Identification and Characterization of Heparan Sulfate-binding Proteins from Human Lung Carcinoma Cells*

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The heparan sulfate proteoglycan/heparin-binding proteins of the human lung carcinoma cell line LX-1 have been identified, partially purified, and characterized. Analysis of the binding of [3H]heparin to membranes isolated from LX-1 cells indicated the presence of two classes of binding sites, with $K_d$ values of $2 \times 10^{-10}$ and $4 \times 10^{-8}$ M and corresponding $B_{max}$ values of $1 \times 10^6$ and $2 \times 10^5$ binding sites/cell. Binding was also observed with isolated heparan sulfate chains and with intact heparan sulfate proteoglycan isolated from two different cell types. With each ligand, binding was inhibited by addition of unlabeled heparin. The binding proteins were extracted from LX-1 cell membranes in detergent solution, and two size classes of binding proteins were identified by overlaying transblots of electrophoretically separated proteins with radioactive ligands. These two classes of binding proteins were shown to contain doublets with estimated molecular masses of ~16 kDa (HSBP1A and HSBP1B) and ~32 kDa (HSBP2A and HSBP2B). The proteins were partially purified by heparin-Sepharose chromatography and shown to bind heparin and heparan sulfate proteoglycan. By amino acid composition, N-terminal amino acid sequence, and reactivity with antibody, HSBP1A was shown to be very similar to histone 2B; HSBP1B may also be related to histone 2A. HSBP2A and HSBP2B, however, did not react with antibodies to the major histones and had compositions different from one another and from HSBP1.

Heparan sulfate proteoglycans are components of plasma membranes (1, 2), basement membranes (3, 4), and interstitial matrices (5, 6). Heparin is a related molecule that has many properties similar to those of the HS1 side chains of HSPGs and is often used as an analogue of HS; however, in vivo, heparin usually occurs as free glycosaminoglycan chains within mast cell granules. The wide distribution of HSPGs has led to a number of studies on the biological functions of these molecules. These studies have indicated potential roles for HSPGs in regulation of several cellular phenomena, e.g. smooth muscle cell proliferation (7), angiogenesis (8), cell attachment (9, 10), and neuromuscular junction formation (11). However, the mechanism of action of HSPGs in these phenomena is not clearly understood.

Studies from several laboratories (12-16) suggest that one possible mechanism of action of extracellular matrix molecules is via interaction with binding proteins or “receptors” on the cell surface. Binding of heparin or HSPGs to a number of cell types, including smooth muscle cells (17), hepatocytes (18), endothelial cells (19), and melanoma cells (20), has been described. However, in most cases, the binding proteins involved have not been characterized. Lankes et al. (21) have isolated and characterized a 75-kDa heparin-binding protein from bovine uteri that may be involved in the inhibition of smooth muscle cell proliferation, and Winer and Ax (22) have isolated three 14-16-kDa heparin-binding proteins from plasma membranes of granulosa cells (22). We have identified a HS/heparin-binding protein from mouse B-16 melanoma cell membranes with a molecular mass of ~14 kDa (20). Interaction of extracellular matrix HSPG with these molecules appears to be involved in the regulation of the production of a collagenase stimulatory factor (23).

In this study, we have identified, partially purified, and characterized two size classes of HSPG/heparin-binding proteins (HSBP1 and HSBP2) with estimated molecular masses of ~16 and ~32 kDa, respectively, from membranes of the human lung carcinoma cell line LX-1. Each of the two classes of proteins can be resolved into two components whose amino acid compositions are similar in some respects, but are not identical. The ~16-kDa proteins (HSBP1A and HSBP1B) are closely related or identical to histones, whereas the ~32-kDa proteins (HSBP2A and HSBP2B) appear to be unique HSPG/heparin-binding proteins.

**MATERIALS AND METHODS**

**Chemicals—**Tritiated heparin (specific activity, 0.34 mCi/mg), Na$^{35}$S (specific activity, 17 $\mu$Ci/$\mu$g), [35S]methionine (specific activity, 1972 $\mu$Ci/$\mu$mol), and Na$^{32}$P (specific activity, 614 $\mu$Ci/$\mu$mol) were obtained from Du Pont-New England Nuclear. Porcine intestinal heparin and chondroitin sulfate from whole cartilage were from Sigma. Heparin-Sepharose was from Pharmacia LKB Biotechnology Inc. Bovine kidney heparan sulfate was from Miles Scientific (Naperville, IL). [35S]HSPGs from bovine aortic endothelial cells and human colon carcinoma cells were generous gifts of Drs. Thomas Wight and Michael Kinsella (University of Washington) and Dr. Renato Iozzo (Jefferson Medical School), respectively. Dermatan sulfate and hyaluronic acid were from Dr. Martin Matthews (University of Chicago). Antibodies to histones were a generous gift from Dr. David Stollar (Tufts University).

Preparation of [35S]HSPG from 3T3 cells has been described previously (20).

**Cell Cultures—**The source of LX-1 cells has been described previously (24). All cells were grown in Roswell Park Memorial Institute culture medium containing 5% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO$_2$ and 95% air.

**Preparation of Membranes and Membrane Extracts—**Cells were collected from monolayer cultures by incubating the culture plates in...
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0.02% EDTA in PBS and then washed with PBS, resuspended in PBS at a cell concentration of \(~2 \times 10^6/100 \mu l\) and centrifuged. The cell pellet was suspended in 0.24 M sucrose in 50 mM Tris, pH 7.4, and sonicated in a Branson sonicator. The suspension was first centrifuged at 500 \(\times g\), and the resulting supernatant was centrifuged at 100,000 \(\times g\) for 1 h.

The membrane fraction prepared as described above was extracted with buffer containing 0.5% deoxycholic acid (sodium salt), 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA in PBS, pH 8.5, by stirring overnight at 4 °C. The extract was centrifuged at 100,000 \(\times g\) for 1 h, and the supernatant was collected.

Binding Assays—The binding assays for whole cells, membranes, and membrane extracts have been described before (20). Briefly, the cells or membranes prepared as described above were mixed with radioactive ligand, incubated, centrifuged, and washed, and the radioactivity associated with the cell or membrane pellet was measured as described (20).

A solid-phase assay was used to measure binding of radioactive ligands to extracts and other soluble fractions. In this assay, the wells of a microtiter plate were first coated with the material to be assayed previously (20). After washing the wells with PBS containing 0.1% bovine serum albumin, they were incubated with radioactive ligand at 25 °C for 30 min and washed again. The radioactivity bound to each well was determined by dissolving the bound material in 2% SDS as described previously (20).

In both assays, specific binding was determined by adding 100 ng of unlabeled heparin to the assay mixture.

Identification of HSBPs by Nitrocellulose Overlay—Samples containing GAG-binding activity were subjected to SDS-PAGE on 12% acrylamide gels (25). The separated proteins were electrophoretically transferred (200 mA overnight at 4 °C) to a nitrocellulose sheet in buffer containing Tris, glycine, and methanol (26). The blot was washed with TBS containing 0.05% Tween 20, blocked with TBS containing 5% horse serum, and washed with TBS containing 0.1% Tween 20 followed by Tris saline only (20). The blot was incubated with radiolabeled heparin or HS (1 \(\times 10^6\) cpm/ml) or HSPG (0.2 \(\times 10^6\) cpm/ml) for 2 h at room temperature. The paper was washed three times with TBS, air-dried, and autoradiographed using Kodak XAR film.

Heparin-Sepharose Chromatography—A heparin-Sepharose column (3-ml bed volume, 1.6 x 1.4 cm) was washed with 10 ml of 0.1% bovine serum albumin in PBS to block nonspecific binding sites and then equilibrated with 0.1% deoxycholic acid (sodium salt) in PBS. Three ml of deoxycholic acid (sodium salt) extract of LX-1 membrane preparations (15 \(\times\) 10^6 cells) was applied to the column and recirculated through the column for ~90 min at a flow rate of ~0.5 ml/min. The column was washed extensively with equilibration buffer followed by washing with 5 ml of 30 mM octyl glucoside in PBS three times. The binding proteins were eluted with a 0-2 M NaCl gradient in PBS at a flow rate of 0.2 ml/min. The volume of the gradient was 40 ml. The peak fraction (2 ml) were collected, and each fraction was monitored for absorbance, binding activity, and protein profiles by SDS-PAGE. The active fractions were pooled, concentrated, and used for further analysis.

Amino Acid Analysis and Partial Sequencing—Heparin-Sepharose column fractions containing ~12 \(\mu g\) of total protein were electrophoresed on 12% acrylamide gels containing 0.1% SDS according to Laemmli (25). After electrophoresis, the gel was soaked in buffer containing 20 mM Tris, 150 mM glycine, and 20% methanol (transfer buffer). The Immobilon membrane was wetted in absolute methanol for 1 min, washed twice with distilled water, and then immersed in transfer buffer. The gel and the membrane were assembled into a blotting apparatus (Bio-Rad), and the protein transfer was carried out at 4 °C by a modification of the procedure of Montelaro (27) using the following voltages and times: 3 V/cm for 16 h and 6 V/cm for 4 h. After transfer, the membrane was washed thoroughly in deionized water; stained with 0.1% Coomassie Blue R-250 in 50% methanol for 2 min, destained in 50% methanol, 10% acetic acid for 2 min (28). The four protein bands corresponding to each component of the two doublets (HSBP1 and HSBP2) were cut out separately and used for amino acid analysis and partial amino acid sequencing. These analyses employed a Waters/Millipore Picotag system and an Applied Biosystems 477A Protein Sequencer with an on-line phenylthiohydantoin analyzer in the protein chemistry facility of the Physiology Department at Tufts Medical School.

Radiolabeling of Binding Proteins—For metabolic labeling, LX-1 cells were grown to confluence, and the media were removed. The dishes were washed with methionine-free Dulbecco’s modified Eagle’s medium and incubated in the same medium for 30 min. The cells were then labeled with \(^{35}\)S methionine (30 \(\mu Ci/ml\) in methionine-free medium for 6 h, after which the culture media were removed. The cell layers were washed with PBS and used for membrane preparation and extraction as described above. The detergent extract of the membranes was loaded on a heparin-Sepharose column and washed, and the bound proteins were batch-eluted successively with 2 column volumes of PBS containing 0.5, 1.0, and 1.5 M NaCl. The eluted fractions were dialyzed, concentrated, analyzed for radioactivity, and then subjected to SDS-PAGE and autoradiography.

RESULTS

Binding Parameters for Heparin, HS, and HSPG—The binding of radioactive GAGs and HSPG to the LX-1 membrane preparation was investigated using optimal conditions for binding that were established in separate experiments. Fig. 1 shows the binding of \(^{3}H\)heparin to membranes derived from LX-1 cells on mixing a constant amount of the membranes with increasing concentrations of ligand. Saturation was obtained, and \(^{3}H\)heparin binding was inhibited completely in the presence of excess unlabeled heparin. Binding was obtained with intact LX-1 cells as well as with isolated membranes (Fig. 1). However, the amount of heparin bound to the cells was lower than that to membranes and varied between experiments. This may have been due in part to the tendency of the intact cells to form aggregates in suspension or to the presence of internal as well as cell-surface binding sites. Binding of \(^{3}H\)heparin to intact cells and to isolated membranes was also measured after washing the cells or membranes with 0.5 M NaCl. This treatment did not significantly alter the amount of binding in either case, implying that the binding sites are tightly associated with the cells and membranes.

Analysis of the \(^{3}H\)heparin binding data (Fig. 2), using the LIGAND computer program of Munson (29), supported a two-site model, with apparent \(K_d\) values of \(2 \times 10^{-10}\) and \(4 \times 10^{-8}\) M. The maximum number of binding sites/cell \((B_m)\) was 1 \(\times 10^7\) for the higher affinity sites and 2 \(\times 10^6\) for the lower affinity sites.

Binding to LX-1 membranes was also obtained using radiolabeled HS, and this binding was also completely inhibited with excess unlabeled heparin. However, binding was considerably less than that for heparin; for this reason and since the specific activity of HS was relatively low, binding could not be measured accurately at lower concentrations of radioactive GAG. However, approximate calculations from the
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**Fig. 2. Analysis of [3H]heparin binding data for membranes from LX-1 cells.** The data from Fig. 1 were analyzed by the LIGAND program of Munson (29). \( K_d \) values of \( 2 \times 10^{-10} \) and \( 4 \times 10^{-10} \) M and \( B_{\text{max}} \) values of \( 1 \times 10^9 \) and \( 1.7 \times 10^8 \) binding sites/cell were obtained.

Scatchard plot gave a single \( K_d \) of \( \sim 1 \times 10^{-7} \) M.

Since HSPG is the natural component of extracellular matrices, we investigated the binding of radioactive HSPG, obtained from two different sources, to LX-1 membranes. Fig. 3 shows the binding of HSPG from bovine endothelial cells and the inhibition of binding by heparin. A similar pattern of binding was obtained with radiolabeled HSPG isolated from human colon carcinoma cells. Inhibition of HSPG binding by heparin was 85-90% at concentrations up to 100 ng, but was only ~70% at 400 ng of added HSPG. The latter result may have been due to the reduced ratio of unlabeled heparin to labeled HSPG at this concentration of HSPG or to participation of the protein core as well as the HS chains of HSPG in binding. Scatchard analysis of these data again gave curvilinear plots, but these were difficult to interpret since HSPG is likely to be a multivalent ligand and since the data suggest the presence of at least two binding sites. Assuming two sites, approximate calculations from the Scatchard plot gave \( K_d \) values of \( 4 \times 10^{-11} \) and \( 6 \times 10^{-10} \) M.

**Specificity of Binding of GAGs and HSPG**—Table I compares the effects of different GAGs in competition for binding of [3H]heparin and [35S]HSPG to LX-1 membranes. Heparin almost completely inhibited the binding of both [3H]heparin and [35S]HSPG. Heparan sulfate was not inhibitory for [3H]heparin binding and was 58% inhibitory for [35S]HSPG binding. The inefficiency of heparan sulfate from bovine kidney in competing for heparin binding has previously been observed by us with B-16 cells (20) and by others with hepatocytes (18). This can probably be explained by differences in the degree of sulfation in different heparan sulfate preparations such as has been observed in binding studies with hepatocytes (18). No significant inhibition of binding of either ligand was obtained with hyaluronic acid, and low levels of inhibition were observed with chondroitin sulfate. Dermatan sulfate showed 46 and 69% inhibition of radiolabeled heparin binding, respectively.

**Identification of Binding Proteins**—To investigate the approximate molecular size of the binding proteins, we performed an overlay binding assay where the proteins were first separated on SDS-PAGE and transblotted onto nitrocellulose membrane and then were incubated with the radioactive ligand. Fig. 4 shows an autoradiograph of such a nitrocellulose transblot of membrane proteins after incubation with radioactive ligands. Binding was seen to occur mainly to two protein doublets, one with an estimated molecular mass of ~16 kDa (HSBP1) and the other with an estimated molecular mass of ~32 kDa (HSBP2). A pattern of binding was obtained with [35S]HS which was similar to that seen with heparin (data not shown). Also, the pattern of binding was not altered by washing the LX-1 cells or the isolated membranes with 0.5 M NaCl prior to processing for the overlay assay. Two other protein bands (22 and 12 kDa) were also observed, but the intensity of these bands was much lower than that of the major bands.

**Table I**

| GAG            | [3H]Heparin | [35S]HSPG |
|----------------|-------------|----------|
| Heparin        | 98          | 80       |
| Heparan sulfate| 3           | 54       |
| Dermatan sulfate| 46       | 69       |
| Chondroitin sulfate| 13       | 28       |
| Hyaluronic acid| 9           | 8        |

**Fig. 3. [35S]HSPG binding to membranes from LX-1 cells.** Membranes (equivalent to \( 0.2 \times 10^8 \) cells) were incubated with different concentrations of [35S]HSPG in the absence (C) or presence (O) of 100 ng of unlabeled heparin. O, difference between the two plots.

**Fig. 4. Binding of [35S]HSPG and [3H]heparin to LX-1 membrane proteins.** LX-1 membranes were dissolved and electrophoresed on 12% SDS-polyacrylamide gel, transblotted to a nitrocellulose sheet, incubated with [35S]HSPG (500 ng of GAG, 0.1 \( \times 10^6 \) cpm) from endothelial cells in the absence (lane 1) or presence (lane 2) of unlabeled heparin (500 ng) or with [3H]heparin (2200 ng, 1 \( \times 10^6 \) cpm) (lane 3), and then exposed to x-ray film. Incubation with [3H]heparin plus unlabeled heparin yielded results similar to those in lane 2. Each lane was loaded with membranes equivalent to \( 0.2 \times 10^8 \) cells.
two bands varied with different membrane preparations, and some times they were not present. One possibility is that these bands represent breakdown products of the binding proteins, but this remains to be investigated. With HSPG, weaker binding was also observed in regions above and below the HSBP2 doublet (lane 1), indicating the possible presence of other HSPG-binding proteins in the LX-1 membranes.

The binding of radioactive HS (lane 2), HS, and heparin (data not shown) to all of these protein bands was almost totally inhibited in the presence of excess unlabeled heparin. The inhibition of [35S]HSPG binding to these bands with unlabeled heparin makes it unlikely that this binding is due to reaction with the core protein of the proteoglycans. Thus, all three ligands bound mainly to two protein doublets, which we have designated as HSBP1A and HSBP1B for the ~16 kDa doublet and HSBP2A and HSBP2B for the ~32-kDa doublet (A signifying the upper band and B the lower band in each case).

The data in Fig. 4 suggest that HSPG binds almost equally to the HSBP1 and HSBP2 doublets, whereas heparin binds preferentially to HSBP1. To investigate this possibility further, we incubated nitrocellulose transblots of the binding proteins with several concentrations of radiolabeled heparin or HS and, after autoradiography, measured the intensity of each band by densitometry. Fig. 5 shows data from such an experiment. It is clear that, at all concentrations tested, heparin binds mainly to HSBP1A and HSBP1B, to a moderate extent to HSBP2B, and much less to the HSBP2 doublet than to the HSBP1 doublet. However, HSPG binds relatively strongly to HSBP1A, HSBP1B, and HSBP2A and moderately to HSBP2B. Thus, the ratio of binding to the HSBP2 versus HSBP1 doublet is proportionately greater for HSPG than for heparin.

Partial Purification of Binding Proteins—For further characterization and partial purification of HS BP1 and HSBP2, we used heparin-Sepharose affinity chromatography. Deoxycholate extract of the LX-1 membrane preparation was applied to a column of heparin-Sepharose, and the column was eluted with a linear gradient of 0–2 M NaCl in PBS. As shown in Fig. 6, the activity was eluted between 1.0 and 1.4 M NaCl, and the peak of activity is present in fractions 10–13. SDS-PAGE of the column fractions and silver staining of the gel indicated that fractions 10 and 11 were enriched with two doublets of estimated molecular masses of ~16 and ~32 kDa (Fig. 7A), the same sizes as the bands seen by the overlay assay of the crude membrane extract (Fig. 4). Fractions 12–14 mostly showed the lower doublet with a trace amount of the upper doublet.

To further confirm the association of binding activity with the two doublets observed in the fractionated material, we pooled the active fractions and performed an overlay assay with [35S]HSPG. As shown in Fig. 7B, radioactive bands were associated with two doublets identical in size to those observed by silver staining in Fig. 7A and by autoradiography of overlays of the crude membrane extracts in Fig. 4. These data strongly indicate that the polysaccharide binding activities mostly reside in the two protein doublets (HSBP1A/B and HSBP2A/B) with molecular masses of ~16 and ~32 kDa, respectively.

In addition to identifying the HSBP bands by overlay assay, we measured binding of [3H]heparin to the pooled active fractions from heparin-Sepharose using a solid-phase binding assay (Fig. 8). Binding increased with increasing concentrations of radioactive ligand until saturation was inhibited by excess unlabeled heparin. The modified Scatchard method of Horejsi and Matousek (30) indicated the presence of two binding sites, with Kd values of ~3 x 10^-9 and ~2 x 10^-8 M, with affinities similar to those obtained with the intact membranes.

Amino Acid Composition and Sequencing—To investigate the relationship of HSBP1A, HSBP1B, HSBP2A, and HSBP2B, we performed amino acid analysis of these proteins.
after separation by SDS-PAGE, blotting to Immobilon, and excision of the four bands from the blot. Since the bands on Immobilon blots are very sharp and clearly delineated from one another, they can be excised cleanly without cross-contamination. Table II shows the total amino acid composition of histone 2B from Ref. 31.

| Amino acid | HSBP1 | HSBP2 | Histone 2B |
|------------|-------|-------|------------|
|            | A     | B     | A         | B         |
| Asp        | 5.2   | 6.0   | 3.3       | 6.4       | 5.1 |
| Glu        | 8.5   | 9.6   | 5.8       | 9.1       | 8.3 |
| Ser        | 10.8  | 6.4   | 9.0       | 10.3      | 10.2 |
| Gly        | 7.7   | 11.3  | 11.6      | 14.2      | 5.8 |
| His        | 2.0   | 2.5   | 0.6       | 1.1       | 2.5 |
| Arg        | 7.0   | 8.7   | 2.7       | 3.8       | 6.6 |
| Thr        | 6.2   | 4.6   | 5.2       | 6.2       | 6.0 |
| Ala        | 10.3  | 11.6  | 20.3      | 12.8      | 10.6 |
| Pro        | 4.4   | 3.7   | 6.1       | 5.0       | 5.1 |
| Tyr        | 3.8   | 3.0   | 1.3       | 2.0       | 4.0 |
| Val        | 6.7   | 5.9   | 4.1       | 5.0       | 7.2 |
| Met        | 0.5   | 0.3   | 0.6       | 1.3       | 1.6 |
| Ile        | 4.8   | 4.3   | 2.0       | 3.3       | 5.1 |
| Leu        | 5.8   | 10.0  | 5.0       | 6.3       | 5.0 |
| Phe        | 1.5   | 1.0   | 1.3       | 2.3       | 1.6 |
| Lys        | 14.4  | 11.0  | 20.8      | 11.0      | 15.0 |

To confirm the similarity of HSBP1A to histone 2B, Western blots were performed with antibodies to the major histones, namely H2A, H2B, H3, and H4. Antibody to histone 2B reacted with HSBP1A, but not with HSBP1B, HSBP2A, or HSBP2B (data not shown). Also, antibody to histone 2A reacted with HSBP1B, but not to the other HSBPs. None of the antibodies showed any reaction with HSBP2A or HSBP2B.

**Association of HSBPs with Cell Surface**—The fact that binding occurs to intact LX-1 cells as well as to isolated membranes and extracts suggests that the HSBPs are present, at least in part, at the cell surface. To investigate this further, we incubated a suspension of LX-1 cells with trypsin before isolating the membranes. Since the cells were intact, only external proteins would be accessible to digestion by trypsin. Fig. 9 shows that treatment of cells with 0.1% trypsin resulted in the almost complete disappearance of HSBP1A and HSBP2 from the membrane preparation, whereas 0.01% trypsin had little effect.

**DISCUSSION**

Our data indicate that human lung carcinoma LX-1 cells possess membrane-associated binding sites that recognize heparin, HS, and HSPG. Analysis of the binding data for heparin suggested two classes of binding sites, with $K_a$ values of $2 \times 10^{-10}$ and $4 \times 10^{-8}$ M and corresponding $B_{max}$ values of $1 \times 10^8$ and $2 \times 10^6$. However, other explanations such as negative cooperativity are also possible. HSPG molecules contain a number of GAG chains attached to a protein backbone, e.g. the endothelial preparation used here contains four to six HS chains/proteoglycan molecule (40). Since each of the HS chains would be expected to bind to an individual binding site, each proteoglycan molecule would interact with multiple sites. Calculations of $K_a$ and $B_{max}$ values for multivalent ligands such as HSPG are difficult to interpret; how-
ever, it is clear from approximate calculations that HSPG
also interacts with the LX-1 cell membranes with high affinity
($K_d = -4 \times 10^{-11}$ and $-6 \times 10^{-10}$ M). It is likely that these
high affinities of interaction are at least in part due to multi-
tivalent interaction since isolated HS chains appear to inter-
act with lower affinity. However, the radiolabeled HS prepa-
ration used here was from 3T3 cells, and the HSPG prepara-
tions were from endothelial and colon carcinoma cells. Since
HS preparations from different sources vary in their com-
position (18), the above interpretation of our results would
require use of HS chains from the same source as the HSPG.
This has not yet been possible due to the difficulty of obtain-
ing sufficient amounts of labeled HS and HSPG of high
specific activity, but current experiments are directed toward
this purpose. Irrespective of these concerns, however, it is
clear from the data presented that the two HS PG preparations
used in this study interact with LX-1 membranes with high
affinity.

Most of the binding of radioactive HSPG as well as of HS
and heparin itself was inhibited by heparin both in the mem-
brane binding assays and in the overlay assays. This implies
strongly that most of the HSPG binding occurred via its HS
side chains, as opposed to its protein core. A low proportion
of the total binding, however, may have been via the core
protein as described recently for hepatocytes (32). The most
logical interpretation of our data is that the major binding
sites recognized by heparin, HS, and HSPG are the same
sites, especially since the same group of proteins (HSBP1 and
HSBP2) were recognized by all three ligands in transblot
overlays. However, HS from bovine kidney did not efficiently
inhibit binding of either heparin or HSPG. The ineffect-
tiveness of this preparation of HS is probably due to its relat-
ively low sulfate content as shown previously with he-
paran sulfate (20). A previous study (20) with B-16 cells also sug-
gested this possibility, but those data were not conclusive in
this purpose. Irrespective of these concerns, however, it is
clear from the data presented that the two HS PG preparations
used in this study interact with LX-1 membranes with high
affinity.

In this study, we have obtained evidence for two classes of
binding sites for heparin, and probably also for HSPG, on
LX-1 cells. A previous study (20) with B-16 cells also sug-
gested this possibility, but those data were not conclusive in
this regard. In agreement with the binding data, we detected
two classes of HS/heparin-binding proteins (HSBP1 and
HSBP2), as defined by their approximate molecular masses,
that are candidates for the two binding sites. However, the
HSPG/heparin binding affinities of the separated proteins
are not yet known. Also, there may be four (rather than two)
different HSBPs since HSBP1A, HSBP1B, HSBP2A, and
HSBP2B appear to have significantly different amino acid
compositions. Recent results indicate that similar classes of
proteins are also present in smooth muscle cell membranes.
The relationship of these proteins to a 78-kDa heparin-bind-
ing protein from bovine uteri that also appears to be closely
associated with the smooth muscle cell surface (21) remains
to be investigated.

The binding of heparin to intact cells and the removal of
all four HSBPs from intact cells by trypsinization indicate that
the HSBPs are localized, at least in part, on the outer
cell surface. However, partial N-terminal sequencing and
reactivity with antibody to histone 2B indicate a strong ho-

The similarity in molecular masses suggests that the 14–16-
kDa heparin-binding proteins from granulosa cell membranes
(22) and the 14-kDa species from B-16 melanoma cells (20)
may also be related to histones. It is possible that nuclear
histones have been released from these cells during culture or
harvesting, have nonspecifically become associated with the
cell surface, and have remained associated with the mem-
branes during isolation, even after extensive washing with 0.5
m NaCl. On the other hand, the presence of histone-like
molecules at the cell surface that bind HSPG may be of
biological importance. Several groups (33, 34, 36) have dem-
onstrated the association of glycosaminoglycans with the nu-
cleus, probably bound to histones. However, recent studies by
Conrad and co-workers (35, 36) indicate that extracellular
HSPG is endocytosed and that a specific subpopulation of
free HS chains becomes associated with nuclei via an un-
known nonlysosomal pathway. A strong inverse correlation
between the level of nuclear HS and cellular growth has been
demonstrated (37). Thus, it is tempting to speculate that
HSBP1 may be involved in transport of this HS from the cell
surface to the nucleus. Detailed studies of the cellular local-
ization and role of HSBP1 will be necessary to clarify this
issue.

Although HSBP1 may be derived from the nucleus or
related to nuclear function, this does not seem likely for
HSBP2. Neither HSBP2A nor HSBP2B was recognized by
antibodies to the major histones (H2A, H2B, H3, and H4),
consistent with their larger size compared with histones. Also,
in recent studies of smooth muscle cells using the same
methodological approaches as those used in this study, we
have found cellular variants that contain both the HSBP1
and HSBP2 doublets and variants that contain only the
HSBP1 doublet, i.e., the presence of HSBP2B was not due to
the membrane preparation method.

The functional significance of the four HSBPs and their
relationship to each other are not yet clear. However, it is
possible that these HSBPs, especially HSBP2, belong to the
growing group of cell-surface receptors for matrix molecules
(12–16) and thus may mediate cellular functions attributed to
HSPG/heparin, e.g., inhibition of smooth muscle and mesan-
gial cell proliferation (7, 38), regulation of c-myc and c-fos
mRNA levels in fibroblasts (39), angiogenesis (8), cell attach-
ment (9, 10), neuromuscular junction formation (11), or the
above-mentioned transport of HS to the nucleus (35–37). We
have also obtained evidence that extracellular matrix HSPG
may regulate the production of a tumor cell-derived factor
that stimulates production of fibroblast collagenase (23). Pos-
sibly, tumor cell HSRPs, such as those described here, are
involved in this phenomenon. Our current work is directed
toward obtaining specific antibodies and cDNAs to facilitate
investigation of the relationship of the various HSBPs, their
localization, and their biological roles.

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