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Endocytic Function, Glycosaminoglycan Specificity, and Antibody Sensitivity of the Recombinant Human 190-kDa Hyaluronan Receptor for Endocytosis (HARE)

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Abstract

The human hyaluronan receptor for endocytosis (hHARE) mediates the endocytic clearance of hyaluronan (HA) and chondroitin sulfate from lymph fluid and blood. Two hHARE isoforms (190 and 315 kDa) are present in sinusoidal endothelial cells of liver, spleen, and lymph nodes (Zhou, B., McGary, C. T., Weigel, J. A., Saxena, A., and Weigel, P. H. (2003) *Glycobiology* 13, 339–349). Here we report the specificity and function of the 190-kDa HARE, expressed without the larger isoform, in Flp-In 293 cell lines (190hHARE cells). Like the native protein, recombinant hHARE contains ~25 kDa of *N*-linked oligosaccharides, binds HA in a ligand blot assay, cross-reacts with three anti-rat HARE monoclonal antibodies, and is inactivated by reduction. The 190hHARE cell lines mediated rapid, continuous ^{125}I-HA endocytosis and degradation for >1 day. About 30–50% of the total cellular receptors were on the cell surface, and their recycling time for reutilization was ~8.5 min. The average K_d for the binding of HA to the 190-kDa hHARE at 4 °C was 7 nm with 118,000 total HA binding sites per cell. Competition studies at 37 °C indicated that the 190-kDa hHARE binds HA and chondroitin better than dermatan sulfate and chondroitin sulfates A, C, D, and E, but it does not bind to heparin, heparan sulfate, or keratan sulfate. Although competition was observed at 37 °C, none of the glycosaminoglycans tested, except HA, competed for ^{125}I-HA binding by 190hHARE cells at 4 °C. Anti-HARE monoclonal antibodies #30 and #154, which do not inhibit ^{125}I-HA uptake mediated by the 175-kDa rat HARE, partially blocked HA endocytosis by the 190-kDa hHARE. We conclude that the 190-kDa hHARE can function independently of other hHARE isoforms to mediate the endocytosis of multiple glycosaminoglycans. Furthermore, the rat and human small HARE isoforms have different glycosaminoglycan specificities and sensitivities to inhibition by cross-reacting antibodies.

Abbreviations: **HA**, hyaluronic acid, hyaluronate, or hyaluronan; **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; **DS**, dermatan sulfate; **ECM**, extracellular matrix; **GAG**, glycosaminoglycan; **HARE**, HA receptor for endocytosis; **hHARE**, human HARE; **HBSS**, Hanks' balanced salt solution; **Hep**, heparin; **HS**, heparan sulfate; **KS**, keratan sulfate; **LECs**, liver sinusoidal endothelial cells; **mAb**, monoclonal antibody; **PBS**, phosphate-buffered saline; **rHARE**, rat HARE; **SK-HARE**, stable SK-Hep-1 cell lines expressing recombinant rat HARE; **Tris**, trishydroxymethylamino methane; **TBS**, Tris-buffered saline; **TBST**, Tris-buffered saline containing 0.05% Tween 20; **BSA**, bovine serum albumin; **DMEM**, Dulbecco's modified Eagle's medium; **Chon**, chondroitin

Multiple extracellular binding proteins and cell surface receptors for HA facilitate many biological activities that are important during complex cellular processes, including development, wound healing, and invasion and metastasis of some cancers (1–5). Due to the rapid turnover of HA in many tissues (6), very high HA levels could occur locally, in lymph fluid, or in plasma. Therefore, efficient mechanisms for HA uptake and degradation are present in mammals to regulate the amount of HA present in a variety of physiological conditions (reviewed in References 2 and 7).

The endocytic clearance receptor for HA, which is designated HARE (HA receptor for endocytosis), is abundant in the sinusoidal endothelial cells of liver, spleen, and lymph nodes (8–10). The biological activity of this clearance receptor was discovered in rodents in 1981 (11–13), and the protein was finally purified by Zhou *et al.* in 1999 (14). Earlier studies using isolated rat LECs indicated that HARE mediates the binding and endocytosis of HA via the coated-pit pathway and that this receptor also recognizes and internalizes chondroitin sulfates (15–17). Unlike the rat HARE proteins, which have been studied extensively in isolated rat LECs, there have been no cellular studies of the human HARE proteins. Human LECs are not available commercially, and to date, no cell lines have been identified that express either the 190- or 315-kDa hHARE isoforms. Consequently, very little is known about the GAG specificity or function of human HARE.

In both rat (14) and human (10), two isoforms of HARE are present with molecular masses of 175 or 300 kDa and 190 or 315 kDa, respectively. The human ~315-kDa HARE is a complex composed of two disulfide-bonded subunits of about 250 and 220 kDa, in a ratio of ~2:1 (10). In contrast, the small hHARE isoform contains a single subunit. All subunits in both hHARE isoforms, although they are different sizes, appear to be derived by proteolysis from the same precursor protein (9), which is Stabilin 2 (18). Full-length Stabilin 2 is a very large putative protein (2551 amino acids), whose synthesis and processing have not yet been fully elucidated. For example, it is not known if the largest subunit in the 315-kDa hHARE corresponds to full-length Stabilin 2 or to a truncated form of the protein. The small rat and human HARE proteins are not encoded directly by mRNA. We recently demonstrated (9) that the native small rHARE isoform contains the C-terminal 1431 residues of the predicted full-length protein and that

this smaller HARE, when expressed in SK-Hep-1 cells in the absence of the large isoform, colocalizes with clathrin as expected for a coated-pit-coupled endocytic HA receptor (19). The two rat HARE species, therefore, appear to be functionally independent isoreceptors for HA.

In the present study, we have created an artificial spleen cDNA for the 190-kDa hHARE to assess its functionality and GAG specificity in stable cell lines. In addition to demonstrating that this smaller hHARE isoform can function as an endocytic, recycling receptor in the absence of the larger hHARE, we found that the human protein has slightly different specificity for various GAGs than the highly related rat 175-kDa HARE isoform. Unexpectedly, several anti-HARE mAbs showed very different reactivity with the rat and human recombinant HARE proteins and were able to block partially the internalization of HA by cells expressing the 190-kDa hHARE.

Experimental Procedures

Materials and Buffers – $Na^{125}I$ was from Amersham Biosciences, and ^{125}I-HA was prepared as described previously (20) using HA oligosaccharides (M_W = 133,000 based on gel permeation chromatography coupled to multiangle laser light scattering analysis), modified only at their reducing ends to contain covalently attached hexylamine. Male Sprague-Dawley rats (200 g) were from Charles River Labs. BSA Fraction V and fetal bovine serum were from Intergen Co. Collagenase was from Roche Applied Science. The preparation and characterization of mouse mAbs raised against the rat 175-kDa HARE were described previously (8). Tris, SDS, ammonium persulfate, *N,N*′-methylenebisacrylamide, and SDS-PAGE standards were from Bio-Rad. Digitonin, from ACROS Organics, was prepared as a 25% (w/v) stock solution in Me_2SO and then diluted into medium as required. Unless noted otherwise, other chemicals and reagents were from Sigma Chemical Co. All GAGs (with the exception of heparin, which came from Sigma) were obtained from Seikagaku Corp. The weight-average molecular masses (determined by gel permeation chromatography coupled to multiangle laser light scattering analysis) for all but two of the GAGs were 14.2–38.8 kDa. The two exceptions were Chon (7.4 kDa) and CS-E (187.7 kDa). Nitrocellulose membranes were from Schleicher & Schuell. HBSS and PBS were prepared according to the Invitrogen catalog formulations. Z-buffer contains 60 mm dibasic sodium phosphate and 40 mm monobasic sodium phosphate, pH 7.0, 10 mm KCl, 1.0 mm $MgSO_4$, and 50 mm 2-mercaptoethanol. TBS contains 20 mm Tris-HCl, pH 7.0, and 150 mm NaCl. TBST is TBS containing 0.05% (v/v) Tween 20.

Construction of hHARE Expression Vector – The 190-kDa hHARE coding region was amplified from pooled lymph node cDNAs (Marathon system; Clontech) using Advantage 2 polymerase (Clontech), and gene-specific forward (5′-GGATCCTCCTTACCAAACCTGCTCATGCGG-3′) and reverse (5′-GGATCCCAGTGTCCTCAAGGGGTCATTG-3′) primers. The PCR product representing the artificial cDNA was then purified by agarose gel electrophoresis using a 0.8% gel containing 0.002% crystal violet for visualization. The band was excised, gene-cleaned, ligated into pCR-XL-TOPO, and transformed into TOP10 *Escherichia coli* cells (Invitrogen). Clones were selected and screened for the full-length insert by restriction digestion and PCR analysis, and a correct clone was used to prepare and isolate the expression vector. The hHARE cDNA was then cut out of the pCR-XL-TOPO plasmid and inserted into the BamHI site of pSecTag/FRT/V5-His-TOPO (Invitrogen). This vector provides a κ light chain secretion signal fused at the N terminus of the hHARE reading frame and two epitope tags (V5 and His-6) fused at the C terminus of the gene product. After transformation into TOP10 *E. coli* cells, several clones were selected and size and orientation of the cDNA insert were verified. The complete sequences of promoter, fusion, and cDNA regions of the final clones were determined and confirmed to be correct.

Selection and Characterization of Stable Transfectants Expressing the 190-kDa hHARE – Flp-In 293 cells (3×10^6; from Invitrogen) were plated in 100-mm tissue culture dishes the day prior to transfection. Cells in 10 ml of antibiotic-free medium were transfected by addition of 750 μl of serum-free DMEM containing 9 μg of pOG44 (which encodes the Flp-In recombinase), 1 μg of pSecTag-190hHARE, and 20 μl of LipofectAMINE 2000 (Invitrogen). Two days post-transfection the medium was replaced with DMEM containing 100 μg/ml hygromycin B (Invitrogen). Due to the build-up of dead cells, the medium was changed every 2–3 days. Visible colonies were observed at days 10–14 and then isolated using cloning rings or collected directly with a plastic pipette tip. Isolated colonies were grown to confluence in 24-well dishes in 1.0 ml of DMEM with 100 μg/ml hygromycin B. After a monolayer of cells had developed from each clone, the cells were scraped and suspended in 1.0 ml of fresh medium. One portion (100 μl) of cells was re-seeded and allowed to grow for subsequent procedures. Another 100 μl of cells was resuspended in DMEM plus 100 μg/ml Zeocin and allowed to grow for 1 week to test for Zeocin sensitivity. A third portion (400 μl) of cells was pelleted and resuspended in 4× Laemmli sample buffer (21) to test for HARE protein expression by SDS-PAGE and Western analysis. The Western blot was probed with anti-V5 antibody (Bethyl Laboratories; 1:5000 dilution) in TBST.

The remaining 400 μl of cell suspension was pelleted and assayed for β-galactosidase activity. Both the Zeocin and β-galactosidase tests indicate whether pSecTag-190-kDa hHARE was inserted correctly and uniquely into the Flp-In recombination site by the Flp-In recombinase encoded by pOG44. The recombinase is lost during subsequent cell divisions, because the encoding plasmid lacks an antibiotic selection gene. For the β-galactosidase assay, a clonal cell pellet was resuspended in 250 μl of 0.5% Triton X-100 in PBS, and 10 μl of cell lysate per well (in a 96-well plate) was combined with 20 μl of distilled deionized H_2O, 70 μl of Z-buffer, and 20 μl of 4 mg/ml *o*-nitrophenyl-β-d-galactoside in Z-buffer. After 15 min at 37 °C, the enzyme reaction was terminated by the addition of 0.1 ml 1 m sodium bicarbonate, and absorbance values were determined at 420 nm. Human embryonic kidney Flp-In 293 cells and 293 cells were included in each assay set as positive and negative controls, respectively. Stable clones with a single plasmid integrated into the correct, unique chromosomal site were those that demonstrated and maintained no detectable β-galactosidase expression, poor or no growth in DMEM containing 100 μg/ml Zeocin, normal cell morphology, and good HARE protein expression. Suitable clones were maintained in DMEM containing 100 μg/ml hygromycin B and 8% fetal bovine serum.

Western and Ligand Blot Assays – Western blotting was performed as described by Burnette (22) with minor modifications. Cell lysates were mixed with equal volumes of 2× SDS sample buffer (21), without reducing agent, to give final concentrations of 16 mm Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% glycerol (v/v), and 0.01% bromphenol blue. After SDS-PAGE, the contents of the gel were electrotransferred to a nitrocellulose membrane overnight at 10 V at 4 °C using 25 mm Tris, pH 8.3, 192 mm glycine, 20% methanol, and 0.01% SDS in a Genie blotter apparatus (Idea Scientific). For the ligand blot assay, the nitrocellulose membrane was treated first with TBS containing 0.1% Tween 20 at 4 °C for 2 h, or TBST overnight, and then incubated with 1–2 μg/ml ^{125}I-HA in 150 mm NaCl, 10 mm HEPES, pH 7.4, and 5 mm EDTA without, or with, a 100- to 150-fold excess of nonlabeled HA (as competitor) to assess total and nonspecific binding, respectively (23). The nitrocellulose membrane was washed with TBST five times for 5 min each and dried at room temperature. Bound ^{125}I-HA was detected by autoradiography using Kodak Bio-Max MS or MR film exposed at –85 °C for 6–48 h. Nonspecific binding in this assay is typically <5%.

For Western analysis, the nitrocellulose membranes were blocked with 1% BSA in TBS at 4 °C overnight either after the ligand blot assay (the membranes were rewet with TBST first) or directly after SDS-PAGE and electrotransfer. The membrane was then incubated with anti-rat HARE mAbs (*e.g.* 1 μg/ml IgG) at 22 °C for 1 h, washed three times for 5 min each with TBST, and incubated with goat anti-mouse Ig-alkaline phosphatase conjugate (1:1500 dilution) for 1 h at room temperature. The nitrocellulose was washed with TBST five times for 5 min each and incubated with *p*-nitro blue tetrazolium and sodium 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine for color development (Bio-Rad), which was stopped by washing the membrane with distilled water.

^{125}I-HA Binding or Endocytosis Assays – Stably transfected 190-kDa hHARE cell lines were grown to confluence in DMEM containing 8% fetal calf serum and 100 μg/ml hygromycin B in tissue culture multiwell dishes (usually 24-well plates). The cells were washed with HBSS and incubated at 37 °C in fresh medium without serum for 30–60 min, the plates were then placed on ice, and the cells washed once or twice with HBSS prior to the experiment. Medium containing 1–2 μg/ml ^{125}I-HA with or without the noted concentration of IgG or other GAG (as competitor) was added to each well, and the cells were incubated either on ice for 60 min to assess cell surface binding or at 37 °C to allow internalization of ligand. To assess HA binding by the total cell receptor population, 0.055% (w/v) digitonin was added to the medium to permeabilize the cells (24, 25). At the noted times, the medium was removed by aspiration, the cells were washed three times with HBSS and lysed in 0.3 n NaOH, and radioactivity and protein content were determined. Values were normalized for cell protein content per well and are presented as cpm/μg of protein. In some cases, HA data are expressed as cpm or femtomoles per million cells. For

Flp-In 293 cells, the mean protein content was determined to be 398 ± 85 μg of protein/10^6 cells (n = 6).

^{125}I-HA Degradation Assay – Degradation of ^{125}I-HA was measured by a cetylpyridinium chloride precipitation assay as described by McGary *et al.* (26). 50-μl samples of medium were mixed with 250 μl of 1 mg/ml HA in 1.5-ml microcentrifuge tubes. Alternatively, 100-μl samples of cell lysate (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 HA. After mixing at room temperature, 300 μl of 6% (w/v) cetylpyridinium chloride in distilled water was added, and the tubes were mixed by vortexing. After 10 min, the samples were centrifuged at 22 °C for 5 min at 9000 rpm in an Eppendorf model 5417 microcentrifuge, using a swinging bucket rotor. A sample (300 μl) of the supernatant was taken for determination of radioactivity, and the remainder was removed by aspiration. The tip of the tube containing the precipitated pellet was cut off then put in a gamma counter tube, and radioactivity was determined. Degradation was measured as the time-dependent increase of nonprecipitable radioactivity. At least 80% of the total radioactivity was precipitable at the beginning of each experiment.

General – Protein content was determined by the method of Bradford (27) using BSA as a standard. SDS-PAGE was performed according to the method of Laemmli (21). ^{125}I radioactivity was measured using a Packard Auto-Gamma Counting system. Digital images were captured using an Alpha Innotech Fluorochem 8000. Images were taken into Corel Photo Paint (version 9.0) as JPG files, cropped and processed identically, and then transferred to Corel Draw (version 9.0) for annotation. N-terminal amino acid sequence analysis was performed by the University of Oklahoma Health Sciences Center, Molecular Biology Resource Facility.

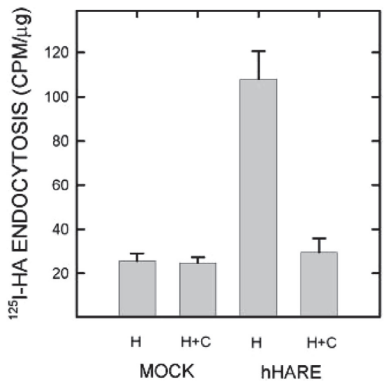


Figure 1. Transiently transfected 293 Flp-In cells express the HA-binding recombinant 190-kDa hHARE protein. 293 Flp-In cells (5×10^4 per well) grown in 24-well plates were transfected with pSecTag-190-kDa hHARE/ExGEN500 or pSecTag/ExGEN500 complexes and allowed to recover for 2 days. The transfected cells were allowed to bind and endocytose 1 μg/ml ^{125}I-HA with (*H+C*) or without (*H*) 100 μg/ml unlabeled HA in serum-free medium for 3 h at 37 °C. Cells were washed with ice-cold HBSS and solubilized in 0.3 m NaOH, and radioactivity and protein were determined (n = 3; ±S.E.).

Results

Expression of Recombinant Human 190-kDa HARE – We recently purified two hHARE proteins, of 190 and 315 kDa, from spleen extracts and then molecularly cloned a partial cDNA from pooled human lymph node and spleen that encoded part or all of the subunits in these two isoforms (10). The 190-kDa hHARE protein is not expressed from a unique mRNA, but rather is encoded by a 4383-bp region (1461 amino acids) at the 3′ end of the full-length *Stab 2* coding region. To express the 190-kDa protein, we created an artificial cDNA for a recombinant 190-kDa hHARE in the pSecTag/FRT/V5/His-TOPO expression vector. For proper membrane orientation and trafficking to the cell surface, the pSecTag vector provides an immunoglobulin κ-chain secretion signal sequence fused at the N terminus of the protein. Transiently transfected Flp-In 293 cells expressed sufficiently high levels of the recombinant 190-kDa hHARE to mediate the specific binding and internalization of ^{125}I-HA (Figure 1). Compared with vector alone, cells transfected with hHARE cDNA internalized ~4 times the amount of HA, and this uptake was completely blocked by unlabeled HA. Specific HA uptake, therefore, was ~80% of the total.

The potential advantage of using Flp-In 293 cells as the parental cell line for generation of stable cell lines expressing hHARE is that all clones should be virtually identical if the plasmid inserts at only the single unique chromosome site containing the engineered integration site. Correct integration at this site interrupts a β-galactosidase gene and a Zeocin resistance gene in the engineered site. Clones containing a single plasmid insertion at the correct engineered site are, therefore, hygromycin B-resistant, negative for β-galactosidase activity, and Zeocin-sensitive. If plasmid insertion occurs at other chromosome sites, rather than the correct engineered site, then clones will express β-galactosidase and be Zeocin-resistant. Out of 41 stably transfected clones that we selected and characterized, three (#9, #14, and #40) had no detectable β-galactosidase activity, were Zeocin-sensitive, and were judged to contain a plasmid insertion at the unique engineered site.

A protein of the correct size for the 190-kDa hHARE was expressed in the three selected stable Flp-In 293 cell lines, and this protein bound ^{125}I-HA with >98% specificity in a ligand blot assay following SDS-PAGE and electrotransfer (Figure 2*A*). The 190-kDa hHARE protein expressed in Flp-In 293 cells had the characteristics previously found for native hHARE purified from spleen (10), and expression of the 190-kDa hHARE did not alter the morphology of Flp-In 293 cells. The recombinant nonreduced protein was recognized in Western blots by the three anti-rHARE mAbs that cross-reacted with native hHARE (mAbs 30, 154, and 159) but not mAbs 28, 174, 235, and 467 (Figure 2*B*, *NR*). Similarly, the reduced 190-kDa hHARE protein reacted with only mAbs 159 and 174 (Figure 2*B*, *R*). Based on its HA-binding activity in these *in vivo* and *in vitro* assays, the recombinant hHARE protein appeared to be folded properly. Consistent with this interpretation, three other characteristics of the recombinant hHARE were identical to those of the native protein (10). Reduction of disulfide bonds resulted in slower migration of the 190-kDa hHARE in SDS-PAGE compared with the nonreduced protein (Figure 2*C*, *lanes 1* and *3, WB*). Reduction of disulfide bonds also caused loss of HA-binding activity (Figure 2*C*, *lanes 1* and *3, AR*). After treatment with endoglycosidase-F to release *N*-linked oligosaccharides, the recombinant protein migrated at a position corresponding to a loss of ~25 kDa (Figure 2*C*, *lanes 3* and *4, WB*). The de-*N*-glycosylated hHARE protein was still able to bind HA in this ligand blot format (Figure 2*C*, *lane 4, AR*). In addition, anti-V5 antibody recognition of the C-terminal epitope provided by the vector was suitable for immunoprecipitation (not shown).

HA Binding and Internalization by Cells Expressing the 190-kDa hHARE – The specific binding of ^{125}I-HA at 4 °C by stable cell lines was typical for a membrane-bound receptor; binding kinetics was hyperbolic and saturated after about 90 min (Figure 3). Essentially no specific binding of ^{125}I-HA occurred in the control cells transfected with empty vector, consistent with the absence of any significant HA receptor activity in 293 cells (Table I). Also, as found for other endocytic, recycling receptors (*e.g.* the asialoglycoprotein and mannose receptors), about 30–50% of the total cellular hHARE population was on the cell surface, and the remainder was intracellular. The native rHARE in isolated LECs is an active endocytic receptor that recycles so that HA can be continually internalized and delivered to lysosomes for degradation over a period of many hours to days (7, 17, 26). To assess the ability of the recombinant 190-kDa hHARE to recycle, cells were allowed

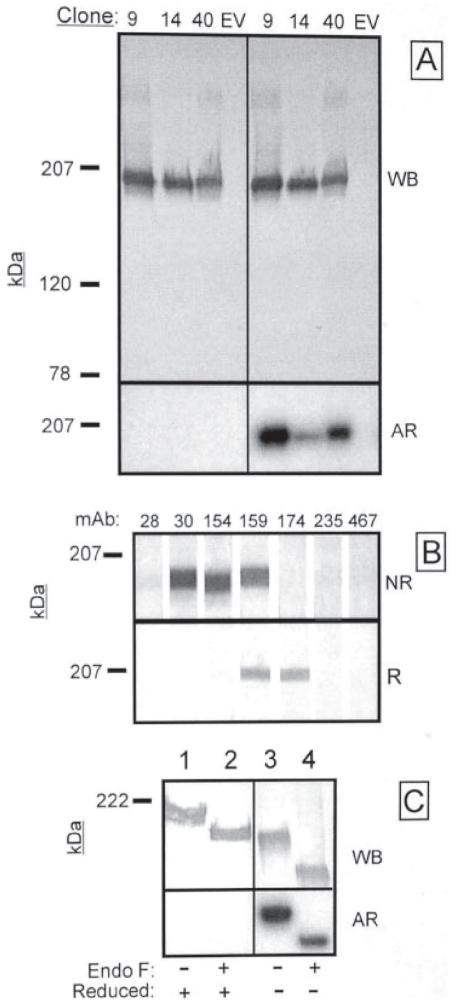


Figure 2. The 190-kDa hHARE is expressed abundantly and is biologically functional in stably transfected 293 Flp-In cells. *A*, whole cell lysates from three clones expressing the 190-kDa hHARE (*9, 14*, and *40*) and one clone transfected with empty vector (*EV*) were subjected to nonreducing SDS-PAGE using a 5% gel, followed by electrotransfer to nitrocellulose. *Bottom panels*: after blocking in 0.1% Tween-20 in TBS for 3 h at room temperature, ligand blotting and autoradiography (*AR*) were performed, using 1.0 μg/ml ^{125}I-HA with (*left*) or without (*right*) 100 μg/ml unlabeled HA, as described under "Experimental Procedures." *Top panels*: the same nitrocellulose strips were then rewetted and blocked with 1% BSA in TBS, and Western blot (*WB*) analysis was performed using a mixture of mAb-30, mAb-154, and mAb-159. *B*, whole cell lysates from clone #9 were subjected to nonreducing (*NR*) or reducing (*R*) SDS-PAGE using a 5% gel and electrotransfer to nitrocellulose. After blocking for 2 h in 1% BSA in TBS, the nitrocellulose was cut into strips and subjected to Western blot analysis with 1 μg/ml of the indicated seven mAbs previously raised against the rat 175-kDa HARE. *C*, cells expressing the 190-kDa hHARE protein were lysed in Laemmli buffer and either treated with endoglycosidase F and/or reduced with 10 mm dithiothreitol. Proteins were separated on a 5% SDS-PAGE. *Bottom panel*: the transfer was incubated with 1 μg/ml ^{125}I-HA with (*left*) or without (*right*) 100 μg/ml unlabeled HA for 2 h at 4 °C and washed, and autoradiography was performed. *Top panel*: the nitrocellulose was then rewetted in 1% BSA in TBS, and Western blot analysis was performed to identify hHARE using anti-V5 antibody.

to internalize ^{125}I-HA for 4 h, and the amount of specific HA uptake was calculated as the number of cell surface receptor equivalents. This estimates the approximate number of times that a cohort of cell surface HARE proteins would have to be used to achieve the observed level of HA uptake. For 190-kDa hHARE Flp-In 293 clones 9 and 14, these recycling ratios were 25 and 32, respectively (Table I). Based on these values of 25–32 surface equivalents of HA internalized in 240 min, the estimated individual receptor recycling time is 7.5–9.6 min, which is identical to the recycling times reported for all the known coated-pit-mediated clearance receptors that recycle (7, 19).

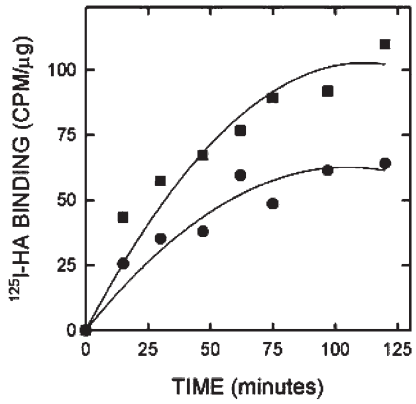


Figure 3. Kinetics of ^{125}I-HA binding by stable cell lines expressing the 190-kDa hHARE. Confluent cell cultures of hHARE expression clones #9 (•) and #14 (▪) were incubated at 37 °C in medium without serum for 60 min. The plates were placed on ice, and the wells were washed once with HBSS. The cells were permeabilized at 4 °C with 0.55% digitonin in PBS to allow access to both surface and internal receptors (24, 25). The cells were washed and then incubated in medium containing 1.5 μg/ml ^{125}I-HA with or without 150 μg/ml unlabeled HA. At the noted times, the cells were washed three times with HBSS and solubilized in 0.3 n NaOH, and protein and radioactivity were determined as under "Experimental Procedures." Data shown represent specific binding; each point is the average of duplicate wells without excess HA (total binding) minus the average of duplicate wells with excess HA (nonspecific binding).

Consistent with the conclusion that the recombinant 190-kDa hHARE is a recycling receptor able to mediate the continuous endocytosis of ligand, the Flp-In 293 cell lines expressing hHARE, but not the vector-alone control, were able to internalize ^{125}I-HA for ≥20 h before cellular accumulation appeared to level off (Figure 4). The apparent saturation of HA uptake is a steady-state situation, however, because cells are still endocytosing ^{125}I-HA while they are releasing radioactive degradation products into the medium at the same rate. The ability of cells to process (*i.e.* internalize, degrade, and secrete degradation products) large amounts of ligand over many hours or days is characteristic of recycling receptors that operate via the coatedpit pathway (7, 19, 28).

Scatchard Analysis of ^{125}I-HA Binding by Recombinant 190-kDa hHARE – Because no ligand binding information exists for the individual hHARE species, equilibrium binding studies were performed using 190hHARE Flp-In 293 clones #9 and #14 to determine total receptor content and the affinity of the HA-hHARE interaction (Figure 5). Based on the concentration of unlabeled HA required for half-maximal competition of ^{125}I-HA binding, the apparent K_m for HA binding is 1–2 μg/ml or ~10 nm (Figure 5*A*). When these data were normalized for the specific radioactivity of the bound ^{125}I-HA at each point, the resulting binding isotherm was hyperbolic, which is typical of many receptor-ligand interactions, and binding approached saturation at >80 μm HA (Figure 5*B*). When analyzed according to the method of Scatchard (29), the data in replicate experiments were best fit by a single straight line ($cc \geq 0.9$), indicating that a single class of noninteracting HA binding sites was present in digitonin-permeabilized cells (Figure 5*C*). Based on two independent experiments with both clones #9 and #14 (n = 8) the mean (±S.D.) B_{max} and K_d values were 196 ± 45 fmol of total HA binding sites/10^6 cells and 7.2 ± 1.2 nm, respectively. The B_{max} value corresponds to ~118,000 total HA binding sites per cell.

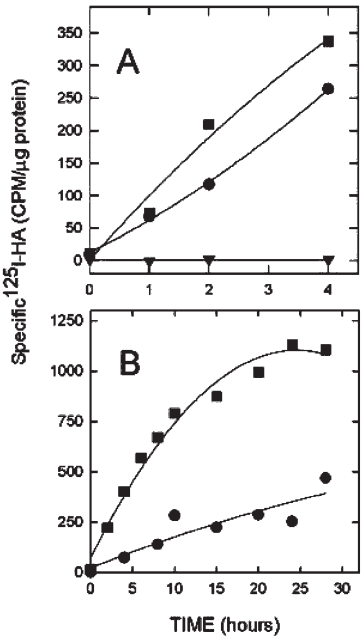


Figure 4. The recombinant 190-kDa hHARE mediates continuous endocytosis and degradation of ^{125}I-HA. *A*, confluent cell cultures of 190hHARE-expressing clones #9 (●) and #14 (■) and empty vector control clone #13 (▼), in 24-well tissue culture plates, were incubated at 37 °C in medium without serum for 30 min. The plates were then placed on ice, and the cells were washed once with HBSS. Medium containing 1.6 µg/ml ^{125}I-HA with or without 160 µg/ml unlabeled HA was added to each well, and the cells were incubated at 37 °Cfor up to 4 h to allow internalization. At the noted times, the medium was removed, and the cells were washed three times with 1 ml of HBSS and lysed in 0.3 n NaOH, and cell protein content and cell-associated radioactivity were determined. The data are shown as specific uptake; the average radioactivity values of duplicates for total uptake minus the average of duplicates for nonspecific uptake. *B*, clone #14, expressing the 190-kDa hHARE, was cultured in 4-well tissue culture plates, and processed as in *A*. In addition, degraded ^{125}I-HA that was cell-associated and in the medium were also measured at the noted times, as described under "Experimental Procedures." The plots show radioactivity (representing intact and degraded HA) associated with the cells (■) and the total degraded ^{125}I-HA (●), *i.e.* products still inside the cell plus those in the medium.

GAG Specificity of the Recombinant 190-kDa hHARE—The GAG specificities of the two hHARE isoforms have not been determined. Using the stable 190hHARE Flp-In 293 cell lines, we examined the ability of individual purified GAG chains to block the endocytosis of ^{125}I-HA. Even at 100 µg/ml, KS, HS, and heparin did not compete for HA binding and uptake at 37 °C, and DS showed a modest ~15% inhibition (Figure 6). This latter slight inhibition by DS appears to be significant, because it was observed in other experiments as noted below. CS-A was the most effective inhibitor, although its blocking ability was not comparable to that of HA, *e.g.* at 30 µg/ml ~45% inhibition was observed with CS-A *versus* ~70% inhibition with HA (Figure 6*A*). Four other GAGs, including chondroitin (Figure 6*A*) and CS-C, CS-D, and CS-E (Figure 6*B*) gave very similar titration profiles, with ~50% inhibition at 100 µg/ml. For comparison, 100 µg/ml HA blocked ^{125}I-HA uptake by ~87%. In contrast to these results at 37 °C, none of the GAGs tested, except for HA, competed for ^{125}I-HA binding to 190hHARE Flp-In 293 cells at 4 °C (Figure 7). ^{125}I-HA binding in the presence of the other nine GAGs ranged within 10% of the no-addition control value. The binding of GAGs other than HA to the 190-kDa hHARE protein, thus, appears to be very temperature-dependent.

The ability of the 190-kDa hHARE to interact with GAGs was also assessed in a ligand blot format in which whole cell extracts were probed, in a Western blot format, with ^{125}I-HA (23). As shown in Figure 1*A*, the level of hHARE protein expression in extracts is high enough to obtain an excellent signal, by autoradiography, in this assay within 6–18 h. The ability of various GAGs to compete for ^{125}I-HA binding to the 190-kDa hHARE in the ligand blot assay (Figure 8) closely paralleled the pattern seen for competition of endocytosis by cells, with the exception of HS. No competition was observed with KS or Hep. As with live cells, Chon, DS, and all the CS types showed significant competition. In contrast, 100 µg/ml HS showed no effect on HA endocytosis in live cells (Figure 6*B*), whereas 50 µg/ml HS inhibited ^{125}I-HA binding by 40% in the *in vitro* ligand blot assay.

It should be informative to compare the GAG specificities of the rat and human small HARE isoforms, because the amino acid sequences of the extracellular domains of these two proteins are 80% identical (10). Such GAG specificity differences might reflect significant differences between species in the biology of HARE or its role in GAG turnover. Figure 9 compares the abilities of various GAGs to compete for ^{125}I-HA endocytosis by isolated rat LECs expressing both rat HARE isoforms, SK-HARE cells expressing the 175-kDa rHARE, and Flp-In 293 cells expressing the 190-kDa hHARE. In each of the three cell types, little or no competition was observed with KS, HS, or Hep. DS competed for HA uptake to the same slight extent (~25–30%) in cells expressing either hHARE or rHARE. The four CS variants competed for HA uptake by both HARE proteins, although the patterns were not identical. The effects of CS-A and CS-D were essentially the same, whereas the preference for CS-C or CS-E was switched between the rat and human HARE proteins. HA-binding to the hHARE was competed better by CS-E, whereas binding to the rHARE was competed better by CS-C. The greatest difference between the rat and human HARE was observed for competition by Chon. The hHARE appeared to interact more strongly with Chon (50% inhibition) than did the rHARE (~10% inhibition).

Table I. Surface and intracellular HA binding and receptor recycling during endocytosis. Stable Flp-In 293 cell lines transfected with empty vector (clone #EV13) or the 190 hHARE cDNA (clones #9 and #14) were grown to confluence, chilled on ice, washed with HBSS, and incubated with medium containing 1.5 µg/ml ^{125}I-HA with or without digitonin as described under "Experimental Procedures" to assess total or cell surface binding, respectively. A set of parallel cell cultures was incubated at 37 °C for 4 h in medium containing 1.5 µg/ml ^{125}I-HA. Nonspecific binding or endocytosis was assessed in the presence of a 100-fold excess of unlabeled HA. Specific values shown are the mean ± S.E. (n = 10 for clones #9 and #14) or the average of duplicates for clone #EV13. The receptor recycling ratio is the amount of specific HA endocytosis divided by the specific cell surface HA binding. Regardless of cell type, when vertebrate cells are treated with digitonin under the conditions used here, ~50% of the total cellular protein is lost, representing the cytoplasmic contents (24). Therefore, the protein yield for permeable cells is about half that for intact cells.

Clone #	Surface	Specificity	Total	Specificity	Endocytosis	Specificity	HARE recycling
	cpm/µg	*%*	*cpm/µg*	*%*	*cpm/_g*	*%*	*Endo/surface*
9	12.4 ± 1.9	63.3 ± 10.0	54.2 ± 15.8	75.2 ± 9.5	300.8 ± 34.8	90.1 ± 2.0	24.8 ± 4.2
14	11.9 ± 3.0	50.7 ± 17.3	62.7 ± 21.7	71.3 ± 10.8	365.4 ± 26.8	90.0 ± 2.1	32.3 ± 8.1
EV13	1.3		12.3		1.6		1.3

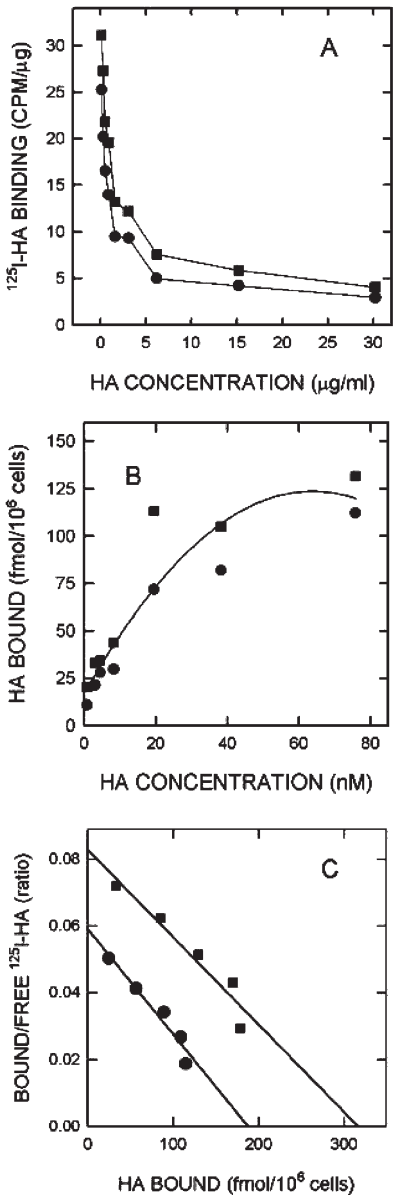


Figure 5. Kinetic and Scatchard analyses of ^{125}I-HA binding to Flp-In 293 cells expressing recombinant 190-kDa hHARE. 190hHARE cell lines #9 (■) and #14 (●) were cultured in 4- or 6-well plates until confluent. The cells were incubated for 60 min at 37 °C in medium without serum and then chilled to 4 °C for all subsequent steps. The cells were washed with HBSS, permeabilized with 0.055% digitonin for 15 min, and then washed with HBSS. At the end of each experiment, cells were washed with HBSS and cell-associated radioactivity and cell protein were determined as described under "Experimental Procedures." *A*, after washing the cells, medium containing 0.1 μg/ml ^{125}I-HA with the indicated amount of unlabeled HA was added to each well, and the cells were allowed to bind the HA on ice for 90 min. Specific binding was ~90%, as assessed in the presence of the highest HA concentration. *B*, the data in *A* for clones #9 and #14 were recalculated as specific HA binding (femtomoles of HA/10^6 cells). The data are the mean ± S.D. of duplicates for each of the two clones (n = 4). *C*, after the cells were allowed to bind HA on ice for 90 min as in *A*, the medium was removed to determine free ^{125}I-HA, and the cells were washed and cell-associated ^{125}I-HA was determined. The specifically bound HA was calculated for the experiment shown in *B*, and the results are presented in the format of Scatchard (29) as the average of duplicates for each cell line.

Inhibition of ^{125}I-HA Endocytosis by Anti-HARE mAbs—We previously developed a panel of eight mouse mAbs against the rat 175-kDa HARE protein to facilitate HARE purification and characterization (8–10, 14). Seven of these mAbs recognize both nonreduced rHARE proteins and were useful for a variety of immunoprocedures. In particular, mAb-174 was extremely useful, because it completely blocks HA binding to the rHARE in LECs (30), in SK-HARE cells (9), or in the ligand blot assay (8). A second mAb, #235, partially inhibited HA binding to rHARE to a level of ~50%, indicating that HA binding likely involves multiple protein regions (epitopes). We recently used mAb-174 to demonstrate that HARE is responsible for the ability of liver to remove circulating HA, because this mAb blocked essentially all ^{125}I-HA uptake in a perfused liver system (30). Although mAb-174 and mAb-235 did not recognize hHARE, three of the seven anti-HARE mAbs (#30, #154, and #159) cross-reacted with both native hHARE isoforms (10) and with the recombinant 190-kDa hHARE (Figure 2*B*). Nonetheless, we tested whether any of the anti-HARE mAbs could inhibit the endocytosis of ^{125}I-HA by 190hHARE Flp-In 293 cell lines (Figure 10). Surprisingly, although mAb-159 had no effect on HA uptake (even at 30 μg/ml), partial inhibition of specific HA endocytosis was observed with both mAb-30 and mAb-154 (Figure 10*A*). Negative controls for these effects included the other four anti-HARE mAbs (#28, #174, #235, and #467), as well as IgG and mouse serum (not shown), any of which caused ≤8% inhibition at concentrations up to 30 μg/ml. Experiments to assess the effects of various mAb combinations on HA uptake at 37 °C showed that the inhibitory mAbs (#30 and #154) were not additive (Figure 10*B*). The maximum partial inhibition of specific HA endocytosis by mAb-30 or mAb-154 was, respectively, ~20–30% and 50–60%.

Discussion

HA and CS turn over continuously in ECMs throughout the body. For humans, the HA turnover rate is so fast (*e.g.* ~24 h in skin) that about one-third of total body HA is degraded and resynthesized daily (6). Partially digested native HA molecules are released from tissue matrices as large HA fragments of ~10^6 Da that would still contain bound aggregating proteoglycans (*e.g.* aggrecan or brevican) and Link proteins (31–33). The released ECM fragments would also contain covalently attached CS and other GAG chains, as well as a variety of bound ECM proteins and growth factors. Thus, multiple components associated with these HA-proteoglycan fragments are simultaneously released from an ECM and then enter lymphatic vessels and flow to regional lymph nodes. Lymph nodes are the initial and primary sites for the clearance of the HA and CS, accounting for ~85% of the HA degradation. Liver is the second clearance site, after the lymph node effluent enters the circulation, accounting for ~15% of the total body HA, and presumably CS, turnover. The clearance and degradation of HA and CS in liver and lymph nodes is mediated by HARE, which is expressed in the sinusoidal endothelial cells of these tissues (8–13).

Although no studies have yet addressed its role in normal health and in various diseases or pathologies, HARE is likely to be important in human physiology. Despite the high turnover rate of HA, the normal steady-state concentration of HA in blood (*i.e.* 10–100 ng/ml) is very low (2, 6). The HA/CS clearance systems utilizing HARE in lymph node and liver, therefore, function very efficiently, indicating that the removal of HA from lymph fluid and blood is important for normal health. First, one would predict that, if HA levels increased, particularly if the HA mass was large, then the increased viscosity of blood might create potentially adverse situations, *e.g.* erythrocyte passage in narrow microcapillaries could be impaired. Second, because HA binds to human fibrinogen (34) and stimulates fibrin clot formation *in vitro* (35), elevated HA levels could alter normal coagulation homeostasis. Finally,

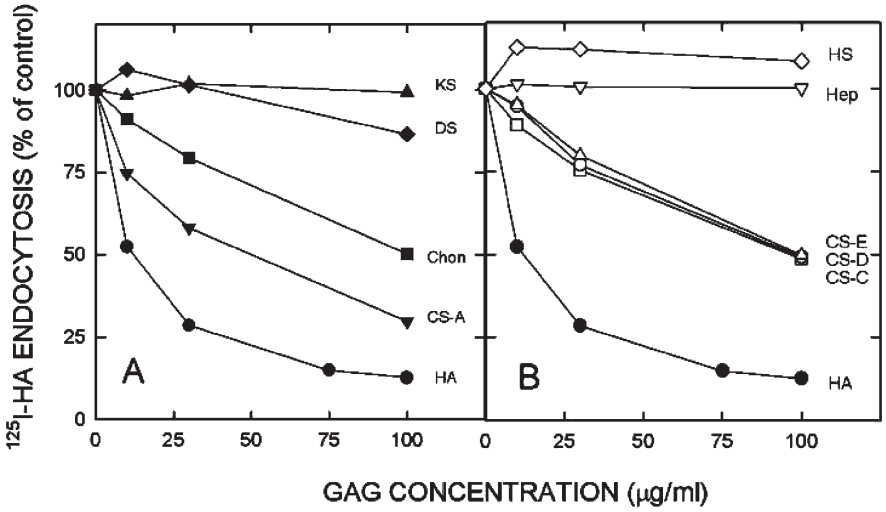


Figure 6. Only some non-HA GAGs compete for ^{125}I-HA endocytosis at 37 °C by stable cell lines expressing the recombinant 190-kDa hHARE. Cells from 190-kDa hHARE Flp-In 293 clones #9 and #14 were incubated at 37 °C for 3 h in medium containing 1.5 μg/ml ^{125}I-HA with 3 to 100 μg/ml of the indicated GAG. The values for competition of ^{125}I-HA internalization by unlabeled GAGs or HA (expressed as a percentage of the no-competitor control) are the average of duplicates from the two clones (n = 4). *A*, keratan sulfate (▲), dermatan sulfate (♦), chondroitin (■), chondroitin sulfate A (▼), HA (●). *B*, heparan sulfate (◊), heparin (▽), chondroitin sulfate E (Δ), chondroitin sulfate D (○), chondroitin C (□), and HA (●).

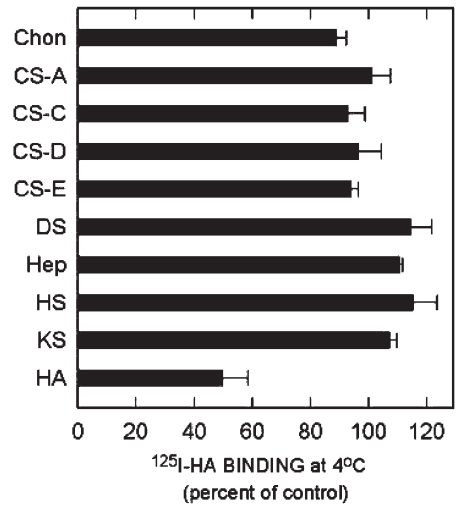


Figure 7. Chondroitin sulfates and other GAGs do not compete well for ^{125}I-HA binding at 4 °C by Flp-In 293 cells expressing the 190-kDa hHARE. After a serum-free incubation and wash with HBSS, cells from 190-kDa hHARE clones #9 and #14 were incubated at 4 °C for 2 h in medium containing 1.5 μg/ml ^{125}I-HA and 50 μg/ml of the indicated GAG. The values for each GAG are the mean of duplicate samples from both clones (n = 4) ± S.E.

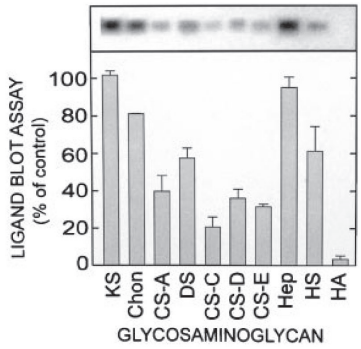


Figure 8. Other GAGs compete for ^{125}I-HA binding to recombinant 190-kDa hHARE in a ligand blot assay. Extracts prepared from 190hHARE clone #14 cells were subjected to SDS-PAGE and electroblotted as described under "Experimental Procedures." The nitrocellulose was blocked with TBS and 0.1% Tween 20 at room temperature for 3 h, and 3-mm strips were cut and placed in Buffer 1 with 0.5% sodium azide, 5 mm EDTA, 0.05 μg/ml ^{125}I-HA, and 50 μg/ml of the indicated GAG or HA. The strips were incubated at 4 °C for 2 h on a rocking platform. The medium was then removed, and the strips were washed extensively with TBST for 20 min, allowed to air dry, and attached to filter paper for autoradiography with BioMax MS film. The exposure shown was for 19.5 h at –85 °C with two intensifying screens. The *graph* shows the average densitometry values ± S.E. from three separate samples for each GAG.

several diseases, including some cancers (36), psoriasis (37), scleroderma (38), rheumatoid arthritis (39), and liver cirrhosis (40, 41), are associated with elevated levels of HA in serum. Over the last decade, numerous studies have suggested that the HA clearance function of liver can be used as a diagnostic tool to detect and monitor liver failure (42). This hepatic function of LECs may also be a prognostic indicator of success in liver transplant patients.

In this study, we created an artificial cDNA to express a recombinant form of the small spleen hHARE isoform in stable cell lines. This enabled us to characterize for the first time the GAG specificity and endocytic activity of the small hHARE isoform in the absence of the larger hHARE isoform. Several key characteristics of the 190-kDa hHARE are very similar to those of the 175-kDa rHARE. Both smaller HARE isoforms are functional endocytic HA receptors with the appropriate, as yet unidentified, sorting signals for targeting HARE to coated pits and then through an intracellular receptor recycling itinerary. Each HARE, although expressed in different cell types, mediated the continuous endocytosis of HA and its delivery to lysosomes for degradation. The rate of hHARE recycling in Flp-In 293 cell lines (*i.e.* one cell surface equivalent per 7–9 min) was comparable to that determined in primary rat LECs (17). The apparently slower rate of rHARE recycling in SK-Hep-1 cell lines (~20 min) is likely due to a decreased capacity of the coated pit pathway in this latter cell line (9, 43, 44), rather than to intrinsic differences between the recombinant rHARE and hHARE. The affinities of the smaller rHARE and hHARE were also very similar, with K_d values of 4.1 and 7.2 nm, respectively.

Two significant differences, which might be related, between the rat and human HARE proteins are their slightly

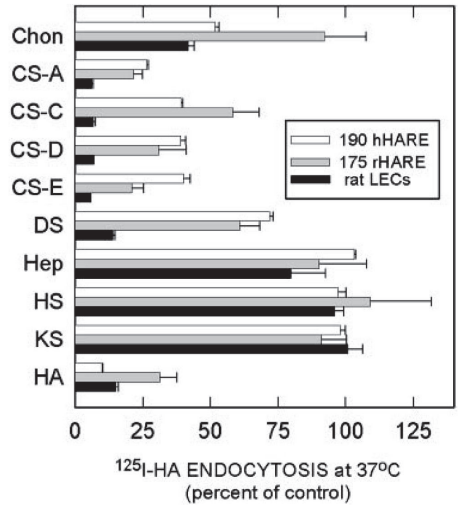
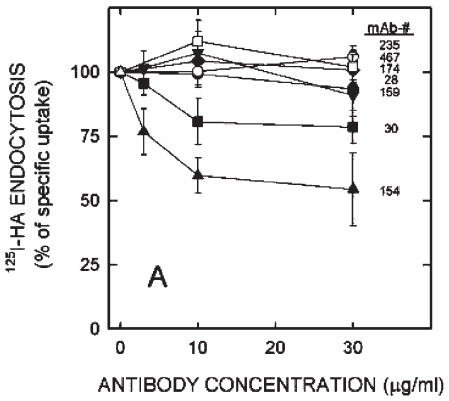


Figure 9. The human and rat small HARE isoforms show different GAG specificities for competition of ^{125}I-HA endocytosis. Flp-In 293 190hHARE clones #9 and #14 (*white bars*), SK-HARE clones #26 and #35 (expressing recombinant the rat 175-kDa HARE), and liver sinusoidal endothelial cells (from freshly perfused rat liver) were incubated at 37 °C for 3 h with medium containing either 1 μg/ml ^{125}I-HA and 30 μg/ml of the indicated GAG (for SK-HARE and liver cells) or 1.5 μg/ml ^{125}I-HA and 100 μg/ml of the indicated GAG (for 190hHARE 293 cells). Cells were then washed and lysed, and the radioactivity and protein content were determined as described under "Experimental Procedures." Each GAG value is the mean ± S.E. of at least four individual wells (*e.g.* two wells for each of two clones or LEC preparations) and is calculated as a percentage of the ^{125}I-HA control (without competitor).



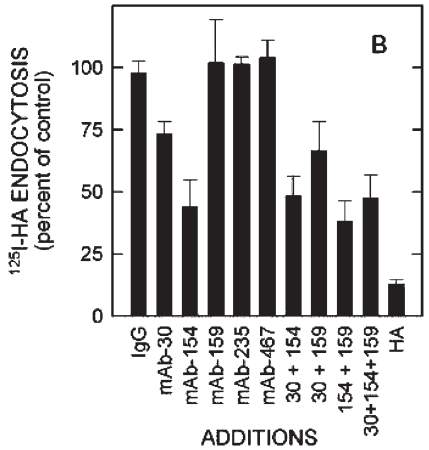


Figure 10. Inhibition by anti-HARE mAbs of ^{125}I-HA uptake by cells expressing recombinant 190-kDa hHARE. Flp-In 293 190hHARE clones #9 and #14 were cultured and processed as described in Figure 4. *A*, the cells were allowed to bind and endocytose 1.5 μg/ml of ^{125}I-HA for 3 h at 37 °C with no additions, or the noted concentration of anti-rat HARE mAbs 28 (●), 30 (■), 154(▲), 159 (▼), 174 (♦), 235 (○), and 467 (□). The cells were processed as described under "Experimental Procedures." The values shown are the average of two replicate wells from each clone (n = 4), expressed as a percentage of the no-addition control specific binding values. Mouse IgG (not shown) at 10 μg/ml was 103 ± 5% of the control value. Specific binding (CPM/μg of cell protein), as assessed in the presence of a 100-fold excess of unlabeled HA, was 87%. *B*, the indicated purified antibodies were used singly or in combination, at concentrations of 20 μg/ml, in an experiment performed as described in *A*. Nonspecific endocytosis was assessed in the presence of 75 μg/ml unlabeled HA.

different GAG specificities and their very different profiles for anti-HARE mAb inhibition of HA binding. The 190-kDa hHARE has a broad specificity for sulfated and nonsulfated GAGs, yet this recognition is not indiscriminant, because HA binding is not affected by KS, HS, or Hep. Thus, the 190-kDa hHARE recognizes HA and chondroitin, the two least negatively charged GAGs, as well as three CS variants with different levels and patterns of sulfation. The two GAGs with the greatest negative charge, HS and Hep, are not recognized. In contrast, all the CS variants tested were able to compete for HA binding. Although the pattern of inhibition by CS variants was similar to that for the 175-kDa rHARE, it was not identical. In particular, the two HARE species differ quantitatively in their recognition of CS-C and CS-E.

The GlcUA-GlcNAc disaccharide units in HA and the GlcUA-GalNAc disaccharide units of chondroitin were recognized by the 190-kDa hHARE. HARE also recognized all of the sulfated CS types tested, despite differences in the position and number of sulfates among their disaccharide units. Chondroitins sulfated at GalNAc positions C4, C6, or C4,6 (*i.e.* CS-A, CS-C, and CS-E) or at C6 of GalNAc and C2 of GlcUA (*i.e.* CS-D) were effective competitors of HA binding, although none were as effective as HA. CS-A was a slightly better competitor than the other CS types, all of which were essentially identical. DS (also called CS-B) was the weakest competitor. KS contains Gal rather than a uronic acid and was not a competitor. Although Hep and HS are very highly sulfated GAGs, they were not able to compete for HA binding to the 190-kDa hHARE. Perhaps HARE can recognize *N*-acetyl groups in the amino sugars of some GAGs, but not the *N*-sulfated glucosamine residues characteristic of Hep and HS.

The greatest difference in GAG specificity between the rat and human proteins was with Chon, which poorly competes for HA binding by the rHARE. The 190-kDa hHARE protein appears to recognize Chon almost as well as most of the CS types. These slight differences between species in relative preference for various GAGs may be reflected in the more dramatic differences in the inhibition of their HA binding ability by mAbs. HA binding and endocytosis by the small (or large) rat HARE proteins is completely blocked by mAb-174 and partially blocked by mAb-235, whereas no inhibition of ligand binding or uptake was observed with the other five anti-HARE mAbs that recognize these proteins in Western blots and various immunoprocedures. In contrast, we found here that mAb-30 and mAb-154 partially inhibit HA binding and endocytosis by the 190-kDa hHARE. The other anti-HARE mAbs, including mAb-174 and mAb-235, were not inhibitory. Thus, although mAb-30 and mAb-154 bind to both rHARE and hHARE, this binding only inhibits HA recognition by the latter protein.

Another distinctive and unusual feature of the 190-kDa hHARE was that GAG inhibition of its binding to HA was temperature-sensitive. Although multiple GAGs were able to block the binding and endocytosis of ^{125}I-HA mediated by

hHARE at 37 °C, none of these GAGs could compete for ^{125}I-HA binding at 4 °C. Presumably, the extracellular domain of the 190-kDa hHARE undergoes a substantial conformational change between 37 °C and 4 °C that virtually eliminates the binding of GAGs other than HA. However, because our binding studies were indirect and only monitored the binding of ^{125}I-HA, it is possible that a conformational change could also create a situation wherein both GAGs might bind to separate sites without interference. Distinguishing between these possibilities must await further direct binding studies between the various GAGs and the purified 190-kDa hHARE or its extracellular domain.

Taken together the above results indicate that amino acid sequence differences between the small rat and human HARE proteins may alter their GAG specificity slightly. These sequence differences may also alter conformations that occur when mAbs bind to the proteins, so that GAG binding is affected indirectly, and in a species dependent way, by formation of a mAb·HARE complex. It is likely that mAb-30 and mAb-154 recognize epitopes that are not directly involved in HA binding by hHARE but that steric factors limit interactions of the protein with HA. The finding that inhibition with either mAb is only partial indicates that hHARE likely contains multiple HA binding regions. It also indicates that each hHARE protein may bind more than one HA molecule. Again, these or other possible explanations will require epitope mapping studies and studies to define the GAG binding regions within the extracellular domain of HARE.

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