Identification and characterization of a divalent cation-dependent glycosaminoglycan-binding protein from rat liver endothelium

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Rat liver endothelial cells (LECs) express a membrane-associated Ca\(^{2+}\)-dependent hyaluronan-binding activity (CaHA-BP) which is distinct from the Ca\(^{2+}\)-independent, endocytic LEC HA receptor (Yannariello-Brown et al., J. Cell Biochem., 48, 73–80, 1992). The CaHA-BP is specific for a subset of glycosaminoglycans, since Ca\(^{2+}\)-dependent binding of \(^{125}\)I-HA (~80 kDa) to LECs was competed with a 100-fold excess (w/w) of HA, chondroitin sulfate, and heparin, but not with chondroitin. The CaHA-BP activity on intact LECs was pH-dependent. Optimal binding occurred at pH 6.0; no binding was detected at pH values \(\leq 5\) or \(\geq 9\). \(^{125}\)I-HA, pre-bound in the presence of Ca\(^{2+}\), could also be dissociated with an acidic buffer (pH 5.0), as well as the divalent cation chelators EDTA and EGTA. \(^{125}\)I-HA binding was stimulated by divalent cations other than Ca\(^{2+}\), such as Mg\(^{2+}\), Mn\(^{2+}\), and Ba\(^{2+}\); with the exception of Zn\(^{2+}\). A photoaffinity crosslinking reagent (\(^{125}\)I-ASD-HA) was used to identify specifically crosslinked polypeptides on LECs. In the absence of Ca\(^{2+}\), and in the presence of EDTA or EGTA, only bands at 175/166 kDa were consistently crosslinked. These bands have been previously identified as the LEC Ca\(^{2+}\)-dependent endocytic HA receptor (Yannariello-Brown et al., J. Biol. Chem., 267, 20451–20455, 1992). In the presence of Ca\(^{2+}\), crosslinking was consistently seen to a 68 kDa polypeptide. Crosslinking was competed with a 100-fold excess (w/w) of HA. These and other data suggest that a 68 kDa protein is the most likely candidate for the CaHA-BP in LECs.

Key words: photoaffinity crosslinking/hyaluronan/calcium/lectin

Introduction

Hyaluronan (HA) is a non-sulfated member of the glycosaminoglycan (GAG) family of polysaccharides, and is composed of repeats of the disaccharide unit \(\beta-(1,4)-\beta-D\)-glucuronic acid-\(\beta-(1,3)\)-N-acetyl-D-glucosamine (Hascall and Hascall, 1981; Laurent, 1989; Laurent and Fraser, 1992). HA is ubiquitously distributed throughout all vertebrate tissues; its highest concentrations are found in such soft connective tissues as cartilage, skin, and vitreous humor. HA is an important regulator of many biological processes, such as cell migration and locomotion (Turley, 1991; Hardwick et al., 1992), cell division (Brecht et al., 1986), tissue organization (Hascall and Hascall, 1981; Yu et al., 1992; Knudson, 1993; Knudson et al., 1993), tumorigenesis, development (Toole, 1981; Turley, 1991), and wound healing (Weigel et al., 1986).

Because of its wide tissue distribution, HA interacts with a variety of cell types. These interactions are mediated by HA-binding proteins found on the cell surface and in the extracellular matrix. These HA-binding proteins/receptors have been detected in a variety of body fluids, tissues and cell types, but only a few have been isolated, extensively characterized, or cloned (Toole, 1990; Turley, 1991; Laurent and Fraser, 1992; Knudson and Knudson, 1993).

Recently, Toole proposed the term 'hyaladherins' for the HA-binding proteins and receptors (Toole, 1990). Some hyaladherins, such as the lymphocyte homing receptor CD44 (Toole, 1990; Knudson and Knudson, 1993) and the RHAMM and HARC complexes described on motile fibroblasts (Turley, 1991), are thought to be involved in cell-matrix and/or cell-cell interactions. Other hyaladherins, such as the cartilage link protein and proteoglycans, are thought to function as organizers of HA-containing matrices (Hascall and Hascall, 1981); recently, direct evidence of a role for CD44 in HA-matrix organization has also been obtained (Knudson, 1993; Knudson et al., 1993). Hyaladherins have also been isolated from the brain (Perides et al., 1989), kidney (Gupta et al., 1991), liver (D’Souza and Datta, 1985), and serum (Yoneda et al., 1990). Some are soluble proteins, e.g., fibrinogen (LeBoeuf et al., 1986) and the 67 kDa protein described in liver (D’Souza and Datta, 1985). Others are membrane-associated like the intracellular HA-binding protein found in rat hepatocytes (Frost et al., 1992), and the divalent cation independent liver endothelial cell HA receptor (Yannariello-Brown and Weigel, 1992) which is responsible for the clearance of degraded HA from the plasma (Smedsgaard et al., 1984).

While these hyaladherins are described as HA-binding proteins/receptors, many can bind other GAGs as well. This degeneracy in ligand binding suggests that each hyaladherin has multiple biological roles, and raises questions regarding the control of their binding specificity.

Interactions between GAGs and their binding proteins are generally thought to be divalent cation-independent; this is true of HA and the hyaladherins. Recently, however, a few exceptions have been noted. A heparin-like ligand has been reported to interact with L-selectin, the lymphocyte homing molecule, in a Ca\(^{2+}\)-dependent fashion (Norgard-Sumnicht et al., 1993), and a cation-dependent hyaladherin has been described in hepatocytes (Frost et al., 1992).

This author has reported a Ca\(^{2+}\)-dependent hyaladherin on rat liver sinusoidal endothelial cells (LECs; Yannariello-Brown and Weigel, 1992; Yannariello-Brown et al., 1992b). While studying the Ca\(^{2+}\)-independent endocytic HA receptor on LECs, Yannariello-Brown et al. (1992b) determined that while HA binding to intact cells or deter-

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gent extracts made from LEC membranes was not dependent on the divalent cation Ca$^{2+}$, the presence of Ca$^{2+}$ (>2.5 mM) enhanced specific HA-binding 4- to 20-fold. A series of experiments led us to the conclusion that the Ca$^{2+}$-independent and the Ca$^{2+}$-dependent activities were distinct, both functionally and biochemically (Yannariello-Brown and Weigel, 1992; Yannariello-Brown et al., 1992b). The Ca$^{2+}$-dependent HA-binding protein (CaHA-BP) is associated primarily, if not exclusively, with the outer surface of the LEC plasma membrane, but is not involved with the internalization of HA (Yannariello-Brown et al., 1992b). The present study further characterizes the LEC Ca$^{2+}$-dependent HA-binding protein (CaHA-BP) biochemically, and identifies a 68 kDa polypeptide as the CaHA-BP in LECs using an $^{125}$I-labelled photoaffinity crosslinking derivative of HA.

Results

Since the HA-binding assays were performed on fibronectin-coated dishes in the presence of bovine serum albumin (BSA), the ability of fibronectin and BSA to bind $^{125}$I-HA in the presence or absence of Ca$^{2+}$ was tested. Neither BSA nor fibronectin bound $^{125}$I-HA when immobilized on tissue culture or bacteriological plastic, or on nitrocellulose in a dot blot assay. Specific LEC CaHA-BP activity was not significantly decreased if BSA was removed from the binding medium, nor was the activity increased if the BSA concentration was increased. Neither BSA nor fibronectin bound HA, even when CHAPS and KCl were added to duplicate conditions in LEC extracts. Anti-fibronectin antibodies conjugated to Sepharose beads did not immunodeplete the CaHA-BP activity from HIGH-KC extracts (not shown).

Effect of various divalent cations and pH on $^{125}$I-HA-binding activity on cultured LECs and soluble LEC extracts

To determine whether divalent cations other than Ca$^{2+}$ could stimulate HA-binding and compare the pattern to that of other divalent cation-dependent HA-binding activities, this activity was measured in cultured LEC and in LEC membrane HIGH-KC extracts. LECs were incubated for 2 h at 4°C with 4 μg $^{125}$I-HA/ml Buffer 1 + 0.15% BSA plus 10 mM EDTA or various divalent cations at a concentration of 10 mM (Figure 1a). The extent of HA-binding stimulated by the various ions was in the order Mn$^{2+}$ > Ba$^{2+}$ > Mg$^{2+}$ > Ca$^{2+}$. This pattern is similar to that of the HABP in hepatocytes (Frost et al., 1990), but completely different from the pattern seen for selectin-ligand interactions (Asa et al., 1992).

The effect of various divalent cations on HA-binding to the LEC HIGH-KC membrane extract was also examined. When the LEC proteins in the HIGH-KC extract were immobilized on nitrocellulose and $^{125}$I-HA binding activity measured using the Dot Blot Assay, almost all the various cations tested stimulated HA-binding at pH 7.0. Stimulation of HA-binding was in the order Mn$^{2+}$ > Ca$^{2+}$ > Mg$^{2+}$ > Ba$^{2+}$ (Figure 1b). However, differences between HA binding in the presence of Ca$^{2+}$, Mg$^{2+}$, and Mn$^{2+}$ were not significant. Three to 4-fold increases in HA-binding were seen compared to the EDTA control. Specific HA-binding was not stimulated in the presence of 10 mM Zn$^{2+}$; HA-binding was actually inhibited. It is interesting to note that the significant differences among cations seen in the intact LECs were not detected in the soluble extracts. The regulatory mechanism(s) controlling cation-dependent HA-protein interactions in the intact cell may be missing from the extracts. For this reason, subsequent HA-binding experiments were conducted on intact LECs, and not on the HIGH-KC extract in the Dot Blot Assay.

Many receptor–ligand interactions are pH-dependent (Drickamer, 1988). Therefore, specific Ca$^{2+}$-dependent HA-binding was determined at various pH values (Figure 2a). Cultured LECs were incubated at the indicated pH values with $^{125}$I-HA in the presence of 10 mM Ca$^{2+}$, Identi
cal dishes were also incubated in the presence of 10 mM EGTA. An identical pattern of pH-dependent HA-binding to LECs was seen in the presence of EGTA as was previously reported by Raja et al. (1988; not shown). Specific CaHA-BP activity could not be detected at pH 4 or 5, or at pH 9. Specific binding was optimal at pH 6, ~600% of that at pH 7. Specific HA-binding at pH 8 was ~200% of the binding at pH 7. That virtually identical results were seen when HEPES replaced MES at pH 6 and TRIS replaced HEPES at pH 7.0 demonstrated that the modulation of a CaHA-BP activity at various pH values was not due to a buffer effect (not shown).

Acidic buffers also removed $^{125}$I-HA pre-bound to LECs in the presence of Ca$^{2+}$ at pH 7.0. Cultured LECs were incubated with $^{125}$I-HA in either 10 mM EGTA or 10 mM Ca$^{2+}$ at pH 7.0 at 4°C. After the unbound ligand was removed by washing at pH 7.0 at 4°C (3 washes x 2 min) a second set of washes was performed at either pH 5.0 or pH 7.0. Figure 2b demonstrates that there is no effect of a low pH wash on Ca$^{2+}$-independent HA-binding to LECs. However, the Ca$^{2+}$-dependent HA-binding was sensitive to a pH 5.0 wash. The low pH wash removed virtually 100% of any Ca$^{2+}$-dependent HA-binding activity; the level of HA-binding was reduced to about that of the Ca$^{2+}$-independent activity.

Carbohydrate specificity of the CaHA-BP

Virtually all HA-binding proteins studied thus far bind other GAGs as well as HA (Toole, 1981; Turley, 1991; Knudson and Knudson, 1993). Therefore, the ability of other GAGs to compete for $^{125}$I-HA binding to CaHA-BP was tested on cultured LECs. In the presence of 10 mM Ca$^{2+}$, HA, chondroitin sulfate, and heparin competed for HA binding by 56, 81, and 92%, respectively (Figure 3). Chondroitin did not compete. This pattern is similar to the pattern seen for CD44-HA interactions (Underhill and Toole, 1979; Lesley et al., 1990).

CaHA-BP activity is not secreted into the culture medium

A series of experiments was conducted to determine if the CaHA-BP was actively shed or secreted into the culture medium in a soluble form. Primary LEC cultures were prepared and plated on fibronectin-coated dishes in RPMI-
Fig. 1. Effect of various divalent cations on HA-binding activity. (A) Cultured cells: cultured LECs were incubated for 2 h at 4°C with 4 μg of 125I-HA/ml Buffer 1 pH 7.0 + 0.15% BSA including either 10 mM EGTA or the indicated divalent cation at a concentration of 10 mM. The binding was performed in the presence or absence of a 100-fold excess of underivatized HA to determine non-specific and total binding, respectively. Specific binding is expressed as the percent of binding in the presence of Ca\(^{2+}\), and was determined as described in Materials and methods. (B) Soluble extracts: the HIGH-KC extract from LEC membranes was diluted in Buffer 1 such that the KCl and CHAPS concentrations were 40 mM and 0.03%, respectively. Ten micrograms of protein were immobilized per spot on 0.45 μm nitrocellulose using a Dot Blot apparatus. The sheets were incubated with 5 μg of 125I-HA/ml Buffer 1 (pH 7.0) + 0.15% BSA plus the indicated divalent cations or EGTA at a concentration of 10 mM. The data is expressed as the percent of the EGTA control.
Fig. 2. Effect of pH on HA-binding activity in cultured LECs. (A) CaHA-BP activity was measured in cultured LECs. The following buffers were used to achieve the indicated pH values: 25 mM MES (pH 4 and 5), HEPES (pH 6 and 7), and Tris-HCl (pH 8 and 9) plus 140 mM NaCl, 8 mM KCl, 10 mM CaCl₂, and 0.15% BSA. Specific binding is expressed as the percent of pH 7.0. (B) To test the stability of [³²P]-HA, prebound at pH 7, to pH 5 wash conditions, LECs were incubated with [³²P]-HA at pH 7, then unbound ligand was washed away, also at pH 7. The cell layers were separated into two groups: half were washed further at pH 7, the other half were washed at pH 5. The cells were then solubilized, radioactivity was quantitated, and the specific binding was calculated.
Fig. 3. Various carbohydrates will compete for CaHA-BP activity. Cultured LECs were incubated for 2 h at 4°C with 4 µg [125I]HA/ml Buffer 1 + 0.15% BSA in the absence or presence of 100-fold excess (w/w) of the specified carbohydrate competitors. The cell layers were then washed at 4°C, solubilized in 0.1% deoxycholate and DNA content was determined. Specific Ca²⁺-dependent HA-binding activity was calculated and is expressed as the percent of control value obtained in the absence of any competitor.

Identification of the CaHA-BP using photoaffinity crosslinking

To identify the CaHA-BP on LECs, the photoaffinity crosslinking reagent sulfosuccinimidyl 2-(p-azido-salicylamide) ethyl-1,3'-dithiopropionate (SASD) was conjugated to the HA-amine derivative, then iodinated. The [125I]-ASD-HA reagent has been used previously to identify the Ca²⁺-independent endocytic HA receptor on LECs (Yannariello-Brown et al., 1992a). As a positive control, cultured LECs were permeabilized with digitonin in the presence of EGTA to detect the Ca²⁺-independent HA receptor. When LECs were crosslinked in the absence of Ca²⁺ and in the presence of 10 mM EGTA, high molecular weight material and a doublet at 175/166 kDa were specifically crosslinked (Figure 4). Single bands were labelled inconsistently, and to a lesser extent at 86, 68 and 55 kDa, as reported previously (Yannariello-Brown et al., 1992a).

When LECs were incubated with [125I]-ASD-HA and crosslinked in the presence of 10 mM Ca²⁺, a similar labelling pattern was seen; however, crosslinking to a 68 kDa polypeptide was greatly enhanced, and a minor doublet was also specifically crosslinked at 42/45 kDa. A 9.3-fold increase in the labelling intensity of the 68 kDa band was seen in the presence of Ca²⁺ versus that in the presence of EGTA, while only a 1.6-fold increase was detected in the 175/166 kDa region when Ca²⁺ was added. The 68 kDa band was competed by 89% with a 100-fold excess of underivatized HA, while the 175/166 bands were competed by 95%. A diffuse area at ~55 kDa was also inconsistently labeled. The crosslinking experiments have been repeated (n = 6) with different preparations of [125I]-ASD-HA and LECs; identical results were obtained.
matography of detergent solubilized LEC membrane extracts, where the major peak of CaHA-BP activity was detected in column fractions corresponding to a MW of ~66-90 kDa depending on the salt and detergent concentration, with a minor peak at ~40 kDa (Yannariello-Brown and Weigel, 1992). The relationship of the 42/45 kDa doublet to the 68 kDa peptide is not known. The smaller peptides may represent degradation products of the 68 kDa band, or may be subunits of a Ca\(^{2+}\)-dependent HA-binding complex, each having the ability to bind HA. Frost et al. (1992) have described two HA-binding activities on hepatocytes, one divalent cation-independent and intracellular, the other Ca\(^{2+}\)-dependent. Crosslinking studies, in the presence of Ca\(^{2+}\), with \(^{125}\)I-ASD-HA in hepatocytes identified a 24 kDa and a 42 kDa molecule with no specific crosslinking to a 68 kDa polypeptide (Frost et al., 1992). It is possible that the 40 kDa polypeptides detected in Sephacryl-400 column fractions (Yannariello-Brown and Weigel, 1992) and the 42/45 kDa bands crosslinked on LECs are due to a minor contamination of hepatocytes in our cultures. A broad band at ~55 kDa was also crosslinked. However, crosslinking was seen in only ~17% of the experiments (n = 6). That labelling to a 55 kDa band was also seen in the absence of Ca\(^{2+}\), and was very prominent upon crosslinking on LEC membranes (Yannariello-Brown et al., 1992a), suggests that the 55 kDa band may be a proteolytic product of the Ca\(^{2+}\)-independent HA receptor.

A number of hyaladherins and GAG-binding proteins have recently been described. It is possible that the CaHA-BP is a previously described hyaladherin or a member of a previously described lectin family. A definitive answer can only be found after isolation and sequencing. However, several lines of evidence suggest that the CaHA-BP is a novel hyaladherin. CaHA-BP does not appear to be related to any of the liver HA-binding proteins previously described. For example, a 67 kDa protein has been described by D’Souza and Datta (1985), but it is soluble and its HA-binding is divalent cation-independent.

The selectins are members of the LEC-CAM family of proteins which mediate the initial interactions between leukocytes and the vascular endothelium (Rosen, 1990; McEver, 1991; Laskey, 1992). Members of this family bind to their respective carbohydrate ligands in a divalent cation dependent manner (Asa et al., 1992). One member, L-selectin, is a GAG-binding protein (Norgard-Sumnicht et al., 1993) which can also be found in a soluble form in serum with a MW of 67 kDa (Schleifenbaum et al., 1992). An argument against a relationship between the LEC CaHA-BP and L-selectin is based on the fact that while the ligands for selectins are found on the vasculature, the selectins are not. It is possible that a serum-derived 67 kDa selectin may be adsorbed to the surface of the liver endothelium, but one would expect this molecule to be removed by a carbonate wash at pH 11, as it would be a peripheral membrane protein. When LEC membranes were incubated in a carbonate buffer at pH 11, the CaHA-BP activity remained with the membranes (Yannariello-Brown and Weigel, 1992). This result argues against L-selectin being responsible for the CaHA-BP activity.

Evidence that the CaHA-BP is not a member of the CD44 family stems from the fact that no CD44 isoforms have ever been localized to the liver endothelium (Picker...
In our hands, CD44 could not be detected on LECs using the following anti-CD44 antibodies: KM201 (Kincade, 1993), K3 (Underhill et al., 1987), or anti-Leu-44 (Serotec; data not shown). CD44 and CaHA-BP do not share the same carbohydrate specificity. HA and chondroitin compete for HA-binding to various forms of CD44, but sulfated GAGs such as chondroitin sulfate and heparin do not (Underhill and Toole 1979; Lesley et al., 1990); chondroitin did not compete for $^{125}$I-HA binding to CaHA-BP, whereas HA, chondroitin sulfate, and heparin competed well (see Figure 3). CD44 binding to HA is not divalent cation dependent. CD44-HA interactions are dependent on disulfide bonds (Kincade, 1993), whereas CaHA-BP activity is not affected by reduction (Yannariello-Brown and Weigel, 1992). Also, the pattern of pH dependence of HA-binding to CD44 is different from that of the LEC CaHA-BP. The interactions between HA and the 85 kDa form of CD44 are pH dependent and have a complex triphasic profile. The 85 kDa CD44 had relatively high levels of HA binding at pH values of 3 and 4, then decreased at pH 5 and 6, increased again at pH 7, then decreased at pH values $>$ 8 (Underhill and Toole, 1980).

While it is also possible that the Ca$^{2+}$-dependent stimulation of HA-binding is not due to a unique binding protein on the cells, but rather that Ca$^{2+}$ affects the HA polymer itself, the data accumulated thus far argue convincingly against this. 1) If the effect were on HA itself, then all cells with Ca$^{2+}$-independent HA-binding activity should have CaHA-BP activity; such is not the case. 2) LECs have both the CaHA-BP and the Ca$^{2+}$-independent endocytic HA receptor as HA-binding activities. These have distinct cellular localization patterns and can be physically separated using differential salt/detergent solubilization and gel filtration chromatography (Yannariello-Brown and Weigel, 1992). 3) It is true that Ca$^{2+}$ has an effect on HA structure (Napier and Hadler, 1978; Carr and Hadler, 1980; Gabriel and Carr, 1989). Light-scattering data suggest that HA molecules become more compact in the presence of 5 mM Ca$^{2+}$ (Gabriel and Carr, 1989). Although HA molecules assume a more compact configuration, they do not aggregate (Gabriel and Carr, 1989). Therefore, the increase in HA-binding observed in the presence of Ca$^{2+}$ is probably not due to an increase in HA self-association. 4) Crosslinking studies with $^{125}$I-ASD-HA in the presence of Ca$^{2+}$ identify a 68 kDa polypeptide on LECs (see Figure 4).

**What is the role of the CaHA-BP?**

The CaHA-BP is localized predominantly, if not exclusively, to the extracellular face of the LEC plasma membrane, and is not involved in the active endocytosis of HA (Yannariello-Brown et al., 1992b). Therefore, the CaHA-BP may play a role in cell-cell interactions or during cell adhesion to HA matrices. The CaHA-BP may facilitate cellular migration through an HA-rich matrix in response to changes in local cell-induced Ca$^{2+}$ concentrations. Alternatively, CaHA-BP may be involved in the organization of an HA-containing matrix. Others have postulated that local cell-induced changes in pH and ion concentrations can cause local structural changes in an HA-rich matrix (Gabriel and Carr, 1989; Scott, 1992). The release of Ca$^{2+}$ or other divalent cations into the matrix could be controlled by the local cell population in response to a variety of physiological signals (Gabriel and Carr, 1989; Grzesiak et al., 1992). Fluctuations in extracellular divalent cations may affect the type of cells entering an HA-rich matrix, their rate of migration, and subsequent re-organization of the existing matrix. Further work is necessary to understand the biological role(s) of the CaHA-BP, and its relationship to other HA-binding proteins.

**Materials and methods**

**Materials**

Cell culture media and reagents were purchased from Gibco (Grand Island, NY) with the exception of BCS, which was from Hazelton Research Products Inc. (St. Lenexa, KS) or Flow Laboratories (McLean, VA). Na$^{21}$I was purchased from Amersham Corp. (10-20 Ci/µg iodine), 1,3,4,6-Tetrachloro-3a, 6a-diphenylglycouril (Iodogen) and SARD were from Pierce. Digitonin was purchased from Kodak Chemical Corp. (Rochester, NY) and 25% (w/v) stock solutions were prepared in dimethyl sulfoxide. Biebnumthane (Hoich dye 33258) was from Behring Diagnostics. Collagenase (Types A, B, and D) was from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tettan pestytes were from Kontes. Sephacryl 400 and PD-10 columns were from Pharmacia (Upsala, Sweden). Nonidet-40 was from U.S. Biochemical Corp. Nitrocellulose paper was from Schleicher and Schuell. BSA fraction V was from Armor Biochemicals (Tarrytown, NY). Centricron devices were from Amicon. All other chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

**General methods**

Buffer 1 contains 10 mM HEPES, pH 7.5, 140 mM NaCl, and 8 mM KCl. Medium 1 contains Eagle’s modified minimum essential medium supplemented with 2.4 mg/ml, HEPES, pH 7.4 and 0.22 mg/ml NaHCO$_3$. Medium 1/BSA or Buffer 1/BSA also contains 0.15% BSA (w/v). Protein concentrations were determined by the method of Lowry et al. (1951) or Bradford (1976), depending on the detergent present, using BSA as a standard. DNA content was determined by the method of Labarca and Paigen using calf thymus DNA as a standard (Labarca and Paigen, 1980). $^{35}$S radioactivity was determined using a Packard Multispect 2 y Spectrometer. SDS-PAGE was performed according to Laemmli (Laemmli, 1970). Purified HA was quaniticated using the modified carbazole assay using glucuronic acid as a standard (Bitter and Muir, 1962). Chondroitin sulfate was de-sulfated using the method of Nagasawa and Inoue (1980).

**HA purification and modification**

Human umbilical cord HA was purified by complexation with cetylpyridinium chloride, fractionation on celite, and ethanol precipitation as described (Yannariello-Brown et al., 1992b). Purified HA contains $<$0.2% protein and $<$0.1% sulfate by weight. HA oligosaccharides have been obtained (Yannariello-Brown et al., 1992b) with an average $M,$ of 80,000 Da (~400-mer), from an individual lot (Sigma) without any further fragmentation. HA oligosaccharides, which normally do not have free amino groups, were uniquely modified at the reducing end of the molecule to produce an allylamine derivative (Raja et al., 1984). This unique modification allowed a subsequent reaction with the Bolton-Hunter reagent to produce a hydroxylphenyl derivative that could be iodinated with Na$^{21}$I using the Iodogen reagent. Using this method, conditions were chosen which routinely achieve specific activities between 80-150 c.p.m./fmol oligosaccharide.

The heterobifunctional, reducible, iodinatable, photoactivatable cross-linking reagent SARD was covalently coupled to HA-amine as described (Yannariello-Brown et al., 1992a). All manipulations with SARDS or the ASD-HA adduct were performed in the dark with the aid of a safe light using a GBX-2 filter (Kodak). The SARD, dissolved in dimethyl sulfoxide, was diluted with 0.1 M sodium carbonate pH 9, then incubated with the HA-amine for 30 min at 23°C; the molar ratio of SARD to free amine was 280:1. Unreacted SARD was removed by centrifugation through a Centricon device with a $M,$ of 280,000. The ASD-HA adduct was iodinated with Na$^{21}$I including the iodogen reagent as described previously (Yannariello-Brown et al., 1992a). The specific activity of the $^{21}$I-ASD-HA photoaffinity cross-linker was ~40 c.p.m./fmol HA.
Soluble CaHA-BP activity was measured using a dot blot assay as previously described (Yannariello-Brown and Weigel, 1992). The KC1 and CHAPS concentrations in the HIGH-KC extract were adjusted with Buffer 1 to 40 mM and 0.03%, respectively. Soluble protein was spotted onto nitrocellulose sheets using a Dot Blot Apparatus (Schleicher and Schuell), then the nitrocellulose sheets were incubated with 3 μg of [3H]-BA/ml Buffer 1/BSA plus or minus a 150-fold excess of undervatized HA in the presence of chelators or various divalent cations. The sheets were then washed, semi-dried, and quantitated. Specific fmol bound were determined by subtracting the fmol bound in the presence of excess unlabeled HA from the total bound in the absence of cold competitor. Specific Ca2-dependent activity was calculated by subtracting the specific binding in the presence of EDTA or EGTA from the specific binding in the presence of Ca2+. This calculation yields the Ca2-dependent activity without the contribution of the Ca2-independent activity.

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References


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Rat liver endothelial cell isolation and short term culture

The method of Smedsrod et al. (1984) was used for LEC isolation from the non-parenchymal cell fraction obtained from collagenase perfused rat livers, with minor modifications (Yannariello-Brown and Weigel, 1992b). Briefly, the non-parenchymal cell fraction was collected from the first series of supranatant centrifugations and then further purified by centrifugation through discontinuous Percoll gradients. LECs were resuspended in growth medium (RPMI 1640 plus 20% BCS, penicillin-streptomycin and glutamine) and plated into 24-well plates (-1 x 105 cells/cm2/well) coated with 50 μg/ml of human plasma fibronectin. After a 120 min incubation at 37°C the medium was removed and the cell layers were washed three times in PBS and then once in growth medium. The cultures were then incubated overnight prior to the start of the experiments. The resulting cultures are >95% endothelial as judged by acetylated low density lipoprotein uptake and non-specific esterase staining (Raja et al., 1988). This protocol routinely yields 40–80 x 104 LECs/30 g male Sprague–Dawley rat.

LEC membrane preparation and detergent extraction

LEC membranes were prepared and extracted as described previously (Yannariello-Brown and Weigel, 1992) with minor modifications. Briefly, LECs were hypotonically swollen in 10 mM Tris pH 8.0, 5 mM EGTA, 5 mM MgCl2 plus a protease inhibitor cocktail, then dounce-homogenized. Nuclei, unbroken cells and debris were removed by centrifuging at 1000 × g for 5 min, then the post-nuclear supernatant was centrifuged at 105,000 × g for 1 h to pellet the total membrane fraction. Membranes were extracted twice with LOW-KC buffer (10 mM HEPES pH 7.5, 5 mM EDTA, 140 mM NaCl, 8 mM KCl and 0.5% CHAPS), to yield the LOW-KC I and II extracts, then once with HIGH-KC buffer (10 mM HEPES pH 7.5, 5 mM EDTA, 140 mM NaCl, 2.0 M KCl and 1.5% CHAPS) to yield the HIGH-KC extract. The HIGH-KC extract contains ~70% of the total extractable CaHA-BP activity and only ~20% of the total extractable protein, and was used exclusively in this study.

Previous experiments have localized the CaHA-BP to the cell surface only (Yannariello-Brown et al., 1992b), therefore, CaHA-BP activity was only measured in the cell surface membranes in any permeabilizing agent. Specific [3H]-HA binding was determined by subtracting the radioactivity bound in the presence of a 100-fold excess of undervatized HA (non-specific binding) from the radioactivity bound in the absence of competitor (total binding). Specific Ca2-dependent activity was calculated by subtracting the specific binding in the presence of EDTA/EGTA from the specific binding in the presence of Ca2+. This yields the specific Ca2-dependent activity without the contribution of the Ca2-independent activity. The data were expressed as specific fmol HA bound/105 cells.

Binding of [3H]-ASD-HA to LECs was performed as described above for the [3H]-HA, except all manipulations were carried out in the dark with 25 μg of HA/ml and 5 μg of [3H]-ASD-HA/ml was used. After the final wash to remove unbound probe, the cell layers were photolyzed with UV light at 254 nm at a distance of 1 cm for 60 s as described (Yannariello-Brown et al., 1992a). The samples were dissolved in 1 L Laemmli sample buffer (Laemmli, 1970), reduced with 5% β-mercaptoethanol (v/v) to release the HA saccharides, then analyzed by SDS-PAGE and autoradiography using an enhancing screen at ~70°C. Labeling was quantitated using a Quik Scan Jr. Densitometer (Helena Labs) and an image analysis system using DNA vision software on a BVT 4000 Biological Analysis system (Biological Vision Inc., San Mateo, CA) as described (Yannariello-Brown et al., 1992a).

Dot blot assay measuring HA-binding activity

Soluble CAHA-BP activity was measured using a dot blot assay as previously described (Yannariello-Brown and Weigel, 1992). The KC1 and CHAPS concentrations in the HIGH-KC extract were adjusted with Buffer 1 to 40 mM and 0.03%, respectively. Soluble protein was spotted onto nitrocellulose sheets using a Dot Blot Apparatus (Schleicher and Schuell), then the nitrocellulose was blocked using a 5% BSA/ml Buffer 1/BSA plus or minus a 150-fold excess of undervatized HA in the presence of chelators or various divalent cations. The sheets were then washed, semi-dried, and quantitated. Specific fmol bound were determined by subtracting the fmol bound in the presence of excess unlabeled HA from the total bound in the absence of cold competitor. Specific Ca2+-dependent activity was calculated by subtracting the specific binding in the presence of EDTA or EGTA from the specific binding in the presence of Ca2+. This calculation yields the Ca2+-dependent activity without the contribution of the Ca2+-independent activity.
Divalent cation-dependent GAG-binding protein


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