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A Novel Ligand Blot Assay Detects Different Hyaluronan-Binding Proteins in Rat Liver Hepatocytes and Sinusoidal Endothelial Cells

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We have developed a sensitive ligand blot assay to detect hyaluronan (HA) binding proteins in cell extracts using ^{125}I-HA. Samples to be tested are electrophoresed using standard, nonreducing SDS-PAGE conditions, electro-transferred to nitrocellulose then blocked in buffer containing Tween 20. After incubation with ^{125}I-HA the nitrocellulose is washed and HA-binding proteins are detected by autoradiography. This method was used to detect different HA-binding proteins in isolated rat liver cell preparations. Two HA-binding bands of 175 kD and 350 kD were detected in sinusoidal endothelial cells. Both bands were competed virtually 100% with nonlabeled HA. Using rat hepatocytes, the assay detected major bands at 85 kD and 180 kD. In addition, histones present in both cell types were readily detected in the low MW region. Thus, two different liver cell types show different HA-binding patterns. The blocking procedure is critical for successful renaturation of HA-binding activity, since substitution of BSA for Tween 20 did not result in detectable ^{125}I-HA-binding. This ligand blot assay will be a powerful tool to detect HA-binding proteins in various other tissues and cell types.

To understand the diverse biological roles played by hyaluronic acid, or hyaluronan (HA),[1] the mechanism by which HA interacts with cells must be elucidated. HA interacts with many different cell types as well as a variety of extracellular molecules collectively known as hyaladherins (1). About 50 hyaladherins have been described. Some are involved in cell-cell or cell-matrix interactions, others are important for HA clearance from the blood or tissues. Some are found in the extracellular spaces and are important in matrix organization. Many hyaladherins have been well characterized biochemically, but only a few have been cloned. Assays to measure HA-binding sites on intact or permeabilized cells as well as in tissue sections, have been described using radiolabeled or chemically modified HA (2–5). Rapid, sensitive procedures, such as dot-blot (6) and precipitation assays (7), have also been developed to measure soluble HA-binding activity. While these assays are extremely useful for the detection of potential hyaladherins, they are limited in that they do not identify the actual molecules interacting with HA. To identify hyaladherins, investigators have utilized immunological techniques such as immunoprecipation and Western blotting, affinity chromatography, and photo-affinity crosslinking. Although these techniques are also very useful, they can be time-consuming and technically challenging, and with immunological methods, one must have a specific anti-hyaladherin antibody. Here we report a ligand blot assay that uses a novel radio-iodinated HA to measure binding activity in SDS-PAGE separated proteins after transfer to nitrocellulose, and demonstrate its use to detect hyaladherins in isolated liver cells.

METHODS

Materials. Cell culture media and reagents were from Gibco. ^{125}I-HA was prepared as previously described (2) using a unique alkylamine derivative of hyaluronic acid, (oligosaccharides of $M_r \sim 60 - 80{,}000$). Male Sprague-Dawley rats (200g)

[1] Abbreviations: HA, hyaluronic acid; LEC, liver sinusoidal endothelial cell; BSA, bovine serum albumin; SDS-PAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; MW, molecular weight; TBS, Tris-buffered saline.

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were obtained from Harlan Breeding Laboratories, Houston, TX. Collagenase was from Boehringer Mannheim Biochemicals. Nitrocellulose paper was from Schleicher & Schuell (0.45 and 0.1 μm). BSA Fraction V was from Armor Biochemicals. All other chemicals and reagents were from Sigma.

Isolation of liver cells. Rat livers were perfused with collagenase as previously described (8). Hepatocytes were obtained by differential centrifugation and filtration of the crude cell preparation. The nonparenchymal cell fraction was utilized for LEC isolation by differential centrifugation and discontinuous Percoll gradients (9). Cells banding at the 25/50 percent interface were removed and washed 3 times in PBS at 4°C.

LEC membrane preparation and detergent extraction. The LEC total membrane fraction was prepared from freshly isolated LECs using hypotonic swelling and dounce homogenization as previously described (9). LEC membranes were extracted twice in buffer 1 (8) containing 0.5% CHAPS, 5mM EDTA and a protease inhibitor cocktail. After gentle stirring for 2 h on ice, the suspension was centrifuged at 105,000xg for 60 min at 4°C and the detergent soluble phase was removed. The pellet was extracted a third time for 2 h at 4°C in the same buffer containing 1.5% Chaps and 2.0M KC1. This detergent soluble phase was designated the high-KC extract.

Hepatocyte membranes and detergent extraction. Membranes prepared from freshly purified hepatocytes as described earlier (6) were carbonate washed and extracted in 50mM KC1, 40mM imidazole, pH 7.4, 1mM EGTA, 4mM $MgCl_2$, 1.5% CHAPS and protease inhibitors by homogenization with a Teflon pestle and gentle mixing at 4°C for 1h. The suspensions were centrifuged at 105,000g for 60 min at 4°C and the detergent-soluble phase was removed. The residual insoluble material was then reextracted for 60 min at 4°C in the same buffer containing 2.0M KC1, centrifuged at 105,000g for 1 h at 4°C and the detergent soluble phase was removed and stored frozen at −70°C. Heparin-agarose was treated with 0.1 mg/ml BSA, washed 3 times with equal volumes of 40mM imidazole, pH 9.0, containing 1mM EGTA, 4mM $MgCl_2$, 0.075% CHAPS, 5% glycerol and 1.2M KC1. Finally, the heparin-agarose was washed 3 times with the same buffer at pH 7.4, with 0.15M KCl (loading buffer) and resuspended as a 1:1 slurry. Two ml of the slurry were then added per 40 ml extract and binding was allowed to occur at 4°C for 90 min, while the samples were rotating. The heparin-agarose was then washed 3 times by centrifugation in loading buffer, and once with the same buffer modified to contain 0.4M KCl at pH 9.0. Specifically bound proteins were then eluted using an equal volume of 1M NaBr in Tris pH 9.0, 1mM EGTA, 4mM MgCl, 0.5% CHAPS, and 5% glycerol. After 30 min at 4°C, the heparin-agarose was centrifuged and the eluted HA-binding proteins were removed.

^{125}I-HA ligand blot assay. Samples were electrophoresed on SDS-PAGE gels (10) without boiling or reducing agent using a modified sample buffer containing 0.1% SDS. Electrophoresis was at 140v for 70 min at <8°C. Separated proteins were electro-transferred onto nitrocellulose (Genie transfer apparatus, Idea Scientific) usually for 2 h at 24V at 4°C with 25mM Tris, pH 8.3, 192mM glycine, 20% methanol and 0.01% SDS. The nitrocellulose was then incubated in TBS containing 0.05% Tween 20 for 1 h at 4°C. The nitrocellulose was cut into identical strips and incubated for 2 h at 4°C with 1–4μg ^{125}I-HA per ml buffer 1 plus 5mM EDTA and 0.15% BSA in the presence or absence of 100-fold excess of unlabeled HA to assess nonspecific and total binding, respectively. The blots were quickly rinsed once in buffer 1 (9) plus 5mM EDTA, washed twice for 5 min each in the same buffer, then washed twice for 5 min each in this buffer containing 0.01% NP40. The blots were quickly rinsed in buffer 1/EDTA and radioactivity was detected by autoradiography on Kodak X-OMAT AR film with an enhancing screen at −70°C. Exposure times were from 2–24 h.

General. Highly purified rat liver nuclei were prepared by the procedure of Blobel and Potter (11). ^{125}I-HA radioactivity was determined using a Packard MultiPrias 2γ Spectrometer. Protein was determined by the methods of Bradford (12) or Lowrey, *et al.* (13) depending on the salt or detergent present in the sample, using BSA as standard.

RESULTS AND DISCUSSION

The ligand blot assay detects high MW HA-binding proteins in LECs. LECs contain a cell surface endocytic receptor for HA and other glycosaminoglycans (14). Although this membrane HA receptor has been studied in whole cells, solubilized and partially purified (14–17), it has not been characterized yet at the molecular level. When an extract from LEC membranes was probed with ^{125}I-HA in the ligand blot assay, two major bands were detected after autoradiography with apparent MWs of 175,000 and 350,000 (Fig. 1). A high level of HA binding was also detected at the dye front, corresponding to low MW proteins, particularly histones, that are known to bind HA (6). The high MW bands were almost completely competed by the presence of a 100-fold excess of nonderivatized HA as specific competitor. Under these conditions, the large signal from histones at the dye front was not effectively competed. In this experiment, we also examined the effect of 1M NaBr in the ligand blot sample buffer (Fig. 1), because it had been included in a protocol developed to prevent hepatocyte membrane proteins from aggregating in solution. Normal SDS-PAGE can be run in the presence of 1M NaBr. Although the two high MW HA-binding proteins were readily detected without NaBr (lane 3), we did notice significant enhancement of binding

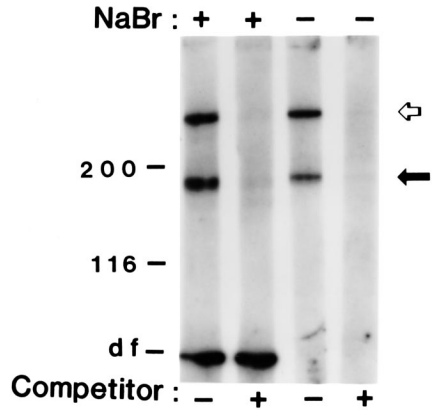

FIG. 1. Identification of the LEC HA receptor using a ligand blot assay. The high-KC extract from LEC membranes was concentrated and de-salted with 20mM Tris, pH 7.0, 5mM EDTA, 0.5% CHAPS and 5% glycerol plus or minus 1.0M NaBr using Centricon devices; the final KCl concentration was <20mM. SDS and bromophenol blue were then added to final concentrations of 0.1% and 0.005%, respectively. The samples were run on a 5% SDS-PAGE gel in Laemmli buffer (10) containing 1.0M NaBr in the absence of a reducing agent, then transferred to nitrocellulose and processed for ^{125}I-HA binding. The 175 (solid arrow) and 350 (open arrow) kD species are indicated.

when samples were run in the presence of NaBr (lane 1). Binding to histones was noticeably absent in the gels run in the absence of NaBr.

Generally, ligand blot sample buffer contained 0.1% SDS. However, no change in the extent of ^{125}I-HA-binding was seen for either the 175 or 350 kD bands with up to 2% SDS (not shown). While samples were usually not boiled, we have noted that HA-binding activity can be recovered in boiled samples, if they are appropriately treated after electro-transfer with solutions containing nonionic detergent.

While the presence of NaBr is not important for ^{125}I-HA-binding to LEC extracts, other parameters were critical for activity. In particular, the protocol for treating the nitrocellulose was very important. If the nitrocellulose was blocked with BSA, rather than Tween 20, then no ^{125}I-HA-binding was observed (Fig. 2). No HA binding is also seen if nitrocellulose strips are blocked with milk solids, gelatin, or other proteins. Binding was only detected if blots were treated with an appropriate detergent such as Tween 20. If blots were first blocked with BSA, HA binding could still be observed after subsequent treatment of the blots with buffer containing Tween 20. HA binding was not restored by treatment with ionic detergents such as SDS, deoxycholate or CHAPS. However, other nonionic detergents, including Triton X-100, NP40, Brij 35, and Tween 85 also gave increased HA binding (not shown), presumably by renaturing the SDS-denatured proteins sufficiently for ligand binding to occur. Reduction of the LEC proteins before electrophoresis using β-mercaptoethanol totally eliminated specific ^{125}I-HA binding, confirming that disulfide bonds are critical for HA-binding by this LEC HA receptor (9).

Previous studies using photo-affinity crosslinking (17), detergent solublization and gel filtration (9) led us to propose that the LEC HA receptor is a disulfide-bonded hetero-dimer composed of 175 and 166 kD subunits; a native receptor might have an apparent MW of 340,000. Detection of a specific HA-binding activity in LEC membranes at 175 kD supports the conclusion that this subunit is responsible for HA binding by the LEC HA receptor.

The ligand blot assay detects different HA-binding proteins in hepatocytes. In earlier studies, we identified a ^{125}I-HA-binding protein in isolated rat hepatocytes (18). During attempts to purify this activity and characterize it further, we discovered that this molecule is also able to ligand blot as observed above for the LEC HA receptor. Extracts prepared from hepatocyte membranes and subject to SDS-PAGE and electro-transfer were probed with ^{125}I-HA (Fig. 3). In addition to the

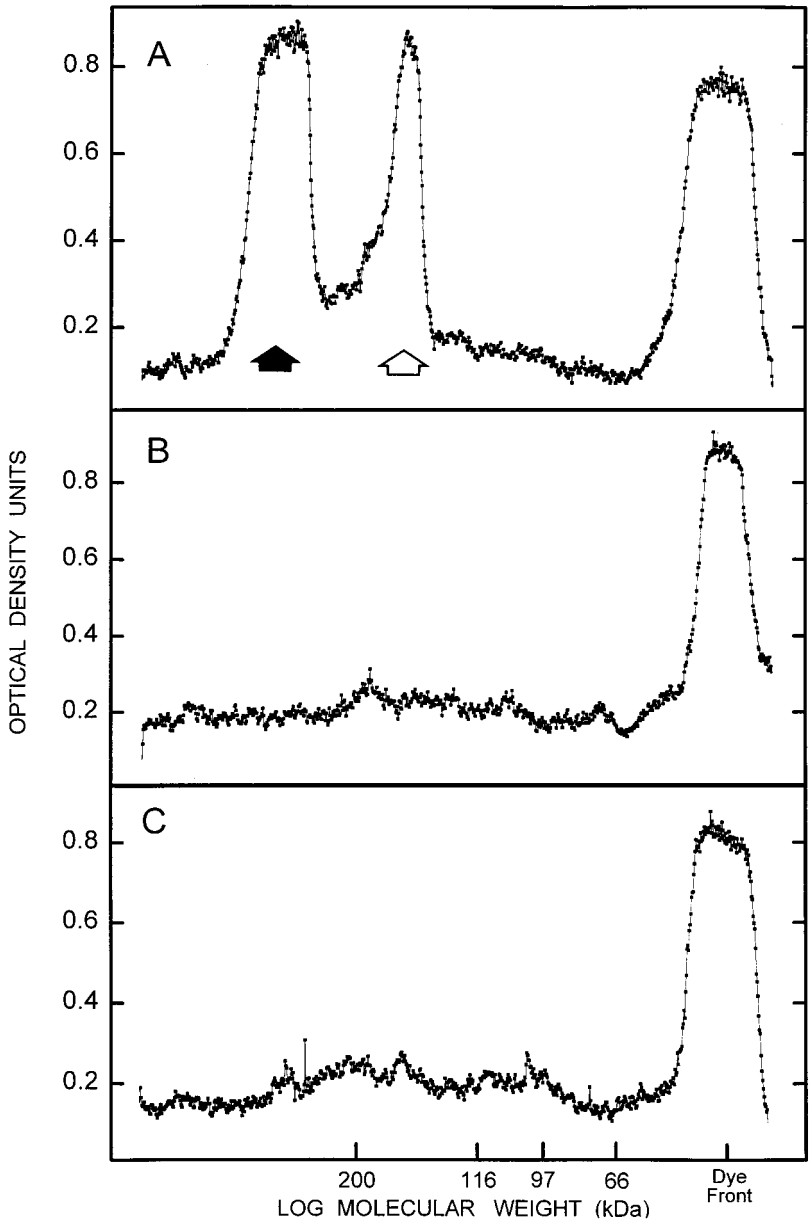

FIG. 2. Effect of Tween 20 on ^{125}I-HA binding to LEC proteins. The high-KC extract of LEC membranes was processed without NaBr as in Fig. 1, subjected to SDS-PAGE and transferred to nitrocellulose. Nitrocellulose strips were blocked with TBS containing 1% BSA alone (B) or 0.5% Tween 20 (A and C). After washing, the strips were processed for ^{125}I-HA-binding as in Methods in the presence (C) or absence (A and B) of excess HA. After autoradiography samples were scanned using a Molecular Dynamics Personal Densitometer SI. The open and solid arrows indicate the regions of the 175 kD and 350 kD proteins, respectively.

expected HA-binding activity of histones in the low MW region, a band was observed at 85–90 kD. This ^{125}I-HA-binding activity was completely eliminated in the presence of a 100-fold excess of nonlabeled HA, which also reduced the signal from histones (Fig. 3A). Electrophoresis using a lower percentage gel revealed several other minor HA-binding activities in this hepatocyte extract

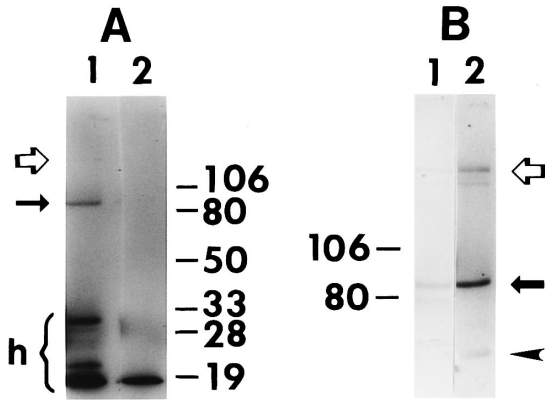

FIG. 3. ^{125}I-HA binding to rat hepatocyte proteins. Detergent solubilized membrane proteins eluted from heparin-agarose were concentrated, subjected to SDS-PAGE (in the presence of 1M NaBr) on a 7.5% gel and transferred to nitrocellulose. **A.** Identical lanes were incubated with ^{125}I-HA either without (lane 2) or with (lane 1) a 100-fold excess of nonlabeled HA. **B.** Another sample was run on a 5% gel: lane 1, protein stained with copper phthalocyanine; lane 2, ^{125}I-HA binding. The 55 (arrowhead), 85 (solid arrow) and 180 (open arrow) kD HA-binding species are indicated. The histone region is marked 'h.'

(Fig. 3B); binding activity was also seen at about 55 kD and 180 kD. The 55 kD HA-binding activity may correspond to the HA-binding protein that was specifically labeled in isolated hepatocytes using an HA photo-affinity derivative (18).

Because of the large amount of histones and large nuclear fraction present in hepatocytes, we wanted to determine whether the ~90 kD HA-binding protein was present in membranes or was actually of nuclear origin. Freshly isolated hepatocytes were homogenized and a nuclear fraction and crude membranes were prepared and analyzed from the same cells (Fig. 4). The ~90 kD ^{125}I-HA-binding activity was only present in the membrane fraction and was specifically competed by nonlabeled HA. The nuclear fraction contained histones ranging in apparent size up to ~30 kD.

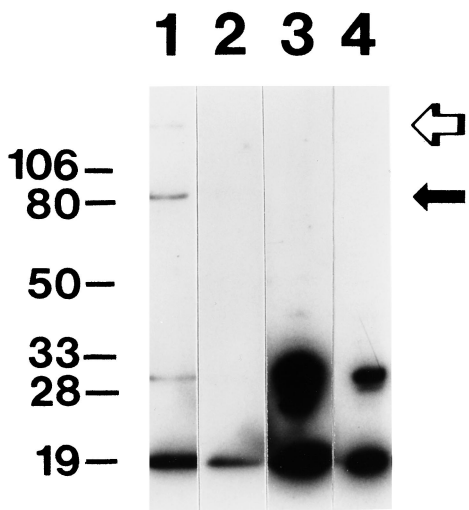

FIG. 4. ^{125}I-HA binding to rat hepatocyte membrane and nuclear fractions. Detergent solubilized hepatic proteins derived from total membranes (lanes 1 and 2) and nuclei (lanes 3 and 4) were subjected to SDS-PAGE (in the presence of 1M NaBr), electro-blotting and ^{125}I-HA binding in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of a 100-fold excess of HA. The 85 (solid arrow) and 180 (open arrow) kD proteins are indicated.

There was no evidence of higher MW HA-binding activity in the nuclear fraction. The hepatocyte HA-binding protein, like that for the LECs, was inactivated by reduction prior to SDS-PAGE. In addition, this activity was also not observed if the blots were blocked with proteins, such as BSA, rather than by exposure to a renaturing detergent such as Tween 20. Detergent treatment was absolutely required to detect the specific LEC and hepatocyte ^{125}I-HA-binding activity in the ligand blot assay.

A 100 kD protein, presumably on the surface of LECs, has been identified by others as a putative HA receptor (19). Recently McCourt, *et al.* (20) isolated a 90 kD protein from whole liver using HA-Sepharose affinity-chromatography as the final purification step. This molecule was identified as ICAM-1 (21) based on partial amino acid sequence. We did not detect a 90 kD polypeptide in LECs with the properties of an HA receptor in the previous crosslinking studies (17) or in the ligand blotting experiments reported here. However, as shown above, hepatocytes clearly have an 85–90 kD HA-binding protein. Since the ICAM-1 protein was isolated from whole liver, it is likely that McCourt, *et al.* (20) isolated a hepatocyte hyaladherin (22), rather than the LEC HA receptor.

We conclude that two of the major cell types in liver, the hepatocytes and LECs, both contain membrane-bound HA-binding proteins that are distinct and that can readily be distinguished by their different characteristics on SDS-PAGE. The sensitivity and ease of this ligand blot assay using ^{125}I-HA will allow the detection and characterization of other, as yet unrecognized, HA-binding proteins and receptors in a variety of other tissues and cell types.

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