Purification and Subunit Characterization of the Rat Liver Endocytic Hyaluronan Receptor*

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The endocytic hyaluronan (HA) receptor of liver sinusoidal endothelial cells (LEC) is responsible for the clearance of HA and other glycosaminoglycans from the circulation in mammals. We report here for the first time the purification of this liver HA receptor. Using lectin and immuno-affinity chromatography, two HA receptor species were purified from detergent-solubilized membranes prepared from purified rat LECs. In non-reducing SDS-polyacrylamide gel electrophoresis (PAGE), these two proteins migrated at 175- and ~300 kDa corresponding to the two species previously identified by photoaffinity labeling of live cells as the HA receptor (Yannariello-Brown, J., Frost, S. J., and Weigel, P. H. (1992) J. Biol. Chem. 267, 20451–20456). These two proteins co-purify in a molar ratio of 2:1 (175:300), and both proteins are active, able to bind HA after SDS-PAGE, electrotransfer, and renaturation. After reduction, the 175-kDa protein migrates as a ~185-kDa protein and is not able to bind HA. The 300-kDa HA receptor is a complex of three disulfide-bonded subunits that migrate in reducing SDS-PAGE at ~260, 230, and 97 kDa. These proteins designated, respectively, the α, β, and γ subunits are present in a molar ratio of 1:1:1 and are also unable to bind HA when reduced. The 175-kDa protein and all three subunits of the 300-kDa species contain N-linked oligosaccharides, as indicated by increased migration in SDS-PAGE after treatment with N-glycosidase F. Both of the deglycosylated, nonreduced HA receptor proteins still bind HA.

HA* is an important extracellular matrix component of all tissues and plays a key role in development, cell proliferation, cell adhesion, recognition, morphogenesis, differentiation, and inflammation (1–4). The daily total body turnover of HA in humans is at least 1 g/day (4). HA degradation and removal in inflammation (1–4). The daily total body turnover of HA in tissues and plays a key role in development, cell proliferation, cell adhesion, recognition, morphogenesis, differentiation, and inflammation (1–4).

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HA is an important extracellular matrix component of all tissues and plays a key role in development, cell proliferation, cell adhesion, recognition, morphogenesis, differentiation, and inflammation (1–4). The daily total body turnover of HA in humans is at least 1 g/day (4). HA degradation and removal in the body occurs via two clearance systems (3): one is in the lymphatic system, which accounts for ~85% of the HA turnover, and another system is hepatic, accounting for ~15% of the total body HA turnover. HA is continuously synthesized and degraded. Very large HA molecules (~10^7 Da) are partially degraded to large fragments (~10^6 Da) that are then released from the matrix and flow with the lymph to lymph nodes. The majority of HA (~85%) is completely degraded in the lymph nodes by unknown mechanisms and the remaining HA (~15%) that passes through the nodes finally enters the blood. Clearance of this circulating HA is presumably important for normal health (3, 4). Elevated serum HA levels are found in several disease conditions such as liver cirrhosis, rheumatoid arthritis, psoriasis, scleroderma, and some cancers (5–7).

LEC s have a very active recycling, endocytic receptor that removes HA and other glycosaminoglycans, such as chondroitin sulfate, from the circulation (3, 8–10). Earlier reports misidentified this LEC HAR as ICAM-1 (11, 12), also known as CD54, which is a 90-kDa protein. This finding was later recognized as an artifact in that ICAM-1 bound nonspecifically to the HA affinity resin employed (13). In two previous studies, one using a photoaffinity derivative of HA (14) and the other using a novel ligand blot assay with 125I-HA (15), we identified two specific HA-binding proteins in isolated rat LECs at 175 and ~300 kDa. In the present study, we have finally purified these two proteins for the first time. Our results show the ~300-kDa HAR protein contains three subunits after reduction but does not contain the 175-kDa HAR protein, which itself contains no other subunits.

EXPERIMENTAL PROCEDURES

Materials—RCA-1-agarose gel was purchased from EY laboratories, Inc. Tris, SDS, ammonium persulfate, N,N'-methylenebisacrylamide, and SDS-PAGE molecular weight standards were from Bio-Rad. Na125I was from Amersham Pharmacia Corp. Nonidet P-40 was from CalBiochem. HA (human umbilical cord) from Sigma was purified as described previously (16). Nitrocellulose membranes were from Schleicher & Schuell. Acrylamide and urea were from U. S. Biochemical Corp. p-Nitrophenyl phosphate was from Kirkegaard & Perry Laboratories. N-Glycosidase F (EC 3.5.1.52), and all other chemicals, which were reagent grade, were from Sigma. TBS contains 20 mM Tris-HCl, pH 7.0, 150 mM NaCl.

Preparation of LECs and LEC Membranes—Male Harlan Sprague-Dawley rats were from Harlan, Indianapolis, IN. LECs were isolated by a modified collagenase perfusion procedure (17), followed by differential centrifugation and then discontinuous Percoll gradient fractionation. Cells were collected from the 25/50% interface and washed three times with phosphate-buffered saline at 4 °C. For preparation of LEC membranes, the cells were hypotonically swollen, homogenized, and centrifuged at 10000 × g. The supernatant was then centrifuged at 105,000 × g to obtain the total membrane fraction (18).

Ligand Blot Assay—Samples were solubilized in an SDS sample buffer: 16 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, and 0.01% bromphenol blue (19). No reducing agent was added unless as noted. Cell or membrane samples were sonicated on ice for 10–20 s. After SDS-PAGE, the gel was electrotransferred to a 0.1-mm nitrocellulose membrane for 2 h at 24 V at 4 °C using 25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol, and 0.01% SDS. The nitrocellulose was treated with TBS, 0.05% Tween 20 at 4 °C overnight and then incubated with 2 μg/ml 125I-HA in TBS without or with a 150-fold excess of HA (as competitor) to assess total or nonspecific binding, respectively. The membrane was washed five times (5 min each) with 0.05% Tween 20 in TBS, dried, and the 125I-HA bound to protein was detected by autoradiography with Kodak BioMax film. Nonspecific binding in this assay is typically <5% (15).

Purification of the HAR—LEC membranes from 18 rats were suspended in 3.6 ml of TBS, 2% Nonidet P-40 and mixed by rotation at 4 °C.
for 2 h. The solubilized membranes were diluted with TBS to 0.5% Nonidet P-40, centrifuged for 30 min at 100,000 × g, and the supernatant was loaded at room temperature onto a RCA-1-gel column (10 ml). The column was washed with 10 volumes of TBS, 0.05% Tween 20. Bound proteins were eluted with 100 mM lactose in distilled water, dialyzed against multiple changes of TBS at 4 °C overnight, concentrated 10-fold using a Centricon-30 (from Amicon), and then passed over an immuno-affinity column (~8 ml) containing monoclonal antibody 175HAR-30 coupled to CNBr-activated Sepharose (~2 mg/ml resin). Details on the preparation and characterization of this and other monoclonal antibodies raised against the rat LEC 175-kDa HAR will be described elsewhere. The antibody column was washed with 10 volumes of 0.05% Tween 20 in TBS and then eluted with 100 mM sodium citrate, pH 3.0. Eluted fractions were neutralized by collection into 1 M Tris. Fractions containing protein were pooled, dialyzed against TBS at 4 °C overnight, and then concentrated using a Centricon-30.

Deglycosylation of HARs with N-Glycopeptidase F—Purified HAR (1.15 μg) was heated with 0.5% SDS at 90 °C for 3 min. Samples (22 μl) were chilled on ice for 4 min and then 0.5 M Tris-HCl, pH 7.2, was added to a final concentration of 10 mM. One-half unit of N-glycopeptidase F (20) and distilled water were added to give a final volume of 25 μl. The samples were incubated at 37 °C overnight, 9 μl of 4-fold concentrated SDS sample buffer was added, and they were heated for 3 min at 90 °C. The samples were subjected to SDS-PAGE, and protein was detected by silver staining, or receptor activity was determined by the 125I-HA ligand blot assay.

Two-dimensional Electrophoresis—Affinity-purified HAR (~1.5 μg) was subjected to SDS-PAGE without reduction, and the gel was stained with Coomassie Blue. The 175- and 300-kDa proteins were excised from the gel, cut into smaller pieces, divided into two portions, and incubated at 90 °C for 4 min with SDS sample buffer with or without 10 mM dithiothreitol followed by 50 mM iodoacetamide. The samples were then subjected to a second dimension of SDS-PAGE without reducing agent. Proteins were visualized by silver staining (21), and HAR activity was assessed by the 125I-HA ligand blot assay.

General—Protein content was determined by the method of Bradford (22) using bovine serum albumin as a standard. Receptor protein content was assessed after precipitation with 5% trichloroacetic acid to remove detergent. SDS-PAGE was performed according to the method of Laemmli (19). 125I-HA was prepared using Iodogen (Pierce) and a uniquely modified hexylamine derivative of HA, synthesized, and radiolabeled as described previously (16). 125I radioactivity was measured using a Packard Cobra Auto-Gamma Counting system, model 5002.

RESULTS AND DISCUSSION

In addition to the normal turnover of HA in tissues throughout the body, a wide range of biomedical and clinical applications use exogenous HA that is also removed from the lymphatics or ultimately from the blood and degraded by the LEC HAR (3, 4). For example, HA is used extensively in eye surgery (23), in the treatment of joint diseases including osteoarthritis (24), and is being developed as a drug delivery vehicle (25). Numerous studies have explored the benefit of HA during wound healing (26, 27). The exogenous HA introduced in these various applications is naturally degraded by the lymph and LEC systems noted above. Despite the very large endocytic and degradative capacity of the LEC HAR (28) and its importance in removing HA from the blood, the HAR had not yet been successfully purified.

The ability to purify the LEC HAR occurred with our discovery that two very active and specific HA-binding proteins, at 175 and 300 kDa, could be readily detected in LECs by ligand blotting using 125I-HA (15). These HA binding activities corresponded perfectly to our previous identification of two HAR proteins on intact LECs using an HA photoaffinity derivative that specifically labeled proteins of 175 and 300 kDa (14). The two HAR species observed by ligand blotting with 125I-HA also showed the same specificity with a panel of polymeric competitors (15) as observed for intact LECs (9, 10).

Sequential Lectin and Immuno-affinity Chromatography Purifies the Two LEC HAR Species to Homogeneity—We have prepared several useful monoclonal antibodies against the rat LEC 175-kDa HAR, including 175HAR-30, which recognizes this protein in Western blots (Fig. 1A) and removes the protein and the HA binding activity from extracts (Fig. 1B). The latter result demonstrates that 175HAR-30 recognizes the bone fide LEC HAR. Another antibody, 175HAR-174, behaves identically to 175HAR-30, but also inhibits specific HA endocytosis in live LECs by >90% (2). 175HAR-30 binds the 175-kDa HAR species and also recognizes the 300-kDa HAR species. However, as described below, the 300-kDa species is not a dimer of the 175-kDa protein and does not contain a 175-kDa subunit. The 175HAR-30 antibody immunoprecipitates both HAR proteins from LEC extracts, thus enabling the 175-kDa HAR and the 300-kDa HAR proteins to be purified for the first time (Fig. 2).

Nonreducing SDS-PAGE analysis showed that these two proteins comprise >98% of the final purified HAR preparations. Based on silver and Coomassie Blue staining of nonreduced gels, the 175-kDa HAR and 300-kDa HAR proteins are purified in an apparent molar ratio of 2:1. The fraction of total staining in the 175-kDa band was 0.47 ± 0.07 (n = 5) and 0.55 ± 0.09 (n = 3), respectively, for Coomassie- and silver-stained gels.

The two purified HAR proteins remained active, as assessed by the ligand blot assay (Fig. 3, lane 1). Both the 175-kDa and ~300-kDa HAR proteins were shifted to a lower mass by treatment with N-glycosidase F, indicating that both HAR species contain N-linked oligosaccharides (Fig. 4, lanes 1 and 2). The de-N-glycosylated 175-kDa HAR and 300-kDa HAR proteins were still capable of 125I-HA binding (Fig. 3, lane 2). Therefore, N-linked oligosaccharides do not appear necessary for the HA binding activity of these receptors. However, the reduced 175- and 300-kDa HAR proteins no longer bind 125I-HA (Fig. 3, lanes 3 and 4).

Subunit Composition of the Two HAR Proteins—To determine whether either protein contains disulfide-bonded subunits, the co-purified 175-kDa HAR and 300-kDa HAR were analyzed by reducing SDS-PAGE (Fig. 4, lanes 3 and 4). After reduction with β-mercaptoethanol, four protein species were evident ranging in apparent size from 97 to 260 kDa. In order...
to determine which HAR species yielded each of these four proteins, the 175-kDa HAR and 300-kDa HAR were first separated by nonreducing SDS-PAGE. The two HAR bands were then excised and reanalyzed by SDS-PAGE with or without reduction (Fig. 5). The reduced 175-kDa HAR yielded no other protein species, but the apparent size of the protein increased to 185 kDa (Fig. 5, lane 3). This shift to higher Mr is typical of membrane receptors with extracellular domains whose compact or tightly folded structures require intraprotein disulfide bridges.

After reduction, the 300-kDa HAR gave rise to three protein species with apparent masses of 260, 230, and 97 kDa, which we designate, respectively, as the a, b, and g subunits of the 300-kDa HAR (Fig. 5, lane 2). None of these three reduced proteins were able to bind 125I-HA (Fig. 3, lane 3). All three subunits contain N-linked oligosaccharides (Fig. 4, lane 3).

Based on Coomassie Blue and silver staining, and their apparent sizes, the molar ratio of the three protein components of the 300-kDa HAR is 1:1:1 (Table I). The 300 HAR could be a (abg) heterotrimer with these three subunits being the products of several different genes. Alternatively, the 300 HAR could be a homodimer of 300-kDa subunits, with one subunit specifically cleaved into 97- and 230-kDa species. In purified HAR preparations, the stoichiometry of the 175- and 300-kDa proteins has consistently been 2–3:1 (175:300). Therefore, the overall stoichiometry of the four proteins in reduced, affinity-purified HAR preparations was 2:1:1:1, respectively, for the 175-kDa protein and the a, b, and g subunits of the 300-kDa complex (Fig. 6).

Although the 175- and 300-kDa species could represent monomeric and dimeric forms of one LEC HAR, the present results show this is not the case. The 175-kDa HAR is not a
TABLE I
Purification of the LEC Endocytic HA Receptor

| Experiment | Protein band |
|------------|--------------|
| 185 kDa | 97 kDa | 230 kDa | 260 kDa |
| fraction of the total staining |
| 0.43 | 0.14 | 0.24 | 0.19 |
| 0.46 | 0.05 | 0.21 | 0.28 |
| 0.51 | 0.10 | 0.24 | 0.15 |
| Mean | 0.47 ± 0.04 | 0.10 ± 0.05 | 0.23 ± 0.02 | 0.21 ± 0.07 |
| Mole ratio | 2.5 | 1.0 | 1.0 | 0.8 |

A 1:1:1 complex of the three 300-kDa subunits might be expected to migrate as a >500-kDa species in nonreducing SDS-PAGE. Although the lack of good standards above 200 kDa makes it difficult to assign relative mass, the 300-kDa HAR appears to migrate anomalously fast (i.e. to a smaller than appropriate size position). We noted earlier (15) that the 175-kDa HAR and 300-kDa HAR are very elongated, not globular, molecules that behave like elongated rods during SDS-PAGE. Their apparent $M_r$ values depend greatly on the pore size of the gels. Therefore, the anomalous migration of the nonreduced 300-kDa HAR may be explained if the two large $\alpha$ and $\beta$ subunits are also very extended or rod-like in the ternary complex (Fig. 6).

We propose that LECs contain the 175- and 300-kDa species as two highly similar but distinct and separate isoreceptors for HA. The consistent 2:1 stoichiometry of the purified 175-kDa HAR and 300-kDa HAR species in LECs may reflect the tight and coordinated regulation of their expression, rather than their physical association. The reason for having two HARs may be related to the great polydispersity of HA. More than one HAR may be required to mediate effective removal from the blood of HA molecules that can vary over a mass range from $10^3$ to $10^6$ Da. Each HA isoreceptor may be specialized to interact with either smaller or larger HA. Ongoing studies to clone and further characterize the four purified HAR subunit proteins will enable us to determine their primary structures and their roles in normal health and a variety of diseases.

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