Identification of a Cell Surface-binding Protein for the Core Protein of the Basement Membrane Proteoglycan*

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We have identified a protein(s) on the surface of hepatocytes that binds to the core protein of the heparan sulfate proteoglycan of basement membranes. These cells attached and spread on substrates prepared from the basement membrane heparan sulfate proteoglycan (HSPG) and its core protein (HSPG-core). Three proteins (Mr = 38,000, 36,000, and 26,000) were found to bind to a HSPG-core affinity column using extracts of iodinated hepatocytes, whereas proteins extracted from isolated membranes contained primarily the larger protein (Mr = 38,000). Similar results were obtained using a solid phase binding technique using labeled HSPG-core. Binding of HSPG-core to the protein (Mr = 38,000) was not altered by the presence of an excess of heparan sulfate, fibronectin, laminin, or collagen IV but was reduced by unlabeled HSPG-core. Similar studies showed that the binding protein (Mr = 3,000) was present in extracts from the membranes of Engelbreth-Holm-Swarm tumor cells, Madin-Darby canine kidney cells, COS cells, melanoma cells, and rat kidney epithelial cells but not in fibroblasts. The protein was found in increased amounts in 3T3 cells treated with retinoic acid. These observations suggest that a variety of cells that contact basement membrane contain the proteoglycan-binding protein.

A large heparan sulfate proteoglycan (HSPG) is the principal proteoglycan in various basement membranes, and accounts for 0.3–3.0% of the dry weight of basement membrane (1). HSPG extracted from the Engelbreth-Holm-Swarm tumor has a large core protein (Mr = 400,000) forming a series of globules in the molecule with 3–4 heparan sulfate chains attached to a globule at one end (1–7). The HSPG isolated from glomerular basement membranes is smaller (8, 9) but may be degradation or alternatively spliced forms (10). HSPG creates ionic barriers on the surfaces of basement membranes preventing the passage of proteins (11, 12). HSPG also binds to other basement membrane components, including laminin and collagen IV (13), and it is possible that HSPG may interact directly with cells through its glycosaminoglycan chains or protein core (1, 14).

Other HSPG have been implicated in cell adhesion and spreading (15–17) and can stabilize focal contacts (18). Because the basement membrane proteoglycan has a large core protein, it seemed possible that it might interact with cells through a specific binding protein. Here, we report the identification of specific cell surface-binding proteins, which are different from the laminin-binding proteins and other matrix receptors, for the core protein of basement membrane HSPG.

EXPERIMENTAL PROCEDURES

Materials—Na125I and the Bolton-Hunter reagent (125I-labeled, specific activity 2200 Ci/mmol) were purchased from Du Pont-New England Nuclear. Protein A-Sepharose 4B was obtained from Pharmacia LKB Biotechnology Inc. n-Octyl β-D-glucopyranoside (octyl-gluco-side), triethanolamine (TEA), lactoperoxidase, and heparan sulfate were from Sigma, and heparin was from Fisher. CHAPS was from Calbiochem. Bacterial collagenase from Clostridium histolyticum was from Boehringer Mannheim. Culture media and fibronectin were from GIBCO.

Laminin was kindly provided by Dr. H. Kleiman (National Institutes of Health, Bethesda, MD). HSPG and collagen IV were purified from the Engelbreth-Holm-Swarm tumor as described (19, 20). The HSPG-core without side chains was prepared by treatment of the purified proteoglycan with heparitinase followed by purification on a Sepharose CL-4B column (3, 10).

Cells and Cell Culture—Adult hepatocytes were isolated from 2-month-old Sprague-Dawley rats using the two-step collagenase perfusion method (21) and then transferred to L15 Leibovitz medium containing 0.2% bovine albumin. The cells were either used immediately or plated in serum-free “199 medium supplemented with 10 µg/ml porcine insulin and 0.2% bovine albumin. The appearance of hepatocyte primary cultures were monitored using a Nikon differential interference contrast microscope.

Fibroblast-like NRK-49F cells and epithelial-like NRK-52E cells were cloned from a mixed culture of normal rat kidney cells. Madin-Darby cells are epithelial-like cells derived from canine kidney. HT-1080 cells are established from a human fibrosarcoma. COS cells are derived from monkey kidney and are transformed by SV40. M2 melanoma cells are from mouse, and NIH/3T3 are derived from NIH Swiss mouse embryo. All cells were obtained from the American Type Culture Collection (Rockville, MD).

Cell Adhesion Assay—Cell adhesion was assayed by adding various amounts of these proteins to 35-mm tissue culture dishes containing 1 ml of serum-free Eagle’s minimal essential media at 37 °C in a 5% CO2 atmosphere. After 2 h, 3% bovine albumin in Eagle’s minimal essential media was added to a final concentration of 1.5% for an additional 30 min. Following removal of the medium, hepatocytes were added in M-199 medium containing 0.02% bovine albumin and 10 ng/ml porcine insulin. After 1 h, plates were gently rinsed twice with 0.1 M phosphate-buffered saline, pH 7.5, to remove unattached cells and then fixed with cold 20% methanol, stained with toluidine blue, and counted with an image analyzer (Optmax V) attached to an Olympus CK2 microscope. Each assay was carried out in triplicate.

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4 The abbreviations used are: HSPG, basement membrane heparan sulfate proteoglycan; HSPG-core, core protein of HSPG; TEA, triethanolamine; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propane sulfonate.

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Radiolabeling of Hepatocytes—Only preparations of hepatocytes that were greater than 95% viable as judged by their exclusion of trypan blue were radioactively labeled. Suspensions of freshly isolated hepatocytes (10 × 10^6 cells) were extensively washed with phosphate-buffered saline at 4 °C and then labeled for 30 min with 2 mCi of Na\(^{125}\)I by lactoperoxidase-catalyzed iodination at 4 °C (22). Hepatocytes were then washed 3 times with phosphate-buffered saline and immediately processed for membrane extraction.

Preparation of Membranes—\(^{125}\)I-Labeled hepatocytes were homogenized in 0.01 M TEA, pH 7.5, containing 8.5% sucrose, 1 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride at 4 °C, then centrifuged at 25,000 × g to prepare crude membranes. The cell membrane pellet was then extracted with 1% CHAPS and 1% octylglucoside in 0.01 M TEA, pH 7.5, containing 1 mM EDTA and 2 mM phenylmethylsulfonyl fluoride for 2 h, at 4 °C and centrifuged at 25,000 × g.

Plasma membranes were obtained using the method of Aronson and Touster (23). Briefly, \(^{125}\)I-labeled hepatocytes were homogenized in cold 0.25 M sucrose and then centrifuged at 1,000 g to separate the nuclear fraction. Supernatant solution was clarified at 33,000 × g for 7.5 min. Membranes were pelleted at 78,000 × g for 100 min, then homogenized in 57% sucrose and 100 mM TEA, pH 7.5, containing 0.15 M NaCl. Membranes were pelleted at 78,000 × g for 1 h at 4 °C and centrifuged at 25,000 × g. The membrane pellet was then extracted with 1% CHAPS and 1% octylglucoside as described above.

Affinity Chromatography of Membrane Proteins on the HSPG-core—HSPG-core affinity chromatography column was prepared at 4 °C by incubating 1 mg/ml HSPG-core with CNBr-activated Sepharose beads in 0.1 M carbonate buffer, pH 8, containing 0.5 M NaCl. After 48 h, beads were incubated with 1 M ethanolamine in the same buffer, then alternatively washed with 0.1 M acetate buffer, pH 4, and 0.1 M carbonate buffer, pH 8, with both solutions containing 0.1% CHAPS. Column was then washed extensively with 0.1% CHAPS in 0.01 M TEA, pH 7.5, 1 mM phenylmethylsulfonyl fluoride. Detergent extracts were dialyzed against the same solution and incubated overnight at 4 °C with HSPG-core-Sepharose beads. The beads were packed in a column and washed extensively with the same buffer containing 0.1 M NaCl. Proteins were eluted first with an increasing linear NaCl gradient (0.15–1 M) in the presence of detergent and then with 0.1 M glycine buffer, pH 2, followed by 8 M urea. Fractions were counted in a γ counter and then extensively dialyzed against distilled water (3 changes) and dried using a Speed-Vac apparatus. Proteins were resolved on a 10% polyacrylamide gel, which was then dried and exposed to an x-ray film.

Binding of \(^{125}\)I-HSPG-core to Membrane Proteins—HSPG-core was iodinated using the Bolton-Hunter reagent (24). Either cell homogenates or detergent-soluble membrane proteins were electrophoresed on a 10% acrylamide gel and transferred to a nitrocellulose filter. The filter was incubated 2 h at room temperature with 0.01 M TEA, pH 7.5, containing 0.15 M NaCl, 1 mM MgCl\(_2\), 3% bovine albumin, and 3% nonfat dry milk. Then, 50 ng/ml of \(^{125}\)I-HSPG-core was added and incubated for 2 h at room temperature. Nitrocellulose filters were

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**Fig. 1. Attachment of hepatocytes to dishes coated with HSPG (C), HSPG-core (O), or laminin (□).** Hepatocytes (0.6 × 10^6 cells in 1.5 ml of serum-free medium) were incubated for 45 min at 37 °C in dishes coated with the indicated amounts of protein. Plates were then washed with phosphate-buffered saline, and attached cells were fixed with methanol, stained, and counted with an image analyzer. The amounts of attached cells are given as the percentage of cells that attached to dishes coated with 50 µg/ml of laminin after 60 min of incubation (an average of 0.45 × 10^6 cells/35-mm tissue culture dish). The values are average of incubation in triplicate dishes.

**Fig. 2. Photomicrographs of hepatocytes after 6 h on HSPG (A), HSPG-core (B), or laminin (C), each at 50 µg, or plastic alone (D).** The cells were maintained in culture in serum-free medium containing bovine albumin and 10 µg/ml insulin and observed with a differential interference contrast microscope. Bar, 20 µm.
Affinity chromatography of hepatocyte membrane fractions on HSPG-core-Sepharose. Cell surface oxidase-catalyzed iodination. The membrane fraction was extracted face proteins of freshly isolated hepatocytes were labeled by lactoperoxidase, pH 7.4, and passed over an HSPG-core-coupled Sepharose 4B column. After washing, proteins were eluted with a linear gradient NaCl (0.15-1 M) in the presence of detergent and then with 0.1 M glycine, pH 2, followed by 8 M urea. Fractions were counted in a γ counter. a, elution profile; b, fractions were pooled as follows: 1 + 2 (A), 3 + 4 (B), 5 + 6 (C), 7 + 8 (D), 9 + 10 (E), 11 + 12 (F), 13 + 14 (G), 15 + 26 (H), 17 + 18 (I), 19 + 20 (J), 21 + 22 (K), and were analyzed on a 10% sodium dodecyl sulfate-acrylamide gel. The gel was dried and exposed to an x-ray film. Standard proteins (kDa × 10−3) are indicated on the left. Un, unbound material.

RESULTS

Attachment of Hepatocytes to Protein-coated Substrata—HSPG, its core protein, or laminin were adsorbed onto plastic wells and assayed for their ability to promote the attachment and spreading of hepatocytes. In the absence of these proteins, few if any cells bound to the uncoated plastic surface. Substrates formed of HSPG and the core protein supported the attachment of the hepatocytes in a dose-dependent manner but to a lesser extent than laminin (Fig. 1). At 50 μg/ml, HSPG and the core protein of HSPG show about half of the cell attachment activity of laminin. Initially, during the first hours in culture, the hepatocytes plated on laminin, HSPG,

Fig. 3. Purification of 125I-HSPG-core-binding proteins by affinity chromatography on HSPG-core-Sepharose. Cell surface proteins of freshly isolated hepatocytes were labeled by lactoperoxidase-catalyzed iodination. The membrane fraction was extracted and solubilized in 1% octylglucoside and 1% CHAPS in TEA buffer, pH 7.4, and passed over an HSPG-core-coupled Sepharose 4B column. After washing, proteins were eluted with a linear gradient NaCl (0.15-1 M) in the presence of detergent and then with 0.1 M glycine, pH 2, followed by 8 M urea. Fractions were counted in a γ counter. a, elution profile; b, fractions were pooled as follows: 1 + 2 (A), 3 + 4 (B), 5 + 6 (C), 7 + 8 (D), 9 + 10 (E), 11 + 12 (F), 13 + 14 (G), 15 + 26 (H), 17 + 18 (I), 19 + 20 (J), 21 + 22 (K), and were analyzed on a 10% sodium dodecyl sulfate-acrylamide gel. The gel was dried and exposed to an x-ray film. Standard proteins (kDa × 10−3) are indicated on the left. Un, unbound material.

Fig. 4. Purification of 125I-HSPG-core-binding protein by affinity chromatography of hepatocyte membrane fractions on HSPG-core-Sepharose. P2 and P3 membrane fractions were purified from iodinated hepatocytes according to the method of Aronson and Touster (23). Membrane proteins were solubilized with 1% octylglucoside and 1% CHAPS, then passed over a HSPG-core affinity chromatography column. Bound proteins were eluted with 1 M NaCl and resolved on a 10% polyacrylamide gel. The gel was dried and exposed 1 day (P3 fraction) or 5 days (P2 fraction) to an x-ray film. Standard proteins are indicated on the left (kDa × 10−3).

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Fig. 5. Binding of 125I-HSPG-core to nitrocellulose filters containing electrophoresed proteins from various cells. Cells were homogenized, and plasma membrane proteins were purified and solubilized in detergent. Proteins from both the cell homogenate (lanes 1, 3, and 4) and the cell membrane preparations (lanes 2 and 5–7) were resolved by electrophoresis on a 10% polyacrylamide gel, then transferred onto a nitrocellulose filter that was incubated with 125I-HSPG-core, and then dried and exposed to an x-ray film. Hepatocytes (lanes 1 and 2), retinoic acid-treated (lanes 3 and 5) or nontreated (lanes 4 and 6) NIH/3T3 cells, and mouse melanoma M2 cells (lane 7) were analyzed.

FIG. 5. Binding of 125I-HSPG-core to nitrocellulose filters containing electrophoresed proteins from various cells. Cells were homogenized, and plasma membrane proteins were purified and solubilized in detergent. Proteins from both the cell homogenate (lanes 1, 3, and 4) and the cell membrane preparations (lanes 2 and 5–7) were resolved by electrophoresis on a 10% polyacrylamide gel, then transferred onto a nitrocellulose filter that was incubated with 125I-HSPG-core, and then dried and exposed to an x-ray film. Hepatocytes (lanes 1 and 2), retinoic acid-treated (lanes 3 and 5) or nontreated (lanes 4 and 6) NIH/3T3 cells, and mouse melanoma M2 cells (lane 7) were analyzed.
proteins were eluted with a linear gradient of NaCl followed by 0.1 M NaCl. Smaller amounts of other proteins were also eluted from the P3 fraction. This result shows that the 38-kDa HSPG-core-binding protein is cell surface-associated and could be directly involved in the binding of the hepatocyte to HSPG-core.

**Binding of Plasma Membrane Proteins to 125I-HSPG-core**

The ability of total cell proteins to bind the core protein of HSPG was tested by electrophoresing hepatocyte lysates, transferring the proteins to nitrocellulose filters and incubating these filters with 125I-HSPG-core. Under these conditions, binding of the labeled HSPG-core was observed to the 38-kDa protein, with less bound by the 36- and 26-kDa proteins (Fig. 4). Using proteins extracted from isolated hepatocyte membranes, binding of 125I-HSPG-core was observed almost entirely with the 38-kDa protein (Fig. 5), indicating that the 38-kDa protein was cell membrane-associated and that the 26-kDa protein was derived from a different site or was lost during the isolation of the membranes.

Various other cell types were tested for the presence of HSPG-core-binding proteins (Table 1). The 38-kDa protein was detected in M2 (Fig. 5), COS, Madin-Darby canine kidney, Engelbreth-Holm-Swarm tumor, and NRK52-E cells. In contrast, no significant labeling was found in HT1080 or NRK49-F cells, and NIH/3T3 cells exhibited only a very small amount of this protein. However, when the NIH/3T3 cells were treated with 10^{-5} M retinoic acid in serum-free medium for 48 h, the amount of the 38-kDa protein was strongly enhanced (Fig. 5).

To investigate the specificity of the binding between HSPG-core protein and the 38-kDa protein, cell membrane extracts were incubated with 125I-HSPG-core, followed by incubation with either cold HSPG-core, laminin, collagen IV, fibronectin, heparin, or heparan sulfate (Fig. 6). Cold HSPG-core was the only compound that significantly displaced the 38-kDa 125I-HSPG-core. This study indicates that the membrane protein recognizes and binds specifically to the protein portion of the proteoglycan and that the heparan sulfate side chains are not important determinants of the interaction of the molecule with this cellular receptor.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Origin</th>
<th>40/36 kDa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal hepatocytes</td>
<td>Rat liver</td>
<td>+</td>
</tr>
<tr>
<td>M2</td>
<td>Dog kidney</td>
<td>+</td>
</tr>
<tr>
<td>COS (transformed fibroblasts)</td>
<td>Monkey kidney</td>
<td>+</td>
</tr>
<tr>
<td>HT1080</td>
<td>Human fibrosarcoma</td>
<td>Undetectable</td>
</tr>
<tr>
<td>EHS</td>
<td>Mouse sarcoma</td>
<td>+</td>
</tr>
<tr>
<td>NIH/3T3</td>
<td>Mouse embryo</td>
<td>Slightly detectable</td>
</tr>
<tr>
<td>M2</td>
<td>Mouse melanoma</td>
<td>+</td>
</tr>
<tr>
<td>NRK-49F (normal fibroblasts)</td>
<td>Rat kidney</td>
<td>Undetectable</td>
</tr>
<tr>
<td>NRK-52E (normal epithelial cells)</td>
<td>Rat kidney</td>
<td>+</td>
</tr>
</tbody>
</table>

**Fig. 6. Specific binding of the HSPG-core protein to the 38-kDa protein.** Hepatocyte cell membrane proteins were electrophoresed on a 10% polyacrylamide gel (5 μg/lane) and then transferred onto nitrocellulose filter. Efficiency of the transfer was checked by Amido Black staining. Filters were cut between 30 and 40 kDa and incubated with 125I-HSPG-core for 2 h. Serial dilutions of either cold HSPG-core, collagen IV, fibronectin, heparin, or heparan sulfate were added to the washing solution. Nitrocellulose filters were dried and counted in a γ-counter. Specificity of the binding was monitored by exposing nitrocellulose fragments to an x-ray film. Values are average of duplicate experiments.

or HSPG-core remained rounded, then slowly spread over the following 6 h (Fig. 2).

**Affinity Chromatography of Detergent Extracts of Hepatocyte Membrane**—To test if specific proteins present on the cell were involved in their attachment to the proteoglycan core protein, hepatocytes were surface-labeled with 125I by the lactoperoxidase method, and the 125I-labeled proteins were solubilized with detergent and applied to a HSPG-core affinity column. The column was washed with TEA and then bound proteins were eluted with a linear gradient of NaCl followed by 0.1 M glycine-HCl (pH 2) and 8 M urea. Two major peaks of proteins were eluted, the first between 0.4 and 0.8 M NaCl and the second with 8 M urea (Fig. 3a). The proteins in each fraction were characterized by electrophoresis (Fig. 3b) which resolved a major protein (M₀ = 38,000) eluting between 0.4 and 0.8 M NaCl. Smaller amounts of other proteins, i.e. M₀ = 28,000–80,000, were also eluted in these same fractions. Urea eluted predominantly a single labeled protein (M₀ = 26,000).

To confirm the cell surface location of the 38-kDa HSPG-core-binding protein, membranes were isolated from iodinated hepatocytes and extracted with detergent, and the extract was passed over the HSPG-core affinity column (Fig. 4). Non-bound materials were washed from the columns with 0.15 M NaCl, and bound proteins were eluted with 1 M NaCl. Both the P2 plasma membrane fraction and the P3 fraction contained the 38-kDa HSPG-core-binding protein. In addition, an 80-kDa binding protein appeared to be enriched in the P2 fraction. Smaller amounts of other proteins (M₀ = 80,000 and 45,000) were also eluted from the P3 fraction. This result shows that the 38-kDa HSPG-core-binding protein is cell surface-associated and could be directly involved in the binding of the hepatocyte to HSPG-core.

**DISCUSSION**

Proteoglycans are a diverse class of macromolecules varying in their protein core and glycosaminoglycan chains (1, 8, 14). They are known to fulfill a variety of functions including a structural role in the matrix and as integral components of cell membranes (25-28). A number of studies indicate that cells have the capacity to interact with a variety of matrix molecules including various collagens and glycoproteins. Also, cells bind to the core protein of a dermatan sulfate proteoglycan and to heparan sulfate proteoglycan. Binding of the dermatan sulfate proteoglycan is involved in its uptake by fibroblasts prior to degradation (29). Binding of the heparan sulfate proteoglycan induces attachment and spreading of the cells (15-17, 30).

We chose to examine the binding of the major proteoglycan of basement membrane to hepatocytes because these cells are known to bind two other components of basement membrane, collagen IV and laminin (31, 32). Hepatocytes in the adult do...
not have a prominent basement membrane. However, various basement membrane components, including collagen IV, laminin, fibronectin, and heparan sulfate proteoglycan, have been detected on the surface of hepatocytes in Dise’s spaces (33–37). Thus, it seemed reasonable to examine the interaction of hepatocytes with various other basement membrane components. These studies showed that hepatocytes were able to bind to and spread on laminin, although not to the extent that the cells bound to a collagen substrate (38). Also, by using core protein freed by heparitinase treatment of heparan sulfate chains, it appeared that the interaction of the hepatocytes was with the core protein rather than with the heparan sulfate chains. Several cell surface proteins were found to bind to the core protein of heparan sulfate proteoglycan.

The major proteins were 38- and 26-kDa proteins. The 38-kDa protein was detected in a variety of normal epithelial cells, transformed cells, and metastatic cells and was increased in NIH/3T3 cells after retinoic acid treatment. Retinoic acids are indeed known to influence cell-to-substratum adhesion in epithelial cells and fibroblasts (39), and it has been shown that retinoic acid specifically enhances the cell attachment of NIH/3T3 to laminin and collagen IV (40).

In addition, it is possible that binding of the various matrix molecules occurs as a concerted process, as seen, for example, with neurites where the differentiation of the nerves is dependent on their interaction with a supramolecular complex that includes laminin and proteoglycan (45, 46).

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