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The ligand-binding profile of HARE: Hyaluronan and chondroitin sulfates **A**, **C**, and **D** bind to overlapping sites distinct from the sites for heparin, acetylated low-density lipoprotein, dermatan sulfate, and CS-E

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Abstract

The hyaluronic acid receptor for endocytosis (HARE)/ Stabilin-2 is the primary systemic scavenger receptor for hyaluronan (HA), the chondroitin sulfates (CS), dermatan sulfate (DS), and nonglycosaminoglycan (GAG) ligands such as acetylated low-density lipoprotein (AcLDL), pro-collagen propeptides, and advanced glycation end products. We recently discovered that HARE is also a systemic scavenger receptor for heparin (Hep) (Harris EN, Weigel JA, Weigel PH. 2008. The human hyaluronan receptor for endocytosis [HARE/Stabilin-2] is a systemic clearance receptor for heparin. *J Biol Chem.* 283:17341–17350). Our goal was to map the binding sites of eight different ligands within HARE. We used biotinylated GAGs and radio-iodinated streptavidin or AcLDL to assess the binding activities of ligands directly or indirectly (by competition with unlabeled ligands) in endocytosis assays using stable cell lines expressing the 315 or 190 kDa HA receptor for endocytosis (315- or 190-HARE) isoforms, and ELISA-like assays, with purified recombinant soluble 190-HARE ecto-domain. For example, Hep binding to HARE was competed by DS, CS-E, AcLDL, and dextran sulfate, but not by other CS types, HA, dextran, or heparosan. ^{125}I-AcLDL binding to HARE was partially competed by Hep and dextran sulfate, but not competed by HA. Two ligands, DS and CS-E, competed with both Hep and HA to some degree. Hep and HA binding or endocytosis is mutually inclusive; binding of these two GAGs occurs with functionally separate, noncompetitive, and apparently noninteracting domains. Thus, HARE binds to HA and Hep simultaneously. Although the domain(s) responsible for Hep binding remains unknown, the Link domain was required for HARE binding to HA, CS-A, CS-C, and CS-D. These results enable us to outline, for the first time, a binding activity map for multiple ligands of HARE.

Keywords: chondroitin sulfate, endocytosis, glycosaminoglycan turnover, heparin, Stabilin-2

Introduction

Scavenger receptors are present in a variety of tissues, particularly the reticuloendothelial systems that clear the lymph and plasma of metabolic waste products and macromolecular debris. Currently, scavenger receptors are organized into five classes and one unclassified group (Yuasa and Watanabe 2003). Scavenger receptors can be broadly expressed or tissue specific, e.g., ranging from MSR1, which is found in macrophages in diverse tissues (Ashkenas et al. 1993), to Macrosialin/CD68, which is expressed only on macrophages found in the liver and peritoneum (Yoshida et al. 1998). The one common feature among scavenger receptors of the various classes is that they all bind low-density lipoprotein (LDL) and/or one or more naturally occurring LDL derivatives: acetylated LDL (AcLDL) and oxidized LDL (oxLDL). Most of these receptors also bind other classical polyanionic ligands such as maleylated bovine serum albumin (BSA), dextran sulfate, chondroitin sulfate (CS), fucoidan, and polyribonucleotides (e.g., poly I, poly G). Although not all of these latter ligands occur naturally, they all share features with natural analogs that facilitate high affinity and specific binding to their respective receptors (Krieger et al. 1993).

In this report, we characterize the ligand-binding profile of the hyaluronic acid (HA) receptor for endocytosis (HARE), also known as Stabilin-2/FEEL-2, which is a scavenger receptor that is highly expressed in the sinusoidal, or noncontiguous, endothelium of liver, lymph node, and spleen (Laurent and Fraser 1992). Fraser et al. (1983) first identified a scavenger clearance activity for HA when they injected mice with ^{14}C-HA and found it sequestered in these organs. In addition, HARE is found in oviduct, corneal and lens epithelium, heart valve mesenchymal cells, ependymal cells lining ventricles in brain, and epithelial cells covering renal papillae (Falkowski et al. 2003). The rat receptor was identified, purified, and characterized first (Yannariello-Brown et al. 1997; Zhou et al. 1999). Human HARE was later cloned and purified (Politz et al. 2002; Zhou et al. 2003), and then characterized in stable cell lines (Harris et al. 2004, 2007). The same protein was initially identified in the human genome database as a Stabilin-1 analog of unknown function (Politz et al. 2002). Human HARE is a 2551-amino-acid, 315 kDa, type-1 transmembrane receptor expressed on the cell surface and intracellular (e.g., endocytic) compartments. Two HARE isoforms (not splice variants) of different mass (e.g., 190 kDa and 315 kDa in human) are found in native tissues (Zhou et al. 1999, 2003; Weigel et al. 2002). During biosynthesis and exocytosis in cells stably expressing full-length human HARE cDNA, a minor subset of the 315 kDa receptor pool is proteolytically cleaved to create the 190 kDa HARE, the C-terminal 1416 aa of the 315-HARE protein (Harris et al. 2007). Although the mechanism and bio-

logical reasons for this protein processing are not yet known, both forms of the receptor are functional (Harris et al. 2004, 2007) and occur in the same tissues.

HARE recognizes a host of different ligands, including HA (Yannariello-Brown et al. 1997; Politz et al. 2002), collagen N-terminal pro-peptides (Hansen et al. 2005), advanced glycation endproducts (Tamura et al. 2003), AcLDL (Adachi and Tsujimoto 2002), CS A-E (Harris et al. 2004), phosphatidylserine (Park et al. 2008), and αMβ2 integrin (Jung et al. 2007). We found recently that HARE is also a clearance receptor for heparin (Hep) (Harris et al. 2008). The receptor is composed of modular repeats of EGF, EGF-like, and Fasciclin domains. A defining feature of HARE is its Link domain, situated near the transmembrane domain, which closely resembles Link domains in other HA-binding proteins such as TSG-6, CD44, and aggrecan (Day and Prestwich 2002).

Our goals in this study were to define which ligands compete with one another, how the binding of one ligand affects the other, and the relative organization within HARE of binding sites for the different ligands. We primarily studied the 190 kDa HA receptor for endocytosis (190-HARE), since it has higher expression as a cell surface receptor or secreted ectodomain than the 315 kDa HA receptor for endocytosis (315-HARE). Both isoforms have very similar, if not identical, ligand-binding profiles.

Results

Antibody inhibition of Hep binding to HARE

hHARE, encoded by the *STAB2* gene, specifically binds to HA and multiple CS types (Zhou et al. 2003; Harris et al. 2004, 2007). We recently reported that HARE/Stab-2 is also the clearance or scavenger receptor for circulating Hep (Harris et al. 2008). Although Hep binds to many proteins, including coagulation factors, no specific receptor for Hep clearance had been identified. Specific Hep binding to purified recombinant-secreted 190-HARE ecto-domain was blocked by a pAb against the purified s190-HARE protein (Figure 1A). At the maximum dose, ~70% of B-Hep binding to s190-HARE was blocked. In contrast, pre-immune Ab at the same concentration did not block Hep binding (not shown). Next, we evaluated the internalization of B-Hep and B-HA by 190-HARE cells in the presence of a saturating amount (30 μg/mL) of rabbit anti-s190-HARE pAb or pre-immune IgG (Figure 1B). Anti-s190-HARE, but not pre-immune, Ab inhibited the uptake of ^{125}I-SA-B-Hep into 190-HARE cells by ~58% (gray bars), whereas ^{125}I-SA-B-HA uptake was inhibited ~90% (black bars). In both cases, pre-immune IgG showed little or no inhibition. The differential inhibition of Hep versus HA binding by the same anti-HARE Ab indicates the possibility that these glycosaminoglycans (GAGs) bind to different sites.

HA and Hep bind to distinct and separate sites in 190-HARE

To determine if HA and Hep bind to the same, to overlapping, or to different sites on 190-HARE, we used an ELISA-like assay with B-GAGs and unlabeled GAGs as competitors (Figure 2A). We used a saturating concentration of B-HA to occupy all binding sites and saturate the HA response signal for this assay (black bar). In parallel, an amount of B-Hep that was not at saturation was used to produce a response signal lower than the HA signal (white bar). When these amounts of

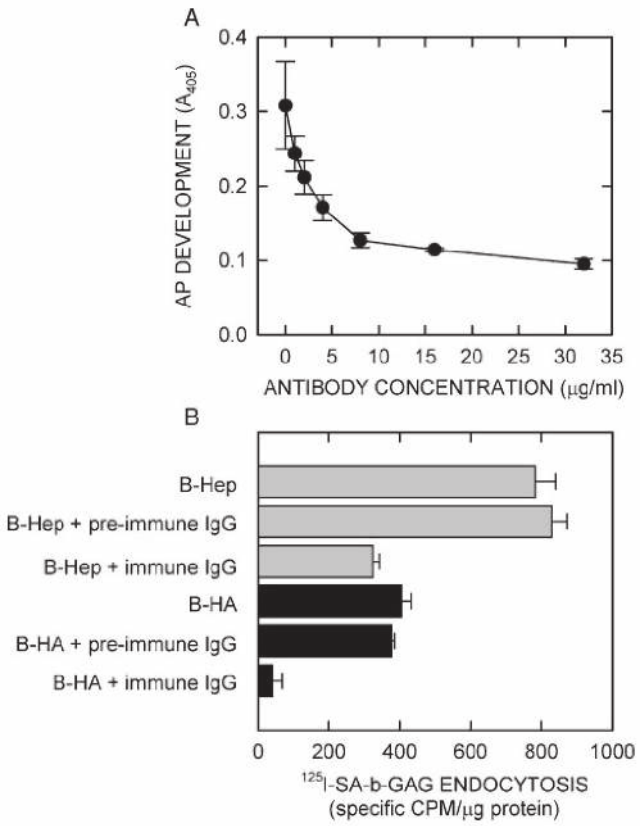

Figure 1. Anti-s190-HARE polyclonal Ab partially blocks both Hep and HA binding and endocytosis by HARE. (**A**) In ELISA-like assays, immobilized purified s190-HARE was incubated for 15 min with increasing concentrations of anti-s190-HARE polyclonal IgG. Following three washes, increasing concentrations of anti-s190-HARE polyclonal IgG and 100 nM B-Hep mixtures were added to the wells and incubated for 2 h at 37°C. After washing and detection with SA-AP, the A_{405} values were quantified and expressed as the mean ± SE (n = 4). (**B**) 190-HARE cells were treated as in *Materials and methods* and then incubated with 30 μg/mL pre-immune IgG or anti-s190-HARE immune IgG for 15 min at 37°C, prior to the addition of 30 μg/mL pre-immune or anti-s190-HARE IgG in combination with either ^{125}I-SA-B-Hep (gray bars) or ^{125}I-SA-B-HA (black bars). The cells were allowed to endocytose GAG complexes for 4 h, washed with HBSS, and lysed with 0.3 N NaOH. Radioactivity and protein were quantified. The values are means ± SE (n = 3) expressed as the cell-associated specific CPM/μg protein.

B-HA and B-Hep were incubated together, they produced an additive signal (upper gray bar) that could be attenuated to the B-HA-alone signal in the presence of unlabeled Hep (lower gray bar). These results strongly indicate that Hep and HA do not bind within the same HARE-binding site and that, unlike TSG-6 which also binds both ligands separately, HARE can bind HA and Hep simultaneously.

To confirm this conclusion based on results from the ELISA-like assay, we performed similar experiments in cells stably expressing either the smaller 190-HARE isoform alone (Figure 2B) or the 315-HARE (Figure 2C); although the cDNA encodes full-length 315-HARE, these cells also make minor amounts of 190-HARE (Harris et al. 2007). The cellular accumulation of ^{125}I-SA-B-GAG in this assay monitors binding to HARE and receptor-mediated endocytosis. In these experiments, the first set of wells contained only saturating amounts

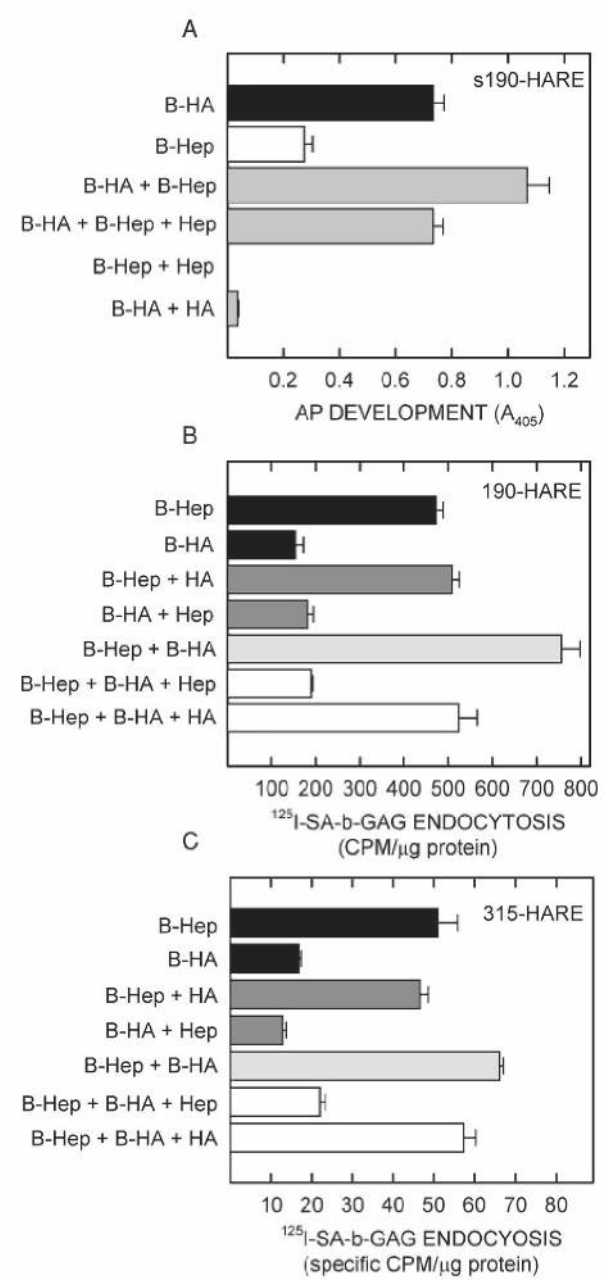

Figure 2. HA and Hep do not compete with each other and specifically and simultaneously bind to HARE. (**A**) In ELISA-like assays, immobilized purified s190-HARE samples were incubated, without competitor, with a saturating amount of B-HA (black bar) or a nonsaturating amount of B-Hep (white bar). These concentrations of B-HA and B-Hep were then incubated together with or without a 10-fold excess amount of unlabeled HA or Hep as competitor as indicated (gray bars). Assays were all performed simultaneously and bound B-GAG was detected using SA-alkaline phosphatase and p-nitrophenylphosphate. The A_{405} values are expressed as mean ± SE (n = 3). For endocytosis assays, 190-HARE (**B**) and 315-HARE (**C**) cells were used to determine HA and Hep uptake and competition. ^{125}I-SA-B-GAG complexes were prepared and then incubated with cells as described in *Materials and methods*. Cells were incubated either with one labeled B-GAG complex alone (black bars) and the other unlabeled GAG (dark gray bars), or with both labeled B-GAGs without (light gray bar) and with one unlabeled GAG as competitor (white bars). All sample wells contained equal amounts of ^{125}I-SA. After 4 h at 37°C, the cells were washed with HBSS three times, lysed in NaOH, and radioactivity and protein were determined. Values are the mean ± SE (n = 3) expressed as the specific CPM/μg protein.

of either ^{125}I-SA-B-Hep or ^{125}I-SA-B-HA (Figure 2, B and C; black bars). In the second set of wells, we also added a 20-fold excess of unlabeled HA to the B-Hep mixture or unlabeled Hep to the B-HA mixture to determine if the binding of either B-GAGs to membrane-bound HARE was cross-competed (Figure 2, B and C; dark gray bars). The results show that Hep and HA do not compete with each other for binding to either membrane-bound HARE isoforms.

Although one ligand might not bind to the other ligand-binding site, occupancy of one site might alter (e.g., in an allosteric-like manner) the binding of the second ligand to its site. In the third set of wells (Figure 2, B and C; light gray bar), we added the ^{125}I-SA-B-HA and ^{125}I-SA-B-Hep mixtures together to determine if both GAGs could bind and be internalized noncompetitively, as in Figure 2A, or competitively (e.g., as in the case of TSG-6). The signals from the combined mixtures were additive (i.e., the sum of the signals obtained with each B-GAG alone), confirming that both GAGs bound to HARE independently and noncompetitively. A fourth set of wells contained both ^{125}I-SA-B-GAG mixtures plus a 20-fold excess concentration of one unlabeled GAG (Figure 2, B and C; white bars). Again, the unlabeled GAG only inhibited the binding of its corresponding B-GAG, without affecting the binding of the other B-GAG. All of the above results support the conclusion that the HA- and Hep-binding sites in HARE are separate and independent.

The hHARE Link domain is required for binding HA, but not Hep

To determine whether the Link domain is involved with HA binding, cells expressing the empty vector (EV), wildtype 190-HARE, or 190-HARE lacking the Link domain (190-HARE(ΔLink)) were assessed for their ability to internalize ^{125}I-HA (Figure 3A). In cells without the Link domain, the binding and endocytosis of ^{125}I-HA was ~90% less than in cells expressing wildtype 190-HARE. However, the small amount of HA internalization by 190-HARE(ΔLink) cells was significantly higher than that for EV cells (p = 0.04; Student's paired t-test); it is not known where this residual HA binding occurs within the 190-HARE(ΔLink) protein. Since many recombinant Link domains bind HA, the results indicate that most of the HA binding to HARE is directly to the Link domain.

To determine if the Link domain is also necessary for endocytosis of Hep, cells expressing EV, 190-HARE, or 190-HARE(ΔLink) were incubated with ^{125}I-SA-B-Hep or ^{125}I-SA alone as a control (Figure 3B). Endocytosis of ^{125}I-SA-B-Hep complexes was not impaired in 190-HARE(ΔLink) cells, indicating that the HARE Link domain is not required for Hep binding. Surprisingly, ^{125}I-SA-B-Hep uptake was actually greater in 190-HARE(ΔLink) cells than in 190-HARE cells. However, this increase was not due to a greater binding stoichiometry of Hep with the receptor, but rather to increased expression of the 190-HARE(ΔLink) protein (Figure 3C). Western analysis of cell lysates showed that 190-HARE(ΔLink) cells express ~50% more receptor than 190-HARE cells. The reason for increased expression is not known, but all three 190-HARE(ΔLink) stable cell lines tested showed similar increases.

Sulfate groups are required for Hep binding with HARE

Since the sulfation of GAGs increases their negative charge and creates multiple types of disaccharide units, we assessed the ability of purified recombinant s190-HARE to bind B-Hep

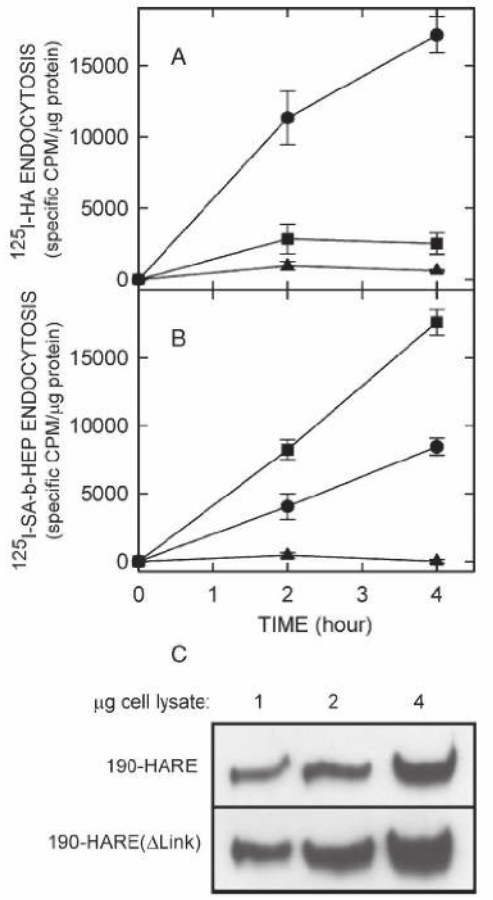

Figure 3. The Link domain is required for HA binding, but not Hep binding. Human 190-HARE (●), 190-HARE(ΔLink) (■), and EV (▲) cells were grown and preincubated in a serum-free endocytosis medium as noted in *Materials and methods*. (**A**) Cells were incubated with ^{125}I-HA alone or with a 50-fold excess of unlabeled HA and processed as in Figure 2 and *Material and methods* to assess total and nonspecific endocytosis, respectively. The differences in uptake (total minus nonspecific) are expressed as the mean ± SE (n = 3) specific cell-associated CPM/μg cell lysate protein. The statistical difference in HA uptake between EV and 190-HARE(ΔLink) cells was significant, as measured by the unpaired Student's t-test (p = 0.04), indicating that 190-HARE(ΔLink) cells do internalize HA above EV control levels. (**B**) The same cells were incubated with 2.5 μg/mL ^{125}I-SA-B-Hep (200 nM) without or with a 10-fold excess of unlabeled Hep to assess nonspecific endocytosis. (**C**) Cell lysate proteins, as indicated, were subjected to SDS–PAGE and Western blotting with anti-V5 Ab, using detection by enhanced chemiluminescence, to determine the relative amounts of 190-HARE and 190-HARE(ΔLink) protein. Based on densitometric analysis of bands using multiple film exposures, the amount of 190-HARE(ΔLink) protein was ~52% greater than wildtype protein in their respective cell lines.

in the presence of excess Hep or heparosan (Figure 4). Heparosan, synthesized by *Pasteurella multocida* Heparosan Synthase I, produces a 200–300 kDa Hep precursor polymer [(α1,4)GlcUA-(β1,4)GlcNAc] with no sulfate modifications (Sismey-Ragatz et al. 2007). A 10-fold excess of heparosan did not affect the binding of B-Hep to s190-HARE, whereas unlabeled native Hep eliminated nearly all B-Hep binding. These data confirm that the sulfates on Hep play a crucial role for binding with HARE.

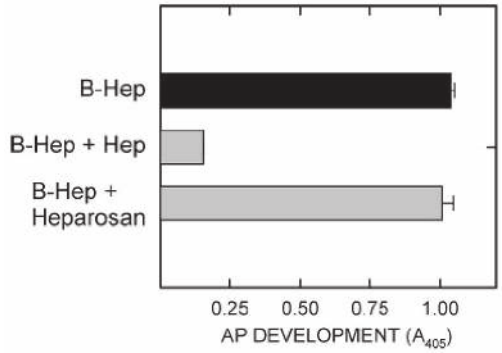

Figure 4. Sulfation of Hep is required for binding with HARE. In ELISA-like assays with purified s190-HARE, 10 nM B-Hep without (black bar) or with (gray bars) 100 nM unlabeled competitor GAGs was incubated for 2 h at 37°C. The strips were washed, incubated with SA-AP, and bound B-Hep was detected with the development of p-nitrophenolphosphate over a 1 h period. All values are the mean ± SE (n = 3) absorbance at 405 nm. The error for the B-Hep plus Hep samples was very small; thus the error bars cannot be seen.

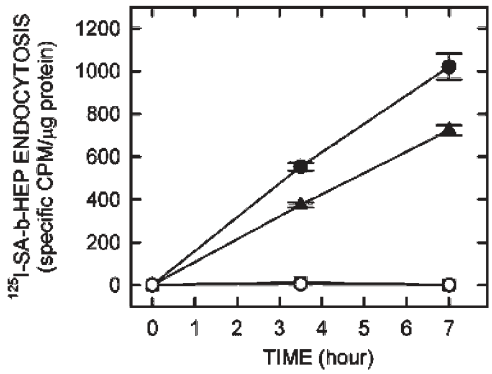

Figure 5. Endocytosis of Hep is partially competed by DS. Human 190-HARE (●, ○, ▲) and EV (■) cells were grown and pre-incubated as described in *Materials and methods*, and incubated with 200 nM ^{125}I-SA-B-Hep preformed complexes without (●, ■) or with 8 μM (40-fold excess) Hep (○) or DS (▲). At the indicated times, cells were washed, lysed, and radioactivity and protein were determined. Values are the mean ± SE (n = 3) specific cell-associated CPM/μg cell lysate protein. The average nonspecific internalization/binding of ^{125}I-SA-biotin (~5% of total) was subtracted from the sample values shown. The symbols and lines for EV cells with ^{125}I-SA-B-Hep (■) and 190-HARE cells with ^{125}I-SA-B-Hep plus unlabeled Hep (○) overlap.

Dermatan sulfate (DS) partially inhibits HARE-mediated endocytosis of B-Hep

We next focused on whether any of the other GAGs compete with Hep and HA for binding and endocytosis mediated by HARE. Since both Hep and DS contain iduronic acid and multiple sulfation sites, we tested if these two GAGs could interact with the same receptor-binding site (Figure 5). 190-HARE and EV control cells were incubated with preformed ^{125}I-SA-B-Hep complexes with no competitors (filled circles and filled squares, respectively). Additionally, 190-HARE cells were incubated with preformed ^{125}I-SA-B-Hep complexes in the presence of excess DS (filled triangles) or Hep (unfilled circles). Both cell lines were also incubated in parallel with ^{125}I-SA (plus biotin) alone to assess nonspecific internalization, which was ~5% of the signal with ^{125}I-SA-B-Hep (not shown). Excess DS decreased the rate of ^{125}I-SA-B-Hep internalization by ~33%. EV cells incubated with ^{125}I-SA-B-Hep alone

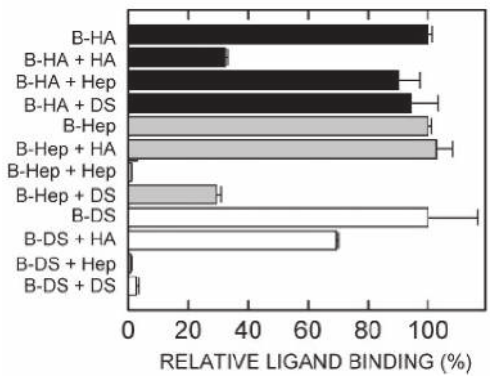

Figure 6. DS competes with both HA and Hep, which do not compete with each other, for binding to HARE. In ELISA-like assays with purified s190-HARE, B-HA (black bars), B-Hep (gray bars), and B-DS (white bars) were incubated alone or with a 10-fold excess of the indicated unlabeled GAG for 2 h at 37°C. Results within each of the three datasets were normalized to 100% for the B-GAG alone. Bound B-GAG was detected and quantified using SA-AP. Colorimetric reaction values are the mean ± SE (n = 3) A_{405}.

(squares) and 190-HARE cells incubated with ^{125}I-SA-B-Hep in the presence of excess unlabeled Hep (open circles) showed essentially no specific internalization.

DS competes with both HA and Hep, which do not compete with each other

Next, we used the ELISA-like assay to examine the relationship among HA-, Hep-, and DS-binding sites on the purified s190-HARE. First, we tested the binding of B-HA in the presence of unlabeled HA, DS, or Hep (Figure 6; black bars). As expected, HA competed effectively (68%), but DS or Hep did not. Second, we tested the binding of B-Hep to s190-HARE in the presence of unlabeled HA or DS (Figure 6; gray bars). B-Hep binding was reduced by Hep (>98%) or DS (71%), but not by HA. The lack of cross-competition between HA and Hep binding to HARE further supports the conclusion that they bind to separate and distinct sites. Third, we tested B-DS binding to s190-HARE in the presence of unlabeled Hep or DS (Figure 6; white bars). B-DS binding was well competed by DS (>97%) and Hep (>99%) but only partially blocked by HA (30%). The results indicate that DS binds within or near the Hep-binding site and also to a lesser degree with the HA-binding site. A similar result was also obtained with 190-HARE or 315-HARE cells incubated with ^{125}I-HA and excess DS; a small, but significant, amount of ^{125}I-HA uptake was competed by DS (Harris et al. 2007).

Dextran sulfate (Dx-S) and CS-E inhibit Hep binding to purified s190-HARE

Dx-S is a synthetic polyanionic polysaccharide, whose degree of sulfation can be varied, and that can mimic some of the sulfation patterns and properties of a GAG, including Hep. In ELISA-like assays with purified s190-HARE, B-Hep binding was progressively inhibited by increasing concentrations of Dx-S (Figure 7A), indicating that Dx-S competes with Hep for a common s190-HARE-binding site. In fact, for equivalent molar amounts, Dx-S was a better competitor for B-Hep binding than unlabeled Hep. This suggests that the right sulfation pattern needed for HARE binding occurs more frequently in Dx-S than in Hep, even though the mass of Dx-S is approximately half of the Hep mass.

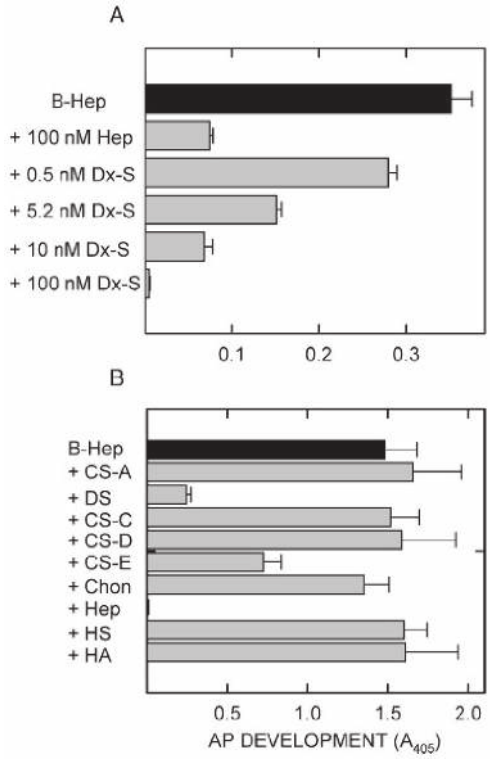

Figure 7. Heparin binding to HARE is competed by Dx-S, DS, and CS-E. B-Hep was incubated in ELISA-like assays with purified s190-HARE, with no addition (black bars) or with the indicated excess unlabeled GAG (gray bars) in two separate experiments. Bound B-Hep was detected as in Figure 4; color development values are the mean ± SE (n = 3) A_{405}. (**A**) B-Hep binding was determined in the presence of excess Hep or increasing concentrations of Dx-S. (**B**) B-Hep binding was determined in the presence of a 10-fold excess of unlabeled Hep or eight other individual GAGs.

We used ELISA-like assays to assess whether CSs A–E, chondroitin (Chon), or heparan sulfate (HS) can also compete for the binding of B-Hep to s190-HARE (Figure 7B). The only GAG that significantly decreased B-Hep binding, other than Hep and DS (CS-B), was CS-E (chondroitin 4,6-disulfate); no significant competition was seen with Chon, CS-A, CS-C, CS-D, HS, or HA. These results indicate that negative charge density is not the only, or even the key, factor involved with Hep binding, but rather the spatial organization and orientation of charge groups likely determines how well the molecule fits into the Hep-binding site(s) of HARE. We also used these ELISA-like and cell-based endocytosis assays to determine if keratan sulfate (KS) affects B-Hep binding and found that KS, like HS and HA, does not compete for B-Hep binding to HARE (not shown).

AcLDL endocytosis by 190-HARE cells is competed by Hep and DS

We compared the ability of EV and 190-HARE cells to endocytose ^{125}I-AcLDL and found that the level of nonspecific binding with excess AcLDL was moderately low (equivalent in both cell lines and ~25% of total HARE-mediated binding). Nonspecific endocytosis was subtracted from the total endocytosis to obtain 190-HARE-mediated specific endocytosis of ^{125}I-AcLDL (Figure 8, black bar). In the same experiment, we

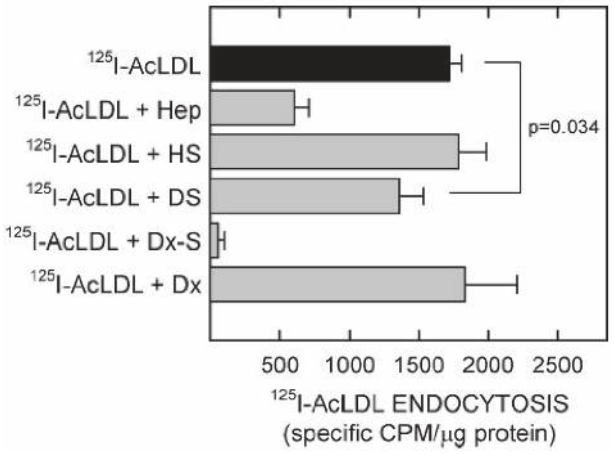

Figure 8. ^{125}I-AcLDL endocytosis is competed by Hep, DS, or Dx-S. 190-HARE (black bar) and EV (not shown) cells were incubated as in Figure 1B with an endocytosis medium containing 2 μg/mL ^{125}I-AcLDL for 4 h at 37°C. To account for nonspecific binding and endocytosis of ^{125}I-AcLDL, the average cell-associated radioactivity of EV cells was subtracted from the 190-HARE cell values. To test the competition by other GAGs for ^{125}I-AcLDL binding and endocytosis, 190-HARE cells were also incubated with excess (4 μM) unlabeled GAG as indicated. The mean ± SE (n = 3) values for the specific cell-associated CPM/μg cell lysate protein were determined as in Figure 1B and *Materials and methods*.

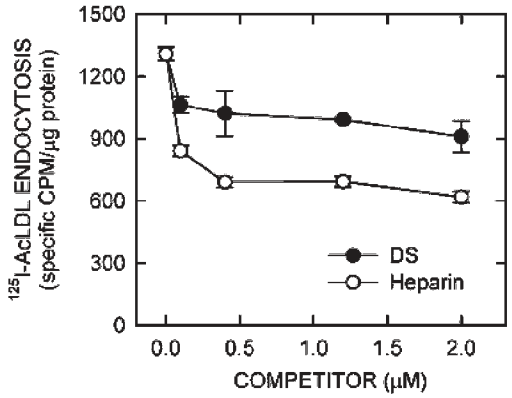

Figure 9. Hep competes more than DS for ^{125}I-AcLDL. 190-HARE cells were prepared for endocytosis assays as in Figure 1B. (**A**) Hep or DS was added, to the indicated concentration, to cells in the endocytosis medium containing 2 μg/mL ^{125}I-AcLDL. After 3 h at 37°C, the cells were washed, lysed, and radioactivity and protein were determined. Values are the mean ± SE (n = 3) cell-associated CPM/μg cell lysate protein.

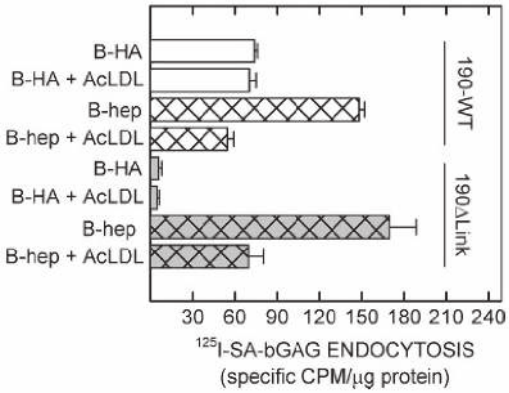

Figure 10. AcLDL competes for Hep, but not HA, binding and endocytosis by both 190-HARE and 190-HARE(ΔLink) cells. 190-HARE (white bars) and 190-HARE(ΔLink) (gray bars) cells were prepared for endocytosis assay as described in Figure 1B and *Materials and methods*. Preformed complexes (100 nM) of ^{125}I-SA-B-HA (solid bars) or ^{125}I-SA-B-Hep (cross-hatched bars) were prepared and added to cells with or without 50 μg/mL AcLDL, as indicated. After 4 h at 37°C, cell-associated CPM/μg cell lysate protein values (mean ± SE; n = 3) were measured as noted in Figure 1B and *Materials and methods*.

assessed the competitive abilities of unlabeled Hep, HS, DS, Dx, and Dx-S (Figure 8, gray bars). Hep and Dx-S, which compete with each other as noted above, inhibited ^{125}I-AcLDL endocytosis by 60% and 95%, respectively, whereas DS reduced ^{125}I-AcLDL internalization by only 20%. HS and nonsulfated dextran had no effect on ^{125}I-AcLDL binding and endocytosis by HARE. Thus, although HS and Hep share many similarities, the results confirm that HS does not bind to HARE at the HA-binding site or the Hep-binding site, which is also shared by Dx-S and AcLDL.

Endocytosis of AcLDL by 190-HARE cells is only partially competed by Hep and DS

Earlier results showed that DS blocks HA uptake only partially by 190-HARE cells (Harris et al. 2004, 2007). Based on results in Figure 7 and Figure 8, DS also competed for Hep and AcLDL binding, with different apparent affinities for HARE. Since DS and Hep bind to the same region of HARE, we tested how increasing concentrations of each affected the binding and endocytosis of a fixed amount of ^{125}I-AcLDL by 190-HARE cells (Figure 9). Both Hep and DS displayed similar dose-response inhibition curves that saturated at ~0.5 μM and resulted in only partial displacement of ^{125}I-AcLDL. Maximum inhibition by DS and Hep plateaued at 25% and 50%, respectively, although the apparent K_i values were similar at ~50 nM. These results indicate that the three ligands (DS, Hep, and AcLDL) have both common overlapping and unique sites of interaction with HARE.

AcLDL blocks endocytosis of Hep, but not HA, by 190-HARE cells

Endocytosis of ^{125}I-SA complexes with B-HA or B-Hep was evaluated in cells expressing either 190-HARE (Figure 10, white bars) or 190-HARE(ΔLink) (Figure 10, gray bars) with or without unlabeled AcLDL. In 190-HARE cells, AcLDL did not decrease B-HA uptake, but reduced B-Hep uptake by >50%. Predictably, AcLDL also decreased B-Hep uptake in 190-HARE(ΔLink) cells. Additionally, the small amount of B-HA accumulation by 190-HARE(ΔLink) cells was not affected by AcLDL. We conclude from this experiment that AcLDL binds within the Hep-binding domain, but not the HA-binding Link domain. Furthermore, the residual HA-binding site in the 190-HARE(ΔLink) mutant receptor also appears to be distinct from the AcLDL-binding site.

Discussion

Recently, we showed that HARE specifically binds with Hep (Harris et al. 2008). It is likely that HARE is the primary scavenger receptor for removing Hep from blood and lymph fluid. In this report, we show for the first time that HARE can

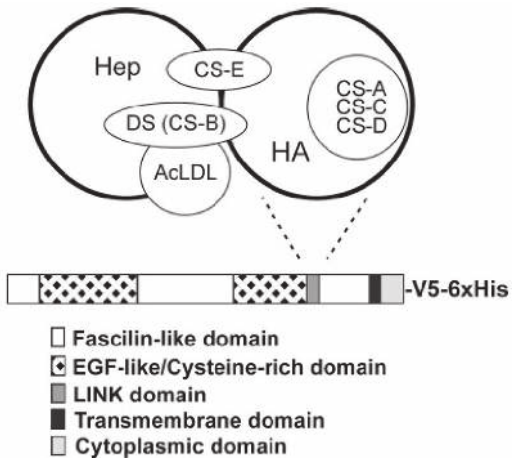

Figure 11. Relative organization of ligand-binding sites of the 190-HARE ecto-domain. The upper schematic binding site model is based on data from this and other studies using ELISA-like or endocytosis assays with different HARE constructs, cell lines, and combinations of the eight ligands shown. The relative size of the circles/ovals approximates the relative binding affinity of a ligand and placement of shape corresponds to relative location along the 190-HARE protein. Ovals indicate binding regions that overlap with the two main binding sites for HA and Hep. The lower linear domain model of the 190-HARE protein shows that the Link domain (dark gray) is required, or partially used, for binding to six of the eight ligands. We do not yet know the locations of the binding sites for Hep, AcLDL, CS-E, and DS within the large region upstream of the Link domain.

specifically and simultaneously bind to both Hep and HA, which do not compete with each other. Five other new findings are (i) HARE binds DS predominantly within part of the Hep-binding site(s) and only to a lesser extent within the HA-binding sites; (ii) AcLDL specifically binds HARE and binding is competed robustly by Hep or Dx-S, partially by DS, but not at all by HA; (iii) at least 90% of the HA-binding activity is due to the HARE Link domain and the remaining ~10% binds to HARE at an unknown site; (iv) HARE lacking the HA-binding Link domain still binds normally with AcLDL or Hep; and (v) only DS and CS-E bind within parts of both the Hep- and HA-binding sites. Previous results showed that CS-A, CS-C, and CS-D all bind within the HA-binding site of HARE (Harris et al. 2004, 2007), and the present results confirm that the Link domain is the major HA-binding site. The Link domain is also required to bind these CS types (data not shown).

Thus, our results show that HARE/Stabilin-2 specifically binds with AcLDL and seven different GAGs (HA, Hep, CS-A, CS-C, CS-D, CS-E, and DS), as well as the synthetic glycan, Dx-S, at two primary independent sites. Based on experiments outlined in this and previous reports, our current model for the organization of these multiple ligand-binding sites illustrates that the HA-binding (Link) domain is completely separate from the Hep- and AcLDL-binding domain (Figure 11). Hep and HA bind to mutually inclusive sites (the two largest nonoverlapping circles, Figure 11) within the 190-HARE isoform (Figures 1B and 2). Although it was assumed that the Link domain is the primary HA-binding site, this is the first report to demonstrate this experimentally (Figure 3A). Figure 3B also confirms that the Link domain is not involved with Hep binding, since cells expressing 190-HARE(ΔLink) internalize Hep. DS competes with the binding of both Hep (Figures 5, 6, and 7B) and HA (Figure 6; Harris et al. 2004, 2007) although there is a higher level of competition with Hep than with HA (Figure 6). CS-E binding to HARE is competed by both Hep (Figure 7B) and HA (Harris et al. 2004) to the same extent; thus bound CS-E may span both binding sites equally. It is also possible that DS or CS-E bind independently, and with different affinities, to parts of the Hep and HA binding sites. CS-A, CS-C, and CS-D all compete only with HA (Harris et al. 2004) and not Hep (Figure 7B). Others reported that HARE is a scavenger for AcLDL (Adachi and Tsujimoto 2002; Tamura et al. 2003). In our direct labeling and competition studies, AcLDL competed with Hep (Figures 8 and 10), DS (Figures 8 and 9), but not with HA (Figure 10). AcLDL binding was also competed with Dx-S (Figure 8), a competitor for Hep binding (Figure 7A), but not with HS (Figure 8). A portion of the AcLDL-binding site lies outside of both the Hep and HA sites (Figure 9) (Figure 11, circles).

The *in silico* structure of the HARE/Stabilin-2 Link domain is predicted based on the structures of other Link-containing proteins (Blundell et al. 2005). Link domains contain a groove for the HA strand to slip into and interact with three Tyr (aromatic) and two Lys/Arg (basic) residues. The Hep-binding site of HARE is still unknown and may be situated within the EGF domains of the protein. It does not appear to be located within a separate binding groove of the Link domain, as seen with TSG-6 (Mahoney et al. 2005). In TSG-6, HA and Hep or HS bind within the Link domain in separate binding grooves. When Hep or HS bind, there is a conformational change in the protein that prevents HA from binding and likewise, if HA binds then the Hep- or HS-binding site is inactive. Thus, HA and Hep both cannot bind simultaneously to TSG-6 (Mahoney et al. 2005). In contrast, despite the high homology (48% identical and 64% similar) between the TSG-6 and HARE Link domains, we do not see a contribution to Hep-binding activity by the HARE Link domain. In space-filling models of the TSG-6 Link domain, the Hep saccharide chain is positioned to interact with basic residues at positions 34, 54, and 56 (Link domain numbering); of these residues, only R56 is present in the Link domain of HARE. Other amino acids possibly involved in electrostatic interactions with the sulfate groups of Hep (Mahoney et al. 2005) are present in Link domains of both HARE (R20, K41, K72, K84) and TSG-6 (K20, K41, K72, R84).

The HARE Link domain functions differently than that of TSG-6, since both HA and Hep bind with HARE simultaneously, and 190-HARE(ΔLink) binds with Hep at least as well as the wildtype receptor. Another difference between the HARE and TSG-6 Link domains is that both Hep and HS bind to the same site on TSG-6 Link, but HARE Link binds only HA and not HS (Figure 7B; Mahoney et al. 2005). Direct binding assays found no HS-binding activity in purified s190-HARE or membrane-bound 190-HARE. One difference between Hep and HS is that Hep is more highly sulfated. Hep sulfation occurs almost entirely along the sugar backbone in contrast to HS, which contains islands of high sulfation between long stretches of low sulfation (Bame 2001). Compared to HA, Hep contains IdoA(2S)-(α1,4)-GlcNS(6S) in greater abundance than IdoA-(α1,4)-GlcNS(6S) and IdoA(2S)-(α1,4)GlcNS, which helps to give Hep a higher sulfation density. GlcNS(6S) residues of Hep also promote more of the rare 3-O sulfation of GlcNS(6S), to form GlcNS(3S,6S), than in HS (Rabenstein 2002). In contrast, HS contains more GlcNAc than GlcNS, rarely any 3-O

sulfate, and the ratio per disaccaride of HS *O*-sulfate to Hep *O*-/*N*-sulfate is 0.2–0.7:2.4. We assume that the specific binding of HARE to Hep, but not HS, involves some of these structural differences between HS and Hep sulfation.

The full-length HARE/Stabilin-2 receptor contains four Cys-rich EGF clusters and seven Fasciclin domains. The 190-HARE encoding region contains the two C-terminal EGF clusters and four of the Fasciclin domains. Since both the full-length 315 kDa and 190 kDa HARE receptor ecto-domains bind Hep to the same extent and the same affinity (Harris et al. 2008), the Hep-binding site(s) must be contained within the 190-HARE-encoding region. Fasciclin domains may not contribute to the Hep-binding sites, since the Fasciclin domain is involved with binding αMβ2 integrins (Jung et al. 2007). The integrin-binding Fascilin domains of the receptor may serve a general adhesive function, since cells with high expression of surface HARE exhibit greater cell–cell and cell–substratum adhesion than EV cells (not shown).

Heparosan, which is the Hep precursor polymer and lacks negatively charged sulfate groups or epimerized uronic acids, does not bind with HARE. Likewise, Dx-S competes with Hep for HARE binding, whereas the unsulfated dextran does not. On the other hand, charged residues alone cannot account for all the binding specificity or activity of HARE for Hep, Dx-S, and DS polymers. If that were the case, then all of the CS species would interfere with Hep binding. CS-A and CS-C have one sulfate (two negative charges) per disaccharide unit and CS-D and CS-E have two sulfates (three negative charges) per disaccharide unit. Based on the results presented here and other supporting data (not shown), only DS and CS-E competed for Hep binding to HARE; CS-A, CS-C, and CS-D did not compete. The different CS types are all highly negatively charged and if sulfate group density were solely responsible for binding to HARE, then most or all the CS species should inhibit Hep binding to various degrees.

All of the classified scavenger receptors bind one or more forms of LDL such as AcLDL (Yuasa and Watanabe 2003). Other groups have used either ^{125}I-AcLDL in direct binding assays with cells expressing Stabilin-2 (i.e., both isoforms) (Tamura et al. 2003) or AcLDL in indirect assays as a competitor for ^{125}I-AGE-BSA binding (Adachi and Tsujimoto 2002). This is the first report to show the direct binding of ^{125}I-AcLDL by the 190-HARE and to assess competition with unlabeled GAGs. We found in this study that ^{125}I-AcLDL endocytosis by cells expressing 190-HARE was completely inhibited by Dx-S and partially inhibited by Hep and DS (Figure 8). The relative binding affinities of two ligands for the same site will determine the equilibrium distribution of both ligands. For example, in Figure 6, B-HA was not competed by excess unlabeled DS, but some of the B-DS was competed by HA. This apparent discrepancy could be explained because HA binding to the HA site has a higher affinity (K_D ~18 nM) than DS binding to the HA site, or there is more than one DS-binding site along the receptor. A plateau effect in dose inhibition curves may indicate that one ligand interacts with only part of the binding site(s) of the other, which is thus still able to bind to HARE with good affinity. All of our data indicate that the AcLDL-binding site overlaps the Hep/DS sites and does not include or overlap with the HA-binding site.

The findings in this report may be very significant physiologically for several reasons. The simultaneous endocytosis of HA/CS and Hep/Hep-like proteoglycans is likely to be very important because these GAGs are continuously shed from extracellular matrixes (ECMs) in tissues throughout the body. That HA/CS and Hep can be simultaneously cleared from circulating lymph or blood by the same receptor indicates a strong biological "need" or evolutionary pressure to have an effective mechanism that removes multiple classes of ECM molecules in a coordinated way. We recently found that HARE mediates the activation of intracellular ERK-signaling pathways in response to HA binding (Kyosseva et al. 2008). Thus, an additional exciting possibility is that in performing its function of clearing multiple GAGs, HARE may also provide a mechanism by which the body senses, evaluates, and responds to ECM turnover and degradation. Normally, proteoglycans, such as aggrecan, are partially degraded and replaced at a slow rate, but turn over much faster during inflammation and tissue remodeling. The large initial degradation products can be internalized by local cells or professional phagocytes in a tissue or flow/perfuse into the general systemic circulation, from which they are removed by HARE. HARE is the primary clearance receptor for CS proteoglycan fragments and Hep-bound proteins in the lymph and circulatory fluids, which constitute the systemic circulation.

Thus, HARE is an efficient scavenger for most of the GAGs, even those that are rare (e.g., CS-E; Ten Dam et al. 2007) and transiently produced in pathological situations. HA, CS, and Hep GAG chains may also serve as "handles" that bind with HARE and indirectly enable the clearance of a multitude of different growth factors, GAG-binding proteins, and other ECM proteins that are in complex with these GAGs. This "piggy-back" clearance role for HARE would allow an efficient removal of the waste proteoglycan-associated material generated during infection, inflammation, development, tissue remodeling, and disease processes in distal tissues (Harris and Weigel 2008).

Materials and methods

Reagents and buffers

Purified unfractionated Hep (UFH) was from Celsus (Cincinnati, OH) and Sigma (St. Louis, MO), weight-average mass = 19 kDa. HA from Genzyme (Framingham, MA) was acid hydrolyzed under mild conditions (Raja et al. 1988), followed by neutralization to obtain a weight-average mass of 100 kDa. CS-A was from CalBiochem (La Jolla, CA) and DS, CS-C, CS-D, CS-E, and HS were from Seikagaku Corp. (Tokyo, Japan). All GAG preparations were analyzed by size exclusion chromatography/multiangle laser light scattering to determine concentration and weight-average polymer mass (Baggenstoss and Weigel 2006). Biotinylated heparin and HA (B-Hep, B-HA) were prepared using the method of Yu and Toole (1995), with slight modifications (Harris et al. 2007). AcLDL was from AbD Serotec (Raleigh, NC). Flp-In 293 cells, culture medium, transfection reagents, and plasmids were from Invitrogen/Gibco (Carlsbad, CA). *pfu* ultra HF was from Stratagene (La Jolla, CA). Classic Blue BX film was from MidSci (St. Louis, MO). Streptavidin (SA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC), biotin-LC-hydrazide, sulfo-NHS-SS-biotin, SnakeSkin dialysis tubing (MWCO 3500) and chemiluminescence reagents, Luminol and Peroxide solutions, were from Pierce (Rockford, IL). Polysorp well strips were from Nunc (Roskilde, Denmark) and *p*-nitrophenylphosphate was from Kirkegaard &

Perry Laboratories, Inc. (Gaithersburg, MD). ^{125}I (100 mCi/mL; specific activity of >0.6 TBq/mg) in NaOH and Sepharose 6 Fast Flow (Nickel NTA) resin were from GE/Amersham Biosciences (Piscataway, NJ). ^{125}I-SA and ^{125}I-HA were prepared as described previously (Weigel 1980; McGary et al. 2003). Concentrator/desalting Centricon devices were purchased from Amicon (Bedford, MA). Protein A/G agarose resin was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Tris-buffered saline containing Tween-20 (TBST) contains 20 mM Tris–HCl, pH 7.0, 150 mM NaCl, and 0.1% (v/v) Tween-20. Coating buffer for ELISA assays contains 15 mM Na_2CO_3, 36 mM $NaHCO_3$, pH 9.5. Blocking buffer is TBST with 2% (w/v) BSA. Endocytosis medium is DMEM containing 0.05% BSA. Hank's balanced salts solution (HBSS) contains 5 mM KCl, 0.4 mM KH_2PO_4, pH 7.2, 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. SA-alkaline phosphatase conjugate and other salts and reagents were from Sigma (St. Louis, MO).

Preparation of s190-, s315-, and 190-HARE(ΔLink) constructs

cDNA constructs encoding the secreted ecto-domains of the 315 kDa and 190 kDa human HARE were produced by the previous method in which the transmembrane and cytoplasmic domains were deleted, while retaining the C-terminal V5 and His_6 epitope tags (Harris et al. 2007). A single primer (5′-GTGACCTTGACCCACACTGGATCCG- AAGGTAAGCCTATC-3′) was used in individual mutagenic reactions with the wildtype HARE cDNAs (315-HARE in pcDNA5/FRT/V5/His, 190-HARE in pSecTag/FRT/V5/His) in an Ericomp thermocycler (18 cycles: 94°C, 30 s; 63°C, 30 s; 71°C, 1 min/plasmid kb) using *pfu* ultra HF. To make the 190-HARE(ΔLink) construct, a single primer (5′-CCACTTCCAGGATACCACTGTTCGGATGAAAGATGT GAACTGCACC-3′) was used with the 190-HARE wildtype template to delete the Link domain (18 cycles: 94°C, 30 s; 62°C, 30 s; 71°C, 1 min/plasmid kb). After these reactions, the plasmids were ethanol-precipitated and resuspended in 17 μL H_2O, 2 μL NEB4 buffer, and 2.5 U of *Dpn*I to cut template plasmids, while retaining intact mutant plasmids. After overnight incubation at 37°C, the digestion mixtures were heated to 95°C for 10 min and immediately transformed into TOP10 supercompetent *Escherichia coli* cells. Bacterial colonies were screened by a miniprep procedure, PCR, and sequencing to confirm the desired mutation. Plasmids containing correct mutations, open reading frames, and promoter regions were used to make stable cell lines as described in Harris et al. (2004, 2007).

Purification of s190-HARE

s190-HARE was purified from the conditioned cell culture medium by immobilized Ni-chelate affinity chromatography. The media (250 mL) were prepared by the addition of 250 mM NaCl, 10 mM imidazole, and 10% glycerol to the indicated final concentration. Two milliliters of a 50% slurry of Ni-chelate resin was added, the mixture was incubated overnight at room temperature with slow rotation, and then passed through a 20 mL column to capture the resin. The resin was washed three times with one bed volume of 500 mM NaCl, 20 mM Na_2HPO_4, and 10 mM imidazole, pH 7.2. The s190-HARE protein was eluted with four successive 1 mL portions of the same buffer containing 400 mM imidazole. For efficient elution of s190-HARE, the first portion was added, the resin was stirred to create a suspension, and after incubation at 22°C for 10 min, the column was then eluted. The next three 1 mL volumes were successively added, incubated for 10 min without mixing, and then eluted. This procedure recovered >90% of the protein associated with the resin. The four eluted fractions were pooled and concentrated to ~0.2 mL using a 30 MWCO concentrator. The s190-HARE preparations were ~10–20% pure, since some serum proteins in the media also adhered to the resin in a Ni-dependent fashion.

To further purify the s190-HARE protein for ELISA assays, the concentrated semi-pure s190-HARE was fractionated by 5% SDS–PAGE. The s190-HARE band, which separated distinctly from other bands, was identified in the gel by negative staining (using 4% $CuCl_2$ in water) and excised with a clean razor blade. The gel was minced to smaller pieces, which were destained with 0.25 M EDTA, 0.15 M Tris–HCl, pH 7.6, placed in a SnakeSkin dialysis bag with 5–10 mL 185 mM glycine, 2.5 mM SDS, and 25 mM Tris–HCl, pH 8.3, and electroeluted using a horizontal gel box for 16 h at 35 V, 4°C. The solution within the dialysis bag was collected, concentrated, and washed as above with phosphate-buffered saline (PBS), and re-concentrated to 0.25 mL. Purity (~99%) was verified by SDS–PAGE, silver staining, and Western blot analysis. Protein concentration was determined by the bicinchoninic acid method (Smith et al. 1985).

ELISA-like assay

The purified s190-HARE protein was diluted in the coating buffer at 1.0 μg/mL and 200 μL was added to each Polysorp well, sealed with Scotch tape, and incubated at 22ºC for 16 h. All the following steps were performed at 37°C. After coating, the Polysorp well surfaces were washed once with PBS and incubated with a 250 μL blocking buffer for 1.5 h. All ligands and SA-AP mixtures were diluted in the blocking buffer. Ligand solutions (200 μL) were incubated in each well for 2 h, removed, and the wells were washed six times with TBST, incubated for 1 h with SA-alkaline phosphatase, washed six times in TBST, and finally incubated with *p*-nitrophenylphosphate. The A_{405} values of the solutions were determined several times within 1 h. Negative controls, used as blanks for the nonspecific binding of SA-alkaline phosphatase, were treated as above except for the addition of B-GAG.

Anti-HARE polyclonal antibody

The soluble ecto-domain of the 190-HARE protein (s190-HARE) was purified by electro-elution, as described above, and used for Ab production by Pocono Rabbit Farm and Laboratory, Inc. (www.prfal.com). Two rabbits were each initially injected with 0.2 mg s190-HARE in Freund's complete adjuvant followed by three boosts with 0.1 mg s190-HARE in Freund's incomplete adjuvant. IgG from the preimmune and postinjected rabbit serum was purified by protein A/G affinity chromatography according to the manufacturer's instructions. The eluted total immune, but not preimmune, IgG recognized the soluble and membrane-bound forms of both 190 kDa and 315 kDa HARE isoforms. Cell lysates separated by SDS–PAGE and probed with immune IgG by Western analysis revealed no cross-reactivity with other proteins (not shown).

Endocytosis of ^{125}I-SA-B-GAG complexes

Stably transfected cell lines were plated in 12-well dishes and grown in DMEM with 8% FBS containing 100 μg/mL Hygromycin B for at least 2 days prior to experiments. Cells were washed with HBSS and incubated at 37°C for 60 min with the fresh endocytosis medium to clear any serum-derived GAGs bound to HARE. During this time, the ^{125}I-SA-B-GAG complexes were formed by incubating 1000 nM B-GAG and 480 nM (25 μg/mL) ^{125}I-SA in the endocytosis medium for 60 min at 22°C. Free biotin (1 μM) was then added to cap any unoccupied biotin-binding sites in ^{125}I-SA-B-GAG, and the preformed complexes were then diluted 10-fold for most experiments. Endocytosis assays were performed at 37°C in the endocytosis medium containing ^{125}I-SA-B-GAG, with or without an unlabeled GAG as a competitor. Nonspecific binding or endocytosis was assessed in two ways, in the presence of an excess of the same unlabeled GAG and with ^{125}I-SA-biotin alone (no B-GAG) (Harris et al. 2007). At the indicated times, cells were washed three times with ice-cold HBSS, lysed in 0.3 N NaOH, and radioactivity (using a gamma counter) and protein content (by the Bradford method) were determined. For the cross-competition experiments in Figure 2, we prepared ^{125}I-SA-B-HA and ^{125}I-SA-B-Hep ligand complexes individually, by mixing ^{125}I-SA with B-HA or B-Hep for 1 h, and then adding excess free biotin before mixing the two complexes together. This prevented SA, which has four biotin-binding sites, from forming dual complexes with both B-GAGs. The amount of ^{125}I-SA was the same in all wells to equalize background CPM levels. Values for endocytosis are presented as the specific CPM/μg protein.

Western analyses

190-HARE or 190-HARE(ΔLink) cells were scraped from the flasks, washed three times by centrifugation with PBS, and lysed in PBS containing 0.5% Triton X-100 and protease inhibitors. Insoluble cellular debris was discarded after centrifugation at 1200 × *g* and protein content in the clarified lysates was quantified by the Bradford method (Bradford 1976). Cell lysate proteins were separated by 5% SDS–PAGE, electro-blotted to nitrocellulose (Burnette 1981), and probed with Ab to V5, an epitope common to all recombinant HARE proteins. Chemiluminescence was used to visualize bands, which were captured on Classic Blue BX film and quantified by densitometry using a FluoChem 8000 (Alpha Innotech).

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Abbreviations

190-HARE	the 190 kDa HA receptor for endocytosis
315-HARE	the 315 kDa HA receptor for endocytosis.
AcLDL	acetylated low-density lipoprotein
BSA	bovine serum albumin
CS	chondroitin sulfate
DS	dermatan sulfate
ECM	extracellular matrix
EV	empty vector
GAG	glycosaminoglycan
HA	hyaluronic acid, hyaluronate, hyaluronan
HARE	HA Receptor for Endocytosis
HBSS	Hanks' balanced salts solution
Hep	heparin
HS	heparan sulfate
LDL	low-density lipoprotein
PBS	phosphate-buffered saline
s190-HARE	soluble ecto-domain of the 190 kDa HARE
s315-HARE	soluble ecto-domain of the 315 kDa HARE
SA	streptavidin
TBST	Tris-buffered saline containing Tween-20

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