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A Hyaluronan Receptor for Endocytosis (HARE) Link Domain *N*-Glycan Is Required for Extracellular Signal-regulated Kinase (ERK) and Nuclear Factor-κB (NF-κB) Signaling in Response to the Uptake of Hyaluronan but Not Heparin, Dermatan Sulfate, or Acetylated Low Density Lipoprotein (LDL)*

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Background: The HARE HA binding Link domain contains *N*-glycans at Asn^{2280}.
Results: HARE(N2280A) cells endocytose HA or Hep normally; NF-κB signaling occurs normally during uptake of heparin, dermatan sulfate, or acetylated LDL but not hyaluronan.
Conclusion: HA·HARE-mediated signaling requires a link domain *N*-glycan.
Significance: A different mechanism for HA·HARE signaling *versus* other ligands indicates a physiologic need for special control of responses to circulating HA.

The human hyaluronan (HA) receptor for endocytosis (HARE; the 190-kDa C terminus of Stab2) is a major clearance receptor for multiple circulating ligands including HA, heparin (Hep), acetylated LDL (AcLDL), dermatan sulfate (DS), apoptotic debris, and chondroitin sulfate types A, C, D, and E. We previously found that HARE contains an *N*-glycan in the HA binding Link domain (at Asn^{2280}), and cells expressing membrane-bound HARE(N2280A) bind and endocytose HA normally (Harris, E. N., Parry, S., Sutton-Smith, M., Pandey, M. S., Panico, M., Morris, H. R., Haslam, S. M., Dell, A., and Weigel, P. H. (2010) *Glycobiology* 20, 991–1001). Also, NF-κB-mediated signaling is activated by HARE-mediated endocytosis of HA, Hep, AcLDL, or DS but not by chondroitin sulfates (Pandey, M. S., and Weigel, P. H. (2014) *J. Biol. Chem.* 289, 1756–1767). Here we investigated the role of Link *N*-glycans in ligand uptake and NF-κB and ERK1/2 signaling. HA·HARE-mediated ERK1/2 activation was HA size- dependent, as found for NF-κB activation. HARE(N2280A) cells internalized HA, Hep, AcLDL, and DS normally. No ERK1/2 activation occurred during HA endocytosis by HARE(N2280A) cells, but activation did occur with Hep. Dual-luciferase recorder assays showed that NF-κB-mediated gene expression occurred normally in HARE(N2280A) cells endocytosing Hep, AcLDL, or DS but did not occur with HA. Activation of NF-κB by endogenous degradation of IκB-α was observed for HARE(N2280A) cells endocytosing Hep, AcLDL, or DS but not HA. We conclude that a Link domain complex *N*-glycan is required specifically for HARE·HA-mediated activation of ERK1/2 and NF-κB-mediated gene expression and that this initial activation mechanism is different from and independent of the initial mechanisms for HARE-mediated signaling in response to Hep, AcLDL, or DS uptake.

The hyaluronic acid receptor for endocytosis (HARE)[2] (1, 2), also designated Stabilin-2 (3) or FEEL-2 (4), is the major systemic clearance receptor for at least 14 ligands (5): oxidized or acetylated LDL (AcLDL), advanced glycation end products, $Gram^-$ and $Gram^+$ bacteria, chondroitin, chondroitin sulfates (types A, C, D, and E), collagen N-terminal pro-peptides, dermatan sulfate (DS), heparin (Hep), hyaluronic acid (HA), αMβ2 integrin (4, 6–9), and phosphatidylserine (10); the latter is a ligand for clearance of apoptotic cells and debris. The HARE ectodomain has two different sites for binding HA or Hep (11). The two functional isoforms of human Stab2 (full-length 315-kDa Stab2 and HARE, a proteolyic 190-kDa product containing the C-terminal 1416 amino acids of Stab2) are highly expressed in the sinusoidal endothelial cells of liver, lymph node, bone marrow, and spleen (1, 12). HARE/Stab2 are also expressed in corneal and lens epithelium, heart valve mesenchymal cells, epithelial cells covering renal papillae, oviducts, and macrophages (10, 13). The 72-amino acid cytoplasmic domain of human HARE/Stab2 contains four endocytic motifs, three of which are needed for optimal clearance of HA, with NPLY being the most important (15).

HARE-mediated ligand endocytosis stimulates several cell signaling pathways. HA uptake by HARE stimulates the activa-

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[2] The abbreviations used are: HARE, 190-kDa hyaluronic acid receptor for endocytosis, the C-terminal half of Stab2; Ab, antibody (IgG); AcLDL, acetylated low density lipoprotein; b-HA, biotinylated hyaluronan; b-Hep, biotinylated heparin; CS, chondroitin sulfate; DS, dermatan sulfate; EV, empty vector; HA, hyaluronic acid, hyaluronate, hyaluronan; Hep, heparin; LUC, luciferase; N2280A cells, cells expressing the HARE(N2280) mutant; SA, streptavidin; Stab2, Stabilin-2; WT cells, cells expressing wild type HARE; pERK1/2, phospho-ERK1/2; tERK1/2, total-ERK1/2; TBST, Tris-buffered saline Tween 20.

tion of extracellular signal-regulated kinases 1 and 2 (ERK1/2) in a dose- and time-dependent manner (16), and anti-inflammatory cytokine release is stimulated in macrophages phagocytosing apoptotic cells via HARE binding to phosphatidylserine (10). We reported that HA·HARE interactions stimulate NF-κB activation of gene expression and that HARE displays an HA size-sensing mechanism for detecting and responding to a narrow size range (40–400 kDa) of HA degradation products (17). We examined seven other HARE ligands for their ability to stimulate signaling and discovered that DS, AcLDL, or Hep uptake also activates NF-κB-mediated gene expression but that CS types A, C, D, and E are inactive (18). As with HA, the latter four ligands also bind to the Link domain, and all four can compete for HA binding. In contrast, DS, AcLDL and Hep are bound at a second independent site and are not competed by HA or the CS types (11). Clathrin mRNA knockdown experiments show that NF-κB activation by any of the signaling ligands is dependent on the coated pit mediated endocytosis of HARE·ligand complexes.

We previously analyzed the *N*-glycans within the human 190-kDa HARE ectodomain, expressed in Flp-In 293 cells, and found that 10 Asn sites are occupied with multiple glycoforms of varying complexity. Most interesting to us was that the site with the greatest diversity of glycans was Asn^{2280} in the Link domain; it contained 15 structures and was the only site with sialylated complex glycans. Cells expressing HARE(N2280A), lacking a Link domain *N*-glycan, endocytosed HA normally.

In the present study, we used stable Flp-In 293 cell lines expressing N2280A or wild type (WT) HARE to investigate the role of the Link *N*-glycan at Asn^{2280} on the ability of HARE·ligand complexes to activate the ERK1/2- and NF-κB-mediated signaling pathways. The results demonstrate a surprisingly ligand-specific requirement for an Asn^{2280} glycan on HA·HARE-mediated ERK1/2 and NF-κB activation. In contrast, Hep·HARE-mediated activation of the ERK1/2 and NF-κB signaling pathways occurred normally in the absence of a Link domain Asn^{2280} glycan.

EXPERIMENTAL PROCEDURES

Cells, Plasmids, Reagents, and Buffers—Flp-In 293 (HEK) cells, DMEM, Lipofectamine 2000, Lipofectamine LTX and PLUS reagents, fetal bovine serum, glutamate, hygromycin B, and Zeocin were from Invitrogen. Streptavidin (SA), biotin-LC-hydrazide, and sulfo-NHS-SS biotin were from Pierce. Plasmid DNA purification and cloning kits were from Qiagen (Valencia, CA) and Fermentas (Glen Burnie, MD). ^{125}I-SA was prepared as described previously (9, 19, 20). Plasmid vectors pGL4.32[luc2P/NF-κB-RE/Hygro], Dual-LUC Reporter Assay System (E1960), and Luminometer Glomax 20/20 were from Promega (Madison, WI). Stable cell lines expressing WT HARE, HARE(N2280A) clone 1, HARE(ΔLink), or empty vector (EV) were generated as described previously (7, 21) using Flp-In 293 cells, which are engineered to contain a selectable recombinase insertion site; the cell lines used here were verified to have the correct single insertion. Unfractionated Hep (~17 kDa) was from Celsus (Cincinnati, OH) or Sigma. CS types A, C, D, and E and DS were from Seikagaku (Japan), and AcLDL was from Kalen Biomedicals (Montgomery Village, MD). Narrow size-range HA preparation of 14, 80, 107, and 178 kDa were made and characterized as described earlier (17). Commercial HA preparations of 51, 741, and 2600 kDa were obtained from Lifecore Biomedical, LLC (Chaska, MN), and 133-kDa Select-HA™ was from Hyalose, LLC (Oklahoma City, OK). All HA weight-average mass values were determined by size exclusion chromatography coupled to multiangle light scattering analysis (17, 22, 23). Enhanced Chemiluminescence substrate for Western blot analysis was from PerkinElmer Life Sciences. Affinity-purified goat anti-V5 polyclonal antibody was obtained from Bethyl Laboratories (Montgomery, TX). Rabbit anti-phospho-ERK1/2 (p44/42; $Thr(P)^{202}$ and $Tyr(P)^{204}$), anti-ERK1/2 and anti-IκB-α, and mouse anti-actin Abs were from Cell Signaling (Beverly, MA). Goat anti-rabbit IgG-HRP, donkey anti-goat IgG-HRP, and donkey anti-mouse IgG-HRP were from Santa Cruz Biotechnology. Protease inhibitor mixture was from Sigma (containing sodium fluoride, sodium orthovanadate, benzamidine, 2-mercaptoethanol, EGTA, EDTA, 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64, sodium pyrophosphate, and Tween 20). Optimum™ autoradiography film was from Life Sciences Products (Frederick, CO), and nitrocellulose membranes were from Schleicher & Schuell. Other reagents were of the highest purity or grade available from Sigma. The compositions of PBS, Hanks' balanced salt solution, lysis buffer, blocking buffer, stripping buffer, TBST, and other buffers were described previously (15, 16). Endocytosis medium contained DMEM with 0.05% bovine serum albumin (no serum). Compete medium contained DMEM plus 8% fetal bovine serum and 100 μg/ml hygromycin B. Transfection medium contained DMEM with 8% fetal bovine serum without antibiotics, and preincubation medium contained DMEM without FBS or hygromycin.

Preparation of ^{125}I-SA·b-HA or ^{125}I-SA·b-Hep Complexes—Radiolabeled complexes for HA (^{125}I-SA·b-HA) or Hep (^{125}I-SA·b-Hep) for endocytosis assays were prepared as noted (9); a 2:1 molar ratio of b-HA or b-Hep and ^{125}I-SA was incubated in 0.5 ml of endocytosis medium for 1 h on a rotary mixer at 22 °C just before the experiment. For nonspecific binding and uptake controls, a 2:1 ratio of free biotin and ^{125}I-SA was used to make ^{125}I-SA·biotin. The pre-formed radiolabeled complexes were diluted in endocytosis medium to the final concentrations indicated.

Endocytosis of ^{125}I-SA·b-HA or ^{125}I-SA·b-Hep—Stable cell lines expressing WT HARE, HARE(N2280A), or EV were plated in 12-well plates and grown in complete medium to 80–90% confluence before experiments. Cells were washed with Hanks' balanced salt solution and incubated at 37 °C for 1 h with endocytosis medium to allow uptake of any bound serum glycosaminoglycans. The medium was aspirated and replaced with endocytosis medium containing 50 nM preformed complexes of ^{125}I-SA·b-HA or ^{125}I-SA·b-Hep. Nonspecific uptake was assessed in parallel samples by incubating cells with ^{125}I-SA·biotin complexes. After the indicated times at 37 °C, the medium was removed by aspiration, and cells were washed 3 times (2 ml each) with ice-cold Hanks' balanced salt solution to remove unbound radioligand and solubilized in 1 ml of 0.3 N NaOH. Radioactivity was measured using a Packard Cobra II gamma counter, and lysate protein content was deter-

mined by the method of Bradford (24) using bovine serum albumin as standard. Specific uptake of ^{125}I-SA·b-HA or ^{125}I-SA·b-Hep by each cell type was calculated by subtracting cell-associated ^{125}I-SA·biotin. HARE-specific binding and uptake of ^{125}I-SA·b-HA or ^{125}I-SA·b-Hep by HARE(N2280A) cells was then determined by normalizing HARE expression relative to WT using Western blot quantification of equal lysate protein samples (15). Result values are expressed as the mean ± S.E. specific fmol/μg of cell protein/HARE.

Cell Culture and Stimulation with HA or Hep for ERK1/2 Activation—WT, N2280A, or EV cells were grown in complete medium till confluence and then plated in 12-well tissue culture plates as described (17). The cells were grown in complete medium for at least 2 days (80–90% confluence) before experiments. Cells were washed with sterile PBS and incubated in fresh medium without serum for 1 h at 37 °C (16). Cells were washed and then incubated at 37 °C in fresh serum-free medium containing 10 μg/ml HA (80 kDa, 125 nM) or Hep (~500 nM) for the indicated times. To determine the HA size dependence for ERK activation, cells were incubated for 20 min with 10 μg/ml HA of different weight-average molar masses (14, 51, 178, 741, or 2600 kDa). Cells were then washed with ice-cold PBS and lysed with lysis buffer. The cell lysates were collected, stored on ice, vortexed repeatedly, and then centrifuged at 12,000 × *g* for 10 min at 4 °C to remove cell debris. Protein concentration in each cell lysate supernatant was determined by Bradford assay (24).

Western Blotting Analysis—Western blotting was performed as noted (16) with some modifications. Briefly, 25 μg of cell lysate protein was resolved in 10% SDS-PAGE and electrotransferred to nitrocellulose using a mini-transfer unit (Bio-Rad) for 1 h at 4 °C at 110 V in 25 mM Tris-HCl, pH 7.4, 190 mM glycine, and 20% (v/v) methanol. Membranes were washed in TBST for 5 min and incubated with 5% nonfat dry milk in TBST for 1 h at 22 °C. For phospho-ERK1/2 (pERK1/2) detection, the preblocked membranes were incubated overnight at 4 °C with the Ab to pERK1/2 (1:1000 dilution in blocking buffer). After washing 3 times for 5 min each in TBST, the membranes were incubated with anti-rabbit IgG-HRP conjugate (1:2500 dilution in blocking buffer) at 22 °C for 1 h. The membranes were washed 3 times for 5 min each with TBST, developed using enhanced chemiluminescence substrate, and exposed to autoradiography film. For total-ERK1/2 (tERK1/2) detection, the same membranes were stripped to remove bound the Ab by washing with water for 5 min and then incubating in stripping buffer at 55 °C for 30 min. After five washings with TBST for 10–15 min each, the blots were reprobed with tERK1/2 (1:1000). For normalizing protein load in each well, the membranes were further stripped and reprobed with anti-actin Ab. Similarly, for determination of HARE expression by WT and N2280A cells, preblocked membranes were incubated with 20 ng/ml anti-V5 Ab (Bethyl Laboratories), washed 3 times for 5 min each in TBST, incubated with the appropriate anti-goat IgG-HRP (1:2000 dilution), and processed as above. Autoradiographic band densities were scanned into digital files and quantified as integrated densitometry values (*i.e.* the sum of all pixel values minus background correction) using an Alpha Innotech FluoroChem 8000 imaging system (Alpha Innotech Corp., San Leandro, CA). ERK1/2 activation in response to HA, Hep, or the control (buffer) treatment is expressed as a ratio (pERK:tERK) and compared with the mock treatment as 100%.

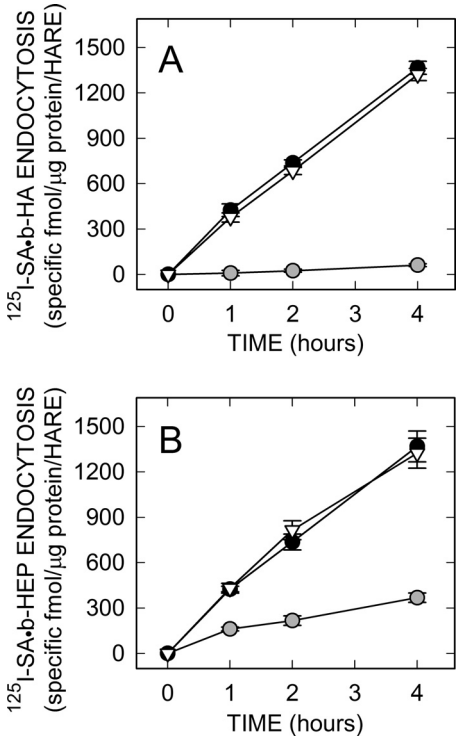

FIGURE 1. **Cells expressing HARE(N2280A) endocytose HA and Hep normally.** WT (●), N2280A (▼), or EV (○) cell lines were incubated with ^{125}I-SA·b-HA (107 kDa; *A*) or ^{125}I-SA·b-Hep (*B*) for the indicated times at 37 °C. Similarly, the three cell types were incubated with ^{125}I-SA·biotin and processed as described under "Experimental Procedures" to determine nonspecific (not shown) and specific endocytosis of HA or Hep. Based on three independent experiments, values are the means ± S.E. (n = 9) of specific cell-associated ligand (fmol/μg of protein/HARE); HARE expression in N2280A cells was normalized relative to WT HARE expression, which was 100%.

Transient Transfection with Firefly and Renilla LUC—WT, N2280A, or EV cells were grown in complete medium till confluence, plated in 12-well tissue culture plates, and grown in complete medium for at least 2 days until cells reached 60–70% confluence. The medium was replaced with fresh transfection medium 10 min before transfection, transfection complexes were generated, and cells were transiently transfected as described (17).

Stimulation of Cell Lines with HARE Ligands and LUC Assays—WT, N2280A, or EV cells transiently transfected with firefly and *Renilla* LUC vectors for 18 h were washed 1 time each with sterile PBS and then with DMEM without serum. Cells were preincubated with fresh serum-free DMEM for 1 h at 37 °C. To assess ligand specificity for HARE-mediated NF-κB-activated reporter gene expression, cells were stimulated for 4 h with low endotoxin HA, Hep, DS, AcLDL, or CS types A, C, D, or E (100 nM) in DMEM. After the indicated times, the medium was aspirated, and cells were processed to determine NF-κB-activated gene expression (17).

IκB-α Degradation Assay—Cells expressing HARE or N2280A were grown to confluence in 6-well plates, washed with sterile PBS, and incubated with serum-free DMEM for 1 h. Cells were then incubated with 1 ng/ml TNF-α, 100 nM HA,

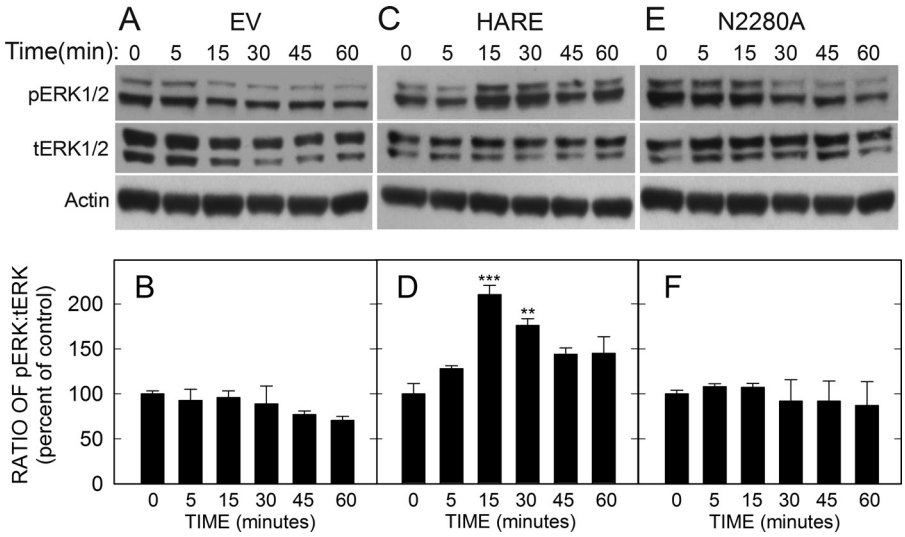

FIGURE 2. **HARE lacking a Link domain *N*-glycan does not stimulate ERK1/2 signaling during HA uptake.** Cells stably expressing EV (*A* and *B*), WT (*C* and *D*), or N2280A (*E* and *F*) HARE were grown to confluence and washed. After 1 h of incubation in serum-free medium at 37 °C, the cells were incubated with 10 μg/ml HA (80 kDa) for the indicated times and processed as in "Experimental Procedures." Sample cell lysates (25 μg) were resolved in 10% SDS-PAGE and transferred to nitrocellulose. Western blot analysis was performed with the Ab against pERK1/2 (*top row*, *top panels*). The same membranes were stripped and reprobed with Ab against tERK1/2 (*middle row*, *top panels*) and then with anti-actin Ab (*bottom row*, *top panels*). Blots from three independent experiments ($n = 3$) were scanned and digitized, and densitometry analysis was performed to determine pERK:tERK ratios at each time point; **, $p < 0.005$; ***, $p < 0.001$.

Hep, DS, or AcLDL or 250 nM CS-A, CS-C, CS-D, or CS-E for 45 min and processed as described previously (18). Equal amounts of cell lysate protein were resolved on 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-IκB-α Ab. The same membrane was reprobed for actin, and the ratio of IκB-α: actin in each sample was determined from the digital images.

Statistical Analysis—Western blot data are presented as the mean ± S.E. based on three independent ($n = 3$) experiments unless noted otherwise, and differences were assessed by analysis of variance as noted below. LUC reporter assay data are presented as the mean ± S.E. based on three independent experiments, each performed in triplicate ($n = 9$). Data were first analyzed by a one-way analysis of variance, and any significant difference in the group was then assessed by individual pair-wise post hoc Tukey's HSD tests using GraphPad Prism v5 statistical software (GraphPad Software, Inc., San Diego). Comparisons were made for none treatment and treatment with the same ligand concentration. Values of $p < 0.05$ were considered statistically significant. Only those samples found to be statistically significant compared with both the no treatment and same ligand or cell type are indicated with a symbol (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$).

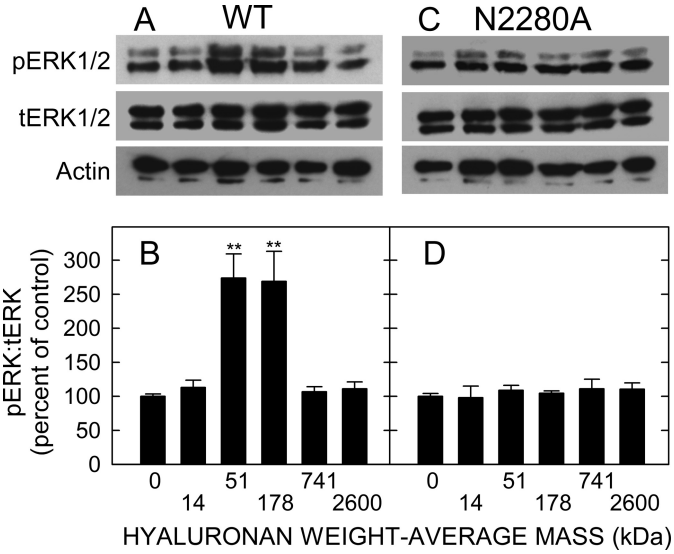

FIGURE 3. **HARE-mediated activation of ERK1/2 is HA size-dependent.** WT and Asn^{2280} cells were treated and processed as in Fig. 2 after incubation for 20 min with 10 μg/ml HA using preparations of different mass as indicated (weight-average molar masses were determined by light scattering as described previously (17, 22)). Normalized data are presented as the mean ± S.E. percent of the pERK:tERK ratio compared with the time 0 (no addition) value as 100%. Values for *p* are based on one-way analysis of variance and pair-wise Tukey's tests as noted under "Experimental Procedures" using pair-wise comparisons of WT or N2280A cells at each time or with each other at the same time. Only the sample sets with significant differences in each case are marked: **, $p < 0.005$.

RESULTS

Endocytosis of HA or Hep Is Not Affected by Loss of the HARE Link Domain N-Glycan—We previously found that Hep and HA bind to distinct, non-overlapping binding sites within the HARE ectodomain (11). Four different CS types also bind to the HA binding site in a competitive manner with HA, whereas DS and AcLDL bind within or near the Hep binding site and compete among themselves to varying extents. Cells expressing EV, WT, or N2280A HARE were assessed for their ability to endocytose complexes of b-HA or b-Hep with ^{125}I-SA (Fig. 1). Flp-In 293 cells were chosen for studies on HARE function because these cells do not express significant levels of other HA receptors, such as CD44 or RHAAM (7). Thus, the level of specific ^{125}I-SA·b-HA uptake by EV cells was very low, whereas the uptake of these complexes by either WT or N2280A cells was much greater and identical (Fig. 1*A*). The presence or absence of a Link domain *N*-glycan had no effect on the ability of HARE to bind and endocytose HA. Similar results were observed for the uptake of ^{125}I-SA·b-Hep by the three cell types (Fig. 1*B*), except that the background level of uptake by EV cells was

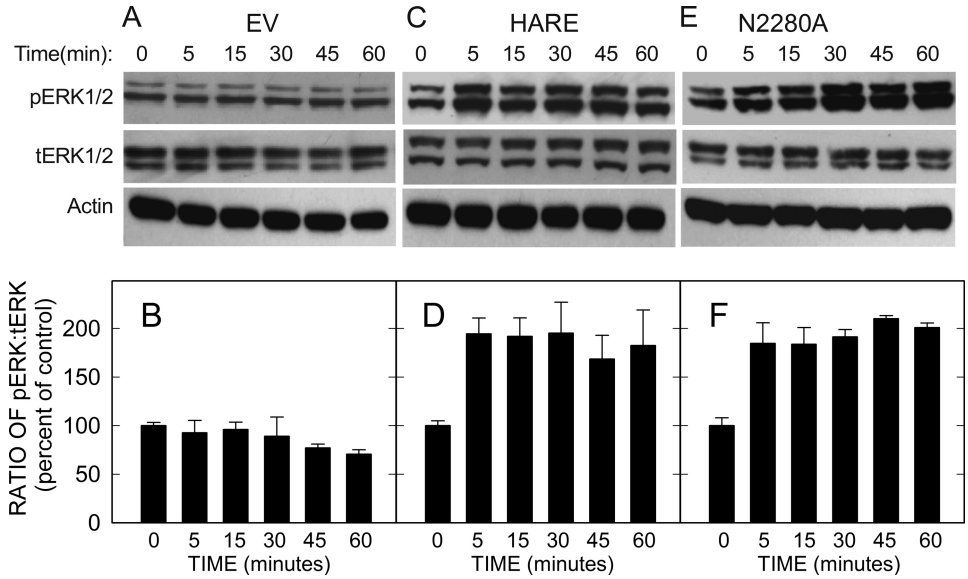

FIGURE 4. **Loss of the Link domain *N*-glycan does not impair Hep-stimulated ERK1/2 activation.** Cells stably expressing EV (*A* and *B*), WT (*C* and *D*), or N2280A (*E* and *F*) HARE were grown to confluence, washed, and incubated for 1 h in serum-free medium at 37 °C. The cells were then incubated with 10 μg/ml Hep (17 kDa) for the indicated times and processed as described under "Experimental Procedures" and in Fig. 2. Western blot analysis was performed with the Ab against pERK1/2 (*top row*, *top panels*), tERK1/2 (*middle row*, *top panels*), and anti-actin Ab (*bottom row*, *top panels*). Normalized data are presented as mean ± S.E. percent ($n = 3$) of the pERK:tERK ratio compared with the time 0 (no addition) value as 100%. Values for p compared EV, WT, and N2280A cells at each time point or with each other. Only the sample sets with significant differences in both cases are marked as noted in Fig. 2 and "Experimental Procedures." For WT (*D*) and N2280A (*F*) cells, all comparisons of time 0 with each indicated time gave p values < 0.005.

greater than for HA, as reported previously (9). The rates of specific ^{125}I-SA·b-Hep endocytosis by WT or N2280A cells were identical and much greater than EV cells. Thus, loss of the *N*-glycan in the HA-binding Link domain does not disrupt the endocytosis of either HA or Hep.

Activation of ERK1/2 in Response to HA·HARE Uptake Does Not Occur When the Link Domain N-Glycan Is Missing—We previously found that HARE-mediated uptake of HA causes activation of ERK1/2 (16), although the downstream components activated via pERK1/2 are not known yet. To assess the effect of Link domain *N*-glycan loss on the ability of HARE to activate ERK in response to HA, we followed tERK1/2 and pERK1/2 in EV, WT, and N2280A cells treated with HA. EV cells showed no changes in pERK1/2 levels over 60 min when treated with HA (Fig. 2, *A* and *B*), whereas WT cells showed a significant biphasic increase in pERK1/2 that peaked at 15 min (Fig. 2, *C* and *D*). However, N2280A cells lacking a Link domain glycan showed no significant changes in pERK1/2 levels in the presence of HA (Fig. 2, *E* and *F*), similar to EV cells.

HARE-mediated Activation of ERK1/2 Is Dependent on HA Size—There is a very pronounced HA size dependence for the ability of HA·HARE complexes to stimulate NF-κB signaling and gene activation (17). The active HA size range is 40–400 kDa, and smaller or larger HA is unable to activate NF-κB even though all HA molecules $> \sim 2$ kDa are bound and internalized. Because the relationship between the ERK1/2 and NF-κB pathways activated by HA·HARE complexes is not known, we assessed whether the activation of ERK1/2 is also dependent on HA size (Fig. 3). Because HA-stimulated ERK1/2 activation was maximal at 15–30 min, we assessed pERK1/2 levels after 20 min in the presence of HA sizes below (14 kDa), within (51 and 178 kDa) and above (741 and 2600 kDa) the activating range. The results showed the same HA size dependence as for NF-κB activation. Only WT cells treated with 51- and 178-kDa HA showed significant stimulation of ERK1/2 ($p < 0.005$). In contrast, treatment of WT cells with smaller (14 kDa) or larger HA (741 and 2600 kDa) did not stimulate ERK1/2 activation (Fig. 3, *A* and *B*). As expected, Asn^{2280} cells treated with any size HA showed no stimulation of ERK1/2 activation (Fig. 3, *C* and *D*).

ERK1/2 Activation in Response to Hep·HARE Uptake Occurs with or without the Link Domain N-Glycan—We also examined the possibility that Hep endocytosis by WT HARE cells stimulates ERK phosphorylation (Fig. 4). WT cells endocytosing Hep showed a similar level of pERK1/2 activation (Fig. 4, *C* and *D*), significantly greater from 5–60 min than EV control cells (Fig. 4, *A* and *B*); interestingly, the response to Hep was sustained longer than the HA response. Unlike the results with HA, ERK1/2 activation by N2280A cells endocytosing Hep (Fig. 4, *E* and *F*) was not altered by Link glycan loss; it was identical with that of WT cells.

NF-κB Activation in HARE(N2280A) Cells Occurs during Endocytosis of Hep but Not HA—Because HARE-mediated endocytosis of either HA or Hep activates NF-κB, leading to expression of a LUC recorder gene (18), we used this system to assess the effect of Link domain glycan loss on cell signaling. EV cells treated with Hep or two different HA preparations, a narrow-size range 107-kDa HA and essentially mono-disperse 133-kDa Select HA™, showed no change in expression of inducible LUC driven by an NF-κB promoter (Fig. 5*A*, *gray bars*). WT cells (Fig. 5*A*, *black bars*) or N2280A cells (Fig. 5*A*, *white bars*) treated with Hep showed essentially identical and significant activation of LUC gene expression ($p < 0.005$). Similarly, WT cells treated with either HA preparation showed significant gene activation ($p < 0.001$). In contrast, the N2280A cells showed no NF-κB-mediated gene activation in the pres-

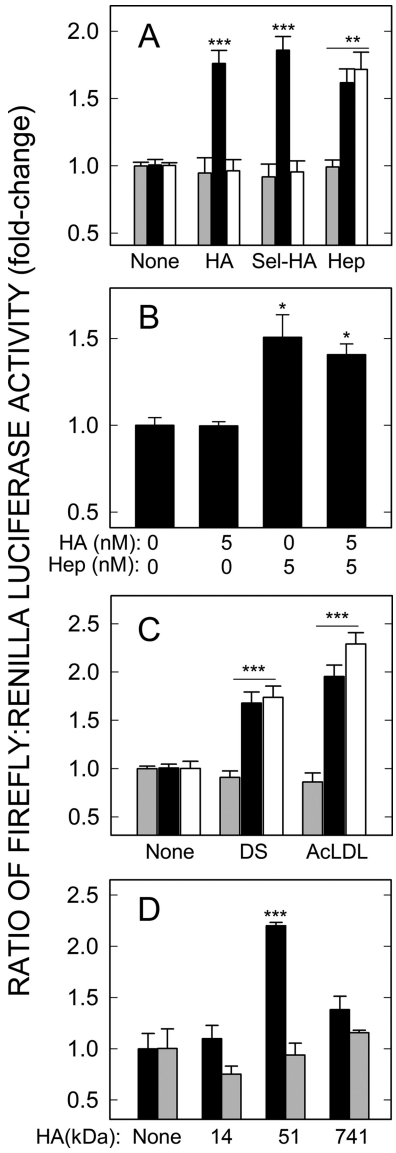

FIGURE 5. **Endocytosis of Hep but not HA by HARE(N2280A) cells stimulates NF-κB activation and gene expression.** Cells expressing EV (*gray*), WT (*black*), or N2280A (*white*) HARE were grown and transfected with LUC recorder gene plasmids as described under "Experimental Procedures." *A*, the cells were treated with 20 nM HA (107 kDa), Select-HA™ (133 kDa), or Hep. *B*, N2280A cells were treated with 5 nM Lifecore HA (51 kDa), Hep, or both. *C*, cells were treated with 20 nM DS or AcLDL. *D*, cells were incubated with Lifecore HA preparations of 14, 51, or 741 kDa. All cells were incubated at 37 °C for 4 h and processed and analyzed for relative LUC activity as described under "Experimental Procedures." Values are the means ± S.E. ($n = 9$) from three independent experiments. Values for *p* compared WT or N2280A cells with EV cells for each condition in *A* and *C*; *p* values in *B* compared the indicated samples with no HA or Hep, and *p* values in *D* compared WT with N2280A cells for each condition (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$).

ence of either HA preparation, indicating the importance of a Link domain glycan for this signaling cascade.

A mixing control with the two ligands was performed to verify that HA did not interfere with Hep signaling in N2280A cells; in WT cells, signaling responses with both HA and Hep are additive (18). The activation of 5 nM Hep was not affected by the presence of 5 nM HA, consistent with the lack of competition for binding by the two ligands (Fig. 5*B*), indicating that the lack of a Link domain *N*-glycan does not affect the independence of the two ligand binding sites.

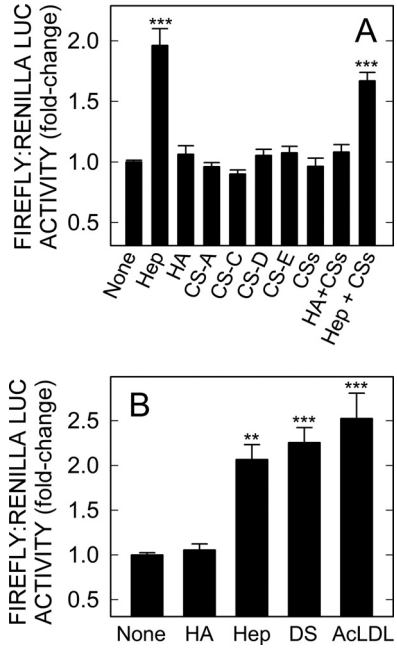

FIGURE 6. **CS types A, C, D, and E do not stimulate NF-κB-mediated gene expression or affect Hep-stimulated NF-κB activation in HARE(N2280A) or HARE(ΔLink) cells.** *A*, N2280A cells were incubated with 100 nM HA, 100 nM Hep, or 250 nM CS-A, CS-C, CS-D, or CS-E separately or in combination (*CSs*) as indicated ($n = 9$). *B*, HARE(ΔLink) cells were incubated with 100 nM HA (51 kDa), Hep, DS, or AcLDL and processed for relative LUC activities as under "Experimental Procedures" and Fig. 4. Values are the mean ± S.E.; *p* values compared the no treatment sample with each ligand treatment sample: **, $p < 0.005$; ***, $p < 0.001$.

We also examined DS and AcLDL, the two other signaling ligands that do not compete with HA for binding. DS and AcLDL were both able to activate LUC gene expression in WT or N2280A cells but not EV cells (Fig. 5*C*), demonstrating that these ligands are endocytosed normally and neither ligand·HARE complex requires a Link domain *N*-glycan for signaling leading to NF-κB activation and gene expression. An additional control was performed to verify that HA·HARE-mediated signaling was completely lost and that the size range for activating HA had not been altered in cells expressing the N2280A HARE variant. We tested HA preparations that were smaller (14 kDa) or larger (741 kDa) than HA in the normal 40–400-kDa signaling range (Fig. 5*D*). None of these HA preparations activated NF-κB signaling in N2280A cells, and only the small-intermediate size HA (*e.g.* 51, 107, and 133 kDa) used here was able to activate signaling in WT cells.

Loss of the HARE Link Domain or the Link Domain N-Glycan Differentially Affects HA Signaling Compared with Other Ligands—We next sought to further define the possible interactions between the Hep and HA binding sites and to determine if *N*-glycan loss alters the signaling potential when CS types A, C, D, and E bind to the Link domain. N2280A cells incubated with 250 nM CS types A, C, D, and E, alone or in combination without or with HA, did not show activation of NF-κB mediated gene expression (Fig. 6*A*). In addition, treatment of these cells with Hep led to significant activation of LUC gene expression ($p < 0.001$), and this was not altered by the presence of the four CS types. To define the requirement of the Link domain for signaling responses to the uptake of HA, DS,

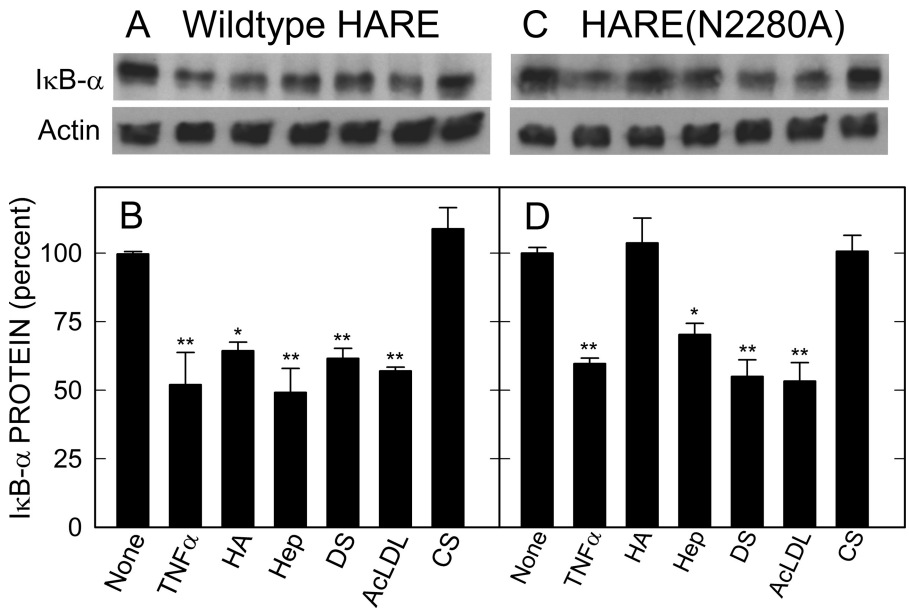

FIGURE 7. **Endocytosis of HA·HARE(N2280A) does not activate degradation of endogenous Iκ-Bα.** WT HARE cells (*A* and *B*) or HARE(N2280A) cells (*C* and *D*) were grown, pretreated, and then incubated with 1 ng/ml TNFα, 100 nM HA (51 kDa), Hep, DS, or AcLDL or with a mixture of CS types A, C, D, and E (250 nM each) as described under "Experimental Procedures." Cell lysates were subjected to SDS-PAGE and Western analysis (*A* and *C*) using Abs against IκB-α (*upper strip*) and then actin (*lower strip*). Western blots from three independent experiments were quantified to determine the IκB-α:actin ratio for each sample (*B* and *D*). Results are presented as the mean ± S.E. ($n = 3$) normalized for IκB-α:actin ratios, relative to the no treatment value as 100%: *, $p < 0.05$; **, $p < 0.005$.

Hep, and AcLDL, we treated HARE(ΔLink) cells, in which the entire Link domain was deleted, with each of the four signaling ligands (Fig. 6*B*). As reported previously (17), HA was unable to initiate signaling, because HA as well as the four CS types (not shown) is not bound and internalized by HARE(ΔLink) cells. In contrast, DS ($p < 0.001$), Hep ($p < 0.005$), and AcLDL ($p < 0.001$) all activated significant HARE-mediated NF-κB signaling, identical to that observed in WT cells. The results also demonstrate that the ability of HARE complexes with DS, Hep, or AcLDL to activate signaling does not require the Link domain. Thus, the results confirm that the binding sites for DS, Hep, and AcLDL are distinct from the HA binding site.

In addition, the above results indicate that the early mechanism by which endocytosed (or endocytosing) HA·HARE complexes initiate activation of the ERK1/2 and NF-κB signaling pathways is different than the early signal activation mechanisms for HARE complexes with Hep, DS, and AcLDL.

Activation of Endogenous IκB-α Degradation Does Not Occur during HA·HARE(N2280A) Endocytosis—Part of the cell signaling cascade leading to NF-κB promoter-driven gene expression is phosphorylation of NF-κB·IκB-α/β complexes; IκB-α/β is a potent intracellular inhibitor of NF-κB (25). The localization of these inactive NF-κB complexes with IκB-α/β is also shifted from the nucleus to the cytoplasm. We previously found that in the absence of the LUC recorder gene plasmids, HARE-mediated endocytosis of HA, Hep, DS, or AcLDL leads to degradation of ~50% of the cellular IκB-α protein in <1 h (18). To determine whether this endogenous signaling cascade is altered by the loss of the Link domain *N*-glycan, we assessed degradation of IκB-α in WT (Fig. 7, *A* and *B*) and N2280A (Fig. 7, *C* and *D*) cells endocytosing the different ligands. When both cell types were incubated with TNF-α as a positive control, cellular IκB-α was significantly decreased (Fig. 7, *B* and *D*) by 45–50% within 45 min ($p < 0.005$). When both cell types were incubated with a mixture of the four nonsignaling CS-types, A, C, D, and E (250 nM each), no IκB-α degradation occurred. In contrast, WT cells responded significantly to the uptake of the three signaling ligands Hep, DS, or AcLDL by degrading 35–51% of the endogenous IκB-α protein (Fig. 7*B*; $p < 0.005$). Similarly, N2280A cells showed significant reduction of cellular IκB-α during the endocytosis of Hep, DS, or AcLDL (Fig. 7*D*; 35–51% degradation, $p < 0.005$–0.05). During HA uptake in WT cells (Fig. 7*B*), endogenous IκB-α levels decreased to 64.4 ± 5.2% of the no addition (buffer only) control ($p < 0.05$). However, the level of endogenous IκB-α did not change in N2280A cells endocytosing HA (Fig. 7*D*). These results confirm that the endogenous NF-κB signaling pathway in the Flp-In 293 cell lines used here is functional and that activation of this pathway during HARE-mediated endocytosis of HA requires a Link domain *N*-glycan. The results also validate the above findings using LUC recorder gene assays.

DISCUSSION

HARE/Stab2 are constitutively recycling clathrin-coated pit-targeted receptors. By definition, constitutive recycling means that receptor internalization occurs continuously and is not coupled to or dependent on ligand binding. The pathways by which these receptors function were well studied 3–4 decades ago and were understood and described by kinetic parameters just as for a typical metabolic pathway (26–31). Because these receptor pathways are continuous, the kinetic constants for the various steps define the fraction of receptors present in the multiple participating surface and intracellular locations (compartments). Thus, HARE molecules continuously move along a fixed surface-intracellular spatial and temporal route designed for efficient large scale interactions with ligands, often at high concentrations. In contrast, most signaling receptors are resident on the surface for much longer times and designed to

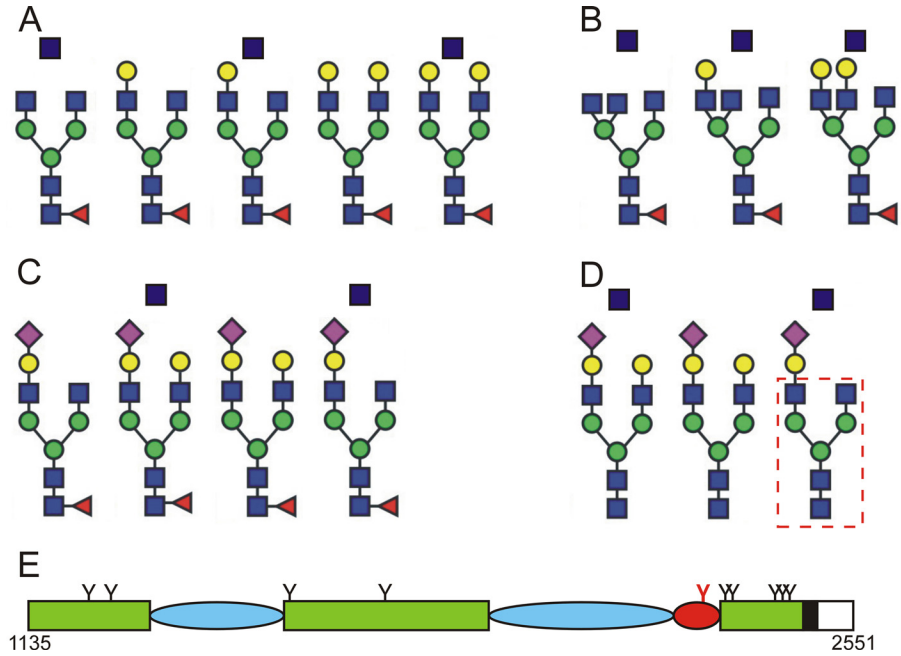

FIGURE 8. **HARE Link domain *N*-glycans and domain organization.** The 15 glycans found at Asn^{2280} (21) are arranged in four groups based on several shared structural features. *A*, bi-antennate, fucosylated and not sialylated. *B*, triantennate, fucosylated and not sialylated. *C*, bi-antennate, fucosylated and sialylated. *D*, bi-antennate, sialylated and not fucosylated. The single GlcNAc (*blue square*) above some structures indicates an additional residue whose position was not determined. The *dashed red box in D* indicates the largest common structure among all species. GlcNAc (*blue square*), Man (*green circle*), Gal (*yellow circle*), Fuc (*red triangle*), sialic acid (*pink diamond*). *E*, the C-terminal ~56% of Stab2 is the 1416-amino acid 190-kDa HARE, which starts at Ser^{1136} and ends at Leu^{2551} and is generated by an unknown protease that cleaves full-length Stab2. The schematic organization of the major human HARE domains shows multiple Cys-rich EGF (*light blue*) and Fasciclin-1 (*light green*) domains and the single Link (*red*), transmembrane (*black*), and cytoplasmic (*white*) domains. Each of the 10 *N*-glycan sites is indicated by *Y*, with the Asn^{2280} glycan in *red*.

respond to relatively small amounts of a special ligand whose concentration is normally low and only increases at relatively long intervals to levels that allow binding and signaling.

The primary function of systemic clearance receptors is the continuous removal of ligands from the lymphatic and vascular circulation systems, so that they can be degraded within cells. As with the asialoglycoprotein, mannose, LDL, and other clearance receptors, HARE/Stab2 do not localize to any plasma membrane domain other than coated pits, which also undergo a dynamic parallel recycling pathway. Consequently, the surface residence time of these receptors is short, and usually ≤30% of total receptors are on the surface at any time. The majority of total receptors (≥70%) travel through many intracellular compartments along the endocytic and receptor recycling pathways. Receptors move to the cell surface, diffuse to a coated pit, are internalized with or without bound ligand, and are recycled back to the cell surface. If ligand is present or not, the same level of receptor recycling occurs, and there is no difference in the time receptor spends on the surface nor its ability to interact with adaptor proteins and be incorporated into a coated pit.

The expression levels and the surface-internal distribution of N2280A and WT HARE are similar, with ~20% of the total on the cell surface (21). More importantly, both receptors are equally functional in being able to mediate continuous ligand uptake, which demonstrates identical times for receptor movement along the constitutive receptor recycling pathway. During HA (Fig. 1*A*) or Hep (Fig. 1*B*) uptake, the surface receptor cohort is recycled and replaced by internal recycling receptors about every 7 min (7).

The functional equivalence of WT and N2280A HARE shows that the protein expression and distribution levels along the recycling pathway are the same, and their functionalities are also the same in terms of their rates of mediating endocytosis, ligand dissociation, and receptor recycling back to the surface. Thus, loss of the Link glycan does not alter receptor localization, trafficking, or endocytic function. This conclusion is further supported by the finding that NF-κB signaling, mediated by the uptake of glycan-deficient HARE complexes with Hep, AcLDL, or DS, is identical to WT receptor. Because NF-κB signaling is dependent on clathrin-mediated endocytosis (18), the overall localization, trafficking, and function of WT and N2280A HARE must be identical; the only effect of not having a Link glycan (or domain) is the loss of HA-HARE-mediated signal transduction.

Specific *N*-glycans on receptors have been linked to cell signaling functions, including a salt-sensing mucin that activates a mitogen-activated protein kinase (32), M3 muscarinic receptor trafficking (33), and the dendritic cell immunoreceptor (34). Several groups reported that *N*- or *O*-glycans can influence the HA binding function of proteins containing a Link module (35–41). It is now generally accepted that *N*-glycans within the Link module of the HA receptor CD44 influence HA binding. In some cases the presence of an *N*-glycan inhibits HA binding. The HA binding ability of the lymphatic receptor LYVE-1 is inhibited by sialylation of *O*-glycans (42), which are not on the Link module, and neuraminidase treatment of sialylated LYVE-1 unmasks HA binding activity. The present results show a clear relationship between the presence of an *N*-glycan in the HARE Link domain and the ability of HA·HARE com-

plexes to activate ERK1/2 and NF-κB. Loss of a Link domain *N*-glycan eliminates the 40–400-kDa HA size-dependent NF-κB signaling; it does not shift the response to a smaller or larger size (Fig. 5*D*).

Early molecular details of how the four signaling ligand·HARE complexes interact with intracellular signaling components must be different for HA *versus* Hep, DS, and AcLDL. The latter three ligands bind at a different site than HA (11), and their ability to initiate ERK1/2 and NF-κB signaling is different and independent of the HA signaling pathway, as demonstrated by their normal signaling in HARE(ΔLink) cells that are unable to endocytosis HA or initiate HA-mediated signaling (Fig. 5*D*). We conclude that, due to the involvement of two different ligand binding regions, there are at least two ligand·HARE complex conformational states during coated pit-mediated endocytosis that are capable of interacting with, and activating, the initial molecular events leading to downstream signaling. One activating conformation is associated with the formation of HARE complexes with Hep, DS, or AcLDL, and the other conformation is associated with HA·HARE complex formation that is somehow dependent on the presence of a Link domain *N*-glycan.

We previously found that all N2280A clones express a major HARE species of WT size and a minor slightly smaller HARE species; both proteins are delivered to the cell surface and bind HA (21). Endoglycosidase H sensitivity shows that WT HARE and the major N2280A HARE have mature *N*-glycans, but the minor N2280A HARE species is shifted to smaller size, indicating multiple immature high mannose *N*-glycans. Both N2280A proteins are shifted to the same smaller size after peptide *N*-glycosidase F treatment. Because half of the 10 glycan sites are downstream of the Link glycan (Fig. 8*E*), these data indicate that loss of the Link glycan slightly increases the chance that some of these five C-terminal *N*-glycans are processed abnormally. Thus, two N2280A HARE isoforms are expressed that are as functional as WT HARE in their ability to bind and endocytose HA, although we cannot test this directly. The minor species is not altered in binding, endocytosis, or signaling, as no differences are apparent with the three other NF-κB signaling ligands between WT and N2280A cells (Figs. 6 and 7), and both the HA and Hep uptake kinetics are identical to WT (Fig. 1).

A plausible mechanism to explain a critical role for an oligosaccharide is that it could enable an HA·HARE complex to create or stabilize a favorable conformation (*e.g.* an allosteric-like response after HA-HARE binding) for interacting with a key initial protein responsible for starting the activation cascade. Support for such a mechanism in the ability of HA·HARE complexes to initiate signaling may be evident in the earlier finding that the transmembrane domain also influences the binding of HA to the Link domain. Purified HARE(N2280A) ectodomain binds little or no HA *in vitro* in ELISA-like, pulldown, or surface plasmon resonance assays (21), whereas WT HARE ectodomain shows robust binding to HA in all assays, with a $K_d \sim 5$ nM. This indicates that an Asn^{2280} glycan stabilizes a conformation needed for the Link domain to bind HA in the absence of the transmembrane region. In contrast, membrane-bound HARE binds and endocytoses HA normally with or without an Asn^{2280} glycan. Although a Link domain *N*-glycan is not required for HA binding and endocytosis, the glycan might, nonetheless, interact with HA directly. Alternatively, the glycan might interact with another region of HARE (in or outside of the Link domain) made available by a conformational change associated with the binding of HA. Either scenario could create a glycan-dependent HA·HARE conformation that is required to initiate signal transduction.

Because a Link domain *N*-glycan is required for HA·HARE signaling, the diversity of glycans at Asn^{2280} was unexpected; especially interesting is that Asn^{2280} has the greatest diversity of glycans and the only sialylated structures of the 10 glycan sites (21). Fifteen glycoforms were identified at Asn^{2280}: 12 are fucosylated in the core chitobiosyl region (Fig. 8, *A–C*), 12 are bi-antennate (Fig. 8, *A*, *C*, and *D*), and 3 are tri-antennate (Fig. 8*B*), 7 are sialylated at the same bi-antennate position (Fig. 8, *C* and *D*), and 10 contain an additional GlcNAc with an ambiguous site of attachment (Fig. 8, *A–D*). This great diversity of glycan structures at a site requiring a glycan for signaling competence indicates either (i) that all the structures possess the basic features needed to activate signaling mediated by HA·HARE complexes or (ii) that there are subpopulations of glycan structures that are not signaling-competent and others that are signaling-competent (perhaps with different levels of signaling responsiveness to HA). We favor the latter possibility because it fits the complexity of HA·HARE-mediated signaling. If the former possibility is correct, however, then one candidate for an activating structure that is common to all 15 glycans is the [$GlcNAc_2Man_3GlcNAc_2$] core region (*red box*; Fig. 8*D*).

In addition to the HA·HARE signal-initiating pathway being distinct from that of other ligand·HARE complexes, this pathway shows a remarkable selectivity for the size of the bound HA that activates signaling. Although HARE binds and endocytoses HA varying in size between ~2 kDa (17) and >8 MDa (43), only HA between 40 and 400 kDa can activate signaling; smaller or larger HA is endocytosed normally but does not activate ERK1/2 or NF-κB (17). Considering the broad HA size ranges in most mammalian sources, the steady-state HA size in the vascular circulation of healthy people (~100 kDa; Ref. 44) is within the activating size range and close to the optimum size (~140 kDa) for HA·HARE signaling (17).

Since its discovery as an HA clearance receptor in 1981 by Laurent and co-workers (45), HARE/Stab2 have been found to clear at least 14 ligands (including bacteria and apoptotic cells). Clearance is achieved by tissue-resident sinusoidal endothelial cells and migratory macrophages. HARE on sinusoidal endothelial cells in lymph node and liver rapidly removes ligands (*e.g.* half-lives of <5 min) from the lymphatic and vascular systemic circulations, respectively. Macrophages clear HARE ligands they encounter in their travels through the circulatory systems and tissues. During HARE/Stab2-mediated phagocytosis, macrophages activate NF-κB leading to new gene expression, in particular TGF-β (10, 16–18).

The discovery that ERK1/2 and NF-κB signaling pathways are activated by a subset of HARE ligands was surprising (18), as this additional function must be coupled to ligand uptake for a different purpose than the housekeeping clearance function (5). The realization that essentially all HARE ligands are derived from tissue biomatrices or cells led us to propose a Tissue Stress

Sensor Model in which the HARE/Stab2 receptor signaling system, operating in parallel with its clearance-turnover function, monitors the status of tissue biomatrix turnover and stress throughout the body (5). The signaling activity and responses of the HARE signaling system during continuous clearance of key indicator ligands (*e.g.* HA, Hep, DS, AcLDL, and phosphatidylserine) could monitor and respond to the pattern of tissue-derived ligands and thus the homeostasis of tissue-biomatrix turnover throughout the body.

The complexity of how the HARE signaling mechanism senses HA supports a growing body of evidence that HA turnover and homeostasis is a more fundamentally important process than just simply the systemic or localized removal of degraded HA. We can understand why this is the case based on the importance of HA size in determining the nature of cellular interactions with, and responses to, HA in the environment. Since the first report in 1985 that small HA oligosaccharides are angiogenic (46), many groups have confirmed the basic finding that size matters; that cellular behaviors are different in the presence of different size ranges of HA (14). The physiologic importance of the ability to monitor tissue HA degradation and homeostasis is underscored by the present finding that the early HA·HARE signaling pathway is also different from the HARE-mediated signaling pathway for other activating ligands.

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