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Characterization of Hyaluronate Binding Proteins Isolated from 3T3 and Murine Sarcoma Virus Transformed 3T3 Cells†

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ABSTRACT: A hyaluronic acid binding fraction was purified from the supernatant media of both 3T3 and murine sarcoma virus (MSV) transformed 3T3 cultures by hyaluronate and immunoaffinity chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis resolved the hyaluronate affinity-purified fraction into three major protein bands of estimated molecular weight ($M_{r,e}$) 70K, 66K, and 56K which contained hyaluronate binding activity and which were termed hyaluronate binding proteins (HABP). Hyaluronate affinity chromatography combined with immunoaffinity chromatography, using antibody directed against the larger HABP, allowed a 20-fold purification of HABP. Fractions isolated from 3T3 supernatant medium also contained additional binding molecules in the molecular weight range of 20K. This material was present in vanishingly small amounts and was not detected with a silver stain or with [^{35}S]methionine label. The three protein species isolated by hyaluronate affinity chromatography ($M_{r,e}$ 70K, 66K, and 56K) were related to one another since they shared antigenic determinants and exhibited similar p*I* values. In isocratic conditions, HABP occurred as aggregates of up to 580 kilodaltons. Their glycoprotein nature was indicated by their incorporation of ^{3}H-sugars. Enzyme-linked immunoadsorbent assay showed they were antigenically distinct from other hyaluronate binding proteins such as fibronectin, cartilage link protein, and the hyaluronate binding region of chondroitin sulfate proteoglycan. The apparent dissociation constant of HABP for hyaluronate was approximately 10^{-8} M, and kinetic analyses showed these binding interactions were complex and of a positive cooperative nature. Tryptic peptide fingerprinting and immunological cross-reactivity suggested the HABP from the virally transformed and the parent cell line sources were closely related. Nevertheless, structural differences were demonstrated by distinct p*I* values and unique peptide sequences. These results are discussed with regard both to the functional significance of hyaluronate–cell surface interactions in transformed as well as normal cells and to the relationship of HABP to other reported hyaluronate binding proteins.

Hyaluronate is one of several glycosaminoglycans that has been implicated in regulating cell behavior during tumorigenesis and embryogenesis (Toole, 1982). Thus, production of hyaluronate is elevated during the morphogenesis of a variety of embryonic tissues (Toole, 1982), in tumor variants selected for enhanced metastatic/invasive capability (Kimata et al., 1983; Turley & Tretiak, 1985), and at the site of invasion of tumors into host tissue (Toole et al., 1979; Kupchella & Baki-Hashemi, 1982; Turley & Tretiak, 1985). Hyaluronate has profound effects on cell behavior in vitro that may contribute to developmental and disease states such as cell overlapping (Turley, 1982), morphology (Toole, 1982), motility (Culp, 1976; Turley & Torrance, 1985), adhesion, and immunogenicity (Toole, 1982). Furthermore, it affects the

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structure of the extracellular matrix which may indirectly influence cell behavior (Toole, 1982; Chakrabarti & Park, 1980).

Hyaluronate appears to exert some of its effects on cell behavior by interacting with receptors on the cell surface (Toole, 1982). Although the molecular identity of these binding sites has not yet been established, a group of small proteins (50K–70K) that bind to hyaluronate (Delpech & Halavent, 1981; Turley, 1982; Blenis & Hawkes, 1984; Underhill et al., 1983), that codistribute with hyaluronate on cell surfaces (Turley & Torrance, 1985), and that are distinct from other characterized matrix proteins (Turley & Moore, 1984) represent possible candidates. These small proteins, like hyaluronate, appear to be regulated during morphogenesis and tumorigenesis (Delpech & Halavent, 1981; Delpech & Delpech, 1984). Further, the ability of these peptides to bind, in addition to hyaluronate, matrix polymers such as fibronectin, laminin, and collagen (Turley & Moore, 1984; Turley et al., 1985) suggests they may also play a role in structuring the extracellular matrix.

Although these hyaluronate binding protein (HABP)[1] fractions (Delpech & Halavent, 1981; Turley, 1982; Underhill et al., 1983; Blenis & Hawkes, 1984) have been isolated from a variety of sources, they have not been extensively characterized other than for their binding preference to hyaluronate and other matrix-associated polymers. We report some of the structural and binding properties of HABP isolated from virally transformed and nontransformed 3T3 cell lines.

Experimental Procedures

Cell Culture. NIH 3T3 and MSV-3T3 cell lines were purchased from American Tissue Type collection. Cells (10^6) were added to roller bottles (850 mm^2) containing "superbeads" (Lux, 0.2 mm, Gibco), 20 mL of DME, and 10% fetal calf serum. Bottles were equilibrated with 8% CO_2 to pH 7.2 and maintained on a rotator at 3 rpm at 37 °C. HABP were routinely isolated from conditioned medium collected after cells had been cultured for 4 days (at which time the "superbeads" were saturated with cells). Cells were then digested for 10 min at 90 °C with 0.1 N NaOH, and a Lowry assay was performed on the digest to quantitate cellular protein (Lowry et al., 1951).

Purity of HA. Each batch of hyaluronate (umbilical cord, grade 1, Sigma) was analyzed electrophoretically (Cappelletti et al., 1979), immunochemically (Oblas et al., 1983), and by gel filtration for contaminating GAG and proteins and to establish its approximate molecular weight. Generally, cellulose acetate electrophoresis of hyaluronate revealed small amounts (<5%) of contaminating chondroitin 4- and 6-sulfate, but protein was not detected, and ELISA (Turley & Moore, 1984) indicated that fibronectin, laminin, and actin were not present. Chromatography of hyaluronate on agarose gels (4%, Bio-Rad) revealed a molecular weight average of 1×10^6 determined in reference to the chromatographic behavior of RNA standards obtained from Dr. G. Gordon, The University of Calgary. Batches of hyaluronate that contained detectable proteins, greater than 10% chondroitin sulfate, or large amounts of hyaluronate fragments (<1000 daltons) were not used for affinity chromatography.

Preparation of [^{3}H]HA. [^{3}H]Hyaluronate was prepared as described previously (Turley & Roth, 1979). Briefly, hyaluronate was dissolved in PBS. Sodium borotritide (10 μCi, 341 mCi/mmol, NEN) was added for 2 h at 20 °C and washed with 10 mM unlabeled sodium borohydride (Pierce Chemicals) for an additional hour. The solution was extensively dialyzed (molecular weight cutoff, 3000) for 36 h against 40 L of distilled H_2O. Samples were lyophilized, chromatographed on a Sephadex G-50 column in 0.1 M ammonium acetate/ethanol buffer (Derby & Pintar, 1978), and collected in the void volume. The specific activity of [^{3}H]hyaluronate was calculated to be 1.5 μCi/mg of hyaluronate.

Isolation of Supernatant HABP. All HABP used for structural and binding analyses were routinely isolated from supernatant medium of high-density cultures, since these gave the greatest yield. HABP were isolated from the supernatant medium of cell cultures as described previously (Turley, 1982) with some minor modifications. Cells in roller bottles were washed several times in DME and then incubated at 37 °C with 10 mL of DME for a total of 4 h. Culture medium was centrifuged at 100*g* at 4 °C for 10 min to remove cells. Medium was placed on ice, and the proteolytic inhibitors, PMSF, leupeptin, antitrypsin, and aprotinin (Sigma Chemicals, 20 μg/mL DME), were added immediately. The medium was then sequentially filtered, at 4 °C, through a 0.22-μm Millipore filter, to remove large protein aggregates such as fibronectin complexes, through an XM50 ultrafilter to exclude proteins of >80 000 daltons and then concentrated with a PM-10 ultrafilter. Additional protease inhibitors were added to the concentrate at this time.

The concentrate containing the binding proteins was applied, twice, to a Sepharose–hyaluronate affinity column [1 × 4 cm, flow rate, 2 mL/min (Turley, 1982)] that had been previously equilibrated with *N*-ethylmaleimide to inhibit any protease activity associated with the hyaluronate (Turley & Moore, 1984) and with propylene glycol (10 mg/mL) to block nonspecific binding to the Sepharose backing. The column was washed with several column volumes of PBS containing the protease inhibitors. Most of the bound protein (60%) was then eluted with 2 M sodium chloride. Guanidinium chloride was more effective in removing HABP (80–90% yield) but compromised the biological activity. Hyaluronate polymer, at effective concentrations, was too viscous to chromatograph, and neither monosaccharides nor *Streptomyces* hyaluronidase digests of hyaluronic acid were effective in removing the protein. The HABP eluted with 2 M sodium chloride was dialyzed (molecular weight cutoff of 3000) against 40 L of distilled H_2O for 12 h, lyophilized, and quantitated according to Lowry et al. (1951). HABP was further purified by immunoaffinity chromatography using polyclonal antibody to the larger proteins (66K–70K) (rabbit, IgG fraction; Turley & Moore, 1984) linked to CN–Sepharose (Sigma Chemicals). Bound protein was eluted from the column with DME (containing the above proteolytic inhibitors) adjusted to pH 11.0.

Radiolabeling of HABP. (*A*) *Metabolic Labeling.* Metabolically labeled HABP was obtained by incubating 10^6 MSV-transformed or 3T3 cells grown in 60-mm dishes (Falcon) with 10 μCi of [^{35}S]methionine (>400 Ci/mmol, NEN), [^{3}H]mannose (40–60 Ci/mmol, NEN), [^{3}H]galactose (20–40 Ci/mmol, NEN), [^{3}H]glucosamine (10–30 Ci/nmol, NEN), or [^{3}H]galactosamine (10–25 Ci/mmol, NEN) for 12

[1] Abbreviations: HA, hyaluronic acid; HABP, hyaluronate binding protein(s); MSV-3T3, murine sarcoma virus transformed 3T3 cells; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; $M_{r,e}$, estimated molecular weight; DME, Dulbecco's modified Eagle's medium; GAG, glycosaminoglycan; ELISA, enzyme-linked immunoadsorbent assay; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline, pH 7.2; BSA, bovine serum albumin; TBS, Tris-buffered saline, pH 7.2; CN, 4-chloro-1-naphthanol; TCA, trichloroacetic acid; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; KLH, keyhole limpet hemocyanin; Tris, tris(hydroxymethyl)-aminomethane; FCS, fetal calf serum.

h at 37 °C in PBS (pH 7.2) containing 1 mg of glucose/1 mL of medium and 10% FCS. The labeled protein was hyaluronate affinity-purified as described above. A previous study demonstrated that metabolically labeled proteins comigrated with native proteins (Turley, 1982). To determine whether the isolated labeled HABP was a cell product, 10 μCi of [^{35}S]methionine was also added to cells for less than 1 min (i.e., zero time point), to fixed cultures, and to cell-conditioned medium for 12 h. Supernatant media was then extracted for labeled HABP as above.

(*B*) *Iodination of HABP.* Fifty micrograms of purified HABP was exposed, for 40 s, to 0.25 mCi of ^{125}I (100 mCi/mL, Amersham) in tubes coated with Iodogen (20 μL of a 1 mg/mL chloroform solution; Pierce Chemicals). The reaction mixture was chromatographed on a Sephadex G-25 column (10-mL bed volume) in PBS (pH 7.2) containing 1% BSA (fraction V, Sigma). One-milliliter fractions from this column were monitored for radioactivity and TCA precipitability using 10-μL aliquots of each fraction. The radioactive peak eluting in the void volume (fractions 4–6, >90% recovery) was pooled, chromatographed on a Sepharose–hyaluronate column, and then eluted from the column with 2 M NaCl. The radioactivity in the eluates was quantitated by using an LKB 1274 automatic γ counter. Recovery from the affinity column was 10% of added ^{125}I-HABP.

SDS–PAGE. Electrophoretic analysis was conducted as described previously (Turley, 1982) on 12% polyacrylamide gels using denaturing and reducing conditions. Ten micrograms of HABP and low molecular weight standards (14K–90K, Bio-Rad) were applied to the gels and electrophoresed. Protein bands were detected by the silver staining method (Sammons et al., 1981).

Gel Filtration of HABP. Either ^{35}S-HABP (1×10^4 cpm) or 10 μg of unlabeled HABP was applied to a Bio-Gel P-100 column (1 × 66 cm, flow rate 0.05 mL/min) in PBS (pH 7.2) at 4 °C. The elution profile was monitored for protein at 280 nm and for ^{35}S label by using scintillation counting. Additionally, unlabeled protein was chromatographed on an LKB TSK 90000 SW HPLC column [1.0 mL/min flow rate, 18 bar pressure, in 0.1 M Na_2HPO_4 buffer at pH 6.8] and detected at 289 nm. Molecular weight standards included thyroglobulin (580K), γ-globulin (160K), bovine serum albumin (68K), and ovalbumin (49K).

Isoelectric Focusing. Two-dimensional analysis of HABP on polyacrylamide gels was conducted as described by O'-Farrell (1975). For the first dimension, protein was applied to isoelectric focusing tube gels (6 cm) containing ampholines pH 9–11 (0.4%) in a buffer of 9.5 M urea, 2% Nonidet 40, 2% ampholine (1.6% of pH 5–7; 0.4% of pH 3–10), and 5 mM DTT. Gels were focused in buffer (upper reservoir 0.2 M NaOH, lower reservoir 0.01 M H_3PO_4) at 400 V for 12–16 h at 4 °C. The pH gradient was measured by cutting an additional gel into 1-cm segments, soaking segments in H_2O for 0.5 h, and then measuring the pH. Gels containing protein samples were placed over a 12% SDS–PAGE and electrophoresed in 0.025 M Tris buffer at 15 mA/gel for 5 h.

Peptide Mapping of HA Affinity-Purified HABP. Peptide maps were obtained as described (Elder et al., 1977). Fifty micrograms of HABP was electrophoresed on SDS–PAGE as above, then stained with 3% Coomassie blue, and destained in acid methanol, and the discernible protein bands were cut out. Gel strips were washed in 10% methanol, lyophilized, and then iodinated (0.5% incorporation) according to the chloramine T method (Elder et al., 1977). Gel strips were again washed in methanol to remove all soluble iodine and lyophilized. Either trypsin or chymotrypsin (50 μg/mL in 0.5 M $NaHCO_3$, pH 8.0) was added to strips for 24 h at 37 °C; the enzyme solution was removed, lyophilized, and then applied, in 20 μL of electrophoretic buffer (8% formalin and 2% acetic acid, pH 2.1), to a thin-layer chromatography plate together with lysine which acted as a tracking dye. Samples were electrophoresed at 400 V for 45 min; the plates were dried and then chromatographed in 30 mL of butanol/pyridine/H_2O/acetic acid (5:4:4:1). Plates were sprayed with ninhydrin to visualize the lysine, then dried, and exposed to Kodak autoradiographic film for 24 h at −70 °C to visualize fragments of ^{125}I-HABP.

Preparation of Antibodies to HABP. Polyclonal antibodies to the larger two HABP from 3T3 and MSV-transformed cells were raised in New Zealand white rabbits (3 kg) as described (Turley & Moore, 1984) after their isolation on SDS–PAGE. Antibodies to HABP had previously been shown not to react immunochemically with several matrix proteins (Turley & Moore, 1984) and were additionally tested here for reactivity toward fibronectin, link protein, and the protein core of chondroitin sulfate proteoglycan. Link protein and the chondroitin sulfate proteoglycan were isolated from bovine cartilage and were the kind gifts of Dr. C. A. Erickson. Antibody toward the hyaluronate binding region of chondroitin sulfate proteoglycan (a kind gift of Drs. V. Hascall and B. Caterson) was also assayed against HABP.

Electrophoretic Transfer of HABP to Nitrocellulose Sheets. Forty micrograms of HABP was electrophoresed on nonreducing PAGE and then transferred to nitrocellulose sheets according to the method of Oblas et al. (1983). Gels containing the protein were applied to nitrocellulose sheets (Bio-Rad) and were electrophoresed at 10 mA in Tris-HCl (200 mM) buffer, at pH 7.4, overnight. Sheets were cut into 1 × 6 cm strips containing the protein and were incubated in PBS containing 3% bovine serum albumin (fraction 5, Sigma) for 1–2 h at 20 °C in plastic containers designed to hold 2 mL of buffer. The inclusion of albumin reduced background staining or labeling in the procedures outlined in subsections A and B below.

(*A*) *Immunoblots of HABP.* Strips were washed 3 times in TBS (50 mM Tris and 200 mM saline, pH 7.2) buffer and then exposed to polyclonal antibodies against HABP (1:40 dilution) in TBS buffer containing 1% BSA for 1 h at 20 °C. Samples were agitated on a rotary shaker (70 rpm) during this time. Strips were washed 3 times with TBS and then incubated in TBS containing peroxidase-linked, goat anti-rabbit IgG (1:350, Miles) for 2 h at 20 °C. Strips were washed to remove excess peroxidase IgG and then exposed to 2 mL of a CN/H_2O_2 solution: 60 mg of CN/100 mL of methanol diluted with TBS (1:4) was mixed with 600 μL of 2% H_2O_2. Color was allowed to develop approximately 0.5 h at 20 °C. The reaction, which was stopped by washing in H_2O, was photographed with a Polaroid Land camera.

(*B*) *Incubation with* [^{3}*H*]*HA.* Strips containing HABP were incubated on a gyratory shaker with 5×10^5 cpm of [^{3}H]-hyaluronate in TBS + 1% BSA for 4 h at room temperature. Strips were washed 3 times with ice-cold TBS, and liquid was removed from the strips by blowing cold air over the membranes which were then cut into 1-cm sections. The position of the protein was judged either by the presence of ^{35}S-labeled HABP in these sections or by cutting the strip lengthwise and staining half with amido black.

Binding Analysis. (*A*) *Binding of* [^{3}*H*]*HA to HABP.* Ten to twenty micrograms of HABP (dissolved in 100 μL of PBS) was added to nitrocellulose-coated multiwells in TBS, pH 7.2

Table I: Purification of HABP from the Supernatant Media of 3T3 Cell Monolayers[a]

purification step	binding/well (cpm)	protein/well (μg)	sp act. of binding (cpm/μg)	total protein in supernatant medium/ purification step (mg)	total estimated binding act./purification step (cpm)	n-fold purification
supernatant medium	1716 ± 603	29.3 ± 3.1	56.9 ± 5.8	10.3 ± 2.3	586173	
0.22-μm filtration	1892 ± 589	23.7 ± 4.3	79.6 ± 6.6	3.6 ± 1.4	286560	1.4
hyaluronate affinity chromatography	4451 ± 562	15.0 ± 1.3	296.7 ± 32.0	0.80 ± 0.26	237360	5.2
immunoaffinity chromatography	11206 ± 361	10.0 ± 0.9	1120.6 ± 60.0	0.35 ± 0.11	392210	19.7

[a] The supernatant medium of 3T3 cultures was collected as described under Experimental Procedures. HABP were purified from this medium by filtration and affinity chromatography steps. An aliuqot from each purification step was obtained which typically contained 20–30 μg of protein. This sample was adsorbed to nitrocellulose-coated multiwells and then exposed to 15 μg of [^{3}H]hyaluronate (specific activity 1.5 μCi/mg of HA) for 1 h at 4 °C. These conditions allowed the quantitation of binding at equilibrium, which was previously shown to be achieved in 0.5 h and maintained for 4 h at 4 °C, and at saturation, which was achieved with 1 μg of [^{3}H]HA/10 μg of adsorbed HABP. Values represent the cpm bound to HABP minus the cpm that bound to control wells which did not contain HABP fractions (300 ± 178 cpm/well). Values represent the mean ● SEM of four replicates from one experimental series.

(96 wells, Millipore), overnight at 4 °C. More than 80% of the added protein (as judged by adsorption of ^{125}I-HABP bound under similar conditions) was thus attached to the nitrocellulose-coated wells. Wells were washed with TBS containing 0.02% Tween and then incubated with 10% propylene glycol (M_r 8000) for 1 h followed by 3% heat-inactivated (boiled for 1 min) calf serum for an additional hour. Both of these treatments reduced background labeling. Wells were washed extensively, incubated with 10000 cpm (≈3 μg) of [^{3}H]hyaluronate in 200 μL of TBS for 3 h, and then washed again with 10 volumes of ice-cold TBS. Each nitrocellulose membrane was cut out and counted in a scintillation counter. Maximal binding of [^{3}H]hyaluronate to 20 μg of HABP occurred at 0.1 μg of hyaluronate. Thus, all assays quantitating the binding of [^{3}H]hyaluronate to adsorbed HABP were conducted at binding saturation. A 100-fold excess (i.e., 300 μg) of hyaluronate, added together with [^{3}H]hyaluronate, reduced binding to adsorbed HABP by 80% (i.e., 2000 ± 623 cpm/well was reduced to 400 ± 89 cpm), indicating that specific binding of hyaluronate to adsorbed HABP was being measured.

(B) Binding of ^{125}I-HABP to HA. Hyaluronate (100 μg) was adsorbed to nitrocellulose-coated multiwells (Millipore) overnight at 4 °C. Wells were washed extensively with TBS containing 0.02% Tween and then incubated with 3% heat-inactivated calf serum and 10% propylene glycol as above to reduce background labeling. Approximately 20% of the added GAG adsorbed to filters, as quantitated by the amount of [^{3}H]hyaluronate bound under similar conditions. HABP isolated by hyaluronate or immunoaffinity chromatography from each cell source and labeled with ^{125}I was incubated in the wells for 1 h at 4 °C in TBS containing 3% calf serum and protease inhibitors (see Isolation of Supernatant HABP). Unbound ^{125}I-HABP was removed by washing with 10 volumes of TBS; nitrocellulose membranes were cut out, and the bound isotope was quantitated with γ spectrophotometry. Four to ten percent of added ^{125}I-HABP bound to 20 μg of hyaluronate in 2 h, representing maximal binding. Binding did not decline for an additional 4 h, and ^{125}I-HABP binding to hyaluronate, as previously shown (Turley & Moore, 1984), was reduced by 70–80% in the presence of a 50-fold excess of unlabeled HABP. Differences in binding capacity were analyzed with a paired Student's *t* test assuming unknown and unequal variance. *P* values of <0.05 were considered significant.

Results

Purification of Hyaluronate Binding Activity. The original supernatant medium collected from 3T3 cultures contained a variety of proteins (Figure 1A), the number of which were reduced by subsequent filtration (Figure 1B). However, filtration resulted in only a marginal increase in hyaluronate binding activity (Table I) probably because of the loss of hyaluronate binding activity (i.e., fibronectin; see Figure 1 and Table I) indicated by the drop in total hyaluronate binding activity after filtration (Table I, column 6). Hyaluronate affinity chromatography of the filtered medium resulted in an approximately 5-fold purification and, generally, isolation of three major proteins (Table I, Figure 1C) of $M_{r,e}$ 70K, 66K, and 56K (Figures 1C and 2). The 66K protein most often represented the major species (i.e., Figure 1C, Figure 2a), but this pattern varied (Figures 2b, and 6) with the three proteins sometimes occurring in equivalent amounts. Further, the estimated molecular weight of the smaller two bands varied between 65K–68K and 54K–57K (i.e., see Figures 1, 2, and 6). The reason for this variability is not known at present but may be due to glycosylation processing. Immunoaffinity chromatography of the three proteins using antibody generated against the larger two protein bands resulted in a 20-fold purification of hyaluronate binding activity and enrichment of the 66K protein band (Table I, Figure 1D). The recovery of the other two proteins, which were generally minor with respect to the 66K protein, after immunochromatography was probably due to low recovery (50%) during this additional

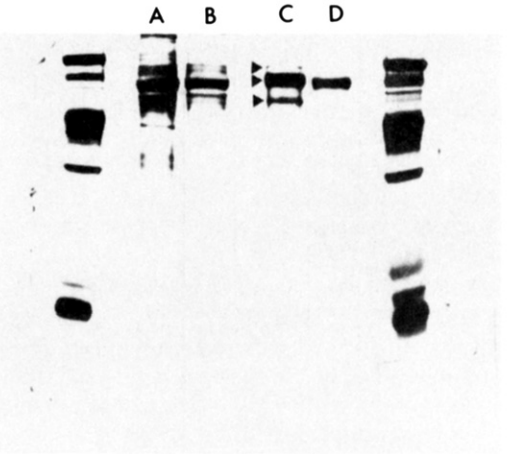

FIGURE 1: SDS–PAGE of proteins occurring in the supernatant media (A–D) collected from 3T3 cultures from which HABP were isolated (B–D). Lane A represents proteins detected in unfiltered medium originally collected from cultures, lane B represents medium that was filtered as described under Experimental Procedures, lane C represents the three typical proteins (marked by arrowheads) collected from hyaluronate affinity chromatography, and lane D represents protein subsequently isolated by immunoprecipitation with polyclonal antibody against the larger two proteins. Only the 66K protein (marked with an arrowhead) is now discernible. Molecular weight standards (14K–95K) were run along the side of the gel. Proteins were visualized by the silver stain method.

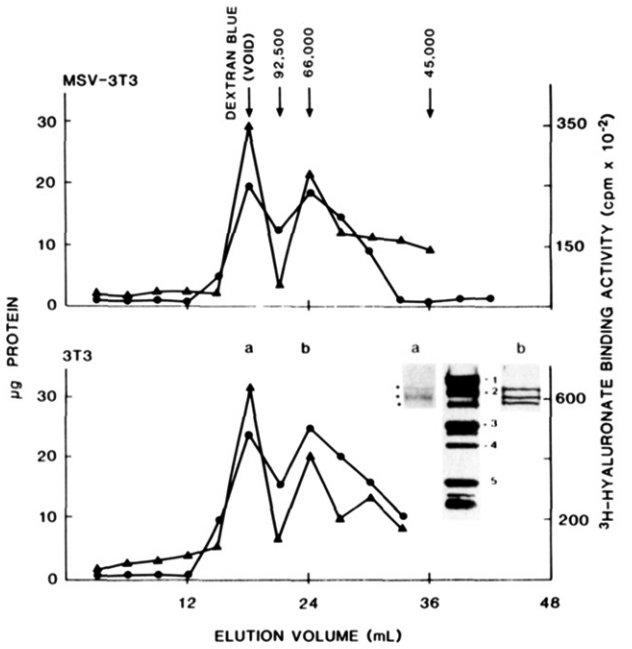

FIGURE 2: Separation of hyaluronate binding fractions with gel chromatography. Hyaluronate binding activity was isolated from supernatant medium of MSV-3T3 and 3T3 cell lines by chromatography on hyaluronate–Sepharose and then separated into several fractions by gel chromatography as described under Experimental Procedures. Protein (▲) that contained the majority of hyaluronate binding activity (●) chromatographed both in the void volume and in the 50K–70K molecular weight fraction. Both protein peaks (a, b) were resolved by SDS–PAGE into three major protein bands (marked by dots) of $M_{r,e}$ 70K, 65K, and 56K. Molecular weight standards included (1) phosphorylase *b* (95.5K), (2) bovine serum albumin (66.2K), (3) ovalbumin (45K), (4) carbonic anhydrase (31K), and (5) soybean trypsin inhibitor (21K).

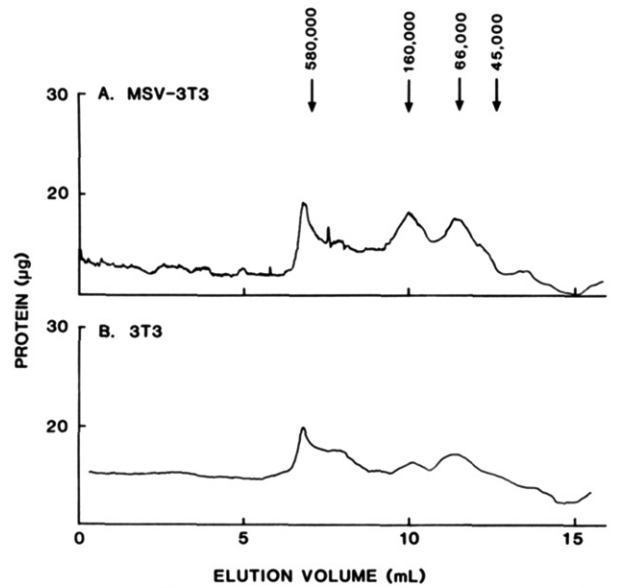

FIGURE 3: HPLC gel chromatography of HABP isolated from MSV-3T3 and 3T3 cells. MSV HABP (A) and 3T3 HABP (B) were applied to an HPLC gel filtration column and chromatographed as described under Experimental Procedures. Effluent protein was detected with a UV monitor at 289 nm. Protein aggregates of up to 580000 daltons were discernible. Molecular weight standards from 45K to 580K were used to size the protein aggregates.

purification step, since all three proteins cross-reacted with the antibody (Figure 6).

Identification of Hyaluronate Binding Activity. As noted above, SDS–polyacrylamide gel electrophoresis of HABP isolated from both MSV-transformed 3T3 and 3T3 cell cul-

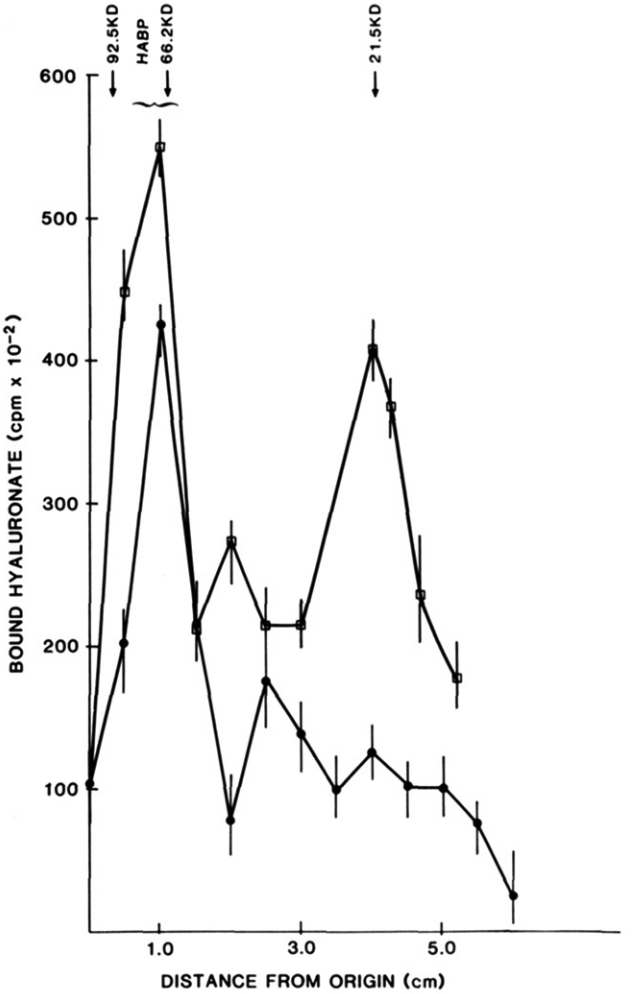

FIGURE 4: Localization of hyaluronate binding activity to the 56K–70K proteins separated by PAGE. MSV HABP (●) and 3T3 HABP (□) were separated on a nonreducing PAGE and then electrically transferred to nitrocellulose sheets as described under Experimental Procedures. Strips of nitrocellulose sheets containing the transferred protein were incubated with 5×10^5 cpm of [^{3}H]hyaluronate (specific activity 1.5 μCi/mg of polymer) in TBS + 1% BSA for 4 h at room temperature. Sheets were washed in ice-cold TBS and then cut in half lengthwise. One strip was stained with Amido black to locate each protein species, and the other was cut into 1-cm strips and counted in a scintillation counter. Values represent the mean of six experimental series ± SEM.

tures by hyaluronate affinity chromatography generally revealed three protein bands of $M_{r,e}$ 70K, 66K, and 56K (Figures 1 and 2a,b, dots) whose motility was not substantially altered by electrophoresis using nonreducing conditions (Figure 6). Gel filtration of these HABP on Bio-Gel P-100 in PBS at physiological pH resulted in the appearance of protein in the void volume which HPLC resolved into molecular weights of up to 580000 (Figure 3). These high molecular weight fractions appeared to represent aggregate formation of the three HABP since they were resolved by SDS–PAGE into the original protein bands of $M_{r,e}$ 70K, 66K, and 58K (Figure 2a,b). However, whether aggregation occurred from protein–protein interactions or other mechanisms (i.e., hyaluronate) is not known at present.

Analyses of the hyaluronate binding activity in the hyaluronate affinity-purified fraction that was subsequently separated by gel chrmomatography or SDS–PAGE are shown in Figures 2 and 4, respectively. Gel filtration separated binding activity into fractions eluting both in the void volume and in the 50K–70K molecular weight range (Figure 2). Both peaks of activity were resolved into 56K, 66K, and 70K pro-

Table II: Characteristics of MSV HABP and 3T3 HABP[a]

source	time of labeling (h)	cpm/μg of cell protein				
		[^{35}S]methionine	[^{3}H]mannose	[^{3}H]galactose	[^{3}H]glucosamine	[^{3}H]galactosamine
(a) 3T3 cultures	12	60300 ± 9350	20800 ± 7356	80900 ± 12891	9815 ± 5231	1896 ± 676
(b) MSV-3T3 cultures	12	83900 ± 11262	13000 ± 1350	73800 ± 11697	3670 ± 781	694 ± 81.2
(c) 3T3 cultures	0	1921 ± 422				
(d) fixed 3T3 cultures	12	3610 ± 59				
(e) medium only	12	2820 ● 73				

[a] Cultures (a and b) were incubated with 10 μCi of either [^{35}S]methionine (specific activity >400 Ci/mmol), ^{3}H]mannose (specific activity 40–60 Ci/mmol), [^{3}H]galactose (specific activity 20–40 Ci/mmol), [^{3}H]glucosamine (specific activity 10–30 μCi/mmol), or [^{3}H]galactosamine (specific activity 10–25 μCi/mmol; NEN) in PBS, supplemented with 1 mg of glucose/mL and 10% fetal calf serum, for 12 h. Medium was collected, and radiolabeled HABP was isolated by affinity chromatography as described under Experimental Procedures. Additionally, several controls were conducted to assess whether labeled protein was due to the activities of cells. Live 3T3 cultures (c) were briefly exposed to [^{35}S]methionine (≈1 min), and then HABP was isolated as above. 3T3 cultures were also fixed with 0.25% glutaraldehyde (d) and then exposed to [^{35}S]methionine for 12 h after which HABP was collected as above. As a final control, [^{35}S]methionine was added for 12 h to medium (DME + 10% FCS) that had been conditioned by 3T3 cells and from which HABP was collected as above. Values repesent the mean ± SEM of four cultures.

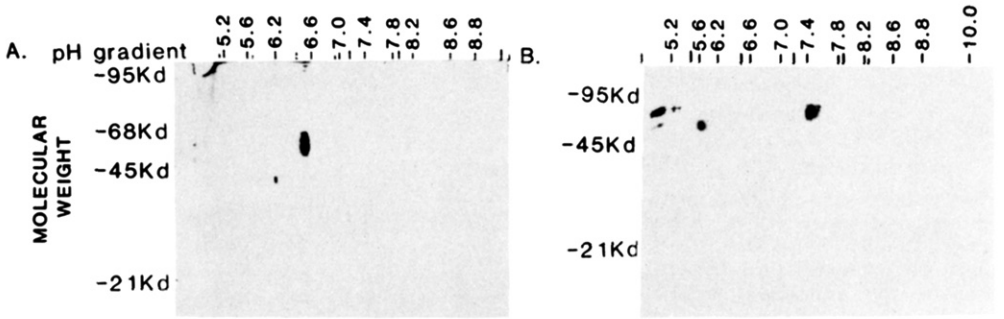

FIGURE 5: Two-dimensional electrophoresis of HABP isolated from (A) MSV-3T3 and (B) 3T3 cell lines. For the first dimension, proteins were applied to isoelectric focusing gels (6 cm) and electrophoresed at 400 V for 16 h at 4 °C over a pH gradient ranging from 4 to 10. Gels were then placed over a 12% SDS–PAGE and electrophoresed at 15 mA per gel to achieve final separation of the proteins. Proteins were visualized with a silver stain.

teins on SDS–PAGE (Figure 2a,b). The transfer of the 56K–70K HABP, after their separation on SDS–PAGE, to nitrocellulose membranes also localized the hyaluronate binding activity to these proteins (Figure 4). In samples isolated from 3T3 cells, additional hyaluronate binding activity was localized to a 20K molecule that did not appear after silver staining of the acrylamide gel or amido black staining of the nitrocellulose membrane.

Characterization of HABP. (*A*) *Radiolabeling.* HABP isolated from cell cultures could be labeled with [^{35}S]-methionine (Table II), suggesting that they were cell-derived proteins. This conclusion was strengthened by the observation that very little [^{35}S]methionine-labeled material was isolated from cultures fixed prior to the addition of label, from 3T3 cultures exposed to label for <1 min, or from cell-conditioned growth media incubated with [^{35}S]methionine (Table II).

The incorporation of ^{3}H-sugars into HABP (Table II) suggested a glycoprotein nature. The relatively small incorporation of [^{3}H]galactosamine provided evidence, additional to the immunoreactivity data given below, that HABP were distinct from proteoglycans.

(*B*) *Isoelectric Points.* The isoelectric points of HABP, isolated from 3T3 and MSV-transformed 3T3 cultures by hyaluronate affinity chromatography, are shown in Figure 5. The three HABP isolated from MSV-3T3 cultures exhibited a p*I* of approximately 6.5 (Figure 5A). In contrast, the 3T3 HABP exhibited several p*I* values of 4.8, 5.2, 5.6, and 7.5 (Figure 5B). All 3T3 HABP molecular weight species focused at pH 4.8 and 7.5 while additional, small amounts of 70- and 56-kDa protein focused at pH 5.2 and 5.6, respectively. These results not only indicated a structural heterogeneity of 3T3-derived HABP, suggestive of varying degrees of glycosylation, but also pointed to structural differences between 3T3- and MSV-3T3-derived HABP.

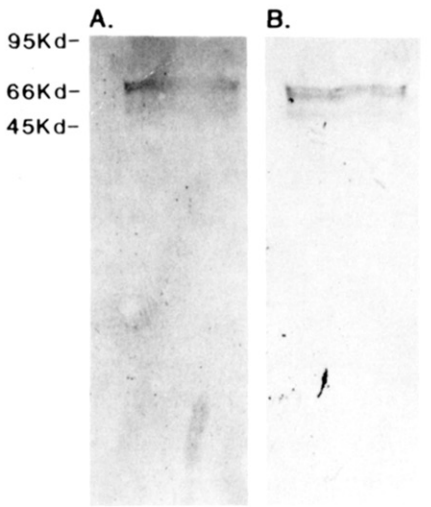

FIGURE 6: Immunochemical characterization of HABP. HABP was isolated from MSV-3T3 (A) and 3T3 (B) cell lines, electrophoresed on nonreducing PAGE, and then electrically transferred from PAGE to nitrocellulose sheets. Blotted proteins were incubated with antibody prepared against the 66K–70K 3T3 HABP and visualized with rabbit IgG linked to peroxidase that was developed with a CN/H_2O_2 solution. All three molecular weight species of HABP from both cell sources cross-reacted with the antibody, indicating they shared common antigenic determinants. Magnification 8×.

(*C*) *Immunological Cross-Reactivity.* Previous ELISA studies showed that 3T3 HABP and MSV HABP were antigenically related to one another (Turley & Moore, 1984). The three protein species present within the HABP fraction isolated by hyaluronate affinity chromatography from both cell sources also cross-reacted with one another (Figure 6), raising the possibility that they were processed fragments of one another. ELISA assays demonstrated that HABP from

Table III: Antigenic Properties of HABP[a]

protein[b]	antibodies to 3T3 HABP[c]	antibodies to hyaluronate binding region of chondroitin sulfate[d] proteoglycan
KLH-3T3 HABP	1.85	0.01
fibronectin	0.05	ND[e]
link protein	0.01	ND
chondroitin sulfate proteoglycan	0.02	2.10

[a] Values represent spectrophotometer readings of an ELISA assay at 405 nm. All values have also been substrated for buffer controls (0.02), and 3T3 HABP has been additionally subtracted for KLH controls (0.09; Turley & Moore, 1984). [b] 0.5 μg of HABP, derivatized to KLH to facilitate its adsorption (Turley & Moore, 1984), was used to coat each well in Linbro multiwell dishes. 20 μg of other proteins was adsorbed, without KLH, to each well. Under these conditions, over 80% of the applied proteins adsorbed to surfaces. [c] Antibodies to the largest 3T3 HABP (66–70 kDa) (1:40 dilution) were used in these experiments. [d] Antibodies to chondroitin sulfate proteoglycan were used at 1:100, a dilution that was sufficient to detect <1.0 μg of proteoglycan. [e] ND, not determined.

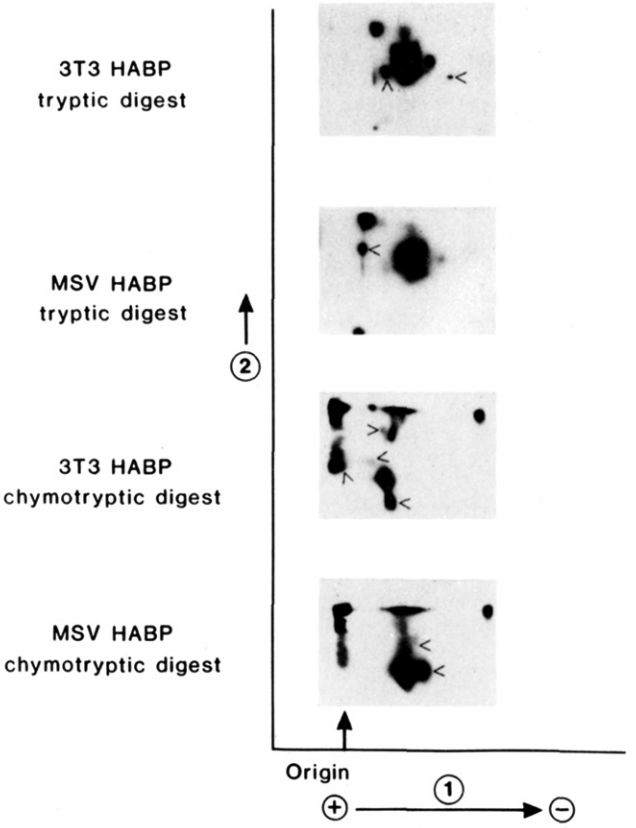

FIGURE 7: Tryptic fingerprints of HABP isolated from 3T3 and MSV-3T3. ^{125}I-HABP prepared from MSV-3T3 or 3T3 cell lines were separated on SDS–PAGE; protein bands were cut out and digested with either trypsin or chymotrypsin. The proteolytic digests were then electrophoresed (1) at 400 V for 45 min in acetic acid/formalin buffer and chromatographed (2) in butanol/pyridine/acetic acid to separate peptide fragments. Autoradiography was used to visualize the tryptic and chymotryptic fingerprints. HABP isolated from MSV-3T3 and 3T3 cell lines exhibited similar peptide fragments although some proteolytic products were unique to both 3T3 HABP and MSV HABP. Unique products are marked with arrowheads.

either cell source were antigenetically distinct from fibronectin, cartilage link protein, and the hyaluronate binding region of the cartilage chrondroitin sulfate proteoglycan (Table III).

(D) Peptide Maps of HABP. HABP from both cell sources contained a number of peptide fragments including a large central segment (Figure 7) that was not apparent when HABP was digested with chymotrypsin. Generally, chymotrypsin digestion of HABP produced smaller peptide segments than

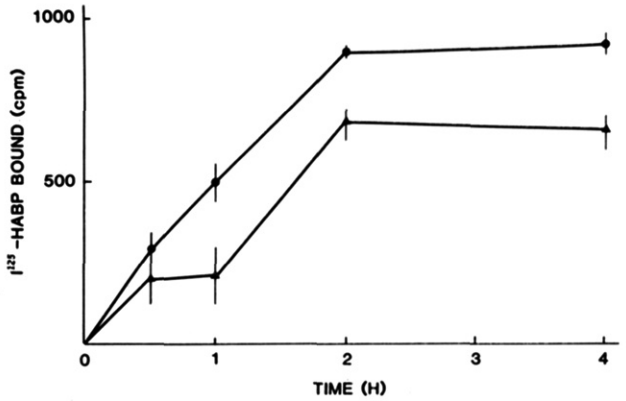

FIGURE 8: Binding equilibrium of ^{125}I-HAPB to hyaluronate. The binding of ^{125}I-HABP isolated from MSV-3T3 (▲) and 3T3 (●) cell lines to hyaluronate was examined over time. ^{125}I-HABP (6×10^5 cpm/well, specific activity 4.5 μCi/μg of protein) was added to 20 μg of hyaluronate adsorbed to nitrocellulose membranes in the presence of 1% BSA at 4 °C. HABP that bound to the hyaluronate-coated nitrocellulose membranes was detected on a γ counter. Values represent the mean ± SEM of 10 replicates.

Table IV: Binding Capacity of ^{125}I-HABP for Hyaluronate[a]

experiment	ng of HABP bound/μg of HA	
	3T3 HABP	MSV HABP
1	42 ± 8.6	6.0 ± 1.1
2	10 ± 2.1	2.4 ± 0.5
3	31.8 ± 12.2	21.6 ± 6.5
4	60.0 ± 15.6	4.5 ± 0.8
mean ● SEM	39.95 ± 20.86	8.6 ± 8.8

[a] ^{125}I-HABP (specific activity 4.0 μCi/μg of protein) was allowed to bind a layer of hyaluronate (20 μg/well) that was adsorbed to nitrocellulose wells as described under Experimental Procedures. Bound ^{125}I-HABP was counted in a γ counter, and binding capacity to hyaluronate was calculated. Values for each experiment (conducted on a single plate) represent mean ± SEM of four samples. A Student's paired t test revealed no significant difference ($t = 2.34$, $df = 3.0$, $p = 0.34$) between the pooled mean binding capacity of 3T3 HAPB compared to MSV HABP for all of the experiments, but all but experiment 3 were statistically different within an experiment ($P < 0.05$).

trypsin (Figure 7). Consistent with results indicating immunological cross-reactivity, the majority of the peptide digests from both cell sources comigrated. However, peptide fingerprinting showed that 3T3 HABP and MSV 3T3 HABP also contained distinct proteolytic products suggestive of structural differences (Figure 7, arrowheads).

Binding Properties of HABP. Specific (i.e., competible; see Figure 9) binding of 3T3 ^{125}I-HABP and MSV ^{125}I-HABP to hyaluronate was maximal by 2 h (Figure 8). Analysis of binding that occurred at this equilibrium was carried out by using three methods: (1) a plot of bound HABP vs. increasing free HABP concentration (Figure 9); (2) a double-reciprocal plot of bound vs. free HABP (Figure 10); and (3) a Scatchard analysis of data in Figure 9 [Scatchard (1949) (Figure 11)]. All of these methods of analysis demonstrated complex kinetic interactions ($K_D \sim 10^{-8}$ M) which were compatible with positive cooperativity between ligand (which in these assays was HABP) and receptor (hyaluronate) as discussed by Boeynaems and Dumont (1975). Apparent differences in binding capacity (Table IV) between HABP isolated from 3T3 and MSV-3T3 cell cultures were not statistically significant using a paired Student's t test to assess the pooled means from four separate experiments. However, a good portion of the variability among experiments could be accounted for by the differential adsorption of hyaluronate to each plate. When results within our experimental series were compared, all but experiment 3 were statistically different ($P < 0.05$), suggesting

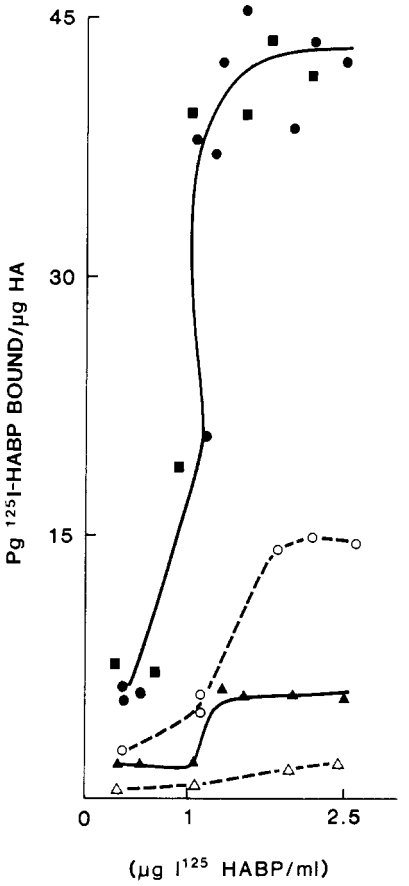

FIGURE 9: Binding isotherm of ^{125}I-HABP to hyaluronate. Increasing concentrations of ^{125}I-HABP (specific activity 4.5 μCi/μg of protein) isolated by hyaluronate affinity chromatography from 3T3 (●) and MSV-3T3 (▲) lines with (open circles or triangles) or without (closed circles or triangles) 50-fold excess of unlabeled HABP were added to hyaluronate adsorbed to nitrocellulose membranes. Increasing amounts of ^{125}I-HABP (specific activity 5.2 μCi/μg of protein) isolated by immunoaffinity from 3T3 cultures (■) were also added to hyaluronate-coated filters and gave a similar binding isotherm. Values represent the mean ± SEM of 10 experiments.

that the binding capacity of 3T3 HABP was greater than MSV-3T3 HABP. Further binding studies using additional assays will be required to further test this. A result was considered statistically significant only if $P \leq 0.05$.

Discussion

This report characterizes some of the properties of several small hyaluronate binding proteins (HABP) isolated from the culture medium of virally transformed 3T3 cells and their parent cell line. We conclude that hyaluronate binding activity is associated with 56K–70K glycoproteins, that these glycoproteins are structurally similar to one another but are distinct from other characterized hyaluronate binding proteins, and that some of their structural properties are altered with viral transformation of the cell source.

Several observations indicate that hyaluronate binding activity is associated with the 56K–70K proteins. First, an enrichment for hyaluronate binding activity is obtained with purification of the HABP. The relatively low level of enrichment achieved with hyaluronate affinity chromatography (5-fold) in comparison to immunoprecipitation of the larger HABP is puzzling but could result from either hyaluronate contamination from hyaluronate affinity columns that interfer with binding assays and is lost after immunoprecipitation or regulation of binding by the smaller HABP or even the 20-kDa fraction. These alternatives are being investigated. Second,

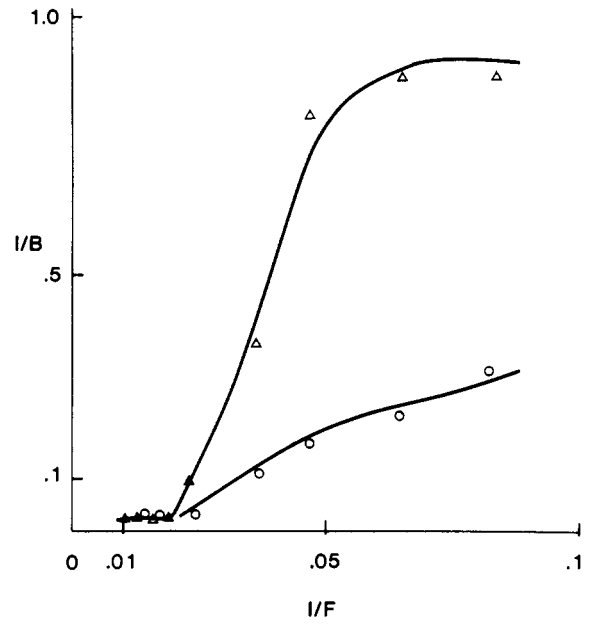

FIGURE 10: Double-reciprocal plot analysis of binding ^{125}I-HABP to hyaluronate. The binding isotherm presented in Figure 8 was analyzed by using the reciprocal plot method: MSV-3T3 HABP (○) and 3T3 (△) HABP.

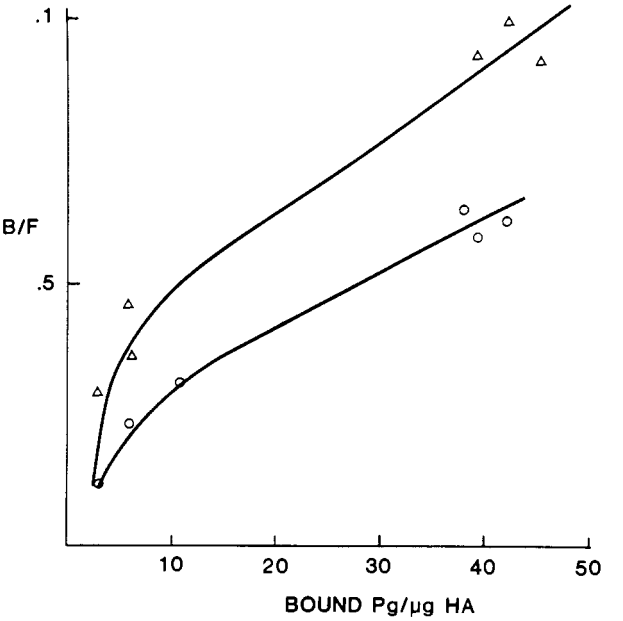

FIGURE 11: Scatchard analysis of the binding of ^{125}I-HABP to hyaluronate. Scatchard analysis of data from Figure 9: MSV-3T3 (○) and 3T3 (△).

[^{3}H]hyaluronate binding activity colocalizes with HABP fractionated by both gel filtration and SDS–PAGE. Third, purified ^{125}I-labeled HABP specifically (i.e., competible with unlabeled HABP) binds to hyaluronate-coated nitrocellulose in a dose-dependent manner.

The HABP described here appear to represent a unique class of proteins that are antigenically distinct from other hyaluronate binding, matrix-associated proteins such as fibronectin, cartilage chondroitin sulfate proteoglycan, and cartilage link proteins. However, the HABP isolated from culture supernatant medium resemble other, small hyaluronate binding proteins isolated from serum and brain tissue in their approximate molecular weight (Delpech & Delpech, 1984) as well as isoelectric point (Delpech & Delpech, 1984) and may be related or identical proteins. Blenis and Hawkes (1984) have also reported hyaluronate binding activity associated with

a 20K trypsin-resistant protein that adsorbs to growth surfaces. This protein could represent a proteolytic fragment of the HABP described here or, alternatively, may be related to the as yet unidentified 20K binding activity isolated from 3T3 cells.

The difference in peptide fingerprints and isoelectric points in HABP isolated from 3T3 and MSV-3T3 cell lines suggests structural differences that occur with viral transformation of the cell source. This observation is consistent with previous reports that note differences in the hyaluronate binding characteristics of SV40-3T3 vs. 3T3 cell lines (Underhill et al., 1983; Goldberg et al., 1983). Goldberg et al. (1983) suggest that these data indicate the occurrence of binding sites on 3T3 cells that are distinct from those on SV40-3T3 cell lines and infer that such differences contribute to these cell line's divergent responses to hyaluronate (Underhill & Dorfman, 1978; Turley & Chapman, 1986). The presence of the additional binding site on 3T3 cells noted here is consistent with this notion. However, the difference in the HABP isolated from 3T3 cells relative to SV40-3T3 suggests that structural changes of one type of binding site also occur with viral transformation that may contribute both to the documented differences in the binding characteristics of hyaluronate for intact 3T3 and SV40-3T3 cells (Underhill et al., 1983) and, ultimately, to the altered response of virally transformed 3T3 cells to hyaluronate relative to their parent line (Underhill & Dorfman, 1978; Turley & Chapman, 1986).

The relationship of HABP described here to endogenous hyaluronate binding sites detected by kinetic studies (Toole, 1982; Underhill et al., 1983; Smedsrod et al., 1984) will require further study. However, evidence that HABP, or antigenically related molecules, represent one class of cell-associated hyaluronate binding sites is as follows. First, HABP occur on cell surfaces, particularly on motile cells (Turley & Torrance, 1985) where they codistribute with newly bound hyaluronate. Second, like endogenous sites, HABP are glycoproteins of about 60–80 kDa that bind to hyaluronate in a Ca^{2+}/Mg^{2+}-independent manner with high affinity and with complex (i.e., cooperative) interactions (Underhill et al., 1983; Smedsrod et al., 1984). That the HABP described here exhibit more complex (i.e., nonlinear Scatchard plots) binding interactions than those reported for the endogenous sits in either intact cells (Underhill et al., 1983; Smedsrod et al., 1984) or lipid vesicles (Underhill et al., 1983) may reflect either differences in the methods used to measure binding kinetics (i.e., solubilized receptors or receptors in a membrane vs. their adsorbtion to cellulose acetate) or differences in the properties of proteins associated with the cell as compared to those released into supernatant medium. Third, the hyaluronate binding activity of HABP fulfills the normal criteria of binding site–ligand interactions including the occurrence of binding that is related to the concentration of the ligand, the occurrence of binding saturation with increasing ligand concentration, and the ability to competitively inhibit binding with unlabeled ligand.

In summary, binding and biochemical properties of a hyaluronate binding protein fraction isolated by hyaluronate and immunoaffinity chromatography from both MSV-transformed 3T3 and the parent cell line are described. Proteins are distinct from other characterized, hyaluronate binding proteins such as fibronectin and cartilage proteoglycans but resemble cellular hyaluronate receptors that have been described by binding analyses and other small hyaluronate binding proteins. Although related, 3T3 and MSV HABP exhibit distinct structural characteristics that may be important to hyaluronate–cell interactions.

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Registry No. Hyaluronate, 9004-61-9.

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