Identification of a 175 kDa protein as the ligand-binding subunit of the rat liver sinusoidal endothelial cell hyaluronan receptor

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The rat liver sinusoidal endothelial cell (LEC) hyaluronan (HA) receptor was previously identified using a photoaffinity HA derivative (J. Biol. Chem., 267, 20451-20456, 1992). Two polypeptides with M_r = 175,000 and 166,000, were consistently crosslinked, suggesting that the LEC HA receptor is an oligomer. Whether one or both subunits participate in HA binding, was not determined. Here we investigate the HA–subunit interactions and the potential oligomeric nature of the LEC HA receptor. When Sephacryl-400 gel filtration chromatography was used to enrich the HA receptor, the 175 kDa polypeptide was the major band seen by SDS–PAGE analysis. Little staining was seen at 166 kDa, suggesting that the 175 kDa protein could be separated from the 166 kDa protein and still retain HA-binding activity. A ligand blot assay was used to determine if each individual subunit could bind HA. LEC proteins were separated by nonreducing SDS–PAGE, and then immobilized onto nitrocellulose. 125I-HA bound to a 175 kDa polypeptide but not to the 166 kDa protein. A high molecular weight band of ~300,000 also bound 125I-HA. 125I-HA binding to the 175 and 300 kDa proteins showed the same specificity of competition with a panel of carbohydrates as the bona fide LEC HA receptor. The 175 kDa HA-binding subunit may be nonglobular (asymmetric), since its apparent size by SDS–PAGE is dependent on the polyacrylamide gel pore size; M_r increases as porosity decreases. LECs were crosslinked to an 125I-labeled photoaffinity HA derivative and the HA saccharides were then released with hyaluronidase. After SDS–PAGE without reduction, radio-labeled bands were seen at 175 and 166 kDa (3:1 ratio), and a high MW (~300,000) species was also detected. These data support an oligomeric model of the LEC HA receptor, and show that the 175 kDa protein possesses HA-binding activity independent from the 166 kDa polypeptide.

Key words: hyaluronan receptor/endocytosis/ligand blot assay/liver sinusoidal endothelial cells

Introduction

An important function of liver sinusoidal endothelial cells (LECs) is to remove the nonsulfated glycosaminoglycan hyaluronan (HA) from the blood via a Class II endocytic receptor (Smedsrod et al., 1990). The receptor also binds and internalizes other glycosaminoglycans, such as chondroitin sulfate (Raja et al., 1988) and heparin (Smedsrod et al., 1984). Therefore, the binding specificity is not restricted to HA; this receptor is specific for a subset of GAGs. Internalization of receptor–HA complexes occurs via a coated pit-mediated pathway (McGary et al., 1989). HA binding and subsequent internalization is not dependent on the extracellular presence of divalent cations such as Ca^{2+} (Yannariello-Brown et al., 1992b). The affinity of the LEC receptor for HA depends on polymer length and is of moderate to high affinity, with K_d values ranging from 10^{-11} to 10^{-8} M (Smedsrod et al., 1990). The molecular nature of the endocytic HA receptor is still being investigated. The receptor was detergent solubilized from LEC membranes using conditions that retain its binding activity (Yannariello-Brown and Weigel, 1992). Biochemical studies on solubilized protein showed that the receptor is thermally labile and behaves as an integral membrane protein. HA receptor activity is sensitive to reducing agents, suggesting that disulfide bonds are critical for maintaining a biologically active conformation, similar to other endocytic receptors (Weigel, 1992). Gel filtration analysis of detergent-solubilized LEC membranes showed HA binding activity in three major peaks with M_r values ~1 x 10^5, 400 x 10^5, and 125 x 10^5 depending on the salt and detergent concentration (Yannariello-Brown and Weigel, 1992). This result suggested that the native LEC HA receptor was either very large or a complex of polypeptides with a tendency to aggregate. When a novel radiiodinated photoaffinity crosslinking derivative of HA was used to identify the HA receptor in LECs, two polypeptides (M_r ~ 175,000 and 166,000) were specifically crosslinked in cultured LECs and in isolated LEC membranes (Yannariello-Brown et al., 1992a). Taken together, the crosslinking, gel filtration, and biochemical analysis led us to propose that the LEC HA receptor is a disulfide-bonded hetero-dimer with a M_r ~340,000 composed of two polypeptides with M_r ~175,000 and 166,000 (Yannariello-Brown and Weigel, 1992; Yannariello-Brown et al., 1992a).

Here we use a novel ligand blot assay for 125I-HA-binding activity to show that the 175 kDa polypeptide of the LEC HA receptor can bind HA independently of the 166 kDa polypeptide and that a high MW species, also has HA-binding activity.

Results

Identification of the LEC HA receptor using a ligand blot assay

LEC membrane extract II was separated by nonreducing SDS–PAGE, transferred to nitrocellulose, and HA-binding proteins were detected using 125I-HA (Figure 1). Two major HA-binding polypeptides were detected with M_r values ~175,000 and ~300,000. When a 150-fold excess of underivatized HA
was added as a competitor, both bands were virtually 100% competed. The HA-binding activity of both proteins was eliminated if the samples were reduced with β-mercaptoethanol prior to SDS-PAGE. The demonstration of HA-binding activity in this ligand blot assay was absolutely dependent on exposure of the electroblotted protein to a detergent such as Nonidet P40 or Tween 20 in the blocking step. No activity was detected if BSA, with no detergent, was used as the blocking reagent (Figure 2, lane B).

Specificity of binding was demonstrated by competition with nonlabeled HA and various other carbohydrates. 125I-HA binding to the 175 and 300 kDa proteins was competed very effectively by HA and chondroitin sulfate, but not as well by desulfated chondroitin, heparin, dextran sulfate, polygalacturonan, DNA, or glucuronic acid (Figure 2, Table I). The differences between HA and chondroitin sulfate versus the other saccharides tested were even greater at lower concentrations. This pattern of competition is essentially identical to that previously observed for 125I-HA binding to the HA receptor in intact or permeable LECs (Raja et al., 1988; McGary et al., 1989). When a variety of heparins from different tissue sources were tested for the ability to compete 125I-HA binding, little or variable competition was detected. Dose–response experiments showed that some heparin preparations tested were unable to block HA binding and, although some showed modest competition (Table I), none competed more than 50%, even at 1.8 mg/ml (not shown). Since heparin preparations can vary in the presence of 5% β-mercaptoethanol as indicated. Resolved proteins were electroblotted onto nitrocellulose, treated with Tween-20 and 125I-HA binding was assessed as described in Materials and methods in the presence or absence of excess HA (as competitor) Molecular weight makers (in kDa) are shown on the left. The solid and open arrows indicate the 175 and 300 kDa proteins, respectively. Df indicates the dye front.

The LEC membrane extract II was concentrated, desalted, separated on a 5% SDS-PAGE gel in a modified ligand blot sample Buffer in the absence of reducing agent and then transferred to nitrocellulose. Strips of nitrocellulose paper containing the separated LEC membrane proteins were incubated in buffer containing Tween 20 with 125I-HA plus or minus a 150-fold excess (w/w) of the following unlabeled saccharide or polyanion inhibitors: HA, hyaluronan; CS chondroitin sulfate; dC, desulfated chondroitin; H, heparin; PG, poly-galacturonan; DS, dextran sulfate; GA, glucuronic acid (monosaccharide). Molecular weight markers (in kDa) are shown on the left. The solid and open arrows indicate the 175 and 300 kDa proteins, respectively. Protein bands are broadened due to incomplete removal of salt. Lane B shows a nitrocellulose strip treated with buffer containing 1% BSA, rather than Tween 20, to block nonspecific binding. In this case renaturation of the HA receptor does not occur and there is no 125I-HA binding.

For example, the pattern of competition for 125I-HA binding to histones, which migrate with the dye front, is much different than that for the HA receptor (Table I). HA binding to histones is presumably mediated by nonspecific charge interactions. The highly anionic polymers such as chondroitin sulfate and heparin were good competitors for this interaction, whereas HA or chondroitin had little effect (Figure 2).

In a series of ligand blot experiments, we noted that the apparent size of the 175 kDa HA-binding protein varied depending on the percentage of the polyacrylamide gel used in SDS-PAGE. Using low percentage gels (5%, w/v) the 125I-HA-binding activity has an Mₐ ~ 175,000. However, as the gel

<table>
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<th>Table I. Specificity of 125I-HA binding in the ligand blot assay</th>
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The LEC membrane extract II was concentrated, desalted, separated on a 6.5% SDS-PAGE gel, and then transferred to nitrocellulose as described in Materials and methods. The sheets were blocked in TBSB₀₀₀₉ + 0.05% Tween 20, then incubated with 2 μg 125I-HA/ml in the absence or presence of 300 μg/ml (a 150-fold excess; w/w) of the various indicated saccharide competitors (CS, chondroitin sulfate; dC, desulfated CS; Hep, heparin; DS, dextran sulfate; pGal, polygalacturonic acid; GA, glucuronic acid). After washing, the bound radioactivity was detected by autoradiography and quantitated by densitometry. Values shown are the average of results from three to seven experiments. The slight effect of dCS is likely due to the incomplete removal of sulfate. df is the dye front, which contains histones that are also able to bind HA.
percentage increased, the migration of this protein decreased relative to the MW standards, so that its apparent size appeared larger (Figure 3). For example, in a 10% gel this HA-binding activity has a $M_r = 225,000$. This behavior indicates an asymmetric, elongated molecule whose rate of sieving during SDS-PAGE is decreased to a greater extent than that of more globular proteins as the pore size of the matrix decreases. The 300 kDa HA-binding protein behaved in a very similar fashion (Figure 3).

HA crosslinking to the LEC HA receptor on cultured LECs:

Previous photoaffinity crosslinker experiments with $^{125}$I-ASD-HA detected two polypeptides of 175 and 166 kDa with no appreciable material at ~300,000 (Yannariello-Brown et al., 1992a). This may be due to the fact that the SDS–PAGE analysis of crosslinked LEC products was performed in the presence of a reducing agent. When using the photoaffinity crosslinker $^{125}$I-ASD-HA to identify HA-binding proteins, one must release the covalently attached HA chains before analysis by SDS–PAGE in order to visualize discrete bands rather than a broad smear (Yannariello-Brown et al., 1992a). This is most easily accomplished by the use of a reducing agent, since SASD has a disulfide bond between the covalently attached HA and the iodinated, photoactivatable arylazide group. Therefore, in order to determine whether high MW oligomers, which could be disulfide-linked, are labeled in such crosslinking experiments, we could not use reduction to release bound HA oligosaccharides. Instead, enzymatic treatment with HAase at 37°C was used to release the crosslinked HA. Since LECs rapidly and efficiently internalize $^{125}$I-ASD-HA-receptor complexes at 37°C, it was necessary to pretreat the cells with 0.4 M sucrose before the start of the experiment. This hypertonic treatment inhibits the formation of coated pits (McGary et al., 1989) and the subsequent internalization of receptor-HA complexes during the 37°C HAase treatment.

Primary cultures of digitonin-permeabilized LECs were incubated with $^{125}$I-ASD-HA, photolyzed, and then incubated at 37°C with HAase to release the covalently attached polysaccharides. After treating cells with HAase (Figure 4), a specifically crosslinked band at 175 kDa, was seen in the absence of competitor; to a lesser extent, a band at 166 kDa was also seen (lane 1). A high MW band at ~300–350 kDa was also detected. The presence of nonlabeled HA as competitor caused a ~98% reduction in crosslinking, as assessed by densitometry (lane 2). In the absence of HAase treatment and reduction only a high MW smear of heterogenous HA-receptor complexes was detected (lane 3). This broad region was effectively competed, however, with nonlabeled HA (lane 4).

Sephacryl-400 gel filtration of solubilized LEC membrane proteins

LEC membrane extract I, which is enriched in HA-receptor activity, was analyzed by Sephacryl-400 gel filtration chromatography (Figure 5A). Protein eluted throughout the resolving range of the column as previously reported (Yannariello-Brown and Weigel, 1992b). However, as assessed by a dot blot...
Fig. 5. Enrichment of the 175 kDa protein after partial purification of the HA receptor. (A) The LEC membrane extract I was chromatographed on a Sephacryl-400 gel filtration column. Even-numbered fractions were tested for $^{125}$I-HA-binding activity using the dot blot assay and odd-numbered fractions were used to determine protein concentration. The total HA-binding activity is shown as c.p.m./fraction. Even and odd numbered fractions were pooled as follows: Pool 1, fractions 14-22; Pool 2, fractions 23-26; Pool 3, fractions 27-40. (B) Extract I and Pools 1-3 from the Sephacryl-400 column were desalted and concentrated using Buffer 1 and Centricon-10 devices. Samples (10 μg protein) were then analyzed by SDS–PAGE under nonreducing conditions using a 6% gel. The polypeptides were visualized by Coomassie R-250. The 175 kDa polypeptide is indicated by the solid circles (●). The positions of the 166 kDa and 300 kDa species are shown by the solid and open arrows, respectively.

The peak of HA-binding activity eluted with an apparent $M_r$ of $\sim 1 \times 10^6$. The column fractions were pooled as indicated, such that Pool 1 contained 5–10% of the total recoverable protein and ~85% of the total recoverable activity. We estimate at least a 25-fold increase in receptor purity based on specific HA-binding activity measured in the dot blot assay (Yannariello-Brown and Weigel, 1992). A significant loss of activity probably occurs during purification without a comparable loss of protein. SDS–PAGE analysis of the various LEC membrane extracts and column fractions support this (Figure 5B). The 175 kDa protein is seen to varying degrees in all lanes but the most intensely stained band is in the Pool 1 fraction, where the 175 kDa protein represents ~10% of the total protein. This band also bound $^{125}$I-HA in the ligand blot assay (not shown). Although the majority of the HA-binding activity was recovered in Pool
Discussion

HA is an anionic polysaccharide with a wide tissue distribution in vertebrates (Laurent, 1989; Laurent and Fraser, 1992). The synthesis and subsequent catabolism of HA must be tightly regulated, and highly efficient, to achieve its remarkably high turnover rate (~1 g/day; Laurent, 1989). The removal of HA fragments occurs to a small extent in the tissues at the site of synthesis, but primarily in the lymphatics, and the liver (Laurent, 1989; Smedsrod et al., 1990; Laurent and Fraser, 1992). The mechanism of HA uptake in the lymphatics is presently unknown. Evidence points to the participation of certain HA-binding proteins such as CD44 being responsible for the degradation of HA in the tissues (Underhill, 1992; Knuudson, 1993), although the mechanism is also unclear. The removal of HA fragments from the bloodstream occurs mainly in the liver and is mediated by the endothelial cells of the hepatic sinusoids (Smedsrod et al., 1990). Several laboratories, including ours, have been investigating the biology and biochemistry of the endocytic LEC HA receptor responsible for this clearance.

Previous studies using photoaffinity crosslinking (Yannariello-Brown et al., 1992a), detergent solubilization, biochemical analysis, and gel filtration analysis (Yannariello-Brown and Weigel, 1992) led us to propose that the LEC HA receptor is a disulfide-bonded hetero-dimer composed of a 175 kDa and 166 kDa subunit in a 1:1 ratio. This means that the LEC HA receptor would have a "native" M₅ ≈ 340,000. This model was primarily derived from two observations. (1) When intact LECs or LEC membranes were crosslinked to the iodinated photoaffinity reagent ¹²⁵I-ASD-HA, two labeled polypeptides were detected under reducing conditions, at 175 and 166 kDa (Yannariello-Brown et al., 1992a). (2) When LEC membrane extracts were analyzed by gel filtration chromatography, HA-binding activity peaks were detected at 1 × 10⁶, 400,000, and 125,000 Da (Yannariello-Brown and Weigel, 1992). The present study also supports an oligomeric model of the LEC HA receptor, and identifies the HA-binding component of the receptor as the 175 kDa subunit.

A 175 kDa protein and the HA-binding activity were also enriched in Pool I from the S-400 column. The estimated MW of the major peak of HA-binding activity in Pool I is ~10⁶, suggesting that the receptor aggregates or is oligomeric. Surprisingly, we detected only small amounts, and occasionally none, of the 166 kDa band in this Pool. If the 166 kDa protein is part of a native heterologomeric HA receptor, this subunit may be dissociable and not required for HA-binding activity per se. Further evidence of the oligomeric nature of the HA receptor was seen in HA crosslinking experiments under nonreducing conditions, and in experiments using the ¹²⁵I-HA ligand-blot assay. In both cases not only was a 175 kDa protein detected, but a much larger protein was also seen with an estimated M₅ ~ 300,000. A minor band of this approximate mobility was also detected in the S-400 Pool I (Figure 5B).

The LEC HA-binding proteins detected in the ligand blot assay showed the same pattern of carbohydrate specificity as the bona fide LEC HA receptor in intact cells. HA and chondroitin sulfate competed for ¹²⁵I-HA binding to the 175 and 300 kDa proteins effectively and equally well, whereas desulfated chondroitin and other polyanions did not compete. Since reduction destroys HA-binding activity (Figure 1B), we cannot yet determine if the 300 kDa protein is a disulfide-bonded oligomer containing the 175 kDa HA binding subunit. Attempts to assess this by following the photoaffinity labeled 300 kDa protein after reduction (as in Figure 3) have been unsuccessful.

A 100 kDa protein has also been identified by others as the putative HA receptor on LECs (Forsberg and Gustafson, 1991). After iodination of intact LECs and detergent solubilization, this protein bound to HA-Sepharose. The 100 kDa protein was very sensitive to proteolysis and yielded a 55 kDa band upon reduction. A multistep isolation protocol was developed to isolate the 100 kDa protein from whole liver extracts; the final step employed HA-Sepharose affinity chromatography (McCourt et al., 1994). Antibodies to the 100 kDa band cross-reacted with a 90 kDa protein eluted from the HA-Sepharose (Forsberg and Gustafson, 1991). Partial amino acid sequence analysis of this 90 kDa band isolated by HA affinity chromatography showed it to be identical to the cell adhesion receptor ICAM-1 (Stad and Buurman, 1994).

The relationship of the 90 kDa ICAM-1 protein to the LEC endocytic HA receptor is uncertain, but it cannot be the endocytic LEC HA receptor for several reasons. During the above purification protocol (Forsberg and Gustafson, 1991; McCourt et al., 1994) the LEC HA receptor would have been inactivated (Yannariello-Brown et al., 1992b) and, therefore, no longer able to bind HA-Sepharose. Since these workers did not assay for HA-binding activity, this possibility was not assessed. We have seen no evidence of a 90 kDa protein with the properties of an HA receptor in our crosslinking, ligand blotting, or affinity chromatographic experiments performed on isolated LECs. We have, however, detected an 85–90 kDa band in isolated rat hepatocytes when using the ligand blot assay to detect ¹²⁵I-HA binding proteins (Yannariello-Brown et al., 1996). Since their purification scheme started with whole liver, rather than isolated LECs, it is more likely that one of the several hyaladherins present in the more abundant hepatocytes (D'Souza and Data, 1985; Toole, 1990; Frost et al., 1992) were recovered instead of the LEC HA receptor. Finally, ICAM-1 is not a clearance receptor; it is on many cell types that do not possess the ability to avidly endocytose HA. There are no reports that ICAM-1 can mediate endocytosis of HA via a coated pit pathway as demonstrated for the bona fide LEC HA receptor (Laurent and Fraser, 1992).

Based on this and our previous studies, we conclude that the LEC HA receptor is probably an oligomer and that the HA-binding activity resides with the 175 kDa protein. One possibility is that the LEC HA receptor is a hetero-dimer composed of a 166 and a 175 kDa protein with a M₅ ~ 340,000. In this model only the 175 kDa subunit has the HA-binding function. The function of the 166 kDa subunit may be regulatory or structural. A second possibility consistent with the data is that the LEC HA receptor is a homo-dimer composed of two 175 kDa subunits, and that the 166 kDa peptide is a degradation product derived from the 175 kDa protein. This could explain why the amount of 166 kDa protein detected is variable. For example, in the crosslinking experiments the ratio of labeling of the two proteins (175:166) varies from 1:1 to 3:1. Occasionally no doublet is detected, only a broad band in the 175–166 kDa region. Future studies with specific antibodies will be required to determine the relationship among the 300, 175, and 166 kDa proteins and their roles in HA receptor function in LECs.
Materials and methods

Materials

Cell culture media and reagents were purchased from Gibco (Grand Island, NY) except for bovine calf serum, which was from Hazleton Research Products, Inc. (St. Leonza, KS) or Flow Laboratories (McLean, VA). Na\textsubscript{231}I was from Amerham Corp. (10-20 mCi/mg iodine). 1,3,4,6-Teizachloro-3,6-diiodohexylglycoluril (Iodogen) and SADW were from Pierce. Fibronectin was a generous gift of Dr. G.Fuller (University of Alabama, Birmingham, AL). Dextrin was purchased from Kodak Chemical Corp. (Rochester, NY), and 25\(^{\circ}\) (w/v) stock solutions were prepared in dimethylsulfoxide. Bisbenzimide (Hoechst dye 33258) was from Behring Diagnostics. Collagenase (Types A, B, and D) and Streptomyces HAase were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Teflon pestles were from Kontes. Sephacyr S-400 was from Pharmacia (Uppsala, Sweden). Nonidet P40 was from U.S. Biochemical Corp. Nicotrocellose paper was from Schleicher and Schuell (0.45 and 0.1 \(\mu\)m). BSA (fraction V) was from Amcor Biochemicals (Tarrytown, NY). All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Buffer 1 contains 10 mM Hepes ph 7.4, 143 mM NaCl, and 6.8 mM KCl.

Purification of HA and preparation of HA adducts

Human umbilical cord HA was purified as described (Yannariello-Brown et al., 1992a). HA oligosaccharides with M\(_2\) -80000 (400-mr) were obtained from a specific lot (Signa) and were used without further fractionation. As described previously, HA oligosaccharides, which normally do not have free amino groups, were uniquely modified at the reducing end of the molecule to produce an alkylamine derivative (Raja et al., 1984). This modification allows subsequent reaction of the free amine with the Bolton-Hunter reagent to produce a benzophenyl derivatice, which is then iodinated with Na\textsubscript{231}I to a specific activity of \(-150\) c.p.m./mol HA. This derivative is designated \(\text{Na}_{231}^{\text{I}}\)-HA.

The synthesis and iodination of the ASD-HA photaffinity adduct has been described (Yannariello-Brown et al., 1992a). Briefly, SADW was dissolved in dimethylsulfoxide and reacted with the HA-amine derivative at pH 8.3 using a molar ratio of SADW to free amine of 280:1. Unreacted SADW was removed by centrifugation using a Centricon filter with a MW cut-off of 30,000. The resulting ASD-HA adduct (250 \(\mu\)g) was iodinated with 1.0-1.5 \(\mu\)Ci of Na\textsubscript{231}I using Iodogen in 500 \(\mu\)L of PBS, pH 7.5, at 4\(^{\circ}\)C for 25 min. Unreacted Na\textsubscript{231}I was removed using a PD-10 gel filtration column eluted with Buffer 1. The specific activity of the \(\text{Na}_{231}^{\text{I}}\)-ASD-HA photaffinity reagent was \(-36\) c.p.m./mol HA. All manipulations with SADW or ASD-HA were performed in the dark with a safe light using a GBX-2 filter (Kodak).

Isolation of LECs and LEC membrane preparation

Male Sprague-Dawley rats were purchased from SasCo. (Oklahoma City, OK) or Harlan Breeding Laboratories (Houston, TX) and used between 6 and 10 weeks of age. Rat livers were perfused with collagenase as previously described (Oka and Weigel, 1987), and the nonparenchymal cell fraction was collected from the first three differential centrifugations. The method of Erlinson et al. (1983) was utilized for LEC isolation with minor modifications (Yannariello-Brown et al., 1992b). The LECs were purified by centrifugation through a discontinuous Percoll gradient prepared in PBS. Cells banding at the 25/50\% interface were removed, washed three times in PBS at 4\(^{\circ}\)C, and then resuspended in Medium 1/BSA, which contains Eagle's modified minimum essential medium supplemented with 2.4 g/HEPES, pH 7.4, 0.22 g NaHCO\textsubscript{3} and 0.15\% BSA (w/v). When isolating LECs for a membrane preparation, the cells were placed in a 15 mL conical tube during the last centrifugation to measure the membrane pellet volume. Membranes were prepared from freshly isolated LECs as previously described (Yannariello-Brown and Weigel, 1992). Briefly, the LECs were hypotonically swollen, Dounce homogenized, and centrifuged at 1000 \(\times\) g, and then the postnuclear supernatant Was centrifuged at 105\,000 \(\times\) g to obtain the total membrane fraction.

LEC membrane detergent extraction

This extraction protocol is a modification of a previously described procedure (Yannariello-Brown and Weigel, 1992). LEC membranes were homogenized in Buffer 1 containing 5 mM EDTA, 0.5\% CHAPS, and protease inhibitors (2 mM DFP, 1 mM PMSF, and 1 mM NEP) in a ground glass Dounce vessel, and then gently stirred for 2 h on ice. The suspensions were then centrifuged at 105\,000 \(\times\) g for 60 min at 4\(^{\circ}\)C, and the detergent-soluble phase was removed and designated membrane extract 1. The residual insoluble material was then reextracted under the same conditions, and the detergent-soluble phase was removed. The residual pellet was extracted for a third time for 2 h at 4\(^{\circ}\)C in Buffer 1 containing 5 mM EDTA, 1.5\% CHAPS, 2.0 M KC1, and protease inhibitors. This detergent soluble phase was designated membrane extract II. The detergent extracts were assayed for protein content and \(\text{Na}_{231}^{\text{I}}\)-HA-binding activity.

Dot blot assay to measure \(\text{Na}_{231}^{\text{I}}\)-HA-binding activity

The dot blot assay was performed as previously described (Yannariello-Brown and Weigel, 1992). Briefly, solubilized membrane proteins or Sephacryl-400 column fractions were spotted onto nitrocellulose sheets (0.45 \(\mu\)m) using a dot blot apparatus (Schleicher and Schuell). The nitrocellulose was then incubated overnight in Buffer 1 containing 5\% BSA at 4\(^{\circ}\C to block nonspecific binding sites. To assess specific \(\text{Na}_{231}^{\text{I}}\)-HA-binding activity, identical sheets were incubated with 4 \(\mu\)g of \(\text{Na}_{231}^{\text{I}}\)-HA/ml in Buffer 1 plus or minus a 125-fold excess of unlabeled HA for 2 h at 23\(^{\circ}\C and then washed quickly three times in Buffer 1 at 4\(^{\circ}\C, once in Buffer 1 containing 0.01\% Nonidet P40, and finally in Buffer 1. Individual spots were cut out with a cork borer, and the radioactivity was quantitated. Specific c.p.m. bound were determined by subtracting the c.p.m. bound in the presence of excess unlabeled HA (nonspecific counts bound) from the c.p.m. bound in the absence of unlabeled competitor (total counts bound).

\(\text{Na}_{231}^{\text{I}}\)-HA ligand blot assay

Membrane extract II was concentrated and desalted using Centricon devices. The KCl concentration was reduced to <20 mM using 20 mM Tris pH 7.0, 5 mM EDTA, 0.5\% Chaps, 5\% glycerol, and 1.0 M NaBr (ligand blot sample buffer). SDS and bromophenol blue were then added from a concentrated stock to a final concentration of 0.03\% Chaps and 40 mM KCl using Centricon-10 devices with a nominal M, cut-off of 10,000 before freezing.

Gel filtration chromatography

Sephacryl-400 gel filtration chromatographic separation of LEC membrane extracts was performed as described (Yannariello-Brown and Weigel, 1992) with the following modifications. The column buffer consisted of Buffer 1 containing 0.03\% CHAPS and 2.0 M KCl without 5 mM EDTA. The column dimensions were 1.0 x 41 cm with a volume of 32 mL. The flow rate was \(-7.0\) mL/h and column fractions were collected at 5 min intervals (<550 \(\mu\)L). Extracts were loaded without adjustment to the CHAPS or KCl concentration in volumes up to 850 \(\mu\)L containing a maximum of 2 mg protein. The fractions were frozen at \(-70\)^\circC or immediately pooled, concentrated, and then desalted with Buffer 1 to give a final concentration of 0.03\% Chaps and 40 mM KCl using Centricon-10 devices with a nominal M, cut-off of 10,000 before freezing.

\(\text{Na}_{231}^{\text{I}}\)-HA-binding assays and crosslinking protocol

Cultured LECs were washed in Buffer 1, then incubated in serum-free Medium 1/BSA for 60 min at 37\(^{\circ}\C. The cells were chilled to 4\(^{\circ}\C, washed with ice-cold Buffer 1/BSA containing 10 mM EGTA, and then either \(\text{Na}_{231}^{\text{I}}\)-HA or \(\text{Na}_{231}^{\text{I}}\)-HA was added at a concentration of 5 \mu g/ml in either Medium 1/BSA or Buffer 1/BSA containing 10 mM EGTA and 0.05\% digorin (w/w). Specificity of binding was assessed in the presence of a 100-fold excess (w/w) of undervatiliated HA or other polysaccharides. The cell layers were incubated at 4\(^{\circ}\C in the dark for 2 h with gentle swirling and washed quickly three times in ice-cold Buffer 1, and then fresh ice-cold Buffer 1 was added to a depth of 1-2 mm. Photolysis was achieved by exposing the cell layers to UV light at 366 nm with a compact 4 W UV lamp (UVF, Inc., San Gabriel, CA). Release of the HA oligosaccharides from the covalently crosslinked complexes was achieved by
incubating cells for 30 min at 37°C in Medium-1/BSA containing 0.4 M sucrose in the presence or absence of 100 U HAase. The presence of 0.4 M sucrose (hyperosmolality) prevents the formation of coated pits, thereby preventing the internalization of HA-receptor complexes (McGary et al., 1989). This is especially important during the 37°C HAase treatment. After HAase treatment, the cell layers were incubated in ice-cold Buffer I plus protease inhibitors, and then scraped from the dishes into fresh cold buffer using a Teflon cell scraper. The cellular material was centrifuged in microfuge tubes using the highest setting on a tabletop microcentrifuge for 5 min at 4°C. The pellets were then solubilized in Laemmli (1970) sample buffer, and heated at 100°C for 3-5 min before analyzing by nonreducing SDS-PAGE.

**General methods**

$^{125}$I-Radioactivity was determined using a Packard Multipias 2 y spectrometer. Protein was determined by the methods of Bradford (1976) or Lowry et al. (1951), depending upon the salt and/or detergent present in the sample, using BSA as a standard. HA saccharides were quantitated by a modified carbazole assay (Bitter and Muir, 1962) using glucuronic acid as a standard.

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**Abbreviations**

LEC, liver sinusoidal endothelial cell; HA, hyaluronan (hyaluronic acid, hyaluronate); HAase, hyaluronidase; PBS, phosphate-buffered saline; SADS, sulforoscinimidyl 2-(p-azidosalicylamido)ethyl-i,3'-dithiopropionate; ASD, 2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate; BSA, bovine serum albumin; EGTA, ethyleneglycol bis(oxaethylenenitriilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazinedehesulfonic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; DFP, diisopropyl fluorophosphate; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

**References**


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