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Hyaluronate-Binding Protein of Simian Virus 40-Transformed 3T3 Cells: Membrane Distribution and Reconstitution Into Lipid Vesicles

Gloria Chi-Rosso and Bryan P. Toole

Department of Anatomy and Cellular Biology, Tufts University Schools of Medicine, Dental Medicine, and Veterinary Medicine, Boston, Massachusetts 02111

Hyaluronate-binding protein (HABP) has been extracted in detergent from the membranes of simian virus 40-transformed 3T3 (SV-3T3) cells (Underhill et al, J Biol Chem 258:8086–8091, 1983). When SV-3T3 cells were treated with trypsin prior to isolation and dissolution of the membranes, no hyaluronate-binding activity could be detected. This indicates that all of the detectable HABP of SV-3T3 cells is located on the external surface of the plasma membrane rather than on internal membranes, which would be inaccessible to the trypsin. The detergent-extracted HABP from SV-3T3 membranes was reconstituted into the membrane of lipid vesicles, which were formed by addition of exogenous phosphatidylcholine and cholic acid to the extracts followed by removal of detergent by dialysis against 0.02 M Tris pH 8.0 in the presence of protease inhibitors. Reconstitution was assessed by sedimentation in a discontinuous sucrose gradient and by gel filtration on Sepharose 4B in the presence and absence of detergent. The characteristics of binding of hyaluronate to the reconstituted HABP were then compared with those studied previously for the original membrane-bound HABP and the detergent-extracted HABP (Underhill et al, J Biol Chem 258:8086–8091, 1983). It was observed previously that binding of hyaluronate to HABP in the cell membranes was of higher affinity and specificity than to HABP in the detergent extracts of these membranes. It was found here that reconstitution of the extracted HABP into the membranes of lipid vesicles led to restoration of affinity of binding to the level observed in the original cell membranes. However, whereas chondroitin sulfate does not compete significantly for binding of hyaluronate to cell membrane-bound HABP, partial competition was observed for the reconstituted HABP as well as for detergent-extracted HABP. Thus, it is concluded that the high affinity of binding of hyaluronate to the plasma membrane of SV-3T3 cells is in part dependent on insertion of the HABP in the membrane, but that other interactions, not duplicated in our reconstitution experiments, must be necessary for the specificity of the HABP.

Key words: hyaluronate receptor, cell-matrix interaction

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Several types of virally transformed cells aggregate via a divalent cation-independent mechanism that is mediated by cell surface-associated hyaluronate (HA) [1–3]. In previous studies we have shown that simian virus 40-transformed 3T3 (SV-3T3) cells exhibit specific, high-affinity sites that bind exogenous HA to and retain endogenous HA at the surface of these cells [4–6], and which also appear to be involved in the above aggregation process [2,7]. When SV-3T3 cells are homogenized and then fractionated by low- and high-speed centrifugation, most of the HA-binding sites are recovered in the high-speed pellet along with the bulk of the cell membranes [8].

Hyaluronate-binding protein (HABP) can then be extracted from these membranes in detergent solutions [8] and has been shown recently to have a molecular weight of approximately 85,000 [9]. However, the soluble HABP binds HA with lower affinity and less specificity than the membranes from which it was derived. In the experiments described herein we demonstrate that, if the cells are treated with trypsin prior to their disruption and the isolation of the membranes, no binding activity is recovered in the membranes. Also we show that detergent-extracted HABP can be reconstituted back into the membranes of artificial lipid vesicles wherein its affinity is restored to that observed for the original cell membranes. Thus, we conclude that the HA-binding protein of SV-3T3 cells is located at the plasma membrane, is intercalated into the membrane via a hydrophobic protein domain, and interacts with HA in a manner that is in part dependent on its insertion in the membrane.

METHODS

Cell Cultures

The SV-3T3 cell line was the same as was used previously [4] and was cultured in 100-mm tissue culture dishes (Falcon) in Dulbecco's modified Eagle's medium (4.5 mg/ml glucose) supplemented with 10% calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a moist atmosphere of 5% CO_2/95% air. In some cases, the SV-3T3 cells were cultured in large roller bottles, and membranes were prepared therefrom at the Cancer Center, Massachusetts Institute of Technology.

Isolation of Cell Membranes

The SV-3T3 cells were harvested from cultures by rinsing twice with Ca^{++}- and Mg^{++}-free phosphate-buffered saline (CMF-PBS) and then incubating the cells in 0.02% ethylenediamine tetraacetic acid (EDTA) in CMF-PBS for 3 min. After centrifugation the cells were washed with CMF-PBS and then suspended in 20 vol 0.01 M Tris pH 8.0 containing 1 mM phenylmethylsulfonyl fluoride (PMSF). After standing in an ice bath for 1 hr to allow the cells to swell, they were lysed with a Polytron (Brinkmann) at a setting of 7 for 15 sec. Two volumes of 0.5 M sucrose in 0.02 M Tris pH 8.0 were added, and the suspension was centrifuged at 2,000g for 15 min to remove nuclei and unlysed cells. The supernatant was recentrifuged at 30,000g for 20 min to pellet the membrane-containing fraction, which was then resuspended in 0.3 M sucrose/0.02 M Tris pH 8.0 at a concentration of 3–5 mg protein/ml as estimated by the method of Lowry et al [10]. These suspensions were stored at −80°C for periods up to 4 months without loss of HABP activity.

Detergent Extraction of HABP

The membrane suspensions were then mixed with an equal volume of 1% deoxycholate DOC (Fisher) in .02 M Tris pH 8.0/1 mM PMSF, stirred gently in an ice bath for 30 min, and centrifuged at 110,000g for 1 hr. The supernatants were used for subsequent experiments.

Hyaluronate-Binding Assay

Unless specified otherwise the following modification of the method of Underhill et al [8] was used. The assay mixture contained 0.02 M Tris pH 8.0, 50–100 μl HABP-containing membrane suspension, vesicle suspension, or DOC extract thereof, and 25 μl of 100 μg/ml ^{3}H-HA in CMF-PBS. The ^{3}H-HA was prepared as described previously [4] and had a specific activity of 1–1.5 $\times$ 10^5 cpm/μg. The assay mixture was adjusted to a volume of 500 μl with CMF-PBS and shaken for 20 min at room temperature. The complex of HA and HABP was precipitated by the addition of 100 μl of 10 mg/ml casein (as protein carrier) plus 600 μl of saturated ammonium sulfate. The mixture was then centrifuged at 9,000g (Beckman Microfuge) for 5 min, and the resulting pellet was washed three times with 50% saturated ammonium sulfate. The pellet was dissolved by heating at 50°C for 10 min in 600 μl 1% sodium dodecyl sulfate, (SDS) and the radioactivity measured in a scintillation counter after the addition of 6 ml of scintillation cocktail. To determine nonspecific background binding, 50 μg unlabeled HA was included in the above assay mixture. All values represent the average of duplicate determinations and are expressed as specific binding, ie, background has been subtracted. Background varied between 10% and 30% of total cpm from experiment to experiment and batch to batch of ^{3}H-HA.

Partial Purification of HABP and Production of Antisera

Detergent-extracted HABP, partially purified by wheat germ agglutinin affinity chromatography, was used as antigen for production of antisera. For chromatography, 5 ml of 0.5% DOC extract (approx 2 mg protein/ml) was applied to a column (0.7 $\times$ 10 cm) containing 2 ml of wheat germ agglutinin-agarose (E-Y Labs), which was pre-equilibrated with .02 M Tris pH 8.0/3.30 μM PMSF/3.33 mM EDTA/1.67 mM NEM/3.30 μM benzamidine (buffer A) containing 0.5% DOC. The column was then washed with 15 ml of 0.5% DOC/buffer A, and the HABP was eluted with 15 ml 0.5% DOC/buffer A containing 0.3 M N-acetylglucosamine. The eluate was dialyzed and concentrated by ultrafiltration under vacuum in a collodion bag immersed in 0.02 M Tris pH 8.0. The recovery of HABP in this procedure was 80–100%, and a 25-fold purification was obtained. SDS-polyacrylamide gel electrophoresis [11] of the preparation followed by Coomassie blue staining showed four major protein bands.

Rabbits were injected with the above preparation at four different sites at 3-week intervals. The first set of injections was performed in complete Freund's adjuvant and subsequent injections in incomplete Freund's. Serum was collected from the animals prior to any injections (preimmune serum) and at 7 days after the third and fourth sets of injections of antigen (antisera I and II, respectively).

RESULTS AND DISCUSSION

Membrane Distribution of HABP

Previous experiments have shown that most of the HABP of SV-3T3 cells is membrane bound [8] and that the HABP is a protein sensitive to trypsin [4]. The

following experiments were performed to determine the proportion of HABP bound to intracellular membranes versus the plasma membrane.

SV-3T3 cells were treated with 0.01% trypsin for 30 min at 37°C in CMF-PBS. As shown previously [4] and as confirmed in two separate experiments here (data not shown), this treatment destroys HABP exposed at the cell surface. However, this treatment would not affect HABP bound to internal membranes since they are not accessible to the trypsin. Cells treated with trypsin as above and cells incubated in parallel in CMF-PBS alone were then washed twice with CMF-PBS containing 0.05% trypsin inhibitor (Sigma) and once with CMF-PBS alone. Total cell membranes were prepared from these two sets of cells as described in "Methods." Both membrane preparations were then dissolved in 0.5% DOC, and HABP activity was measured also as described in "Methods." As can be seen from Table IA, activity was obtained for the membranes prepared without previous exposure of the cells to trypsin, but the membranes from the trypsinized cells exhibited no activity.

In a second type of experiment, SV-3T3 cells were incubated with or without trypsin as above and washed; then the whole cells were dissolved in 0.5% DOC, centrifuged at 110,000g for 1 hr, and HABP activity was measured in the resulting supernatants and pellets. This protocol provides a measure of soluble as well as membrane-bound HABP in the SV-3T3 cells. It also provides a control for the possibility that trypsinization causes the membrane-bound HABP to become redistributed in the membrane preparation procedure used in the first protocol above. Activity was only found in extracts of the cells that were not treated with trypsin (Table IB). No activity was present in the pellets from centrifugation of the DOC extracts. The lower specific activity obtained for extracts of the nontrypsinized cells compared to that obtained in the previous experiment using only the isolated membranes (Table IB vs IA) was due to the far greater amount of protein in the whole-cell extract than in the membrane extracts.

It is concluded from the above experiments that all of the detectable HABP activity in SV-3T3 cells is exposed at the outer surface of the plasma membrane (and thus is accessible to the trypsin), and that intracellular pools are too small in amount to be detected or are in inactive form.

Reconstitution of HABP Into Lipid Vesicles

DOC extracts of membranes prepared as described in "Methods" were mixed with phosphatidylcholine and cholic acid (4:1), then the resulting solutions were

TABLE I. Membrane Distribution of HABP in SV-3T3 Cells*

Source of DOC extract	Experiment no.	Specific binding (cpm/mg protein) Nontrypsinized cells	Trypsinized cells
A. Cell membranes	1	38,210	0
	2	54,000	0
B. Whole cells	1	8,220	0
	2	7,750	0

*DOC extracts were prepared from either A) membranes derived from SV-3T3 cells that were preincubated in the presence or absence of trypsin or B) whole cells preincubated in the presence or absence of trypsin (see text for details). All binding values are averages of duplicates and have been corrected for background. Variation in the duplicate values was less than 5% in all cases.

dialyzed to remove detergent and centrifuged [12,13]. Routinely, 5 ml of extract was mixed with 8 mg of dipalmitoyl phosphatidylcholine (Sigma), 2 mg of cholic acid (Sigma), and a trace of ^{14}C-dipalmitoyl phosphatidylcholine (New England Nuclear) to monitor phospholipid distribution in subsequent manipulations. The mixture was then dialyzed twice against 1 liter of 0.02 M Tris pH 8.0/1 mM PMSF/10 mM EDTA/5 mM N-ethylmaleimide/1 mM benzamidine at 4°C for 48 hr to remove the DOC detergent, thus leading to the formation of lipid vesicles [12]. The dialyzed solution was centrifuged at 110,000g for 1 hr, and the pellet, which contained the reconstituted lipid vesicles, was suspended in 0.02 M Tris pH 8.0. The HA-binding activities of this resuspended pellet fraction and the supernatant from the centrifugation were then measured, and virtually all of the HABP activity was found to be present in the vesicle fraction. In three experiments the recovery of HABP in the vesicle fraction averaged 88%.

To further document the reconstitution of HABP into the vesicles, they were centrifuged in a discontinuous sucrose gradient constructed from solutions of 60%, 30%, and 17% sucrose. The vesicles were found to band at the interface of the 17% and 30% sucrose as determined by the distribution of ^{14}C-phosphatidylcholine (Fig. 1B). Most of the HABP comigrated with the vesicles (Fig. 1A). The recovery of HABP activity from the gradient was approximately 80%. The fractions containing both the vesicles and HABP were then pooled as indicated by the bar in Figure 1 and applied to a Sepharose 4B column in the presence or absence of detergent [14]. Figure

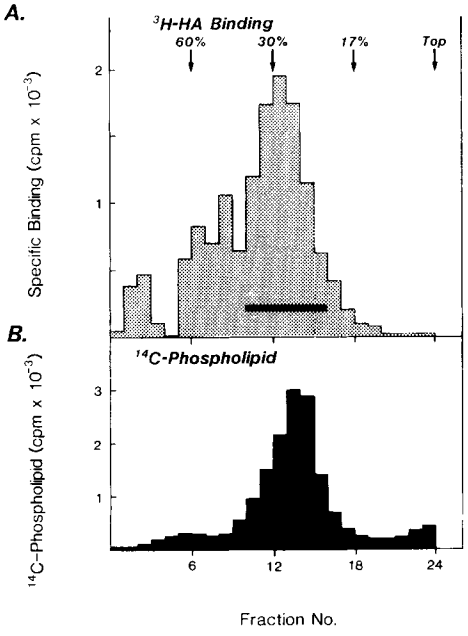

Fig. 1. Sucrose density gradient fractionation of reconstituted lipid vesicles. A discontinuous gradient was constructed using layers of 2.5 ml each of 60%, 30%, and 17% sucrose (the arrows indicate the top of each of these layers). The sample (2.5 ml) containing reconstituted vesicles (see text) was applied to the top of the gradient, and the gradient was then centrifuged at 26,000 rpm for 16 hr in a Spinco SW27 rotor. Fractions (400 μl) were collected, and samples of each (100 μl) analysed for A) HA-binding activity; B)^{14}C-phosphatidylcholine content. Fractions 10–16 were pooled (as indicated by the bar in A) for subsequent experiments.

2 shows the results of chromatography of the vesicles without the addition of detergent. Virtually all of the vesicles, as measured by ^{14}C-phospholipid distribution, were eluted in the void volume (Fig. 2B), as was approximately 65% of the HABP (Fig. 2A). In three separate experiments, the proportion of HABP eluting in the void volume varied between 65% and 100%. This variability was presumably due either to inefficient reconstitution or to partial proteolytic cleavage of the HABP in some cases. Redissolution of the vesicles in 0.5% DOC followed by chromatography in the presence of 0.5% DOC caused the phospholipid to elute in the total volume (Fig. 3B) and the HABP to elute with a K_{av} of 0.77 (Fig. 3A). The recovery of HABP activity from these columns was approximately 70%. These results indicate that most of the HABP had become associated with the lipid vesicles during reconstitution from the cell membrane extracts and that retreatment of the vesicles with detergent gave rise again to dissolution of the lipid, thus allowing its separation from the HABP on the Sepharose 4B column in the presence of detergent.

To determine the proportion of the HABP that was intercalated into the reconstituted lipid vesicle membrane rather than being nonspecifically trapped inside the vesicles, the HABP activity of reconstituted vesicles was measured before and after treatment with trypsin, detergent, or both (Table IIA). The activity of the intact, nontrypsinized vesicles represents the amount of membrane-bound HABP with its binding sites projecting externally. As expected, all of this HABP was accessible to and was thus destroyed by trypsin (Table IIA). The activity of the detergent-solubi-

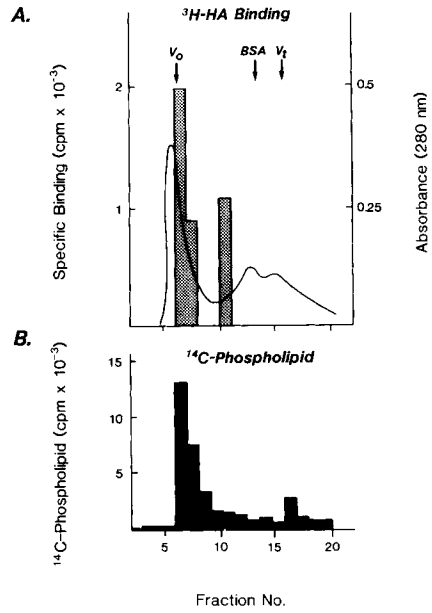

Fig. 2. Sepharose 4B gel chromatography of reconstituted lipid vesicles. Pooled fractions from sucrose gradient centrifugation containing reconstituted vesicles (see Fig. 1) were applied to a column of Sepharose 4B (1.5 × 28 cm) equilibrated in buffer A (0.02 M Tris pH 8.0/3.30 μM PMSF/3.33 mM EDTA/1.67 mM NEM/3.30 μM benzamidine). Fractions (1.4 ml) were collected and assayed for: A) HA-binding activity (shaded bar) and absorbance (line); B) ^{14}C-phosphatidylcholine content. The column was calibrated with ^{3}H-hyaluronate (Vo), ^{3}H-acetate (Vt), and bovine serum albumin (BSA) (MW: 68,000).

lized, nontrypsinized vesicles represents the total amount of HABP (ie, internal plus external), whereas that of the solubilized, pretrypsinized vesicles represents internal HABP, which is inaccessible to the trypsin. Addition of the values for internal and external activities should give a figure that is the same as that obtained for total HABP. As can be seen from Table IIA, the total activity obtained in the detergent-solubilized, nontrypsinized vesicles was 4,371 cpm compared to 3,771 cpm for internal (trypsinized then solubilized) plus external (intact, nontrypsinized). Compar-

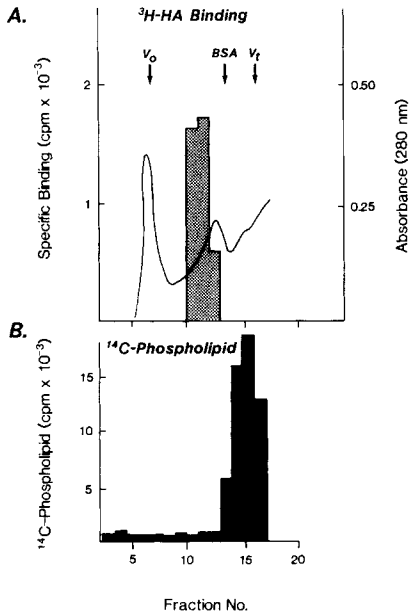

Fig. 3. Sepharose 4B gel chromatography of reconstituted lipid vesicles subsequent to redissolution in detergent. Reconstituted vesicles obtained from the sucrose gradient fractionation (see Fig. 1) were mixed with DOC to give a solution of 0.5% DOC, then applied to a column of Sepharose 4B as in Figure 2 except that the column was equilibrated in buffer A containing 0.5% DOC. Fractions were collected and analyzed for A) HA-binding activity (shaded bars) and absorbance (line); B) ^{14}C-phosphatidylcholine content.

TABLE II. Organization of HABP in Reconstituted Lipid Vesicles*

	Specific binding (cpm)	
	Nontrypsinized	Trypsinized
A. Detergent dissolution		
Intact vesicles	2,817	0
Detergent-solubilized vesicles	4,371	954
B. NaCl treatment		
Nontreated vesicles	870	—
NaCl-treated vesicles	1,078	—

*In A, reconstituted vesicles were first incubated in the presence or absence of 0.01% trypsin in CMF-PBS at 37°C for 30 min, washed with 0.05% trypsin inhibitor in CMF-PBS followed by CMF-PBS alone, then binding of hyaluronate was measured with or without prior dissolution in 0.5% DOC. In B, the vesicles were incubated at 4°C for 5 min in the presence or absence of 2 M NaCl, then centrifuged, washed, and the HA-binding activity of the pellets was measured. All measurements are averages of duplicates and are corrected for background. Variation between duplicates was less than 5%.

ison of the value for external HABP, ie, 2,817 cpm (Table IIA), with the average of the above two values for total binding, ie, 4,071 ± 300 cpm, shows that approx 70% of the total HABP in the reconsituted vesicles is membrane bound, with the binding site projecting externally. To ensure further that this external HABP was retained in the vesicle membrane by hydrophobic interactions rather than being absorbed to the surface by ionic bonds, the HA-binding activity of the vesicles was also tested after treatment with 2 M NaCl. No loss of activity from the surface of the vesicles occurred as a result of this treatment (Table IIB).

It is concluded from the above that the HABP contains a hydrophobic domain that enables its insertion into lipid membranes and a hydrophilic domain that extends out from the membrane and contains the HA-binding site.

Properties of HABP Reconstituted Into Lipid Vesicles

In a previous publication [8] we found that detergent-solubilized HABP exhibited a lower affinity for binding of HA than the membrane-bound HABP from which it was derived. If the solubilized and membrane-bound HABP are identical moieties, we would predict that reconstitution of HABP into vesicle membranes would lead to recovery of the higher affinity seen in the original cell membrane. Figure 4 compares Scatchard plots of the binding of HA to HABP in the original cell membranes and detergent extracts to that in reconstituted vesicles. It can be seen that the K_d obtained for the reconstituted vesicles (0.67 nM) was considerably lower than that for the detergent-solubilized HABP (2.85 nM) and was almost identical with that for the original cell membranes (0.53 nM). Thus, as expected, reconstitution into the vesicle membranes led to increased affinity of binding, possibly owing to the greater statistical probability that individual HA molecules would react with multiple HA-binding sites held in juxtaposition in the membrane as opposed to HABP molecules dispersed in solution [8]. An alternative possibility is that HABP-lipid interactions alter the conformation of HABP such that it has a higher affinity for HA.

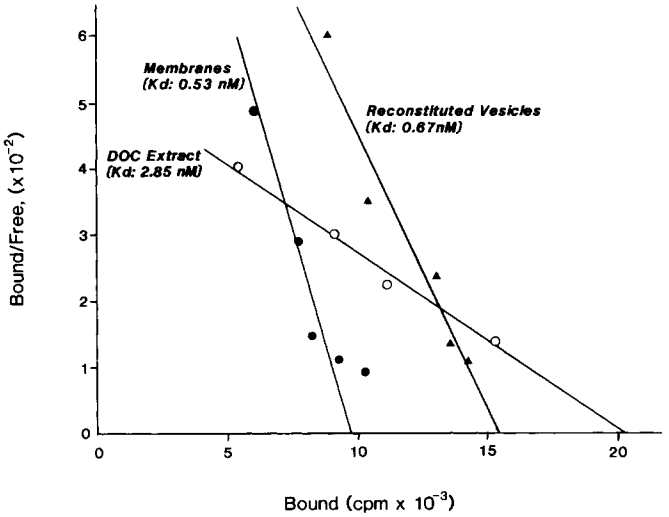

Fig. 4. Effect of reconstitution of HABP into lipid vesicles on binding affinity. Scatchard plots are given for the original cell membranes (●), DOC-extracted HABP (○), and reconstituted HABP in lipid vesicles obtained from the sucrose density gradient fractionation (Fig. 1) (▲).

It was also found previously [8] that detergent solubilization gave rise to a loss of specificity of the HABP, in that chondroitin sulfate competed significantly with binding of HA to the solubilized HABP but not to the membrane-bound HABP. Thus experiments were performed to determine whether reconstitution of HABP into the vesicles (at the same protein-to-lipid ratios as in the experiments above) caused recovery of specificity for HA. However, chondroitin sulfate was found to compete for HA binding to the vesicles to a similar extent to that found previously for the detergent-solubilized HABP (Fig. 5). Thus, reconstitution did not lead to recovery of specificity.

Since the detergent solubilized HABP did not exhibit the same specificity for HA as the original membrane-bound HABP, even after reconstitution into vesicle membranes, we sought further evidence that they were in fact the same or related molecules. To do this we prepared antisera to detergent-extracted HABP that had been partially purified by wheat germ agglutinin affinity chromatography (see "Methods") and tested its effect on binding of HA to intact cells and to the reconstituted vesicles. In both cases, complete inhibition of binding was obtained with 25 μl of antiserum, whereas no inhibition occurred with preimmune serum from the same rabbit (Table III). This result, the virtually identical K_ds of the membrane-bound and reconstituted HABP, and the efficient recoveries of HABP activity during dissolution and reconstitution argue against the possibility that the detergent-solubilized HABP activity is due to a different molecular moiety from that in the cell membrane preparations.

In conclusion, our results indicate: 1) that the majority of the HABP of SV-3T3 cells is exposed at the outer surface of the plasma membrane; 2) that the HABP has the properties of an integral membrane protein in that detergents are required for its extraction [8] and in that it can be reconstituted back into lipid vesicle membranes; 3) that the high affinity of binding of HA to the plasma membrane is in part dependent

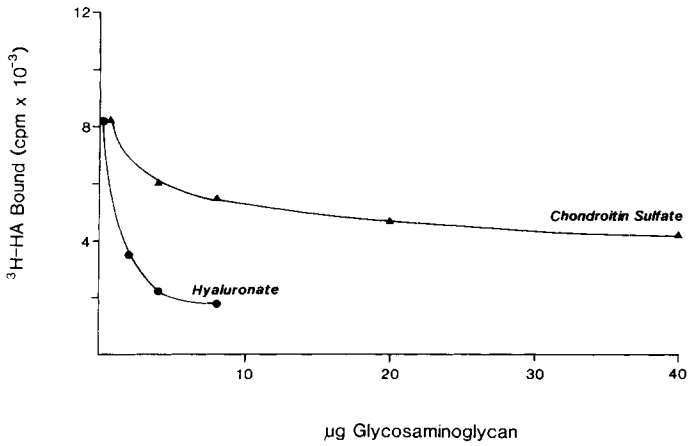

Fig. 5. Effect of reconstitution of HABP into lipid vesicles on specificity of binding. Competition curves are given for the effect of various concentrations of HA (●) and chondroitin sulfate (▲) on the binding of ^{3}H-HA to reconstituted vesicles obtained from the sucrose gradient fractionation (see Fig. 1). The degree of competition by chondroitin sulfate found here was similar to that found previously for HABP dissolved in detergent [8] but was considerably greater than that found for cell membranes or whole cells [4,8].

TABLE III. Antiserum Inhibition of HABP Binding to SV-3T3 Cells and Reconstituted Vesicles*

Experiment no./ serum added	Specific binding (cpm)	Percent inhibition
A. Whole cells		
1. Preimmune	1,352	—
Antiserum I	−4	100
2. Preimmune	1,811	—
Antiserum II	92	95
B. Reconstituted vesicles		
3. Preimmune	1,162	—
Antiserum II	8	99

*For each measurement, 2.5×10^6 cells (A) or an amount of reconstituted vesicles containing similar HA-binding activity (B) were preincubated with 25 μl of serum for 30 min at 37°C in CMF-PBS, and then the suspension was assayed for binding of HA by standard procedures [5,6] using 1.5 μg ^{3}H-HA. Antiserum I was collected 7 days after three sets of injections of antigen, and antiserum II was collected 7 days after 4 sets of injections (see "Methods"). Each measurement is the average of duplicate determinations and is corrected for background. Variation between duplicates was less than 5%.

on insertion of the HABP into the membrane; and, 4) that other interactions in the membrane, not duplicated in our reconstitution experiments, must be necessary for the specificity of the binding protein for HA.

Recent studies indicate that similar HA-binding sites to those described herein appear on the surface of mesenchymal cells during their differentiation in the chick embryo limb bud [15] and heart [16], as well as on adult rat liver endothelial cells [17]. The latter are clearly involved in clearance of hyaluronate [17,18] and chondroitin sulfate [19] from the circulation. However the precise relationship of the HABP from SV3T3 cells to these HA-binding sites and to other HABPs recently described in other laboratories [20–23] is not yet known.

ACKNOWLEDGMENTS

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