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Expression, Processing, and Glycosaminoglycan Binding Activity of the Recombinant Human 315-kDa Hyaluronic Acid Receptor for Endocytosis (HARE)

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Abstract

The hyaluronic acid (HA) receptor for endocytosis (HARE; also designated stabilin-2 and FEEL-2) mediates systemic clearance of glycosaminoglycans from the circulatory and lymphatic systems via coated pit-mediated uptake. HARE is primarily found as two isoforms (315- and 190-kDa) in sinusoidal endothelial cells of the liver, lymph node, and spleen. Here we characterize the ligand specificity and function of the large stably expressed 315-HARE isoform in Flp-In 293 cell lines. Like human spleen sinusoidal endothelial cells, Flp-In 293 cell lines transfected with a single cDNA encoding the full-length 315-HARE express both the 315-kDa and the proteolytically truncated 190-kDa isoforms in a ratio of ~3–4:1. The 190-kDa HARE isoform generated from the 315-kDa HARE and the 315-kDa HARE specifically bound ^{125}I-HA. Like the 190-kDa HARE expressed alone (Harris, E. N., Weigel, J. A., and Weigel, P. H. (2004) *J. Biol. Chem.* 279, 36201–36209), the 190- and 315-kDa HARE isoforms expressed in 315-HARE cell lines were recognized by anti-HARE monoclonal antibodies 30, 154, and 159. All 315-HARE cell lines could endocytose and degrade ^{125}I-HA. Competition studies with live cells indicate that 190-HARE and 315-HARE bind HA with higher apparent affinity (K_d ~10–20 nm) than chondroitin sulfate (CS) types A, C, D, or E. Only slight competition of HA endocytosis was observed with CS-B (dermatan sulfate) and chondroitin. Direct binding assays with the 315-HARE ectodomain revealed high affinity HA binding, and lower binding affinities for CS-C, CS-D, and CS-E. A majority of each HARE isoform was intracellular, within the endocytic system, suggesting transient surface residency typical of an active endocytic recycling receptor.

Abbreviations: **GAG**, glycosaminoglycan; **BSA**, bovine serum albumin; **CS**, chondroitin sulfate; **CS-A**, chondroitin 4-sulfate; **CS-C**, chondroitin 6-sulfate; **CS-D**, chondroitin 2,6-sulfate; **CS-E**, chondroitin 4,6-sulfate; **ECM**, extracellular matrix; **HA**, hyaluronic acid, hyaluronate, hyaluronan; **HARE**, HA receptor for endocytosis; **hHARE**, human HARE; **HBSS**, Hanks' balanced salts solution; **HS**, heparan sulfate; **mAb**, monoclonal antibody; **PBS**, phosphate-buffered saline; **s190-HARE**, soluble 190-kDa HARE ectodomain; **s315-HARE**, soluble ectodomain of the 315-kDa HARE; **190-HARE**, the 190-kDa HA receptor for endocytosis; **315-HARE**, the 315-kDa HA receptor for endocytosis; **s190- and s315-HARE**, soluble 190- and 315-HARE, respectively; **ELISA**, enzyme-linked immunosorbent assay; **DMEM**, Dulbecco's modified Eagle's medium; **FBS**, fetal bovine serum

The glycosaminoglycan (GAG) hyaluronic acid (HA) is a protein-free polymer of disaccharide units containing glucuronic acid and *N*-acetylglucosamine (1, 2). HA is involved in many physiological processes (3), such as wound healing, development, and metastasis of some cancers (4–8). The typical molecular mass of the polysaccharide ranges from just a few thousand Da (tens of sugars) that are thought to be important in cellular signaling (6) to several million Da (tens of thousands of sugars). These larger forms of HA are present throughout the body and are particularly concentrated within the bursa of major joints, such as the knee, where they help to provide shock absorbance in cartilage or lubrication in synovial fluid (9, 10), and the eye, where HA maintains structural integrity of the vitreous humor (11). The adult human body contains ~15g of HA, of which about 5 g are turned over daily (12). Partially degraded HA perfuses from extracellular matrices (ECMs) and enters the lymphatic and vascular circulation systems, where it is catabolized to shorter fragments. This active maintenance of HA turnover must be efficient in order to maintain homeostatic conditions for total body HA.

All of the other GAGs, including the chondroitin sulfates (CSs), heparan sulfate (HS), and keratan sulfate, are linked to core proteins (as proteoglycans) that help to form ECMs, such as the basement membranes of tissues, or structural components of organs, such as the vitreous humor. There are over 30 known core proteins that are essential for a diverse array of functions, such as neural development, growth factor signaling, and pathogen recognition (13). These core proteins are found as prevalent components of tissue ECMs or as specialized components needed for the development of microenvironments that interface a specialized tissue cell type with the ECM. Both the proteoglycans and their attached GAG chains may combinatorially interact with ligands and contribute to modulation of the functional aspects of a particular microenvironment (*e.g.* CS interacting with apolipoprotein E for uptake of β-very low density lipoprotein in hippocampal neurons) (14). Although numerous studies have focused on how the inhibition of some CS proteoglycans enhances neural development, especially in injured spinal cord models, there is very little information on how CS and HS are catabolized. The current model is that extracellular chondroitinases, heparinases, and proteases ini-

tially break down these GAGs and proteoglycans, and their final digestion can then take place intracellularly at the local tissue level. However, in many cases, some of these GAGs and proteoglycan fragments will probably find their way into the lymphatic and circulatory systems, especially during injury or disease. Without some type of efficient GAG-clearing mechanism, these fluid circulatory systems may get overwhelmed with large amounts of debris derived from tissue ECMs throughout the body.

Although the synthesis and catabolism of HA are generally the most understood pathways of any of the GAGs, the details of each pathway currently remain largely unknown. In the early 1980s, it was observed that liver sinusoidal endothelial cells endocytose circulating HA (15–19). We know now that HA·HARE complexes are endocytosed via the clathrin-coated pit pathway (20, 21), and their formation is cation-independent (22), specific, and of high affinity (23, 24). Like the mannose, low density lipoprotein, and asialoglycoprotein receptors, HARE is a recycling receptor that moves through an intracellular itinerary every ~10–15 min (25). This receptor system was shown to be responsible for the physiological turnover of about one-third of the total body HA per day (12, 26). Most of the partially degraded HA perfusing from a tissue ECM encounters this HA receptor first in lymph nodes, which catabolize about 85% of the HA turning over daily. The remaining 15% of the HA drains from the lymphatics into the circulatory system and is removed by the same receptors present in liver sinusoidal endothelium. This rapidly recycling HA receptor is also present in high copy number in spleen, where it presumably mediates additional, but unknown, HA/GAG activities other than systemic clearance.

The primary scavenger receptor for systemic HA turnover is HARE. Orthologues of this receptor are present in other mammals, including cow, pig, guinea pig, rat, mouse, and human (27). Human and rat HARE are primarily found in the sinusoidal endothelial cells of the lymph nodes, liver, and spleen (28–31). Additionally, mouse HARE/Stab-2 has been detected in specialized tissues, such as the corneal and lens epithelium, mesenchymal cells of heart valves, and prismatic epithelial cells covering the renal papillae (27), and bovine HARE has been reported in the oviduct (32).

The HARE proteins were first purified from rat liver (33) and human spleen (30) and then molecularly cloned from rat and human RNA pools (29–31). The hHARE is encoded by a single gene found on chromosome 12q23.3 spanning 180.2 kb and containing 69 exons. The gene encodes a 2551-amino acid glycoprotein with a molecular mass of ~315 kDa in SDS-PAGE. A primary function of this receptor is to bind and internalize HA for turnover, although other GAGs, such as the CSs (20, 34–37) (this report) are also internalized by this receptor, and advanced glycation end products appear to be ligands as well (38, 39). The internalized receptor traffics through the early endocytic pathway and is recycled to the cell surface (21, 29, 37, 39). A 20-min recycling time was observed for the rat 175-kDa HARE expressed in SK-Hep1 cell lines, whereas a 7–9-min cycle rate was observed with the 190-kDa hHARE expressed in Flp-In 293 cells. These inconsistent receptor recycling times may be dependent on intrinsic cell machinery differences.

Although our group has studied the biological activity of rat and human HARE, we have focused primarily on the smaller 190-kDa HARE isoform. Other groups have focused on the full-length HARE in human, mouse, pig, and cattle and overlooked the smaller isoform as a breakdown product or an experimental anomaly. Thus, confusion has arisen about this particular receptor, including the existence of the smaller form (175 kDa in rat, 190 kDa in human), where it comes from, whether it is functional, and, if so, what the biological implications may be (40, 41). For example, the ratio of rat 175-HARE to 300-HARE in the liver is higher than in the spleen. Thus, the levels of each isoform appear highly regulated (30, 33), but it is still not known why or how this occurs. We recently confirmed that the 190-kDa hHARE is, in fact, a functional endocytic recycling receptor that can mediate GAG uptake in the absence of the larger 315-kDa hHARE (37).

In this report, we stably expressed the recombinant full-length 315-kDa human HARE, using cDNA derived from human lymph node, and show that a small fraction of the total parent receptor is proteolytically cleaved to produce a functional 190-kDa receptor. Thus, the 315-HARE expressed in stable Flp-In 293 cell lines produces two receptors that bind to and internalize HA. The secreted ectodomain of the 315-kDa receptor was glycosylated; was reactive with anti-HARE monoclonal antibodies (mAbs); bound HA, CS-C, CS-D, and CS-E well; and bound more weakly to CS-B and chondroitin. When expressed in Flp-In 293 cells, most of the receptors are found intracellularly, suggesting that as the receptor cycles between plasma membrane and endocytic vesicles, the time spent on the surface is quite short.

Experimental Procedures

Materials, Solutions, and Buffers—Tris-HCl, glycine, and acrylamide were obtained from Research Products International (Mt. Prospect, IL); $MgCl_2$, SDS, and methanol were from EMD (Gibbstown, NJ); Triton X-100, *o*-nitrophenyl-β-d-galactoside, digitonin, Tween 20, saponin, cetylpyridinium chloride, secondary antibodies conjugated to alkaline phosphatase, heparin, ampicillin, and imidazole were obtained from Sigma. Formaldehyde without methanol for cell fixation was from Polysciences, Inc. (Warrington, PA). All GAGs except HA and heparin were from Seikagaku Corp. (Tokyo, Japan) and characterized by size exclusion chromatography/multiangle laser light scattering. HA, prepared from bacterial fermentation, was obtained from Genzyme Corp. (Cambridge, MA). Flp-In 293 cells, serum, DMEM, hygromycin B, zeocin, glutamine, plasmid expression vectors, supercompetent TOP10 *Escherichia coli*, and Lipofectamine 2000 were from Invitrogen. Anhydrous Me_2SO and $CuCl_2$ were obtained from Acros Chemical (Morris Plains, NJ). ^{125}I (100 mCi/ml; specific activity of >0.6 TBq/mg) in NaOH and Sepharose 6 Fast Flow (nickel-nitrilotriacetic acid) resin were from Amersham Biosciences. ^{125}I-HA and ^{125}I-streptavidin were prepared as described previously (42, 43). Streptavidin, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl, biotin-LC-hydrazide, and sulfo-NHS-SS-biotin were purchased from Pierce. *p*-Nitrophenyl phosphate reagent was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD), and ELISA strips were from Nunc (Roskilde, Denmark). Affinity-purified polyclonal goat anti-V5 antibody and goat anti-V5 antibody resin were obtained from Bethyl Laboratories (Montgomery, TX). Affinity-purified anti-V5

mAb was from Serotec (Oxford, UK). Anti-actin mAb (I-19) and Protein A/G Plusagarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). *Taq* polymerase for DNA end modifications and screening was from Continental Laboratory Products (San Diego, CA), and *pfu Ultra* for cloning was from Stratagene (La Jolla, CA). We purified DNA from agarose or aqueous solutions using Qbiogene Bio 101 DNA purification kits (Carlsbad, CA). We used either colorimetric or chemiluminescence detection of blotted protein for Western blot analysis. Colorimetric reagents (*p*-nitro blue tetrazolium and sodium 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine) and the chemiluminescence reagents (Luminol and peroxide solutions) were from Pierce. Concentrator/desalting cartridges were from Amicon (Bedford, MA). TBST contains 20 mm Tris-HCl, pH 7.0, 150 mm NaCl, and 0.1% Tween 20. TBST/BSA is TBST with 1.0% (w/v) BSA. Coating Buffer for ELISA assays contains 15 mm Na_2CO_3, 36 mm $NaHCO_3$, pH 9.5. HBSS 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 7.0 with HCl. Z-buffer for the β-galactosidase activity assay contains 60 mm Na_2HPO_4, 40 mm NaH_2PO_4, 10 mm KCl, 1.0 mm $MgSO_4$, 50 mm 2-mercaptoethanol, pH 7.0.

Construction of the 315-HARE Expression Vector—Due to the large size of the cDNA, the 315-kDa hHARE coding region was amplified from lymph node cDNA pools (Marathon System; BD Biosciences) using *pfu Ultra* in two sections that overlapped by 484 nucleotides. The 5′-half of the cDNA was amplified using gene-specific forward (5′-GGATCCATGATGCTACAACATTTAGTAATTTTTTGTCTTGG-3′) and reverse (5′-GGTCATTATGGAGAAAGAAGCTCAGGAAATAGGAG-3′) primers, whereas the 3′-half of the cDNA was amplified using gene-specific forward (5′-TCCTTACCAAACCTGCTCATGCGGCTGGAACAG-3′) and reverse (5′-GGATCCCAGTGTCCTCAAGGGGTCATTGCC-3′) primers. Both cDNA sections were purified, mixed, denatured, and allowed to hybridize together, and the complementary strands were filled in using *pfu Ultra*. Using the forward primer for the 5′-half and the reverse primer for the 3′-half, we then amplified the entire 315-HARE encoding cDNA (35 cycles, T_m = 55 °C, extension time = 8 min). Immediately following the amplification, 2.5 units of *Taq* polymerase and 2 mm $MgCl_2$ were added to the reaction mixture, which was incubated for 10 min at 72 °C to add free deoxyadenylate on the 3′-ends of the PCR product for compatible cloning in the TOPO line of expression vectors. The PCR product representing the full-length 315-HARE cDNA was purified by agarose gel electrophoresis using a 0.8% (w/v) gel containing 0.002% crystal violet for band visualization. The band was excised with a clean razor blade, gene-cleaned, ligated into pcDNA5/FRT/V5/His_6-TOPO, and transformed into TOP10 *E. coli* cells. Colonies growing on LB-ampicillin plates were screened for cDNA inserts by visualizing total extracted nucleic acid on 1% agarose gels stained with ethidium bromide. Colonies with potential HARE cDNA were verified by PCR using gene-specific forward (5′-GTTCCATCTACGATCGCCACTGGGCCAG-3′) and vector-specific reverse (5′-CGTAGAATCGAGACCGAGGAG-3′) primers. The complete sequences of the promoter and complete coding regions of cDNAs in the final clones were determined to be correct before they were used in subsequent experiments. This full-length HARE DNA sequence obtained was identical to that of NCBI accession entry NM_017564 with the exception of silent mutations located at codons 833, 1112, 1700, 1937, 2005, and 2175 and one missense mutation, which created a D1276A mutation. However, Ala^{1276} was reported in an earlier entry for stabilin-2 (accession number AJ295695) and was also present in the 190 hHARE clones reported by Harris *et al.* (37). The D1276A change does not appear to affect the biological activity of the receptor and probably represents a polymorphism in the human *STAB2* gene. All protein expression experiments were performed using this NM_017564-like cDNA.

Selection and Verification of 315-hHARE Cell Lines—Cell lines were produced and characterized as described by Harris *et al.* (37) with minor modifications. Flp-In 293 cells (3×10^6; Invitrogen) were plated in a 100-mm dish 2 days prior to transfection. Cells incubating in 10 ml of fresh antibiotic-free DMEM supplemented with 8% fetal bovine serum were transfected by the addition of 750 μl of serum-free DMEM containing 1.0 μg of pcDNA5/FRT/315-HARE V5/His_6, 9.0 μg of pOG44 (encoding the Flp-In recombinase) and 20 μl of Lipofectamine 2000. Starting 24–36 h post-transfection, the medium was replaced every 2–3 days with DMEM plus 8% FBS containing 100 μg/ml hygromycin B. Visible circular colonies were present about 2 weeks post-transfection and isolated using a P20 pipette with P200 tips. About 40 colonies were isolated by gently nudging with the tip while aspirating no more than 10 μl at a time. Medium on the plate was changed after picking every five colonies to reduce the chance of aspirating free-floating cells. Isolated colonies were grown in 24-well dishes in DMEM plus 8% FBS with 100 μg/ml hygromycin B. After a monolayer of cells had developed from each clone, cells were scraped in 1 ml of medium and partitioned into four portions. One portion (100 μl) was reseeded in fresh medium and allowed to continue growing in the 24-well dish. The second portion (100 μl) was reseeded in 100 μg/ml zeocin to test for zeocin sensitivity. The third portion (400 μl) was pelleted and resuspended in 50 μl of 4× Laemmli sample buffer (44) to test for HARE expression by SDS-PAGE followed by Western analysis using anti-V5 antibody.

The remaining 400 μl of cell suspension was pelleted and assayed for β-galactosidase activity. In addition to the zeocin sensitivity test, the β-galactosidase test indicates whether the HARE-encoding plasmid integrated specifically and correctly into the genomic FRT site, which is the unique feature of the Flp-In cell line series. The cell pellet for this assay was resuspended in 200 μl of PBS containing 0.5% Triton X-100 and 20 μl of cell lysate per well of a 96-well plate was combined with 70 μl of Z-buffer and 20 μl of 4.0 mg/ml *o*-nitrophenyl-β-d-galactoside in Z-buffer. After 15–30 min at 37 °C, the absorbance values were measured at 405–420 nm. Lysates from HEK Flp-In 293 and HEK 293 cells were used as positive and negative controls, respectively. Stable cell lines were selected for further study only if they grew well in DMEM containing 8% FBS and 100 μg/ml hygromycin B and showed HARE expression, hygromycin B resistance, zeocin sensitivity, no β-galactosidase activity, and normal cell morphology as determined by phase-contrast microscopy.

Endocytosis and Degradation of ^{125}I-HA—The C-terminal epitope tags do not interfere with HA binding, internalization, or trafficking mediated by the 190-kDa HARE (37). Stably transfected cell lines expressing the 315-HARE were plated in 12-well dishes and allowed to grow in DMEM with 8% fetal calf serum supplemented with 100 μg/ml hygromycin B at least 2 days prior to an experiment. Cells were washed with HBSS and incubated for 60 min with fresh medium containing 0.075% BSA to allow HARE-mediated internalization of any serum GAGs that might be bound to the cell surface. The same DMEM-BSA medium containing 1–2 μg/ml ^{125}I-HA was then incubated with cells with or without the noted GAG as a competitor. Endocytosis or binding only of ^{125}I-HA was measured by incubating cells at 37 or 4 °C, respectively. To determine HA binding by the total cell receptor population, cells on ice were permeabilized with 0.055% (w/v) digitonin (using a 25% stock solution) dissolved in anhydrous Me_2SO (45). At the indicated times, cells were washed three times with ice-cold HBSS and lysed in 0.3 n NaOH, and radioactivity and protein content (46) were determined.

Degradation of ^{125}I-HA was measured by a cetylpyridinium chloride precipitation assay, as reported previously (47). Briefly, 50 μl of sample medium was mixed with 0.25 ml of a 1.0 mg/ml solution of HA (as a carrier) in 1.5-ml microcentrifuge tubes. Alternatively, 100-μl samples of cell lysates in 0.3 n NaOH were mixed with 47 μl of 0.6 n HCl, 28 μl of distilled water, and 125 μl of 2.0 mg/ml carrier HA solution. After mixing at room temperature, 300 μl of 6% (w/v) cetylpyridinium chloride in distilled water was added to each tube and vortexed. The samples were allowed to precipitate for 10 min, followed by centrifugation at 8600 × *g* for 5 min in an Eppendorf 5417 using a swinging bucket rotor. A 300-μl sample of the supernatant was taken for radioactivity determination, and the remaining supernatant was removed by aspiration. The bottoms of the tubes containing the pellet were cut off and placed in 12 × 75-mm plastic tubes for radioactivity determination using a Packard Cobra II γ counter. Degradation was calculated as the time-dependent increase of nonprecipitable radioactivity. At least 80% of the total radioactivity was precipitable at the beginning of each experiment.

Western and Ligand Blot Assays—Cell lysates or immunoprecipitates were mixed with 4× Laemmli sample buffer (44) without reducing agent and analyzed by SDS-PAGE using a 5% gel. Protein electrotransfers to nitrocellulose were performed using a Bandit Electroblotter (Owl Scientific; Rochester, NY) for 1.5 h at 4 °C at 110 constant V in 25 mm Tris, 192 mm glycine, pH 8.3, and 15% methanol, pH 8.3. Subsequent steps were at room temperature. For Western analysis (48), the nitrocellulose membranes were treated with TBST/BSA for at least 2 h to block nonspecific binding sites followed by incubation in TBST/BSA with 1 μg/ml anti-rat HARE mAb or 1:5000 anti-V5 antibody. After washing three times with TBST, the blots were incubated with the appropriate secondary antibody conjugated with alkaline phosphatase or horseradish peroxidase, followed by three washes, and then detection with the appropriate reagents. When necessary, the blots were stripped of bound antibodies by incubating the membranes in 100 mm 2-mercaptoethanol, 2% SDS, 62.5 mm Tris-HCl, pH 6.7, at 55 °C for 30 min. After several washes in TBST, the blots were reprobed with anti-V5 mAb. Densitometry was performed using an Fluor-Chem 8000 gel visualization and analysis system (Alpha Innotech Corp., San Leandro, CA).

For ^{125}I-HA ligand blots (49), the nitrocellulose membrane was incubated with TBST for at least 2 h at 4°C followed by an incubation with 1 μg/ml ^{125}I-HA in PBS containing 150 mm NaCl, 10 mm HEPES, pH 7.4, 5 mm EDTA, with or without 100 μg/ml unlabeled HA as competitor to assess nonspecific binding. Following a 2-h binding incubation at 4 °C, the membrane was washed with excess TBST at least five times over 30 min and dried at room temperature. Bound ^{125}I-HA was detected by autoradiography using Eastman Kodak Co. MS film exposed at –80 °C for 24–48 h.

The method for biotin-GAG ligand blots was a modification of the above HA ligand blot procedure in which the membrane with blotted proteins was incubated with a specific amount of biotin-GAG, washed, and detected with ^{125}I-streptavidin. Biotin-GAGs were made according to the protocol of Yu and Toole (50), and a biotin quantification kit (Vector Laboratories, Burlingame, CA) was used to determine the number of biotins/GAG chain. The molar mass and concentration of each GAG were determined by size exclusion multiangle laser light scattering before and after the biotinylation reactions to ensure the addition of 1–2 biotins per GAG chain, without GAG degradation, and to calculate the final yield of each biotin-GAG.

Endoglycosidase F Treatment and Immunoprecipitation—HARE was immunoprecipitated from cell lysates or media containing 0.5% Nonidet P-40 and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, 30 μg/ml phenylmethylsulfonyl fluoride) using anti-rat HARE mAbs 30, 154, and 159 attached to CNBr-activated Sepharose (0.3 mg/ml each mAb) and allowed to rotate overnight at 4 °C. If the sample was to go straight to Western analysis, the resin was washed twice with PBS and eluted with an equal volume of 4× Laemmli buffer and centrifuged briefly to pellet the resin. If the sample was to be treated with endoglycosidase F, the centrifuged resin was washed twice with PBS and incubated with an equal volume of PBS containing 1% SDS to disrupt antibody-HARE binding. The supernatant recovered after centrifugation was adjusted to 1% Nonidet P-40 and incubated with 1.0 unit of *N*-glycosidase F (Roche Applied Science) overnight at 37 °C. The sample was then mixed with 0.25 volumes of 4× Laemmli buffer, separated by 5% SDS-PAGE, and analyzed by Western blot assays.

Fluorescence Microscopy—Cells were grown on 18-mm glass coverslips in complete medium for at least 2 days prior to fixation. Cells were washed twice with PBS and fixed in PBS containing 1% formaldehyde for 1 h at room temperature, followed by two rinses in PBS. Nonspecific antibody interactions were blocked with PBS containing 0.25% BSA for 30 min. Alternatively, cells were permeabilized (45) with 0.05% saponin for 30 min at room temperature after the blocking procedure. Cells were incubated with both primary and secondary antibodies in PBS containing 0.25% BSA for 1 h and washed between incubations. Glass coverslips were mounted onto glass slides immediately 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.

Secreted Receptor Constructs—cDNA constructs for the secreted ectodomains of both the 315- and 190-HARE were produced by deleting the transmembrane and cytoplasmic domains, while retaining the V5 and His_6 tags. A single primer, 5′-GTGACCTTGACCCACACTGGATCCGAAGGTAAGCCTATC-3′, was used in individual mutagenic reactions with the wild type HARE cDNAs (315-HARE in pcDNA5/FRT/V5/His, 190-HARE in pSecTag/FRT/V5/His) in an Ericomp thermocycler for 18 cycles (94 °C for 30 s, 63 °C for 30 s, 71 °C for 1 min/plasmid kb) using *pfu Ultra*. After the reaction, the plasmid was ethanol-precipitated and resuspended in 17 μl of H_2O, 2 μl of NEB4 buffer, and 2.5 units of DpnI to cut the template plasmid while retaining the intact mutant plasmid. After an overnight incubation at 37 °C, the digest mix was heated to 95 °C for 10 min, cooled, and immediately transformed into TOP10 supercompetent *E. coli* cells. Bacterial cells containing the plasmid of choice were screened by a miniprep procedure, and PCR and DNA sequencing confirmed the mutation. Plasmids containing the desired mutation were used to make stable cell lines, which were created and evaluated as described above.

Soluble HARE Purification—s190-HARE or s315-HARE was purified from pooled cell growth media (DMEM containing 8% FBS and 100 μg/ml hygromycin B) via the His_6 tag. To remove particulate matter, conditioned medium (incubated with cells for at least 2 days) was centrifuged at 1500 rpm for 4 min. Clarified medium was supplemented with the following to the indicated final concentrations: 10% glycerol, 250 mm NaCl, 10 mm imidazole. A 1% volume of 50% slurry of Ni^{2+}-charged Sepharose 6 Fast Flow resin (Amersham Biosciences) was added, and the mixture was incubated overnight at room temperature with gentle rotation. The resin was captured in a 25-ml column, washed with 10 column volumes of PBS containing 10 mm imidazole, and then eluted with 4 column volumes of PBS containing 400 mm imidazole. The partially purified HARE was concentrated using a 30-kDa molecular mass cut-off concentrator and separated by SDS-PAGE on a 5% gel. The gel was stained with a 4% $CuCl_2$ solution to visualize protein bands, and the HARE band was cut out of the gel with a clean razor blade, minced, and electroeluted overnight in dialysis tubing (35 V, 4 °C) in 185 mm glycine, 25 mm Tris-HCl, pH 8.5, 0.07% SDS. The final purified HARE preparation was concentrated to 0.1–1.0 mg/ml as above, washed twice with PBS, and stored at –20 °C until use in subsequent assays.

ELISAs—All steps were carried out at 37 °C. A set amount of HARE protein in Coating Buffer (200 μl) was incubated in each well of a Polysorb strip for 2 h. The surface of the well was then blocked with TBST supplemented with 2% BSA and 0.1% Tween 20 for 1.5 h. After washing, increasing concentrations of biotin-HA were incubated for 2 h, washed six times with TBST (without protein), incubated for 1 h with streptavidin-alkaline phosphatase, washed six times in TBST, and finally incubated with *p*-nitrophenyl phosphate. The A_{405} of the strips were determined each hour for 3 h.

Cell Surface Biotinylation and Immunoprecipitation—Four different 315-HARE clones (17.5, 29, 30, and 36) were grown to confluence in T-25 tissue culture flasks. After a 60-min incubation in serum-free medium, the cells were washed twice with cold PBS and incubated in the presence or absence of 0.05% digitonin (45) in PBS for 10 min at 4 °C. The cells were then washed twice with cold PBS and treated with 2 mm sulfo-NHS-SS-biotin in PBS for 30 min at 4 °C. The biotinylation reaction was terminated by washing and then incubating the cells with 100 mm glycine, pH 8.0, in PBS for 20 min at 4 °C to quench unreacted sulfo-NHS-SS-biotin. Washed cells were then lysed in 0.5% Nonidet P-40 PBS containing 200 μm phenylmethylsulfonyl fluoride, 1.0 μg/ml pepstatin, and 1.0 μg/ml leupeptin for 1 h at 4 °C, and cell lysates were cleared by centrifugation at 14,000 × *g* for 10 min at 4 °C. The resulting supernatant was used for immunoprecipitation experiments. Anti-rat HARE mAb-159 (2 μg/ml) was added to 1.0 ml of supernatant, which was incubated with rotation for 2 h at 4°C, and immune complexes were then collected with 20 μl of 250 μg/ml Protein A/G-Plus-agarose overnight at 4 °C. The resin was washed three times with cold PBS, and the pellet was resuspended in 30 μl of 2× Laemmli sample buffer without reducing agent, incubated for 3 min at 90 °C, and subjected to SDS-PAGE and Western analysis.

Results

Two Isoforms of HARE Are Expressed from a Single 315-HARE cDNA—Results from previous studies showed that HARE is a cell surface receptor that clears HA, CS, and advanced glycation end products from the circulatory and lymphatic systems. Studies of HARE or stabilin/FEEL-2 in other laboratories focused only on the large isoform, disregarding the possibility of a smaller isoform (*i.e.* the 190-kDa HARE). However, we have shown that the smaller isoform of the recombinant rat (29) or human HARE (37) is a recycling endocytic receptor specific for HA and multiple CS types that operates via the coated pit endocytosis pathway. To study the specificity and function of the large human HARE, we amplified and cloned the open reading frame from pooled lymph node cDNA and inserted the full-size 315-HARE cDNA into the expression vector pcDNA5/FRT/V5-His-TOPO. Except for the absence of the secretion signal, this is the same vector that we used previously to express and characterize the independent function of the 190-HARE.

The full-length 315-kDa HARE type I membrane protein contains an extracellular domain with four cysteine-rich fasciclin/epidermal growth factor-like domains of unknown function, an extracellular LINK domain, a cytoplasmic domain, and a single membrane domain (Figure 1*A*). The 190-HARE is identical to the C-terminal 1417 amino acids of the full-length HARE (Figure 1*A*), which we previously suggested arises by proteolytic cleavage (29, 37).

The six cell lines used in this study each contain a single 315-HARE cDNA integrated at the single correct recombinase-specified site in Flp-In 293 cells, as confirmed by their resistance to hygromycin B, lack of β-galactosidase activity, and sensitivity to zeocin. The amount of 315-HARE expression in all six cell lines was only ~12% of that observed for recombinant 190-HARE expressed alone in the same cells using the same promoter and growth conditions (37). Nonetheless, all six cell lines mediated specific HA endocytosis over 4 h that was well above the background level of the parental Flp-In 293 cells or cells transfected with vector alone (not shown).

Unexpectedly, all cell lines expressing the single cDNA encoding full-length 315-kDa HARE produced two individual

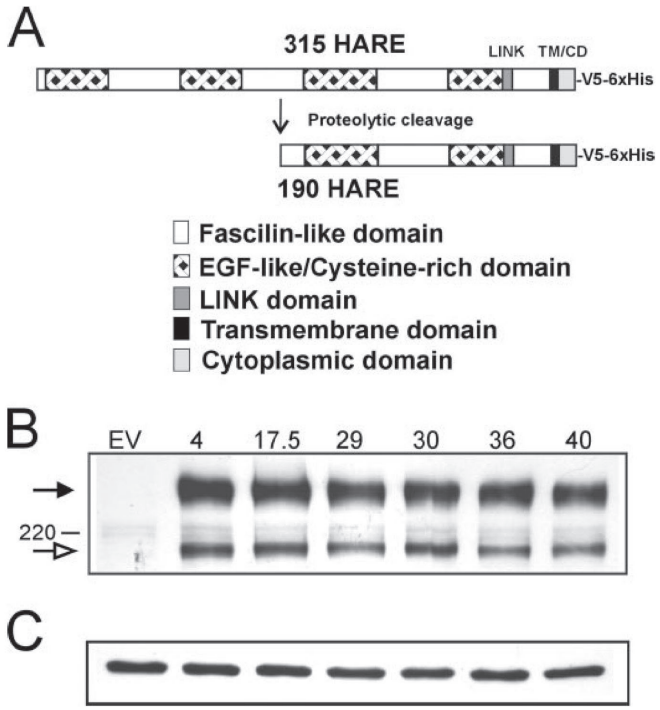

Figure 1. Schematic of the two HARE isoforms and their expression in stable cell lines. *A*, the scheme illustrates the domain organization of the 315- and 190-kDa HARE proteins. The four Cys-rich domains are 37–39% identical (~50% similar) to each other. Each receptor isoform contains one LINK domain, one transmembrane region, and one cytoplasmic domain. The *arrow* indicates the N terminus of the 190-kDa HARE (Ser^{1136}), which may be the site of cleavage to create this isoform. *B*, in order to assess the cellular ratios of 315- to 190-HARE isoforms, cells were scraped from T-25 flasks, washed, and resuspended in PBS with 0.5% Nonidet P-40 and protease inhibitors. The cell lysates were incubated with rotation at 4 °C for 1 h and centrifuged to remove cell debris, and total protein content of each sample was quantified using the Bradford assay (46). Lysate protein (20 μg) was analyzed by SDS-PAGE and Western blots. The 315-HARE (*filled arrow*) and 190-HARE (*open arrow*) were detected by chemiluminescence using polyclonal anti-V5 antibody. *C*, the blot was stripped and reprobed with anti-actin antibody to allow samples to be normalized to cellular protein content.

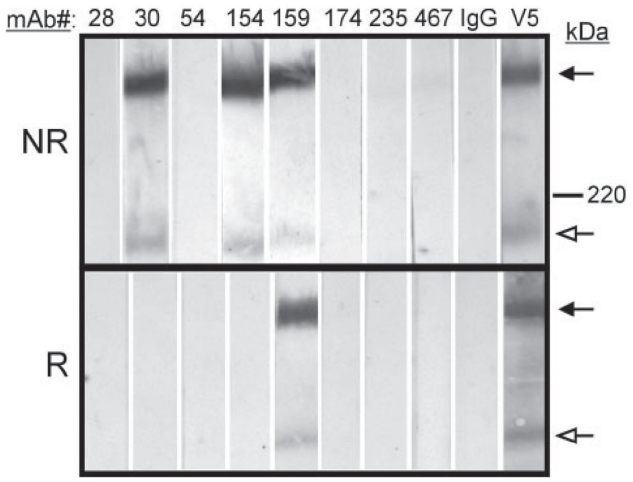

Figure 2. Both recombinant human 315- and 190-kDa HARE isoforms cross-react with rat anti-HARE mAbs. Whole cell lysates were subjected to reducing (*R*) or nonreducing (*NR*) conditions, separated by 5% SDS-PAGE, and electrotransferred to nitrocellulose as described under "Experimental Procedures." Nitrocellulose was cut into strips and blocked with TBST/BSA, and each strip was incubated with 1 μg/ml mouse IgG or one of the eight indicated mAbs raised against the rat 175-kDa HARE (28, 33). The 315-kDa HARE (*filled arrows*) and the 190-kDa HARE (*open arrows*) proteins were recognized by mAbs 30, 154, and 159 in the nonreduced samples, whereas both receptors reacted with only mAb 159 when reduced. The anti-V5 lane (*far right*) was a positive control for mAb detection.

HARE proteins with molecular masses of ~315 and 190 kDa as detected in Western blots using an antibody against the C-terminal V5 epitope (Figure 1*B*). The native spleen and recombinant 190-HAREs migrated identically in 5% SDS-PAGE with the 190-kDa species in these 315-HARE cell lines (not shown). The HARE expression patterns among all the cell lines were virtually identical, and the majority of the recombinant protein migrated at ~315 kDa with a lesser amount migrating at 190 kDa (Figure 1*B*). All cell lines produced approximately the same amount of 315-kDa receptor relative to cellular actin (1.9 ± 0.2 for the six samples in Figure 1, *B* and *C*) and produced similar ratios of the 315-kDa to 190-kDa HARE (2.7 ± 0.4 for the experiment in Figure 1*B*). The 315/190-HARE ratio was typically 3–4:1 in multiple Western analyses using detection by either chemiluminescence or alkaline phosphatase.

Recombinant HARE Appears to Be Folded and Glycosylated Correctly and Specifically Binds HA—Although we do not know why expression of the recombinant full-length HARE was lower than the independently expressed 190-HARE, we suspect that overexpression of such a large protein containing 202 cysteines might overwhelm the endoplasmic reticulum systems that facilitate protein folding and disulfide bond formation. The two expressed receptors appear to be folded correctly based on their reactivity with three anti-rat mAbs that cross-react with human HARE (Figure 2) and the specific HA binding activity of each recombinant protein in ligand blot and cell culture assays. mAbs 30, 154, and 159 detected both the larger and smaller HARE proteins in whole cell lysates of 315-HARE-transfected cells (Figure 2, *NR*). Reduction of the proteins with dithiothreitol resulted in detection by only mAb 159 (Figure 2, *R*), confirming that mAbs 30 and 154 recognize conformational (*e.g.* sulfhydryl-dependent) epitopes, perhaps within the Cys-rich epidermal growth factor-like domains that are conserved between rat and human HARE proteins. Using SDS-PAGE to resolve the two HARE species, we verified that both receptors specifically bind ^{125}I-HA in a ligand blot assay (Figure 3*A*). The above results demonstrate that the 190-kDa protein produced by 315-HARE cell lines is a HARE isoform and not an artifact of the assays performed. In addition, stable cell lines containing the empty vector alone do not show mAb- or HA-binding bands at these M_r positions (not shown).

Reduction by dithiothreitol, followed by alkylation to block refolding via disulfide bond formation, dramatically shifted the migration of the 190-HARE species to apparently larger mass (Figure 3*B*). The decreased migration of the reduced 315-HARE through 5% SDS-PAGE was less apparent, since migratory shifts in a protein of this size are more difficult to demonstrate. Also, the rod-like geometry of the nonreduced and reduced full-length receptor may contribute to their similar migration patterns (51). We conclude from Western blot and silver stain (not shown) analyses of reduced

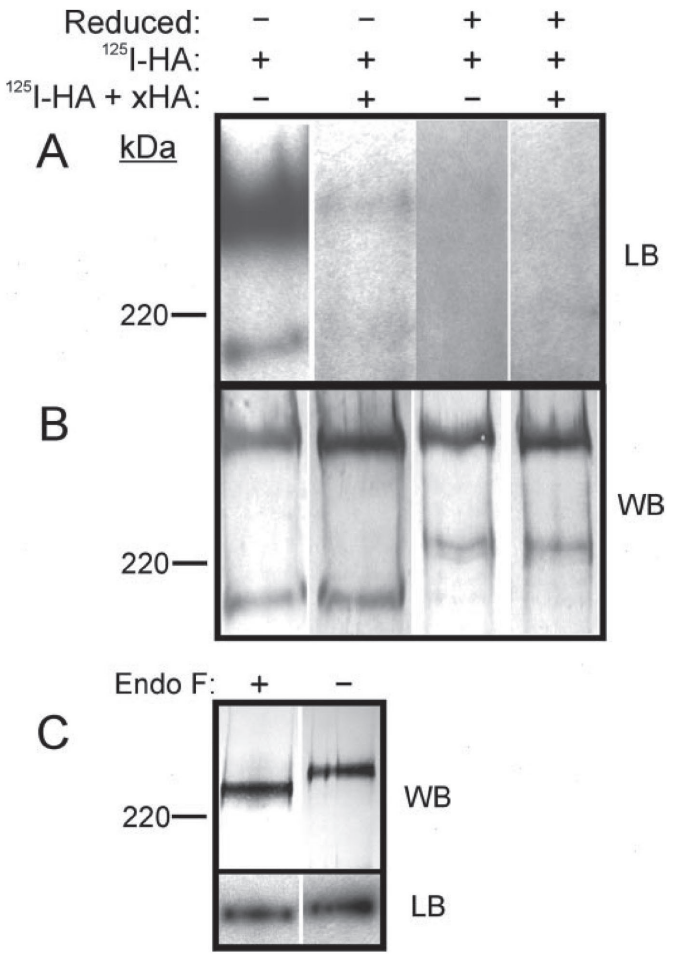

Figure 3. The nonreduced 315- and 190-kDa HARE proteins specifically bind HA. Whole cell lysates from cells stably expressing recombinant 315-kDa HARE were subjected to either reduction and then alkylation or nonreducing conditions, followed by 5% SDS-PAGE and electrotransfer to nitrocellulose membranes. *A*, a ligand blot (*LB*) assay was first performed by incubating the nitrocellulose with 1 μg/ml ^{125}I-HA either alone or mixed with a 100-fold excess of unlabeled HA, washing, and autoradiography as described under "Experimental Procedures." *B*, following the ligand blot, the nitrocellulose membrane was rehydrated in TBST/BSA, and Western blot (*WB*) analysis was performed using anti-V5 antibody to identify hHARE. *C*, the 315-HARE protein was immunoprecipitated from cell lysates with mAb 30, eluted from the resin with 0.1% SDS, and digested overnight with endoglycosidase F. After electrophoresis and electrotransfer to nitrocellulose, the blot was subjected to the ^{125}I-HA ligand blot assay followed by Western blot analysis with anti-V5 antibody.

and nonreduced samples that the recombinant human 315-HARE is a single polypeptide and not composed of multiple subunits linked together by disulfide bonds.

When the full-length hHARE was treated with endoglycosidase F to remove *N*-linked oligosaccharides, a more pronounced migratory shift was observed (Figure 3*C*), which was similar to that observed previously for the recombinant 190-HARE and the native rat and human receptor isoforms. Endoglycosidase F treatment did not affect ^{125}I-HA binding, revealing that *N*-linked glycans are not required for HA binding.

Due to the low amount of recombinant 190-kDa HARE in the 315-HARE stable cell lines, we were not able to obtain enough purified 190-HARE for N-terminal amino acid sequencing. However, we purified the 190-HARE from a human spleen sample and obtained the sequence NH_2-LLPNLLMRL. Except for the first amino acid, this sequence is identical to the presumed sequence for the 190-HARE starting at Ser^{1136} of the full-length protein. The first Leu in our sample may be a sequencing error or a real mutation (polymorphism) in the genome of the individual spleen donor.

Cells Expressing HARE Endocytose and Degrade ^{125}I-HA—Recombinant HARE encoded by the full-length cDNA is biologically active, since cells expressing the protein can endocytose HA. Four different cell lines (17.5, 29, 30, and 36) were allowed to endocytose ^{125}I-HA with and without competing nonlabeled HA over a 26-h period (Figure 4*A*). Unlike the parent Flp-In 293 cells or cells transfected with vector alone, 315-HARE cells specifically internalized HA. HA uptake increased and reached a steady state accumulation after about 24 h. In addition to internalization, the Flp-In 293 cells were able to deliver HA to lysosomes, where it was degraded (Figure 4*B*), presumably by the combined actions of endogenous lysosomal hyaluronidases, β-*N*-acetylglucosaminidase, and β-glucuronidase. Unlike the previously described 190-HARE cell lines, the 315-HARE clones varied more in their HA endocytic rates and degradation.

We and others showed previously that internalization of HARE in primary cell cultures or stable cell lines occurs via a clathrin-coated pit pathway (21, 29, 35, 39). To confirm that the recombinant 315-kDa HARE mediates endocytosis via coated pits, we incubated 315-HARE clone 30 cells with ^{125}I-HA for 4 h in medium containing increasing amounts of sucrose (Figure 4*C*). Under hyperosmotic conditions (*e.g.* ≥0.4 m sucrose), receptor-mediated endocytosis decreased by >90%, indicating that HA uptake depends on clathrin assembly into coated pits (52–54).

The s315- and s190-HARE Ectodomains Bind ^{125}I-HA—The secreted s315-HARE ectodomain was purified from cell culture medium via either the His_6 tail using nickel-chelate affinity chromatography or anti-V5 immunopurification. Partially purified s315-HARE appeared to be folded correctly, since it bound HA in the ligand blot assay (Figure 5*A*), whereas reduction nullified HA binding, and deglycosylation did not affect HA binding, which were the same results seen with the membrane-bound 315-HARE. Expression and glycosylation of the s190 and s315-HARE ectodomains in cell culture were virtually identical. In addition, the recognition of the soluble receptors by mAbs 30 and 154 was the same as the membrane receptors in Western blot or immunoprecipitation assays (not shown). Both HARE ectodomains also bound comparable amounts of ^{125}I-HA in the ligand blot assay (Figure 5*B*).

Since the s315-HARE has HA binding capability comparable with the wild type receptor, we used an ELISA-like assay to estimate the HA binding affinity. Purified s315-HARE or s190-HARE was plated on Polysorb strips, nonspecific binding sites were blocked with BSA, and the strips were incubated with increasing amounts of biotin-HA. Bound biotin-HA was detected with streptavidin-alkaline phosphatase. The binding of biotin-HA to either HARE ectodomain was almost identical (when normalized to pmol of protein). The data were fit as second order binding curves (Figure 6) with a calculated K_d value of 9.9 ± 1.2 nm ($p < 0.0001$) for the s315-HARE and 20.7 ± 6.4 nm ($p = 0.0004$) for the s190-HARE. These K_d values are very close to the dissociation constant

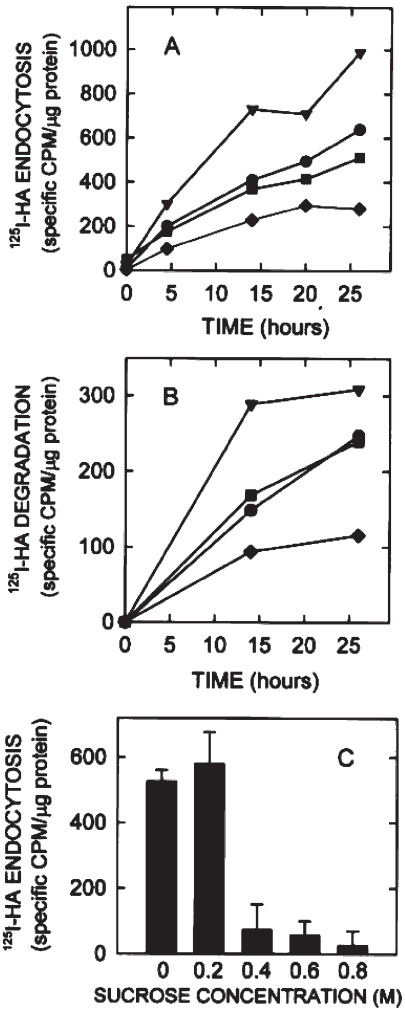

Figure 4. Endocytosis and degradation of ^{125}I-HA by cells expressing HARE. Four 315-HARE cell lines (clone 30 (▼), clone 29 (●), clone 36 (■), and clone 17.5 (♦) were plated in 24-well plates and grown to confluence. *A*, after a 60-min incubation in serum-free medium, the cells were washed with 1 ml of HBSS and allowed to endocytose 1.5 μg/ml ^{125}I-HA in DMEM supplemented with 0.05% BSA, with or without 150 μg/ml unlabeled HA. At the noted times, medium was aspirated, and the well was washed three times with 1 ml of HBSS. The cells were lysed in 0.5 ml of 0.3 n NaOH, and radioactivity and protein were determined. The values shown are the average of duplicates (typically within 10%) for specific cell-associated cpm/μg of protein: total uptake (no excess HA) minus the nonspecific uptake (with excess unlabeled HA). Specific uptake and degradation values, which varied depending on the cell line and time point, were 77–87 and 62–87%, respectively. *B*, cell-associated degradation of ^{125}I-HA was determined at the noted times by a cetylpyridinium chloride precipitation assay, as described under "Experimental Procedures," using a portion of the neutralized cell lysate from the samples in *A*. Degradation values are the average of duplicates. *C*, 315-HARE cells mediate HA internalization by a clathrin-coated pit pathway. 315-HARE clone 30 cells were incubated in medium with 1 μg/ml ^{125}I-HA with (to assess nonspecific uptake) or without (to assess total uptake) a 100-fold excess of unlabeled HA and increasing concentrations of sucrose, as indicated. A stock 1.2 m sucrose solution was made in DMEM-BSA and diluted in DMEM-BSA to give the final concentrations indicated. Prior to the experiment, cells were incubated at 37 °C in DMEM-BSA for 1 h and then DMEM-BSA/sucrose for an additional 15 min. At time 0, the ^{125}I-HA mixes in DMEM-BSA/sucrose were added, and after 4 h the cells were washed, and protein content, radioactivity, and specific HA uptake were determined as described under "Experimental Procedures." Specific ^{125}I-HA endocytosis without sucrose was 70%. Specific cpm/μg of protein values for endocytosis are means ± S.D. (n = 3).

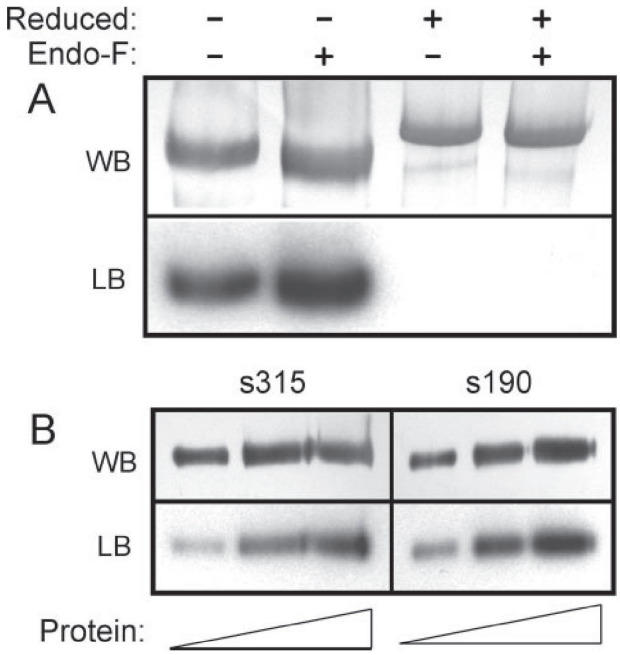

Figure 5. The secreted 315-kDa HARE ectodomain is glycosylated and functional. *A*, the s315-HARE was purified by Ni^{2+} affinity chromatography and reduced with dithiothreitol or deglycosylated with endoglycosidase F treatment as described under "Experimental Procedures." The deglycosylated receptor retains HA binding activity as seen in the ligand blot (*LB*) assay, in contrast to the reduced receptor, which does not bind ^{125}I-HA. Following the ligand blot, the same membrane was subjected to Western blot (*WB*) analysis using anti-V5 antibody. *B*, the s190-HARE and s315-HARE demonstrate comparable ^{125}I-HA-binding activities in the ligand blot assay. A ligand blot assay was performed with increasing amounts of purified s190-HARE or s315-HARE protein after SDS-PAGE and electrotransfer.

value of 7 nm determined for HA-receptor complexes in cells expressing recombinant 190-HARE (37).

HARE Binds to a Subset of GAGs in Addition to HA—We previously reported that other GAGs compete with HA for binding to the 190-HARE. Since it is not known how HARE binds to its multiple ligands or what domains within the protein interact with each ligand, the GAG-binding properties of the larger 315-kDa HARE may be different from those of the smaller HARE. We therefore tested the GAG-binding and internalization properties of cells expressing the 315-HARE to discern whether they were different for this larger HA-binding receptor. This was tested in endocytosis assays in which 190-HARE (clone 14) and 315-HARE (clone 30) stable cell lines were allowed to endocytose ^{125}I-HA in the presence of an excess of different competing GAGs (Figure 7*A*). These results showed that the GAG-binding profiles are very similar between cells independently expressing the 190-HARE and cells expressing the 315-HARE (and smaller amounts of 190-HARE). The ability of all of the CS types to compete for HA uptake indicates that these GAGs bind at or near the HA binding site(s) in either HARE ectodomain.

Thus far, all of our binding experiments have used ^{125}I-HA and competing nonlabeled GAGs in live cell assays to obtain evidence that the other GAGs bind to HARE. Since negative results in these indirect assays do not rule out the presence of independent binding sites for the other GAGs, we developed direct binding assays to determine whether

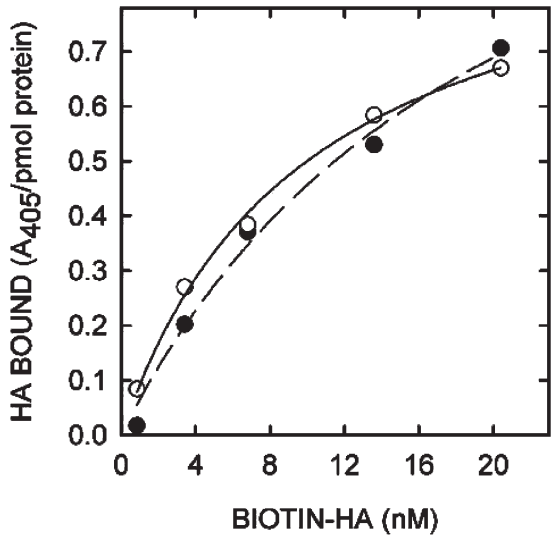

Figure 6. The s315- and s190-HARE ectodomains bind HA with high affinity. The s315-HARE and s190-HARE ectodomains were purified by affinity chromatography and electroelution, plated on Polysorb strips in duplicate (0.5 μg/well), and allowed to bind increasing amounts of biotin-HA as described under "Experimental Procedures." After washing, bound biotin-HA was detected with streptavidin-alkaline phosphatase using *p*-nitrophenyl phosphate as substrate, and A_{405} values were normalized per pmol of protein plated. The lines are regression analyses for s315-HARE (*solid line*, *open circles*) and s190-HARE (*dotted line*, *closed circles*) data calculated using the ligand binding curve-fitting and analysis of variance functions of SigmaPlot version 10.0. The K_d values for biotin-HA binding to s190- and s315-HARE under these experimental conditions were 20.7 ± 6.4 (S.E.) nm (p = 0.0004) and 9.9 ± 1.2 (S.E.) nm (p < 0.0001), respectively.

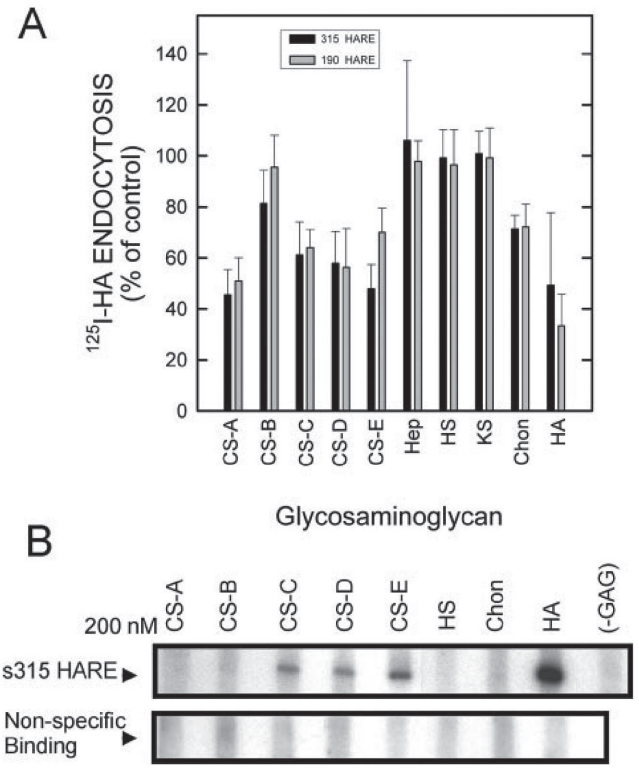

Figure 7. 315- and 190-HARE cell lines display similar patterns for glycosaminoglycan competition of HA endocytosis. *A*, 315-HARE clone 30 and 190-HARE clone 41 cells were plated in 12-well plates and grown to confluence. After a 1-h incubation in serum-free medium, the cells were washed with 2 ml of HBSS and allowed to endocytose 1.5 μg/ml ^{125}I-HA for 4 h at 37 °C in DMEM supplemented with 0.05% BSA with or without 30 μg/ml of the noted GAG. The medium was aspirated, the well was washed three times with 2 ml of HBSS, and the cells were lysed in 1 ml of 0.3 n NaOH. Radioactivity and protein per sample were determined. The data are expressed as a percentage of the radioactivity in cells with no other GAG addition. The mean ± S.D. (n = 6) of duplicates from three separate experiments is shown. *B*, direct binding of a GAG was assessed by a ligand blot assay in which the indicated biotin-GAG was incubated alone or with a 4-fold excess of unlabeled GAG. After washing the membrane strips containing the protein-GAG complex, the biotin-GAG remaining was detected by autoradiography after incubation with 2.5 μg/ml ^{125}I-Streptavidin. CS-C, CS-D, CS-E, and HA bound with HARE with the highest affinity, whereas CS-B and chondroitin (*Chon*) bound with a low affinity. No binding was detected with CS-A or HS. The negative control (*far right lane*, *top*) containing the same amount of s315-HARE was not treated with any biotin-GAG but was treated with ^{125}I-streptavidin.

other GAGs can bind HARE. To test this possibility, we prepared a panel of eight biotin-GAGs (*i.e.* chondroitin, CS-A, CS-B, CS-C, CS-D, CS-E, HA, and HS) and assessed the GAG binding to HARE using a ligand blot procedure similar to that described above for ^{125}I-HA (Figure 7*B*). In this experiment, the s315-HARE was purified by Ni^{2+}-chelate chromatography followed by 5% SDS-PAGE, blotted to nitrocellulose, and incubated with biotin-GAG with or without an excess of the same nonbiotinylated GAG to determine nonspecific binding. Binding of the biotin-GAG was then detected using ^{125}I-streptavidin and autoradiography. The results showed specific binding of the s315-HARE with CS-C, CS-D, CS-E, and HA, suggesting that HARE is a clearance receptor for multiple GAGs (Figure 7*B*). Biotin-HS did not bind to the s315-HARE. We know from other experiments (and longer exposure times of this experiment) that CS-B and chondroitin also bind to HARE but with a lower affinity that has yet to be measured (not shown).

A Majority of HARE Resides on Intracellular Vesicles—Since endocytic recycling receptors are found on the cell surface and in multiple early endosomal compartments along their intracellular itinerary, we wanted to determine the distribution of HARE between surface and intracellular compartments. The 190-HARE, for example, is distributed roughly equally, with ~50% on the cell surface (37). This is similar to the rat small HARE isoform stably expressed in SK-Hep1 cells, which co-localized with early endocytic markers, such as clathrin, but not later endosomes or lysosomes (29). An initial assessment of receptor localization was performed using fluorescence microscopy in which cells were either permeabilized with 0.05% saponin (Figure 8*A*) or intact (Figure 8*B*), and the fixed cells were probed with mAb-30 against the 315- and 190-HARE proteins. The permeabilized cells contained numerous bright intracellular vesicles with a faint ring of staining representing the cell surface. In contrast, nonpermeabilized cells showed only distinct perimeter fluorescence, indicating surface expression (Figure 8*B*). The fluorescence output from a field of permeabilized cells was ~4–5 times greater than a field of nonpermeabilized cells, confirming that most HARE is intracellular.

Next, we used a biotin-labeling technique to determine the ratio of internal to surface receptor, because differences in this ratio could explain the clonal differences in endocy-

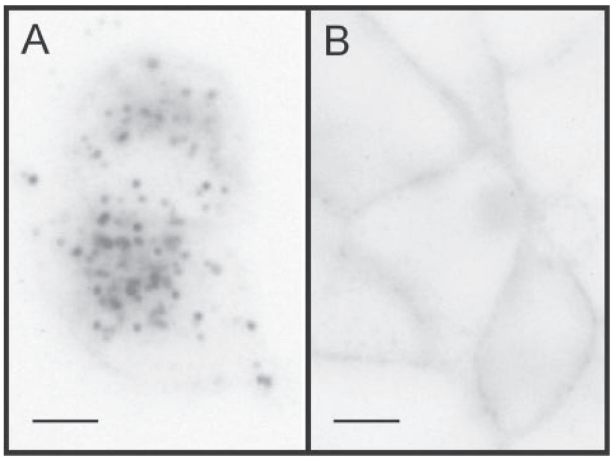

Figure 8. Cellular localization of HARE. 315-HARE clone 30 cells were grown on glass coverslips, fixed, and treated either with (*A*) or without (*B*) saponin as described in Reference 45 and under "Experimental Procedures." Anti-HARE mAb 30 followed by rhodamine-labeled anti-mouse secondary antibody was used to detect the receptor. Detergent-permeabilized cells were about 4–5 times brighter than the untreated cells, indicating that most of the receptor resides in intracellular membrane vesicles. Images of the cells were taken in color, converted to grayscale, and then black-and-white-inverted to visualize more clearly the cellular details seen in the color images. The cells shown were representative of all of the cells in a typical field at ×600 magnification. *Bars*, 10 μm.

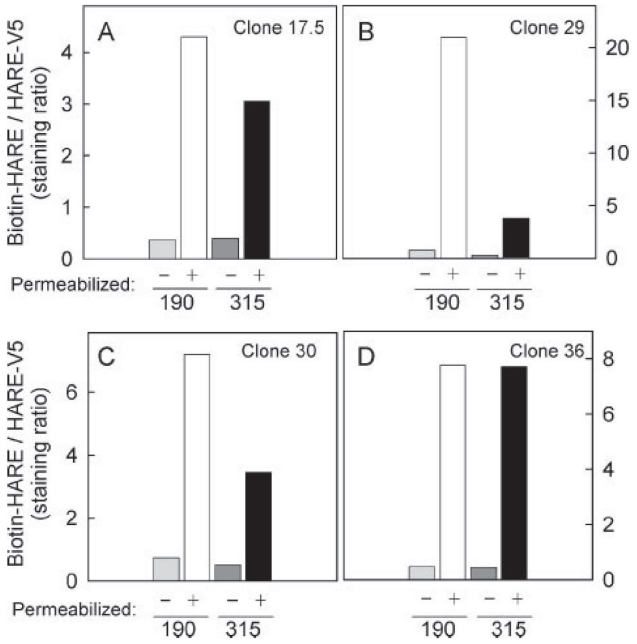

Figure 9. Biotinylation analysis of the cellular distribution of the two HARE isoforms in 315-HARE cell lines. 315-HARE cell lines 17.5 (*A*), 29 (*B*), 30 (*C*), and 36 (*D*) were plated in tissue culture flasks and grown to confluence. Cell surface or total cellular receptors were biotinylated using 2 mm sulfo-NHS-SS-biotin for 10 min at 4 °C, respectively, in the absence (not permeabilized) or presence (permeabilized) of 0.05% digitonin (45). Cells were solubilized with 0.5% Nonidet P-40 plus protease inhibitors and then processed for immunoprecipitation with mAb 159 and analysis by SDS-PAGE as described under "Experimental Procedures." After electrophoresis, the proteins were electrotransferred to nitrocellulose and probed with streptavidin-horseradish peroxidase conjugate, and the same blots were then stripped and reprobed with anti-V5 antibody. The blots were scanned, and the digital data were expressed as the average (n = 4) ratio of streptavidin/V5 detection (band densities). Shown are 190-kDa HARE (unpermeabilized (*light gray bar*) and permeabilized (*white bar*)) and 315-kDa HARE (unpermeabilized (*dark gray bar*) and permeabilized (*black bar*)).

tosis of HA among cell lines. Cells from four different 315-HARE cell lines were incubated with biotinylation reagent on ice to prevent constitutive endocytosis and without (non-permeabilized) or with (permeabilized) 0.05% digitonin to allow perfusion of the biotinylation reagent to the cell interior. Total HARE was then immunoprecipitated from cell lysates, and Western analysis was performed to compare the amount of biotinylated HARE (using streptavidin-horseradish peroxidase) and total HARE (using anti-V5 antibody). The results demonstrated that a majority of the 190-HARE and 315-HARE in all four clones resides on the interior of the cell. Cells not treated with digitonin had only their cell surface receptors biotinylated, and the ratio of those receptors compared with the total receptor content (*i.e.* anti-V5 staining) was low (Figure 9, *A–D*, *light* and *dark gray bars*). In contrast, most of the HARE in permeabilized cells was biotinylated, and the ratio of biotin-HARE to V5 staining was quite high, indicating a much greater receptor content inside cells relative to the surface (Figure 9, *A–D*, *open* and *solid bars*). Based on these biotin-labeling and the preceding fluorescence experiments, we estimate that only about 5–10% of the total cellular HARE, for either isoform, is at the cell surface.

Discussion

HARE, which has also been named stabilin-2, Feel-2, and Fex-2 by other laboratories, is the primary receptor for the clearance of HA and probably CS from the circulatory and lymphatic systems. HARE binds not only HA but also other GAGs, particularly the CS types, with high specificity. HA, free CS chains, and probably CS proteoglycan fragments, all of which continuously flow from tissue ECMs, first enter the lymphatic system and encounter the endocytic, recycling HARE in the lymph nodes, where most of these ECM components are degraded. This normal GAG turnover process might be accelerated in wound healing, injury, growth, and other disease states, including some cancers. Most of the high molecular mass (>10^6-Da) HA and, presumably, the other CS-type GAGs are cleared in the lymph nodes, and the remaining GAGs that enter the blood stream are cleared by liver sinusoidal endothelium-associated HARE. The activity of this GAG clearance system was first observed in experiments using animal models to study HA catabolism (17, 18, 34, 55). In addition to its scavenger activity, HARE located in the spleen, which is not a clearance organ, may play an important yet unknown role in immune system function.

In this report, we note that human HARE is expressed as two isoforms that are encoded from the same cDNA (*i.e.* an mRNA not subject to alternate splicing). The 190- and 315-kDa isoforms are recognized by the same subset of anti-rat HARE mAbs, and both soluble ectodomains bind HA to the same extent and with almost the same affinity. Both HARE isoforms also recognize multiple CS types as well as HA. We know that cells expressing only the 190-kDa isoform can function in the absence of the 315-kDa isoform to bind and internalize HA and other GAGs (37). When both the 315- and 190-kDa HARE are present in the same cells within a tissue,

we do not yet know whether they can function as independent receptors, as heterooligomeric complexes, or as both.

Based on our experiments with the recombinant HARE isoforms expressed in stable cell cultures, either together or the 190-HARE alone, it seems that the biological activities of these two receptors are remarkably similar. Both HARE isoforms contain the same LINK domain and the two C-terminal Cys-rich domains (Figure 1*A*). The larger isoform has the additional two N-terminal Cys-rich domains. These four domains are highly conserved, especially their Cys alignments, which indicates that their disulfide bond pattern and overall folding are essentially identical. Although the function of these Cys-rich domains is not known, it is tempting to speculate that they are involved in GAG binding, perhaps with multiple similar binding sites in each domain. Studies are in progress to test this possibility. Both HARE isoforms also contain the same cytoplasmic domain for potential intracellular signaling (*e.g.* endocytic trafficking).

Thus far, it has not been possible to study the activity of the membrane-bound 315-HARE independently of the 190-kDa receptor, since proteolytic cleavage appears to be the normal processing pathway for the full-length protein in 293 and other cells (*e.g.* CHO), as it appears to be *in vivo*. Despite the consequent complication of not being able to characterize the GAG binding and endocytic ability of the 315-kDa HARE by itself in live cells, we were able to demonstrate that the soluble ectodomain of the 315-HARE binds directly to CS-C, CS-D, and CS-E in addition to HA. We also detected a lower level of s315-HARE binding to CS-B and chondroitin, but the sensitivity of the ligand blot assay may not be sufficient to quantify these interactions. We are currently developing alternative assays to address this issue.

The only inconsistency we have observed in the GAG-binding activity of HARE occurred with CS-A. In live cell assays, CS-A competes very effectively with labeled HA (20, 23, 29, 36, 37); however, we could not detect a CS-A-HARE interaction by ligand blot assays, and we obtained similar sporadic binding results in ELISA-like assays (not shown). The binding activity of CS-A may be sensitive to the conditions of our *in vitro* assays, or the affinity may be too low to retain a good signal with the washing protocol in these assays. We are currently working to optimize the level of detection with these assays as well as testing whether the 190-HARE and 315-HARE have different GAG binding profiles due to the additional protein domains of the larger isoform.

Expression of the full-length 315-HARE cDNA produces two protein products of different size but similar function. The processing of the 315-HARE appears to be a tightly regulated process, since the ratio of 315- to 190-HARE was consistently ~3–4:1 in multiple experiments with the six cell lines used here. Earlier results also demonstrated a consistent ratio of the two isoforms in rat liver or human spleen and the possibility that isoform ratios might differ between tissues (30, 33). From our protein sequencing data, the 190-HARE purified from human spleen begins at Ser^{1136}. This N-terminal region does not contain a consensus sequence or motif for known proteases, and the biological implications of such an activity are unknown at this time. A goal for future studies is to prevent processing of the 315-HARE, by use of inhibitors or mutagenesis, in order to study the cellular function of this larger isoform in the absence of the 190-kDa HARE. In ongoing studies, mutagenesis of a possible adjacent furin-like site (KK^{1131} to AA^{1131}) did not eliminate the proteolytic processing in 293 cells. Similarly, studies with a variety of commonly used protease inhibitors (*e.g.* cathepsin L, GM6001, EDTA, E-64, aprotinin) failed to identify a potential protease. We are currently investigating how proteolytic cleavage of HARE occurs, what protease is involved, or whether HARE itself might be autocatalytic.

Observance of the smaller HARE isoform is not unique to our laboratory. A report on murine stabilin-2 and human stabilin-1 provided evidence that human stabilin-2/HARE is processed to generate the smaller isoform, although the authors did not discuss its presence or consider it relevant (39). The highly similar protein stabilin-1 also has a smaller isoform of about 140 kDa (56; E. N. Harris, unpublished observations). Potentially, the smaller isoforms of both stabilin-1 and HARE may have different ligand-binding profiles or a different subset of interacting effector molecules than their full-length counterparts.

Unlike the large native rat liver HARE isoform, which contains two large disulfide-bonded subunits of 220 and 250 kDa (33), the recombinant 315-kDa human HARE is a single polypeptide. By Western analysis, the 315-kDa protein band did not decrease in size after reduction with dithiothreitol, and multiple bands were not detected. Reduction of immunoaffinity-purified 315-HARE produced a single band detected by silver staining. In previous studies with purified 315-HARE from human spleen, we found that this isoform contained two large disulfide-bonded subunits. Although it is possible that Flp-In 293 cells do not have the capability of sinusoidal endothelial cells to post-translationally modify HARE in the same fashion, in more recent experiments with fresher spleen tissue, we did not find multiple subunits in the large HARE isoform. It thus appears likely that variable extraction or storage conditions influence nonspecific cleavage of the large protein isolated from tissues. Therefore, we presently believe that each human HARE isoform is composed of only one type of polypeptide subunit. These results do not exclude the possible presence of nondisulfide-bonded HARE homo-oligomers or hetero-oligomers, as noted above.

The expression level of the recombinant 190-HARE in earlier studies (37) was 8–10-fold higher than HARE expression in any of the 315-kDa HARE cell lines. Since the same cytomegalovirus promoter drives expression of either HARE isoform, we initially thought there might be a difference in translation efficiency related to the use of two different signal sequences. To test this, we put the 315-HARE-encoding cDNA, without the native signal sequence, into the pSecTag vector containing the Ig-κ chain signal sequence. This is the same signal sequence used to express the 190-HARE cDNA (37). The change in signal sequences did not make any difference in 315-HARE expression (not shown), indicating that lower expression is probably a protein folding or trafficking issue rather than a difference in transcription or translation.

The 315-HARE has 202 Cys residues, compared with 104 for the 190-HARE, and may simply be much more difficult to fold and align correctly for disulfide bond formation. Thus, a higher fraction of the 315-HARE protein may be misfolded and degraded in the endoplasmic reticulum, compared with the 190-HARE. The lower expression of 315-HARE may also account for the greater variability in HA endocytic activity

among the six cell lines expressing the 315-HARE. This variability may seem surprising given the single uniform insertion site of the HARE cDNA in Flp-In 293 cells. However, the background genotype will not be identical for any cloned cell lines, due to random mutations in other genes. Endocytosis and degradation rates may be sensitive to changes in hundreds of different proteins involved in coated pit cycling, receptor recycling, endosomal, lysosomal, and other intracellular trafficking pathways. Since the signal (*i.e.* HARE-mediated HA uptake) is lower, such mutations may have more noticeable inhibitory effects.

The human HARE LINK domain is 50% identical (65% similar) to the *Gallus gallus* (chicken) TSG-6 LINK domain and 26% identical (44% similar) to the human CD44 LINK domain. The LINK domain in HARE is a primary candidate for an HA-binding domain, although we do not yet know if the epidermal growth factor-like Cys-rich domains facilitate binding to HA or to some of the other CS types. The LINK domains from HARE, CD44, and TSG-6 contain four highly conserved cysteines, which may be required for proper folding to facilitate HA binding. Based on structural studies and computer modeling, most of the LINK amino acids involved with HA binding are Tyr (57). Since the results in Figures 5*B* and 6 show that the HA-binding ability of the s190- and s315-HARE are essentially the same, the common LINK domain could be responsible for HA binding. Preliminary results show that HA endocytosis by cells stably expressing a 190-HARE LINK-deletion variant is inhibited greatly compared with wild type, but is not eliminated. Currently, we are investigating whether the HARE LINK domain binds to HA or CS and if potentially key amino acids identified by NMR comparison studies of LINK modules are involved.

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