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Ed Harris
University of Nebraska - Lincoln, eharris5@unl.edu

Janet A. Weigel
University of Oklahoma Health Sciences Center

Paul H. Weigel
University of Oklahoma Health Sciences Center, paul-weigel@ouhsc.edu

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The Human Hyaluronan Receptor for Endocytosis (HARE/Stabilin-2) Is a Systemic Clearance Receptor for Heparin

Edward N. Harris, Janet A. Weigel, and Paul H. Weigel

Department of Biochemistry and Molecular Biology, and The Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190
Corresponding author — P. H. Weigel, tel 405 271-1288, fax 405 271-3092, email paul-weigel@ouhsc.edu

Abstract

The hyaluronic acid receptor for endocytosis (HARE; also designated Stabilin-2) mediates systemic clearance of hyaluronan and chondroitin sulfates from the vascular and lymphatic circulations. The internalized glycosaminoglycans are degraded in lysosomes, thus completing their normal turnover process. Sinusoidal endothelial cells of human liver, lymph node, and spleen express two HARE isoforms of 315 and 190 kDa. Here we report that the 190- and 315-kDa HARE isoforms, expressed stably either in Flp-In 293 cell lines or as soluble ectodomains, specifically bind heparin (Hep). The K_d for Hep binding to purified 190- and 315-kDa HARE ectodomains was 17.2 ± 4.9 and 23.4 ± 5.3 nm, respectively. Cells expressing HARE readily and specifically internalized ^{125}I-streptavidin-biotin-Hep complexes, which was inhibited >70% by hyperosmolar conditions, confirming that uptake is mediated by the clathrin-coated pit pathway. Internalization of Hep occurred for many hours with an estimated HARE recycling time of ~12 min. Internalized fluorescent streptavidin-biotin-Hep was present in a typical endocytic vesicular pattern and was delivered to lysosomes. We conclude that HARE in the sinusoidal endothelial cells of lymph nodes and liver likely mediates the efficient systemic clearance of Hep and many different Hep-binding protein complexes from the lymphatic and vascular circulations.

Abbreviations: **Hep**, heparin; **Ab**, antibody; **AP**, alkaline phosphatase; **aa**, amino acids; **BSA**, bovine serum albumin; **EV**, empty vector; **GAG**, glycosaminoglycan; **HA**, hyaluronic acid, hyaluronate, hyaluronan; **HARE**, hyaluronan receptor for endocytosis; **HS**, heparan sulfate; **HSPG**, heparan sulfate proteoglycans; **LDL**, low density lipoprotein; **PBS**, phosphate-buffered saline; **s190-HARE**, soluble 190-kDa HARE ectodomain; **s315-HARE**, soluble ectodomain of the 315-kDa HARE; **SA**, streptavidin; **TBS**, Tris-buffered saline; **190-HARE**, the 190-kDa HA receptor for endocytosis; **315-HARE**, the 315-kDa HA receptor for endocytosis; **b-Hep**, biotin-heparin; **DMEM**, Dulbecco's modified Eagle's medium; **ELISA**, enzyme-linked immunosorbent assay

Heparin (Hep) is the most anionic proteoglycan, due to the extensive sulfation of its glycosaminoglycan (GAG) chains, and contains many different sulfated disaccharide isomers of *N*-acetylgalactosamine and glucuronic acid or iduronic acid. Hep binds to many different soluble, matrix, and cell surface proteins and receptors, and has many functions, including its roles as an anti-coagulant and as a co-receptor for some growth factors (1, 2). Hep is a highly prescribed drug in surgical patients and those at risk for thromobosis. The genes and metabolic pathway for Hep biosynthesis in mast cells are understood reasonably well, and many of the biological and clinical activities of Hep have been well studied for several decades (3–6). In contrast, we know less about the catabolism of Hep and how total body homeostasis of this multifunctional proteoglycan is maintained. In particular, the mechanisms controlling systemic turnover of Hep, whether as endogenous proteoglycan or free chain drugs, are not known.

Although receptors for Hep have been characterized on a variety of cell types (*e.g.* macrophages, vascular smooth muscle cells), none of these mediate substantial clearance of Hep (7–9). A few reports implicated a role for Kupffer cells in Hep clearance, but were not followed up (10, 11). The possible contribution of liver sinusoidal endothelial cells to Hep uptake in these primary cell preparations was not examined. Because Hep is a widely prescribed drug, it is even more important to understand the factors that control its clearance and, therefore, pharmokinetics. Animal studies of Hep clearance, except by renal function, have been difficult to perform, because Hep binds to so many soluble, cell surface or matrix proteins and become widely distributed after injection.

Unfractioned Hep (3000–30000 Da), low molecular mass Hep (300–8000 Da), and the pentasaccharide, Fondaparinux, are the three classes of Hep drugs used to treat venous thrombosis, acute myocardial infarction, trauma, obesity, and coronary and peripheral vascular procedures; all situations wherein patients need immediate platelet anti-coagulation therapy (12). Following an intravenous bolus, unfractioned Hep has a half-life of ~1 h and is cleared from the circulation by the liver and kidney (13). Low molecular mass Hep and the pentasaccharide, subcutaneously injected, have half-lives of ~3–6 and ~17 h, respectively (14). Larger more structurally diverse Hep is more readily cleared than the lower molecular weight fragments. Although clinical handbooks declare that Hep is metabolized and cleared by the reticuloendothelial system, the mechanisms for Hep clearance are not known.

The primary scavenger receptor for systemic turnover of HA and most types of chondroitin sulfate, but not HS, is HARE/Stab-2, which mediates most of the total body HA turnover per day (15–17). HARE is found primarily in the sinusoidal endothelial cells of the lymph nodes, liver, and

spleen (18–21), and also in specialized tissues such as corneal and lens epithelium, mesenchymal cells of heart valves, prismatic epithelial cells covering the renal papillae (17), and in oviduct (22). Partially degraded HA perfuses from extracellular matrices in tissues and enters the lymphatic and hepatic vascular circulation systems where it is endocytosed by HARE and catabolized. The partially degraded HA perfusing from tissues first encounters HARE in lymph nodes, which mediates ~85% of the daily HA turnover. The remaining 15% of HA drains from the lymphatics into the circulatory system and is removed by HARE in liver sinusoidal endothelium.

The large full-length human HARE, encoded by the *STAB2* gene (accession number NM_017564 on chromosome 12q23.3 (NCBI data base), is a ~315-kDa 2551-aa glycoprotein (21, 23). The smaller 190-kDa HARE is not a splice variant, but rather it is an isoform derived from the full-length 315-kDa HARE by proteolysis (23). This 190-kDa HARE is, in fact, a functional endocytic recycling receptor that mediates GAG uptake in the absence of the larger 315-kDa HARE (24). A primary function of these two HARE isoforms is to bind and internalize HA and most of the chondroitin sulfate types (24–28); advanced glycation end products are also ligands (29, 30). The internalized HARE-ligand complexes traffic through the early endocytic pathway. After dissociation, the receptor recycles back to the cell surface, whereas the GAG ligand is delivered to lysosomes for degradation (19, 24, 30, 31).

In this report, we identify HARE as the receptor that specifically binds, internalizes, and mediates degradation of Hep. We demonstrate specific and high affinity HARE-Hep binding under a variety of conditions and active HARE-mediated endocytosis of Hep in stable cell lines. We conclude that Hep clearance by the body is mediated, probably predominantly, by HARE in the sinusoidal endothelium of the liver and lymph nodes.

Experimental Procedures

Materials, Solutions, and Buffers—Hep (free chains) was from Celsus (Cincinnati, OH) and Sigma. Flp-In 293 cells, fetal bovine serum, high glucose Dulbecco's modified Eagle's medium (DMEM), hygromycin B, Zeocin, glutamine, plasmid expression vectors, super-competent TOP10 *Escherichia coli*, LysoTracker DND-99, SA-Alexa 488, and Lipofectamine 2000 were from Invitrogen. ^{125}Iodine (100 mCi/ml; specific activity of >0.6 TBq/mg) in NaOH and Sepharose 6 Fast Flow (nickel-nitrilotriacetic acid) resin were from GE Healthcare. PolySorP strips were from Nalgene Nunc, Int. (Rochester, NY). EpranEx plates were from Plasso, LLC (UK). Streptavidin (SA) was from Pierce. ^{125}I-SA was prepared as described previously (32, 33). The protocol of Yu and Toole (34) was used to make biotin-Hep (b-Hep), and the number of biotins/chain was quantified using a QuantTag™ Biotin Kit (Vector Labs, Burlingame, CA). Size exclusion multiangle laser light scattering was performed to measure Hep molar mass and concentration, and to verify the addition of 1–2 biotins per Hep chain, without degradation (23). Western blot analysis was by colorimetric or enhanced chemiluminescence detection of blotted protein. Other materials, reagents, and kits were obtained as described recently (23). TBS contains 20 mm Tris-HCl, pH 7.0, 150 mm NaCl; TBST is TBS with 0.1% Tween 20. TBST/BSA is TBST with 1.0% (w/v) BSA. Coating Buffer for ELISA contains 15 mm Na_2CO_3, 36 mm $NaHCO_3$, pH 9.5. PBS contains 137 mm NaCl, 8 mm Na_2HPO_4, 1.5 mm KH_2PO_4, 2.7 mm KCl, pH 7.2. Hanks' balanced salts solution contains 5 mm KCl, 0.4 mm KH_2PO_4, 0.8 mm $MgSO_4$, 137 mm NaCl, 0.3 mm Na_2HPO_4, 5.5 mm glucose, 1.26 mm $CaCl_2$, 0.5 mm $MgCl_2$, and 28 μm phenol red; at the time of use, 3.5 g/100 ml of $NaHCO_3$ was added and the pH was adjusted to 7.2 with HCl. Endocytosis medium contains DMEM with 0.05% BSA.

Constructs and Cell Lines Expressing Membrane-anchored or Soluble 190-HARE, 190-HARE(ΔLink), or 315-HARE—Preparation of cDNA constructs and vectors for the creation of stably transfected 293 Flp-In cell lines expressing, the full-length 315-HARE, 190-HARE, or the secreted ectodomains of the 315-HARE or 190-HARE (lacking transmembrane and cytoplasmic domains) were described by Harris *et al.* (23, 24). Creation of 190-HARE(ΔLink) cDNA constructs and stable cell lines was described recently (35). All recombinant HARE proteins contain C-terminal V5 and His_6 epitope tags. Final clones were selected based on criteria of normal growth and morphology, good HARE expression, and that a single cDNA had inserted at the recombinase-mediated integration site provided in the Flp-In cell lines. SDS-PAGE using 5% gels and Western analysis using anti-V5 Ab were performed as described (24).

Preparation of ^{125}I-SA·b-Hep Complexes—For cell-based assays, 2:1 molar ratios of b-Hep and ^{125}I-SA were mixed, at 40–50-fold higher concentrations than in experiments, in 0.5 ml of Endocytosis medium for 30 min on a rotary mixer at room temperature just prior to an experiment. For controls with ^{125}I-SA alone, the same proportional amounts of free biotin and ^{125}I-SA were used. After mixing, the ^{125}I-SA·b-Hep or ^{125}I-SA·biotin complexes were diluted into Endocytosis medium. Final concentrations of b-Hep and ^{125}I-SA complexes in assays were 100 and 96 nm (2.5 μg/ml), respectively.

Binding and Endocytosis of ^{125}I-SA·b-Hep—Stably transfected cells, clone 9 (24), expressing 190-kDa HARE, were plated in 12-well dishes and grown in DMEM with 8% fetal calf serum and 100 μg/ml hygromycin B for at least 2 days prior to experiments. Cells were washed with Hanks' balanced salt solution and incubated at 37 °C for 60 min with Endocytosis medium (no serum) to allow HARE-mediated internalization of any bound serum GAGs. Endocytosis assays with these washed cells were performed at 37 °C in Endocytosis medium containing pre-formed complexes of ^{125}I-SA·b-Hep with or without unlabeled Hep as competitor. Binding of ^{125}I-SA·b-Hep to cells was measured at 4 °C. Specific binding or endocytosis was assessed in the presence of excess unlabeled Hep and, in most cases, in cells incubated in parallel with only ^{125}I-SA-biotin to determine background counts/min values. These values were subtracted from all data points to determine specific ^{125}I-SA·b-Hep endocytosis. To determine the amount of ^{125}I-SA·b-Hep binding by the total cell HARE population, cells on ice were permeabilized with 0.055% (w/v) digitonin, using a 25% stock solution dissolved in anhydrous dimethyl sulfoxide (36). At the indicated times, cells were washed three times with ice-cold Hanks' balanced salt solution, lysed in 0.3 n NaOH, and radioactivity and protein content (37) were determined and expressed as counts/min/μg of protein.

Ligand Blot Assay for b-Hep Binding—Cells expressing 315- or 190-HARE were scraped from T-75 flasks in the presence of serum and lysed in 0.5% Nonidet P-40. After rotating for 1 h at 24 °C, cell debris was removed by centrifugation, and the cell lysate was immunoprecipitated with anti-V5 Ab, which recognizes this fusion epitope on both recombinant proteins. Following 5% SDS-PAGE, the proteins were transferred to nitrocellulose membranes and blocked with TBST, 1% BSA at 4 °C for 2 h. The method for detecting b-Hep binding to HARE is a modification of a ligand blot procedure for HA binding (38). Nitrocellulose membranes were incubated with TBST/BSA for 2 h at 4 °C, washed, and incubated with 200 nm b-Hep in TBST/BSA, with or without 2000 nm unlabeled Hep as competitor to assess nonspecific binding. After incubation for 1.5 h at 4 °C, the membrane was washed with excess TBST at least 5 times over 30 min, and incubated with 2.0 μg/ml ^{125}I-SA for 30 min. After washing with TBST at least 5 times over 30 min, the membrane was dried at 22 °C and bound ^{125}I-SA was detected by autoradiography using Kodak MS film exposed at -80 °C for 1–18 h.

Western Blot Analysis—Following the ligand blot procedure, the membranes were rehydrated in TBST/BSA, incubated with 20 ng/ml anti-V5 Ab (Bethyl Labs, Montgomery, TX), washed, incubated with the appropriate secondary Ab conjugated with horseradish peroxidase, and detected by ECL. The ECL signal was captured using Classic Blue Film BX (MIDSCI, St. Louis, MO). Densitometry on both the Western and ligand blot films was performed with an Alpha Innotech FluorChem 8000.

Fluorescence Microscopy—Cells were grown on 18-mm glass coverslips in complete medium for at least 2 days prior to the experiment. B-Hep (100 nm) or unlabeled Hep was combined with 1 μg/ml SA-Alexa 488 in 0.5 ml of DMEM and rotated for 30 min prior to the experiment. Live cells were incubated with SA-Alexa 488·(biotin)-Hep conjugates in Endocytosis medium for 6 h at 37 °C. The medium was replaced with fresh Endocytosis medium containing 50 nm LysoTracker and the cells were incubated for 1 h. Live cells on glass coverslips were washed by dipping slides into PBS 3 times and immediately mounted onto glass slides prior to visualization using a Nikon Diaphot 300 fluorescence microscope. Images were captured with a DXM1200 side-mounted camera operated by Act-1 software version 2.6.3.

sHARE Purification—s190-HARE and s315-HARE were purified from pooled growth media by Ni^{2+} chelate affinity chromatography, followed by SDS-PAGE and electroelution as described by Harris *et al.* (24). Final purified HARE preparations were concentrated to 0.1–1.0 mg/ml using Amicon concentrators (Millipore), washed twice with PBS, and stored at -20 °C until use in subsequent assays.

ELISA-like—A set amount of HARE protein in Coating Buffer (200 μl) was placed in each well of a PolySorP strip, sealed to prevent evaporation, and incubated overnight at room temperature. All subsequent steps were carried out at 37 °C. The surfaces of the wells were then blocked by incubation with TBST and 2% BSA for 1.5 h. After washing, increasing concentrations of b-Hep were added and the plates were incubated for 2 h, washed 6 times with TBST, incubated for 1 h with SA-AP, washed 6 times in TBST, and finally incubated with *p*-nitrophenol phosphate. The A_{405} values of the strips were determined every 15 min for 1 h using a SpectraMax 340 (Molecular Devices) plate reader.

Biotinylation and Iodination of BSA—BSA (2 mg) was biotinylated using Sulfo-NHS-LC-biotin (Pierce) according to the manufacturer's instructions and then dialyzed extensively against PBS to eliminate excess free biotin. A 10:1 molar ratio of biotin-to-BSA molecules was used and the final b-BSA product contained an average of 5 biotins/BSA (a 50% efficient reaction), as assessed with the QuantTag Biotin Kit (Vector Labs). B-BSA (0.4 mg) was then iodinated as described previously using 0.5 mCi of ^{125}I for 15 min at 22 °C (39). ^{125}I-b-BSA was separated from free iodine on a PD-10 column, and 0.5-ml fractions were collected and assessed for radioactivity using a γ-counter. Fractions in the first void volume peak were pooled, and protein content and radioactivity were determined.

Degradation Assay—190-HARE and EV cells were grown to 80–90% confluence in 12-well plates prior to experiments. In initial trials, ternary complexes of ^{125}I-b-BSA·SA·b-Hep were formed using numerous ratios of the three components, and then tested for specific endocytosis by 190-HARE cells. These results indicated an optimal ratio of 100 nm ^{125}I-b-BSA, 200 nm SA, and 100 nm b-Hep. The ternary complexes were allowed to form while mixing by rotation at room temperature for 2 h just prior to an experiment. Similar mixtures containing ^{125}I-b-BSA and b-Hep without the SA linker or the ternary complexes with a 100-fold excess of unlabeled Hep were used as controls for nonspecific endocytosis. One hour prior to an experiment, cells were incubated in DMEM containing 2% BSA to block nonspecific binding sites and to allow endocytosis of any serum-derived GAGs bound to cell surface HARE. Cells were then incubated in DMEM/BSA with 0.5 ml of the ternary complexes or the control mixtures for 4 h, washed 3 times with 2 ml of cold Hanks' balanced salt solution, and incubated for 15 h in 0.5 ml of fresh DMEM plus 2% serum. The collected chase medium was cleared of cell debris by centrifugation and loaded on a PD-10 (G-25 Sephadex) column and eluted with PBS. Forty-eight 0.5-ml fractions were collected and radioactivity in each fraction was determined using a γ-counter.

Results

The possibility that HARE is the major systemic clearance receptor for Hep in the vascular and lymphatic circulatory systems has great physiological significance and would be an important finding. We, therefore, sought to test this using a variety of protein- and cell-based assays.

The Purified s315- and s190-HARE Ectodomains Bind B-Hep—To determine whether Hep can bind to both HARE isoforms, we used direct ELISA-like binding assays with the purified recombinant 190-HARE and 315-HARE ectodomains. As the b-Hep concentration increased, the amount of b-Hep bound to s190-HARE increased sharply and saturated above ~100 nm (Figure 1*A*, *solid line*). These data were readily fit by nonlinear regression analysis to a hyperbolic binding curve, indicating a single class of Hep binding sites with a K_d of 17.4 ± 4.9 nm ($p < 0.0001$). Binding of b-Hep was specific, because the presence of excess unlabeled Hep eliminated by >95% the binding of SA-AP (not shown). A similar

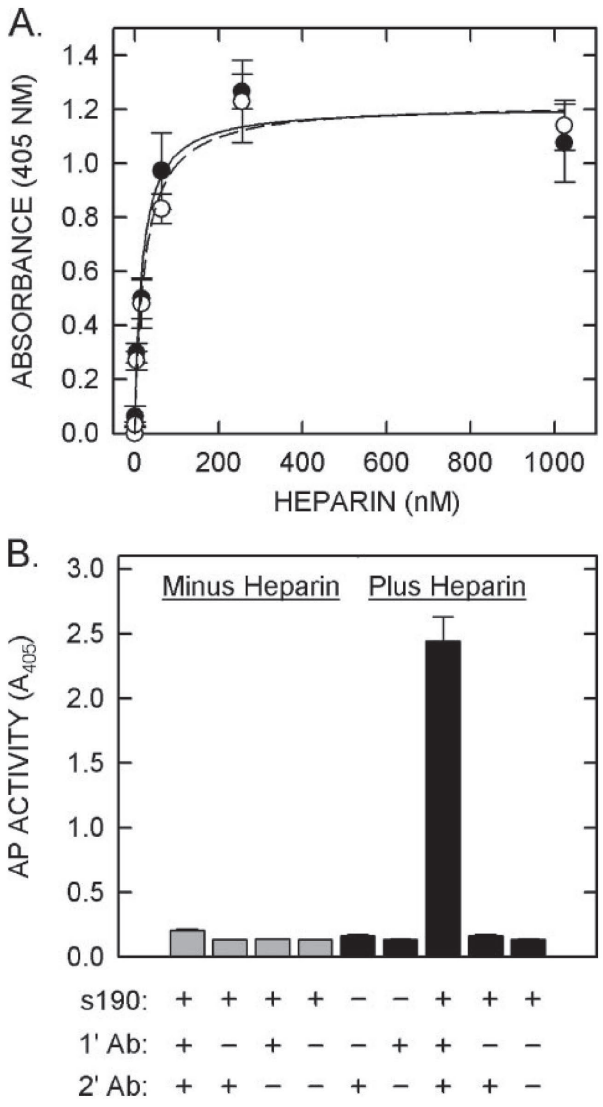

Figure 1. Hep binding to the purified ectodomains of 190-kDa and 315-kDa HARE is specific. *A*, equal molar amounts of purified s190- (○) and s315-HARE (•) proteins were coated onto PolySorP strips. Strips were blocked with 2% BSA to prevent nonspecific binding, washed, and incubated with 1.0–1024 nm b-Hep for 1.5 h as indicated. Bound b-Hep was detected with SA-AP and *lines* were calculated by regression analysis (SigmaPlot version 10). The K_d for b-Hep binding to s190-HARE (*solid line*) was 17.4 ± 4.9 nm ($p < 0.0001$) and the K_d for b-Hep binding to s315-HARE (*dotted line*) was 23.2 ± 5.3 nm ($p < 0.0001$). *B*, unlabeled Hep was coated onto EpranEx plates according to the manufacturer's instructions (*black bars*). Purified s190-HARE (4 μg/ml) was incubated in each well (except as indicated) for 2 h at 37 °C. Bound s190-HARE was detected with primary (*1′ Ab*) anti-V5 Ab (+) or buffer only (-) and after washing, the wells were treated with buffer (-) or secondary (*2′ Ab*) anti-goat Ab-AP conjugate (+) as indicated. Values are the mean ± S.E. ($n = 3$) A_{405}.

experiment with the 315-HARE ectodomain also showed a hyperbolic binding curve for b-Hep (Figure 1*A*, *dashed line*) with a K_d of 23.2 ± 5.3 nm ($p < 0.0001$), indicating virtually identical binding kinetics for the s190- and s315-HARE proteins. Thus, the additional N-terminal portion of the s315-HARE, consisting of 1135 aa, is not involved in Hep binding. The results confirm that Hep specifically binds with high affinity to the purified s190-HARE and s315-HARE ectodomains *in vitro*.

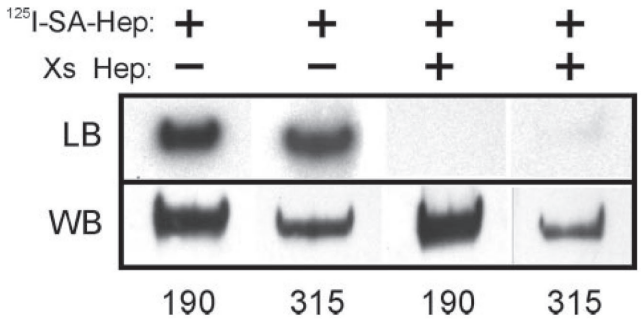

Figure 2. Hep binds specifically to the 190-HARE and 315-HARE membrane proteins. 190- and 315-kDa HARE proteins from cell lysates were immunoprecipitated with anti-V5 Ab, separated on 5% SDS-PAGE, and electrotransferred to nitrocellulose. For the ligand blot (*LB*), strips of nitrocellulose were incubated with 200 nm b-Hep with or without unlabeled excess (*Xs*) Hep, and b-Hep binding was detected with ^{125}I-SA followed by autoradiography. Western blot (*WB*) analysis was then performed on the same blot using anti-V5 Ab to identify both isoforms of HARE followed by ECL detection.

A "Reverse" ELISA-like Assay Validates the Specific HARE-Hep Interaction—To ensure that the biotin tag was not producing false positive signals in our *in vitro* and endocytosis assays, we assessed specific Hep binding using a reverse ELISA-like assay. Unlabeled Hep was coated onto EpranEx plates according to the manufacturer's instructions and then allowed to bind with purified s190-HARE protein. Primary anti-V5 Ab, directed against the HARE C-terminal epitope tag, followed by a secondary Ab-AP conjugate, was used to detect HARE binding with Hep (Figure 1*B*). Because Hep is highly anionic, and sticks nonspecifically to many proteins, we included controls to ensure that the primary (1′) or secondary (2′) Abs were not randomly adhering to Hep or to the plastic. All controls showed about the same low values (~10% of maximum), indicating that the signal output from this assay is not due to nonspecific Hep binding. This assay also validates results from the ligand blot and endocytosis assays that use pre-formed ^{125}I-SA·b-Hep complexes.

The Membrane-anchored 315- and 190-HARE Proteins Bind B-Hep—The ability of the intact 190-HARE and 315-HARE isoforms to bind Hep was assessed in membrane extracts after nonreducing SDS-PAGE and transfer to nitrocellulose. The HARE proteins re-nature after electrotransfer and incubation with Tween 20, and can be detected by a ligand blot assay with ^{125}I-HA (38). Nitrocellulose strips containing 190-HARE or 315-HARE were incubated with b-Hep (Figure 2), with or without an excess of unlabeled Hep, to determine nonspecific binding, and b-Hep binding was assessed using ^{125}I-SA and autoradiography. The results show that b-Hep binds to both HARE membrane proteins and binding is inhibited ≥95% by unlabeled Hep.

Cells Expressing 190-HARE Specifically Endocytose Hep—Because it is difficult to radioiodinate Hep without substantial modification, we developed a protocol to measure binding or internalization using ^{125}I-SA complexed to b-Hep. Pre-formed ^{125}I-SA·b-Hep complexes (using an optimum 2:1 molar ratio of SA:b-Hep) are readily taken up by cell lines expressing the 190-HARE alone (Figure 3*A*, *circles*). The rate of Hep endocytosis was linear for 8 h and then plateaued, indicating a steady-state accumulation of ligand. Because Hep naturally "sticks" to many substances, specific HARE-mediated

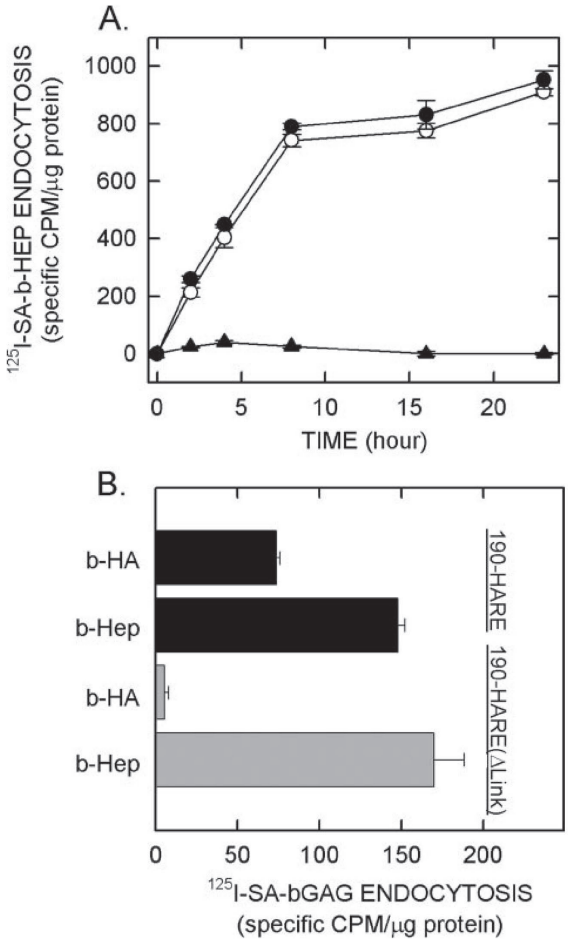

Figure 3. Endocytosis of Hep by cells expressing recombinant 190-HARE. *A*, two independent human 190-HARE cell lines (●, ○) and EV cells (▲) were grown to ≥80% confluence, washed, and incubated in Endocytosis medium for 1 h to clear receptors of any serum-derived GAGs. To measure endocytosis, cells were incubated with 2.5 μg/ml pre-formed complexes of ^{125}I-SA·b-Hep or ^{125}I-SA-biotin alone in Endocytosis medium for the indicated times. Values are the mean ± S.E. (n = 3) of specific cell-associated counts/min/μg of cell lysate protein. The average nonspecific cell-associated ^{125}I-SA-biotin was essentially the same (≤5%) in all cell lines and was subtracted from the total to obtain the sample values shown. *B*, 190-HARE (*black bars*) and 190-HARE(ΔLink) (*gray bars*) cells were allowed to internalize either ^{125}I-SA·b-HA or ^{125}I-SA·b-Hep at 37 °C for 4 h in parallel with the nonspecific ^{125}I-SA-biotin controls. Cells were processed and values presented as above in *A*.

internalization of ^{125}I-SA·b-Hep into cells was assessed with three controls. First, the parent cell line expressing the empty vector (EV) was incubated with ^{125}I-SA·b-Hep to determine whether any other native receptor on the cell surface was able to bind and internalize Hep (Figure 3*A*, *triangles*). Second, a 10-fold molar excess of Hep was added to HARE-expressing cells incubated with ^{125}I-SA·b-Hep to determine the specificity of Hep binding to HARE (≥90%; not shown). Third, under the same experimental conditions in parallel, both EV and HARE expressing cells were incubated with ^{125}I-SA-biotin (without Hep) to verify that the radioactive signal with ^{125}I-SA·b-Hep was a function of HARE-b-Hep interactions and not nonspecific binding by either biotin or ^{125}I-SA to the cells (not shown). All three above controls to assess nonspecific endocytosis of ^{125}I-SA-biotin were low (≤5%) and virtually identical for both cell lines.

The HARE Link Domain Is Required for HA, but Not Hep, Endocytosis—Because we recently showed that the Link domain of HARE is required for HA binding (35), we wanted to determine whether it was also needed for Hep endocytosis. Using PCR-based techniques, we made a 190-HARE mutant lacking the 93-amino acid Link domain and created stable 190-HARE(ΔLink) cell lines. To compare the Hep uptake abilities of 190-HARE(ΔLink) and wild type 190-HARE, we incubated both types of cells with either ^{125}I-SA·b-HA or ^{125}I-SA·b-Hep (Figure 3*B*). Nonspecific endocytosis of ^{125}I-SA-biotin was subtracted to assess specific internalization of HA or Hep by both cell lines. Cells expressing the 190-HARE (Figure 3*B*, *black bars*) internalized both b-HA and b-Hep complexes, as expected. In contrast, cells expressing 190-HARE(ΔLink) (Figure 3*B*, *gray bars*) internalized a similar amount of b-Hep, but very little b-HA. These results confirm, for the first time, that the HARE Link domain is necessary for HA, but not Hep, binding and internalization.

190-kDa HARE Is a Recycling Endocytic Receptor for Hep Internalization—HA uptake involves binding to HARE, internalization, and then dissociation in maturing acidic endosomes. The "free" receptor is recycled back to the cell surface and undergoes multiple rounds of HA internalization. We used 190-HARE and EV cells to compare cell surface binding *versus* cellular accumulation of Hep to determine whether HARE also is able to release Hep and recycle back to the cell surface to bind and internalize more Hep in a continuous manner (Figure 4*A*). At 4 °C Hep binds to 190-HARE on the cell surface, which comprises ~10% of the total cellular receptor number (23, 24). To assess receptor recycling, we compared values for surface binding to Hep (*i*.e. number of surface Hep receptors) with total cellular accumulation of Hep by endocytosis at 37 °C over 3 h. After subtracting the nonspecific values for both cell lines, 190-HARE cells internalized ~15 times the amount of total Hep compared with the surface-bound Hep. We estimate that the receptor recycling time is ~12 min: to bind, internalize, and release Hep, and then return to the cell surface for another endocytic cycle.

Hep Endocytosis Is Mediated by the Clathrin-coated Pit Pathway—The clathrin-coated pit pathway is one of the most efficient and rapid methods employed by cells to internalize ligands. Endocytosis via the clathrin-coated pathway can be temporarily and reversibly inhibited by hyperosmolar conditions, *e.g.* ≥400 mm sucrose as an osmolite (40–42). Because, our previous data show that HARE utilizes this pathway for internalization of HA (23, 24, 31), our goal was to determine whether HARE retains this property when bound with Hep. Cells expressing 190-HARE were incubated with ^{125}I-SA·b-Hep in DMEM, with increasing concentrations of sucrose, or with non-labeled Hep as competitor for 4 h (Figure 4*B*). Under hyperosmotic conditions (*e.g.* ≥0.4 m sucrose), specific receptor-mediated endocytosis of ^{125}I-SA·b-Hep decreased by >70%, indicating that Hep uptake depends on clathrin assembly into coated pits. Cells incubated with ^{125}I-SA·b-Hep and excess unlabeled Hep represented the nonspecific baseline (~10%) for Hep binding and internalization. In conclusion, these data demonstrate that the internalization of Hep is dependent on the receptor (HARE)-mediated clathrin-coated pit pathway.

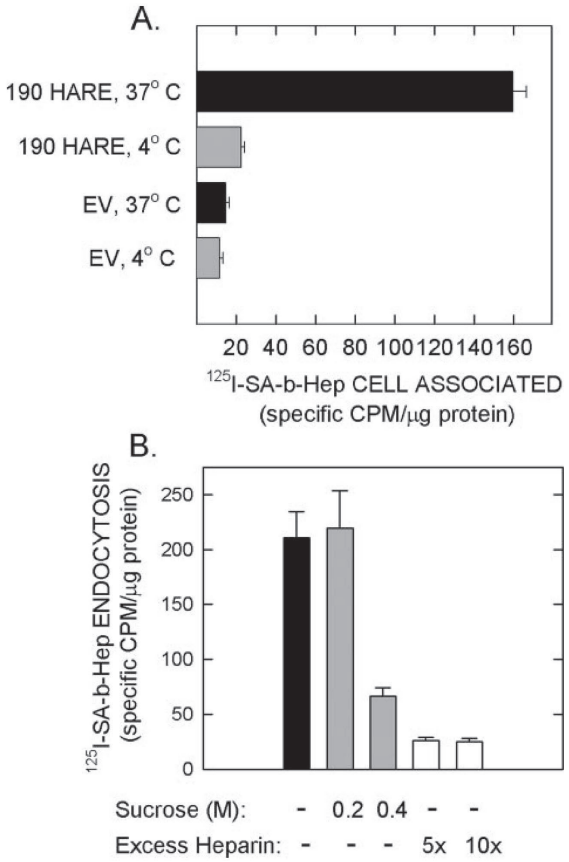

Figure 4. HARE mediates specific continuous clathrin-dependent endocytosis of Hep. 190-HARE cells and EV cells were grown and prepared as described in the legend to Figure 3. *A*, preformed ^{125}I-SA·b-Hep complexes in Endocytosis medium were added and 190-HARE and EV cells were incubated for 3 h at either 37 (endocytosis, *black bars*) or 4 °C (cell surface binding, *gray bars*). Cells were then washed, lysed, and radioactivity and protein content determined. *B*, to test the effect of hyperosmolarity on endocytosis, cells were preincubated in either isosmotic or hyperosmotic (with 0.2 or 0.4 m sucrose) Endocytosis medium for 15 min, and then incubated for 4 h with ^{125}I-SA·b-Hep in isosmotic (*black bar*) or hyperosmotic (*gray bars*) conditions or isosmotic conditions with excess unlabeled Hep (*white bars*) as a nonspecific internalization control. Values are the mean ± S.E. (n = 3) cell-associated specific counts/min/μg of protein.

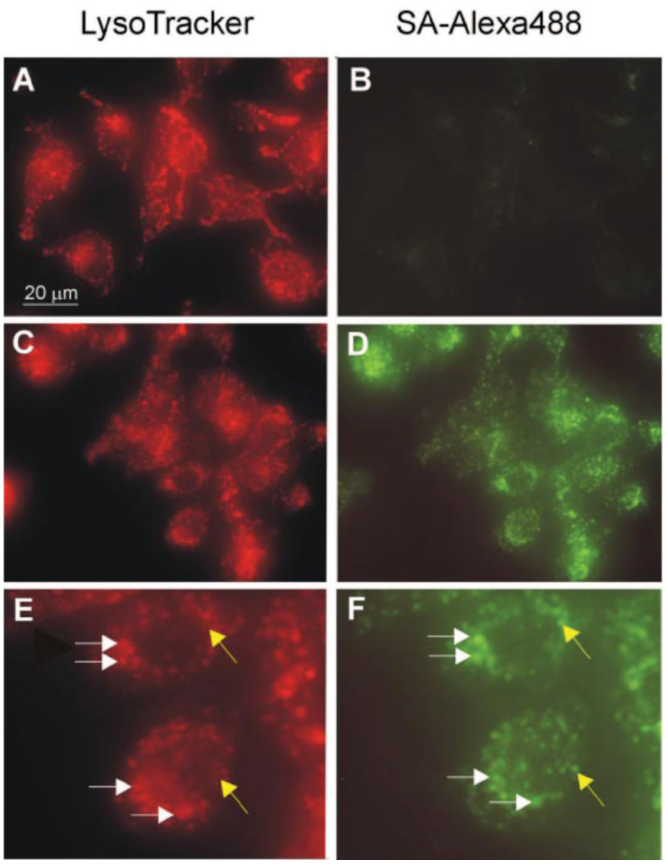

Figure 5. Cells expressing 190-HARE deliver internalized Hep to lysosomes. 190-HARE cells were incubated for 6 h with SA-Alexa 488 in the presence of unlabeled Hep (*A* and *B*) or SA-Alexa 488 bound to b-Hep (*C–F*). Live cells were visualized at ×200 magnification (*A–D*) or ×400 magnification (*E* and *F*) as described under "Experimental Procedures." Cell images were captured in two different color channels: LysoTracker-containing vesicles (*A*, *C*, and *E*) were visualized in the *red channel*, whereas SA-Alexa 488 in the presence of unlabeled Hep (*B*) or bound to b-Hep (*D* and *F*) was visualized in the *green channel*. The *white arrows* point to *red* lysosomes that are also loaded (and likely co-localized) with *green* SA-Alexa 488·b-Hep. In contrast, the *yellow arrows* indicate vesicles containing SA-Alexa 488·b-Hep that are not acidic (*E* and *F*). Cells expressing EV did not internalize SA-Alexa 488·b-Hep (not shown).

Internalized Hep Is Delivered to Lysosomes—Previous studies with both the rat and human HARE isoforms showed that HARE binds, internalizes, and delivers HA to lysosomes (19, 24, 31). To confirm that Hep is also internalized and delivered to lysosomes, we incubated 190-HARE cells with SA-Alexa 488 bound to b-Hep or free in the presence of unlabeled Hep, as a negative control (Figure 5). After 6 h, the medium was replaced with medium containing 50 nm LysoTracker (Invitrogen), a non-toxic, membrane-permeable, weakly basic dye, which is fluorescent when protonated in acidic compartments (*e.g.* lysosomes). Lower magnification shows a fairly homogenous population of cells expressing HARE (Figure 5). It is evident that 190-HARE cells have numerous lysosomes, as indicated by LysoTracker (Figure 5, *A, C*, and *E*). SA-Alexa 488-b-Hep was also present in many internal vesicles, some of which also stained with the LysoTracker dye (Figure 5, *D* and *F*). This is expected, because many compartments along the intracellular endocytic pathway become saturated relatively quickly with the internalized Hep and then wait to be delivered to lysosomes, where the slower degradation processes occur. To ensure that fluorescence was not due to nonspecific internalization of SA-Alexa 488, cells were incubated with the same amount of SA-Alexa 488 and unlabeled Hep. By uncoupling the Hep from the fluorescent tag, the cells could be assessed for nonspecific endocytosis of SA-Alexa 488. Most cells internalized a tiny amount of SA-Alexa 488, as expected, but overall cell fluorescence decreased >95% and was difficult to capture, even at a higher camera sensitivity (Figure 5*B*). Thus, the vast majority of the SA-Alexa 488·b-Hep conjugate is internalized specifically via HARE (Figure 5, *D* and *F*).

Degradation of internalized b-Hep is difficult to demonstrate directly, because all cells have other biotin-containing molecules. Therefore, we verified that b-Hep is degraded after endocytosis by an indirect approach using ternary complexes of ^{125}I-b-BSA·SA·b-Hep (Figure 6). ^{125}I-SA·b-Hep could not be used, because SA is extremely resistant to proteases after uptake by cells or *in vitro* (*e.g.* treatment of ^{125}I-SA

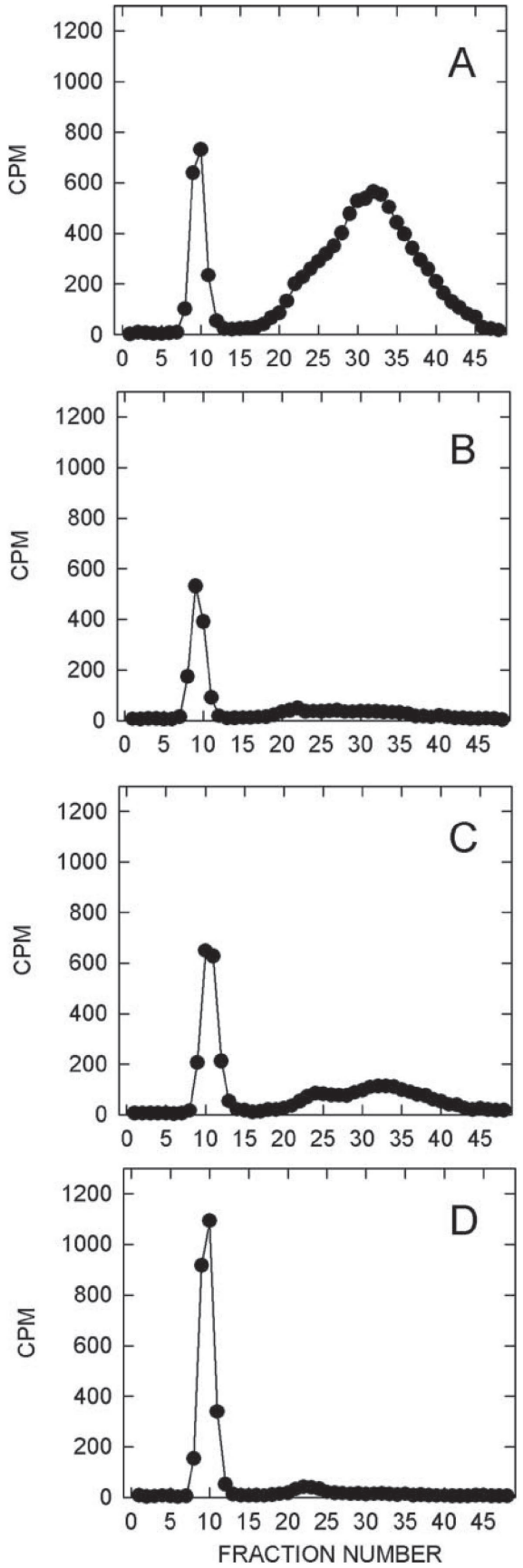

Figure 6. Hep·BSA complexes are degraded after HARE-mediated endocytosis. To assess the HARE and Hep dependence of degradation, the following combinations of cell type and ^{125}I-b-BSA complexes were used: 190-HARE cells were incubated with ^{125}I-b-BSA·SA·b-Hep complexes without (*A*) or with (*B*) a 100-fold excess of unlabeled Hep, or with ^{125}I-b-BSA and b-Hep in the absence of SA (*C*), EV cells (*D*) were incubated with ^{125}I-b-BSA·SA·b-Hep complexes. Cells were incubated at 37 °C for 4 h, washed, and then incubated for 15 h with fresh medium. Medium samples were chromatographed over PD-10 columns and 0.5-ml fractions were assessed for radioactivity. The first peak (V_0; fraction 10) represents a small amount of intact ^{125}I-b-BSA, coupled or uncoupled to SA·b-Hep. The large broad peak (fractions 20–45) represents degraded ^{125}I-b-BSA fragments that were released into the medium, after lysosomal digestion, during the chase period. Data points are the average of three separate samples.

with pronase, trypsin or proteinase K for 2 h at 37 °C did not alter band intensity or position; by SDS-PAGE, silver staining, and autoradiography). In contrast, BSA was 100% degraded under the same conditions (not shown).

To assess degradation, we used ternary complexes of ^{125}I-b-BSA·SA·b-Hep in an indirect approach in which biotin-BSA is degraded only following Hep-mediated endocytosis (Figure 6). Complexes of ^{125}I-b-BSA·SA·b-Hep were formed under conditions that optimized specific Hep-dependent endocytosis by 190-HARE cells. 190-HARE or EV cells were incubated with these ternary (or other control) complexes for 4 h to load up intracellular endocytic vesicles and then chased for 15 h with fresh medium to allow time for delivery to lysosomes, degradation, and release of degradation products. Gel filtration of ^{125}I-b-BSA·SA·b-Hep chase medium samples showed most of the ^{125}I-labeled radioactivity in the included second peak, indicating that lysosomal enzymes had digested ^{125}I-BSA to a variety of smaller sizes (Figure 6*A*). A small amount of radioactivity was present in the void volume; probably partially digested or undigested complexes. ^{125}I-b-BSA degradation products were virtually absent in the EV cell control, indicating that cells not expressing HARE do not internalize Hep, and thus do not degrade ^{125}I-b-BSA·SA·b-Hep complexes (Figure 6*D*). To ensure that the ^{125}I-b-BSA·SA·b-Hep degradation peak was the result of Hep-mediated endocytosis, 190-HARE cells were incubated with these complexes in the presence of a 100-fold excess of Hep (Figure 6*B*). The amount of degraded products decreased by 96% (after subtracting the small EV cell background), confirming that HARE is required for the observed degradation. As a second control, 190-HARE cells were incubated with "uncoupled" complexes (*i.e.* ^{125}I-b-BSA and b-Hep) to which SA was not added to connect b-Hep with ^{125}I-b-BSA (Figure 6*C*). Again, degradation of ^{125}I-b-BSA was decreased 81%, consistent with the conclusion that ^{125}I-b-BSA·SA·b-Hep internalization requires HARE-mediated binding to Hep.

Discussion

HARE (also designated Stabilin-2 and Feel-2) is the primary receptor for the clearance of many GAGs from the circulatory and lymphatic systems. HARE is a recycling receptor that operates via the coated pit endocytosis pathway to mediate internalization, and the subsequent delivery of ligand to lysosomes for degradation (19, 23, 24, 31, 43). Full-length 315-kDa HARE is a type I membrane protein (2551 aa) with a small cytoplasmic domain (72 aa), one membrane domain, and a large extracellular domain (2458 aa). The 190-kDa HARE is identical to the C-terminal 1417 aa of the 315-HARE, and is generated *in vivo* and *in vitro* in sta-

bly transfected cells by proteolytic cleavage (19, 24). We and others report that recombinant HARE behaves similarly in *in vitro* cell culture and in the sinusoidal endothelium of liver, lymph node, and spleen (28, 30, 44, 72). We show in this report that purified s190-HARE ectodomain specifically binds Hep (Figures 1 and 2), that cells expressing the recombinant protein internalize labeled Hep (Figures 3 and 4), and that Hep is trafficked to lysosomes (Figures 5 and 6).

This is the first report that HARE is also involved in the systemic clearance and homeostasis of Hep. For several decades, the anticoagulant properties of Hep have been used to treat thrombosis. However, newly recognized Hep properties may be useful as inhibitors of cancer metastasis and HIV-1 replication (45, 46). As with any soluble circulating factor designed to stimulate cellular responses, there must be efficient (and often redundant) mechanisms to dampen the signal and restore normal homeostasis. Despite thousands of basic and clinical studies on the biology of Hep, few reports have implicated specific receptors or cell types that are involved in the important process of removing Hep quickly and efficiently after its biological actions have been initiated.

In contrast to HA, little is known about the systemic turnover of Hep, although a current model is that extracellular heparanases and proteases partially degrade Hep and HSPGs, and the released GAG fragments could then be internalized and completely degraded at the local tissue level (47). Extracellular matrix and intracellular heparanases are important for HSPG turnover, Hep catabolism, and extracellular matrix remodeling (47–51). During extracellular matrix remodeling, a majority of the resulting free GAGs and proteoglycan fragments enter the lymphatic and circulatory systems, especially during injury or disease. Without an efficient GAG-clearing mechanism, these fluid circulatory systems would be overwhelmed with large amounts of debris derived from tissue extracellular matrixes throughout the body. We propose that the scavenger receptor HARE in sinusoidal endothelial cells of liver and lymph node, and perhaps spleen, recognizes and internalizes Hep for eventual degradation in lysosomes.

The liver is a primary organ for Hep homeostasis involving Hep-binding activities in the sinusoidal or non-continuous endothelium (52), Kupffer cells (10), and parenchymal cells (53). All these cell types contain scavenger receptors such as SCARB1 on parenchymal cells (54, 55) and macrosialin (56) and LOX-1 (57) on Kupffer cells. HARE is found in sinusoidal endothelial cells, not in Kupffer or parenchymal cells. HARE is also one of the few scavenger receptors identified to be associated with clathrin-coated pits (19, 30, 31), which contributes to much higher endocytosis and recycling rates (*e.g.* Figure 4*B*). In the past 20 years, many scavenger receptors have been cloned and, by definition, their common ligands are LDL or its derivatives, acetylated LDL and oxidized LDL (58). Scavenger receptors often bind more than one ligand and some (*e.g.* macrosialin and Lox-1) bind Hep, whereas MARCO, SREC, and CL-PI do not (58). HARE, like other scavenger receptors, binds acetylated LDL (E. N. Harris and P. H. Weigel, unpublished results, 29, 59). Despite much accumulated ligand binding data on these scavenger receptors, Hep binding has been assessed only as a competitor for binding of LDL, acetylated LDL, or oxidized LDL; labeled Hep was not used in direct binding studies. Negative results in indirect binding studies do not exclude the possibility that Hep binds to other independent sites on MARCO, SREC, and CL-PI. This is the first report to show directly that Hep binds with high affinity to both purified recombinant HARE *in vitro* and to live cells expressing HARE.

Thus far, direct binding studies have shown that HARE binds with HA (18), advanced glycation end products (29), several chondroitin sulfates (23), and Hep (this study). In addition, HARE is expressed in prime locations (*e.g.* the endothelium of liver and lymph nodes) to be a front-line receptor able to internalize multiple ligands. Hep binding to vascular endothelium after intravenous injection into animals was first reported by Mahadoo *et al.* (60). Follow-up studies showed Hep binding and endocytosis by cultured vascular endothelial cells, but no receptor was ever identified (61, 62). Moreover, these studies were from the perspective of how Hep binding to vascular endothelium affected soluble protein mediators in the coagulation cascade, and they concluded that the vascular endothelium is involved with partial neutralization of the anticoagulant activity of Hep. Both groups concluded that Hep binding sites on the vascular endothelium were nonspecific.

In contrast, the present study shows that both isoforms of HARE specifically bind Hep with high affinity and mediate its rapid internalization. Because HARE is a continuously recycling receptor, it clears Hep very efficiently. The estimated recycling time for HARE-mediated uptake of Hep is very similar to the values for HARE-mediated uptake of HA (31) and those for the asialoglycoprotein (43), mannose (63), transferrin (64), and LDL (65) receptors.

Because HA and Hep are both natural ligands for HARE, an important question is do they compete with each other for binding. Data from ongoing studies, show that HA and Hep do not compete with each other, consistent with reports using labeled Hep and HA in animals (E. N. Harris and P. H. Weigel, unpublished results, 66, 67). Unlabeled HA or chondroitin sulfate did not affect clearance of labeled Hep, indicating the binding sites for Hep were not the same as for HA. Liver sinusoidal endothelial cells have two active but different binding activities for HA and Hep, and the natural assumption was that two different receptors must be involved. In the present report we show that, in fact, the human receptors for both Hep and HA are the same protein: HARE. Importantly, both activities are in the 190-HARE and 315-HARE isoforms. Furthermore, we find that the Link domain of HARE is crucial for HA internalization, but is not required for Hep internalization (Figure 3*B*). We also find that HA and Hep can bind to HARE simultaneously; these results will be presented elsewhere in a more extensive analysis of the HA and Hep binding sites of HARE, and their relationships with other ligands (E. N. Harris and P. H. Weigel, unpublished results).

Our published and unpublished results show that HS, in contrast to the more highly sulfated Hep, does not bind to HARE (23, 24). This is significant, because HSPGs are found throughout the developing hematopoietic system (68), and if HARE bound HSPGs, it might interfere with development of these cells in spleen and, possibly, bone marrow. Exclusive binding of Hep, but not HSPG, by HARE could be advantageous in that by-products of coagulation and infection are readily cleared from the circulation, keeping the systemic

fluids relatively clear of these undesired products in contrast to their higher concentrations in wound or infected areas. For instance, tissue factor pathway inhibitor counteracts tissue factor and inhibits factors VIIa and X (69). Under normal conditions, factor Xa, a proteolytic product of factor X, in the presence of factors VII and VIIIa, will bind tissue factor pathway inhibitor on endothelial cells and be internalized via an unknown receptor (70). In the presence of Hep, however, tissue factor pathway inhibitor binds Hep and is released from the endothelium to form complexes that can include factors VIIa and X (71). These complexes may then bind via the Hep, and be internalized and cleared from blood by cells expressing HARE.

Based on the discovery that HARE mediates the rapid internalization of Hep, an intriguing possibility is that Hep may be the "vehicle" for the turnover of dozens of different Hep-binding proteins (*e.g.* growth factors) found in blood, lymph, and interstitial fluids. Hep-protein complexes in the vascular and lymphatic circulation systems could be cleared by HARE and degraded. Such a general clearance mechanism might be more physiologically practical than the utilization of dozens of different parallel receptor systems in which individual proteins in protein-Hep complexes were recognized and cleared from blood.

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