280	39
		H2-7	40	12,127 ± 1,119	119
		H2-280	80	8,866 ± 639	87
		Hermes-3	140	9,758 ± 486	96
		BU52	40	13,630 ± 843	134
		J173	80	8,631 ± 164	85
	2	None (control)	0	39,078 ± 116	100
		K-3	40	19,270 ± 475	49
		Hermes-1	40	19,776 ± 282	51
			80	18,104 ± 252	46

SV-3T3 (mouse) and HCV-29 (human) cells were extracted in DOC buffer, and preincubated with the various antibodies. The extracts were then assayed for [^{3}H]hyaluronate binding activity (Batch 1 was used in experiments 1 and Batch 2 in experiment 2). Nonimmune mouse IgG had no effect on the binding.

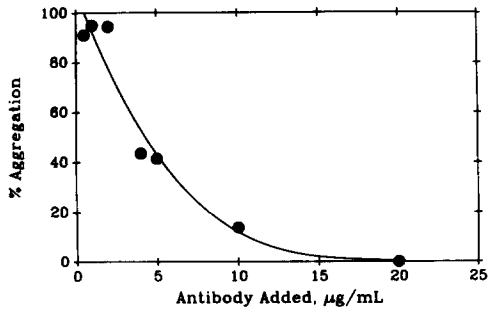

Figure 4. The effect of the KM-201 mAb on the aggregation of SV-3T3 cells. SV-3T3 cells were suspended in 0.5 M NaCl, 0.02 M Tris, pH 7.3, and then swirled in the presence of varying amounts of the KM-201 mAb. After a period of 30 min, the extent of aggregation was determined with a counter (Coulter Electronics Inc.). The initial counter reading (i.e., time = 0) was 19,809 ± 1,405/0.5 ml of diluted sample. Each point represents the average of triplicate determinations and varies by < 10%. The aggregation was totally abolished (i.e., % aggregation = 0) by the addition of 10 μg/ml testicular hyaluronidase.

receptor on mouse cells. This suggested that this mAb might also be capable of preventing hyaluronate dependent aggregation of mouse cells. To test this possibility, varying amounts of KM-201 were added to suspensions of SV-3T3 cells. After swirling these cells for 30 min, the extent of aggregation was determined with a counter (Coulter Electronics Inc.). Fig. 4 shows that relatively small amounts of the KM-201 mAb inhibited the hyaluronate dependent aggregation of the cells. Neither control mouse IgG, nor Mel-14 had any detectable effect on the extent of aggregation even at much higher concentrations (data not shown). These results corroborate the ability of the KM-201 mAb to block the binding of hyaluronate to the receptor.

Hyaluronate Does Not Participate in the Binding of Lymphocytes to High Endothelial Venules

As described above, the Hermes-3 mAb had no effect on hyaluronate binding. Previous studies have shown that this mAb blocks lymphocyte binding to mucosal endothelial cells (17). Taken together, these results suggest that the binding of lymphocytes to mucosal endothelial cells involves determinants on CD44 that are not required for hyaluronate binding. However, other studies have shown that polyclonal antibodies to CD44 are capable of blocking lymphocyte binding not only to mucosal but also to peripheral lymph node and synovial high endothelial cells (3), suggesting that additional determinants of CD44, not effected by Hermes-3, may also participate in the recognition of endothelial cells. Thus, it remained possible that the hyaluronate binding activity of CD44 may also participate in binding to mucosal or other high endothelial venules. To test this possibility, the binding of lymphocytes to frozen thin sections of high endothelial venules was examined in the presence and absence of the K-3 and KM-201 mAbs, both of which very effectively block the binding of hyaluronate to the receptor. As shown in Table IV, neither of these mAbs had a major effect on the binding of lymphocytes to high endothelial venules. The K-3 mAb had no detectable effect on the binding of hamster lymphocytes, while the KM-201 mAb produced only a minor reduction in mouse lymphocyte binding to the venules of Peyer's patches and did not influence the interaction with venules of peripheral lymph node (Table IV). These results suggest, but do not prove, that hyaluronate is not involved in this adhesion process.

To evaluate further the possible role of hyaluronate in the homing of lymphocytes, the lymphocyte binding assay was performed in the presence of excess hyaluronate and hyaluronidase, both of which also inhibit hyaluronate dependent adhesion of cells (35, 37). Table V shows that hyaluronate, even at relatively high concentrations, had only a modest effect on the binding of lymphocytes to high endothelial cells, and this effect may be due to the increased viscosity of the medium. Similarly, enzymatic degradation of hyaluronate on the frozen sections did not significantly inhibit the binding of lymphocytes (Table V).

Discussion

The results of this and other studies suggest that the hyaluronate receptor is identical to CD44. First, both of

Table IV. Effect of Various mAbs on the Binding of Mesenteric Node Lymphocytes to High Endothelial Venules (HEVs)

Source of lymphocytes	Experiment No.	Treatment	Binding of lymphocytes to high endothelial venules			
			Peripheral lymph node		Peyer's patches	
			Lym/HEV	Control	Lym/HEV	Control
				%		%
Hamster	1	Control	10.8 ± 1.4	100	6.2 ± 0.3	100
		K-3	9.4 ± 0.4	87	5.6 ± 0.3	90
Mouse	1	Control	14.7 ± 0.5	100	5.4 ± 0.5	100
		K-3	17.9 ± 0.8	122	6.5 ± 0.6	120
	2	Control	15.6 ± 1.7	100	6.4 ± 0.5	100
		KM-201	17.0 ± 1.0	109	4.6 ± 0.5	72
	3	Control	–	–	6.9 ± 0.5	100
		KM-201	–	–	5.6 ± 0.4	81

Lymphocytes were prepared from the Peyer's patches of hamsters or mice and preincubated with the indicated mAb at saturating concentrations (20 μg in 100 μl). This mixture was then gently agitated with frozen sections of either peripheral lymph nodes or Peyer's patches from mice. After fixation, the number of lymphocytes bound per high endothelial venule was determined (Lym/HEV).

Table V. Effect of Hyaluronate and Hyaluronidase on the Binding of Mesenteric Node Lymphocytes to High Endothelial Venules (HEVs)

Treatment	Concentration	Binding of lymphocytes to high endothelial venules			
		Peripheral lymph nodes		Peyer's patches	
		Lym/HEV	Control	Lym/HEV	Control
	μg/ml		%		%
Hyaluronate	0	13.6 ± 1.1	100	5.5 ± 0.6	100
	5	14.4 ± 1.1	103	5.2 ± 0.6	95
	50	14.6 ± 1.1	107	5.3 ± 0.6	96
	500	11.0 ± 0.9	81	4.9 ± 0.7	89
Hyaluronidase	0	10.2 ± 1.1	100	8.6 ± 0.9	100
	1	10.0 ± 1.8	98	7.8 ± 1.3	91
	10	13.0 ± 2.5	127	8.2 ± 1.1	95
	100	10.6 ± 1.9	104	10.4 ± 1.6	121

In the first experiment, lymphocytes from mice were preincubated with varying concentrations of hyaluronate, and then assayed for binding to high endothelial cell venules in the continued presence of hyaluronate. After fixation, the number of lymphocytes bound per high endothelial venule was determined (*Lym/HEV*). Results are from a representative experiment of two performed with similar results. The inhibition seen at high concentrations of hyaluronate is not statistically significant. In the second experiment, frozen section of mouse peripheral lymph nodes and Peyer's patches were pretreated with varying amounts of hyaluronidase, washed, and then examined in the lymphocyte binding assay.

these proteins have a very similar type of tissue distribution, being present on a number of cell types including macrophages and the actively dividing epithelial cells. Second, the hyaluronate receptor is similar to CD44 with respect to molecular weight, isoelectric point, presence of phosphate groups, and behavior on molecular sieve chromatography. Third, a number of mAbs directed to CD44 cross react with the hyaluronate receptor from both mouse and human cells. And fourth, one of these mAbs, KM-201, directly blocks the binding of hyaluronate to the receptor and prevents hyaluronate-dependent aggregation of cultured mouse cells.

Several recent studies have provided additional evidence for the identity of the hyaluronate receptor and the CD44. Lesley et al. (21) have shown that some, but not all, lymphocyte cell lines which express CD44 can bind to dishes coated with hyaluronate and that this binding to hyaluronate could be blocked by mAbs directed against CD44 (21). Furthermore, Aruffo et al. (2) have used the techniques of molecular biology to create a soluble hybrid of CD44 and immunoglobulin protein, which was capable of binding specifically to hyaluronate including that associated with high endothelial cells in culture. Collectively, these observations indicate that at least some members of the CD44 family of glycoproteins can bind to hyaluronate, and thus are similar or identical to the hyaluronate receptor.

At present it is unclear whether CD44 represents a single protein or a series of closely related proteins. This latter possibility is suggested by a number of studies. For example, Flanagan et al. (6) have shown that two types of CD44 can be separated from a single cell type by ion exchange chromatography. Additionally, Picker et al. (28) have shown that the CD44 from squamous epithelia differs from that present on lymphocytes, fibroblasts and glial cells with respect to molecular weight and isoelectric point. And finally, Lesley et al. (21) have found that only a fraction of the cell lines that express CD44 can attach to a hyaluronate-coated substratum. Thus, it is possible that only a subset of the CD44 family of molecules is capable of binding hyaluronate.

Despite the uncertainty concerning the homogeneity of CD44, it is apparent that this protein (or family of proteins) is capable of binding to at least three different ligands; hyaluronate, collagen types I and VI (4), and the mucosal vascular addressin, a protein present on specialized high endothelial cells that is believed to support lymphocyte extravasation in the gut-associated lymphoid tissues (3, 34). The ability of CD44 to bind to more than one ligand is in keeping with it being a member of the cartilage link protein superfamily of proteins. The link protein itself is capable of binding to three ligands; hyaluronate, the cartilage core protein and collagen (5).

The interaction of CD44 with these different ligands appears to be responsible for distinct types of cellular adhesion. For example, the interaction of CD44 with hyaluronate has been shown to mediate the divalent cation independent aggregation of macrophages and fibroblasts (11). In addition, it also appears to be responsible for the binding of some types of cultured lymphocytes to stromal cells from bone marrow, which can be blocked by the KM-201 mAb (24). In the present study, this mAb has been shown to inhibit hyaluronate binding. Indeed, recent experiments have indicated that in some cases, the adhesion between cultured lymphocytes and stromal cells is specifically inhibited by treatment with hyaluronidase, confirming the role of hyaluronate in this adhesion process (25).

In contrast, the interaction of CD44 with hyaluronate does not appear to be involved in the binding of lymphocytes to high endothelial cells. Initially, CD44 was believed to be responsible for the homing to most lymphoid tissues since polyclonal antibodies to CD44 inhibited lymphocyte binding to mucosal, lymph node as well as synovial high endothelial cells (17). However, subsequent research indicated that CD44 was responsible for the selective homing of lymphocytes to the mucosal as opposed to peripheral lymphoid tissue, since a lymphoid cell line lacking CD44 (Jaukat) was still capable of attaching to endothelial cells of peripheral lymph nodes (33). This conclusion was further supported by the fact that the mAb Hermes-3 selectively blocked lymphocyte binding to mucosal endothelial cells, but not to peripheral lymph nodes (3). This mAb also inhibited the interaction of purified CD44 with isolated mucosal vascular

addressin, which is postulated to be the recognition signal in this tissue (3). In the present study, the Hermes-3 mAb was found to have no effect on the hyaluronate binding activity of CD44, and conversely mAbs KM-201 and K-3 that block hyaluronate binding activity, did not substantially inhibit lymphocyte interactions with the high endothelial venules of either mucosal or peripheral lymph nodes. Furthermore, neither hyaluronate nor hyaluronidase inhibited the binding of lymphocytes to the venules. Taken together, these results suggest that the hyaluronate binding region of the CD44 molecule is distinct from the Hermes-3 epitope that is involved in lymphocyte homing, presumably through its interaction with mucosal addressin. This is further supported by sequence analysis of CD44 that has revealed that its NH_2-terminal domain is homologous to the cartilage link protein (9, 30), suggesting that the hyaluronate binding site is located on the distal region of the molecule. The Hermes-1 mAb, which had some inhibitory effect on the binding of [^{3}H]hyaluronate, recognizes an epitope in this NH_2-terminal domain (our unpublished observations). In contrast, the epitope for Hermes-3 appears to be present on a more proximal region of the CD44 molecule that is heavily glycosylated (9).

It is possible that the CD44 glycoprotein serves a number of different functions during the lifetime of lymphocytes. Through its Hermes-3 epitope, CD44 may help lymphocytes to recognize and bind to the high endothelial cells lining the venules of mucosal lymphoid tissue. Once the lymphocytes have migrated through the endothelial cells and enter the connective tissue, the CD44 protein may interact with hyaluronate, collagen and potentially other molecules in the stroma to allow the lymphocytes to migrate through the extracellular matrix.

In conclusion, the apparent identity of the hyaluronate receptor and CD44 unifies a large body of research concerning this cell adhesion molecule. Collectively, these studies yield a detailed picture of the CD44 molecule with respect to its interaction with hyaluronate (36, 39), addressin (3), and collagen (4), its tissue distribution (1, 6, 10, 28, 36), its physical properties (6, 18, 39), its amino acid sequence (9, 30), carbohydrate component (6), and its association with actin filaments (15, 19, 20). Undoubtedly, future research will reveal additional characteristics for this very interesting molecule.

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