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The Hyaluronan Receptor for Endocytosis Mediates Hyaluronan-Dependent Signal Transduction via Extracellular Signal-Regulated Kinases

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Abstract

The hyaluronan (HA) receptor for endocytosis (HARE) mediates the endocytotic clearance of HA and other glycosaminoglycans from lymph and blood. Two isoforms of human HARE, 315- and 190-kDa, are highly expressed in sinusoidal endothelial cells of liver, lymph node, and spleen; HARE is also in specialized cells in the eye, heart, brain, and kidney. Here we determined whether HA binding to HARE initiates intracellular signaling in Flp-In 293 cells stably expressing either the 315- and 190-kDa HARE or the 190-kDa HARE alone. HARE was co-immunoprecipitated with extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal protein kinase (JNK), and p38 members of the mitogen-activated protein kinase signaling cascade. ERK phosphorylation increased in a dose- and time-dependent manner when HA was added to cells expressing full-length or 190-kDa HARE, but not cells with vector-only or a HARE(Δ Link) construct with greatly decreased (~90%) HA uptake. HA did not induce phosphorylation of JNK or p38. A maximum increase in phospho-ERK1/2 occurred within 30 min at 5 μ g/ml HA, and the response was dampened at >20 μ g/ml HA. HA binding did not increase the level of HARE-ERK complexes, but did increase HARE phosphorylation. These findings demonstrate a novel functional response, when HARE binds HA, that leads to activation of ERK1/2, important mediators of intracellular signal transduction.

Abbreviations: HA, hyaluronan; aa, amino acids; BSA, bovine serum albumin; CS, chondroitin sulfate; ECM, extracellular matrix; ERK, extracellular signal-regulated kinase; EV, empty vector; GAG, glycosaminoglycan; HARE, HA receptor for endocytosis; hHARE, human HARE; HRP, horseradish peroxidase; JNK, c-Jun N-terminal kinase; MAP kinase, mitogen-activated protein kinase; mAb, monoclonal antibody; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; pAb, polyclonal antibody; pERK, phosphorylated ERK; pJNK, phosphorylated JNK; p-p38 kinase, phosphorylated p38 kinase; RHAAM, receptor for HA-mediated mobility; tERK, total ERK protein; tJNK, total JNK protein; 190-HARE, the 190-kDa HA receptor for endocytosis; 315-HARE, the 315-kDa HA receptor for endocytosis

Hyaluronan (HA), a nonsulfated GAG made up of repeating β (1,4)-d-glucuronic acid- β (1,3)-N-acetyl-d-glucosamine disaccharide units, is present in the ECM of all vertebrate tissues.

HA plays important roles in matrix assembly, cell differentiation, migration, morphogenesis, and wound healing (1–3).

Elevated levels of HA are associated with various pathologies, such as arthritis, inflammation, and cancer (4–7). The average adult human contains ~15 g of HA, of which ~5 g is synthesized and degraded daily in tissues throughout the body (1). Most of the HA and CS types released from tissues during this turnover process are ultimately cleared from the circulation and lymph fluid by the HA Receptor for Endocytosis (HARE) (8, 9), also known as Stabilin-2 (10) or FEEL-2 (11). Human HARE is encoded by the 180-kb STAB2 gene, found on chromosome 12, consisting of 69 exons, and is abundantly expressed in the sinusoidal cells of lymph nodes, liver, and spleen (8–10, 12, 13). Rat and hHARE in these tissues are present as two isoforms (8, 12, 13), e.g. hHARE isoforms are ~190 and ~315 kDa. Although HARE in the sinusoidal cells of liver and lymph node has a known endocytic clearance function, it may also have other, not yet described, functions. HARE is also expressed in corneal and lens epithelium, in mesenchymal cells of heart valves, in ependymal cells lining the ventricles in the brain, in epithelial cells covering the renal papillae (14), and in oviduct (15). The functions of HARE in these latter tissues are unknown and might be different from local GAG clearance.

We have stably expressed the recombinant 190-kDa (16) and full-length 315-kDa (17) HARE proteins in Flp-In 293 cell lines, using cDNA derived from human lymph node. These Flp-In cell lines have one unique, recombinase-mediated integration site. The full-length 315-kDa HARE is a type I membrane protein that contains a 2458-aa extracellular domain (with four cysteine-rich fasciclin/epidermal growth factor-like domains of unknown function and a LINK domain), a transmembrane domain, and a 72-aa cytoplasmic domain. The 190-kDa HARE is identical to the C-terminal 1417 aa of the 315-kDa HARE and is derived from the full-length protein by proteolytic cleavage (12, 13, 17).

HARE binds and mediates internalization of HA as well as four types of CS (16, 17) via the clathrin-coated pit-mediated endocytic pathway (18, 19). Recent studies demonstrate that endocytosis plays an important role in the activation and propagation of signaling pathways, such as ERK 1 and 2 (20, 21), which are members of the mitogen-activated protein (MAP) kinase cascade. Multiple reports have documented that HA binding to HA receptors CD44 and RHAMM

(CD168) triggers signal transduction events through activation of ERK1/2 (22, 23). MAP kinases are important mediators of signal transduction and play key roles in the regulation of many cellular processes, such as cell growth and proliferation, differentiation, and apoptosis.

In mammalian cells, three major groups of MAP kinases have been identified: ERK, JNK, and p38 (24). A common feature of the three MAP kinases is that they are typically organized together in three-kinase modules. The usual ERK signaling cascade proceeds through activation of Raf, MEK, and then ERK1/2. MAP kinases are stimulated via receptor and nonreceptor protein kinases and G-protein-coupled receptors. MAP kinase cascades are differentially activated by a variety of extracellular stimuli, including growth factors, cytokines, stress, and ECM components (23, 24). After activation, MAP kinases are translocated to the nucleus where they phosphorylate transcription factors that regulate expression of immediate-early response genes. This cascade of responses results in altered levels of proteins that lead to functional and morphological changes of the cell.

HA signaling through CD44 and RHAMM involves several signaling pathways, including the MAP kinase cascade, that lead to HA-dependent functions in various cells and tissues; *e.g.* HA binding to CD44 may regulate hematopoiesis by activating the p38 MAP kinase (25). In addition, HA-CD44 interactions affect matrix metabolism in articular chondrocytes via activation of transcription factor NF κ B and the p38 MAP kinase (26, 27). Furthermore, CD44 and RHAMM play pivotal roles in activating oncogenic signaling and HA-mediated tumor cell functions. Overexpression of RHAMM strongly correlates with overexpression of Ras and ERK, and could be a useful prognostic marker for breast cancer progression (28). CD44 and RHAMM form complexes with ERK1/2 in invasive breast cancer cells with high basal motility (29). An HA-CD44-mediated oncogenic mechanism also occurs in ovarian cancer progression (30).

In the present study, we found that the receptor HARE was able to mediate HA-dependent intracellular signal transduction and increase the phosphorylation of HARE and ERK1/2. This unexpected cell signaling activity of HARE indicates that this receptor is involved in multiple intracellular routing or signaling pathways.

Experimental Procedures

Materials, Solutions, and Buffers—Tris-HCl, glycine, and acrylamide were obtained from Research Products International (Mt. Prospect, IL); SDS, Nonidet P-40, and methanol were from EMD (Gibbstown, NJ). Protease inhibitor mixture (catalog number P8340 containing 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, leupeptin, bestatin, pepstatin A, and E-64), sodium pyrophosphate, sodium fluoride, sodium orthovanadate, benzamidine, 2-mercaptoethanol, EGTA, EDTA, and Tween 20 were obtained from Sigma. HA, prepared by bacterial fermentation, was obtained from Genzyme Corp. (Cambridge, MA). 125 I-Iodine (100 mCi/ml; specific activity of >0.6 TBq/mg) in NaOH was from GE Healthcare. 125 I-HA, modified at the reducing end was prepared as described previously (31), Flp-In 293 cells, fetal bovine serum, Dulbecco's modified Eagle's medium, and hygromycin B were from Invitrogen. Affinity purified goat

anti-V5 pAb was obtained from Bethyl Labs (Montgomery, TX). Rabbit pAbs against pERK (Thr(P) 202 and Tyr(P) 204) 1 and 2 (44- and 42-kDa, respectively), ERK1/2, pJNK (Thr(P) 183 and Tyr(P) 185), JNK, p-p38 MAP kinase (Thr(P) 180 and Tyr(P) 182), p38 MAP kinase, and PD 98059 (a MEK inhibitor) were obtained from Cell Signaling Technology (Beverly, MA). Protein A/G Plus-agarose, goat anti-rabbit IgG-HRP, goat anti-mouse IgG-HRP, donkey anti-goat IgG-HRP, goat anti-actin (I-19) pAb, antERK2 conjugated to agarose, and rabbit anti-ERK2 (C-14, which also recognizes ERK1) pAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-Tyr(P) (PY-20) mAb was from BD Biosciences (San Jose, CA). Western Lightning Chemiluminescence Reagent Plus was from PerkinElmer Life Sciences. Nitrocellulose membranes were from Schleicher & Schuell (Dassel, Germany). Classic blue autoradiography film BX was from MIDSCI (St. Louis, MO). Coomassie Protein Assay Reagent was obtained from Pierce. TBS contains 20 mM Tris-HCl, pH 7.0, 150 mM NaCl. TBST is Tris-buffered saline with 0.1% (v/v) Tween 20. Lysis Buffer contains 20 mM Tris, pH 7.2, 1 mM sodium orthovanadate, 3 mM benzamidine, 2 mM sodium pyrophosphate, 5 mM sodium fluoride, 2 mM EGTA, 5 mM EDTA, 1 μ g/ml of protease mixture inhibitor, and 0.5% (v/v) Nonidet P-40.

Cell Lines Expressing 315- or 190-kDa HARE—The construction of 190- and 315-HARE expression vectors, and the selection and characterization of stable Flp-In 293 cell transfectants are described in detail by Harris *et al.* (16, 17). To make the 190-HARE(Δ link) construct, a single primer (5'-CCACTTCCAGGATACCCTGTTCCGGATGAAAGATGTGAAGTGCACC-3') was used with the 190-HARE wild type template to delete the coding region of the 93-aa Link domain. Mutagenesis was performed with *pfu* Ultra (Stratagene) in an EriComp thermal cycler (18 cycles: 94 $^{\circ}$ C, 30 s; 62 $^{\circ}$ C, 30 s; and 71 $^{\circ}$ C, 1 min/plasmid kb). After these reactions, both template and new plasmids were ethanol precipitated (0.1 volume of 3 M potassium acetate, pH 5.5, 2 volumes of 95% ethanol), resuspended in 17 μ l of H $_2$ O, 2 μ l of NEB4 buffer, and 2.5 units of DpnI to digest template plasmids, while retaining the intact mutant plasmids. After overnight incubation at 37 $^{\circ}$ C, the digestion mixtures were heated to 95 $^{\circ}$ C for 10 min and immediately transformed into TOP10 supercompetent *Escherichia coli* cells. Bacterial cells were screened by a Miniprep procedure, PCR, and sequencing to confirm the desired mutation. Plasmids containing correct mutations, open reading frames, and promoter regions were used to make stable cell lines as described (16, 17). Cells stably expressing recombinant full-length 315-HARE also produce the 190-HARE from the full-length protein by proteolysis. The cloned and amplified open reading frame for the full-length 315-HARE from pooled lymph node cDNAs (Marathon system; Clontech) was inserted into expression vector pcDNA5/RFT/V5-His-TOPO. Except for the secretion signal, the same strategy was used for the 190-HARE. Only stable clones with a single plasmid integrated into the correct, unique recombinase-mediated chromosomal site were selected and used, as described (16, 17).

Cell Lines and Cell Stimulation—Cells expressing full-length 315-HARE, 190-HARE alone, Flp-In 293 parent cells, or cells transfected with vector alone were grown in Dulbecco's modified Eagle's medium with 8% fetal bovine serum

supplemented with 100 µg/ml hygromycin B for at least 2 days prior to experiments, to 80–90% confluence. Cells were washed 2 times with phosphate-buffered saline and incubated in fresh medium without serum for 1 h at 37°C prior to starting the experiment. Based on preliminary results (not shown) the 1-h incubation in serum-free medium was optimal for observing subsequent HA-induced protein phosphorylation. The cells were washed, received fresh serum-free Dulbecco's modified Eagle's medium, and incubated with HA at various concentrations for various times at 37 °C, as indicated. All experiments used non-animal-derived HA with a weight-average molar mass of 580 kDa. For ERK inhibition assays, after the 1-h incubation in serum-free medium, cells were pretreated with different concentrations of the MEK inhibitor PD 98059 for 30 min and then treated with 5 or 10 µg/ml of HA for 20 min at 37 °C. Cells were then collected by scraping, washed with ice-cold phosphate-buffered saline, and centrifuged at 1,600 × g for 5 min. Cell pellets were incubated with mixing for 1 h at 4 °C in Lysis Buffer, and the lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C. The cell lysate supernatants were used for experiments after determination of protein content (32).

Immunoblot Analysis—Cell lysates (25 µg of protein) were subjected to 5 or 10% SDS-PAGE (33) and electrotransfer to nitrocellulose membranes (34) using a mini-transfer unit (Bio-Rad) for 2 h at 4°C at 80 V in 25 mm Tris-HCl, pH 7.4, 190 mm glycine, and 20% (v/v) methanol. To confirm that any differences in band intensity following Western blot analysis were not due to differences in protein loading, the membranes were first stained with 0.1% Ponceau S water-soluble stain and photographed. Membranes were then washed 4 times for 5 min each with water and incubated with 5% nonfat dry milk in TBST for 1 h at room temperature. The washed membranes were incubated overnight at 4 °C with pAbs (diluted 1:1000 in 5% milk/TBST) to pERK1/2, or ERK1/2, or Abs (diluted 1:1000 in TBST containing 5% BSA) to pJNK, JNK, p-p38 MAP kinase, or p38 MAP kinase. After washing 3 times for 10 min each in TBST, the membranes were incubated with anti-rabbit IgG-HRP conjugate, diluted 1:2000 in TBST containing either 5% milk (for ERK Abs) or 5% BSA (for JNK and p38 Abs) for 1 h at room temperature.

For Western blot analysis using anti-V5 pAb, membranes were incubated in TBST containing 1% BSA for 1 h at room temperature, washed, incubated with 1:4000 goat anti-V5 Ab for 1 h, washed, and incubated with secondary anti-goat Ab conjugated to HRP diluted 1:6000. We used 5% BSA-containing buffers for blocking nonspecific binding sites and for incubations with anti-Tyr(P) (PY20) mAb, diluted 1:1000. After four washes for 10 min each in TBST, ECL and exposure to film was utilized for detection according to the manufacturer's instructions. Autoradiography films were scanned into digital files, and band densities were quantified, using an Alpha Innotech FluoroChem 8000 imaging system (Alpha Innotech Corporation, San Leandro, CA), as integrated densitometry values (*i.e.* the sum of all pixel values minus background correction). The results are expressed as a ratio, *e.g.* pERK divided by tERK.

In Figures 1, 2, 5, 6, 7, and 8, the blots shown in the same panel were successively stripped and stained with different Abs to normalize the results. After one round of detection,

blots were stripped by washing with water and incubating in 62.5 mM Tris-HCl, pH 6.7, 100 mM β-mercaptoethanol, 2% SDS at 55 °C for 30 min. After five washings for 10–15 min each with TBST, the blots were reprobed with a different Ab as above. As a control for gel loading accuracy, bound Abs were stripped from the blot and the blot was reprobed with anti-actin Ab. The ECL and film method was utilized for detection. Figures show a representative set of Western blots from two or usually three independent experiments, which gave similar results.

Immunoprecipitation Assays—Rabbit pAb against ERK2, JNK, p38, or mouse anti-Tyr(P) mAb (1 µg/ml) were added to cell lysates (200 µg of protein) and incubated with rotation for 2 h at 4 °C. Immune complexes were then collected by adding 20 µl of 250 µg/ml Protein A/G Plus Agarose and incubated with rotation overnight at 4 °C. The resin was washed three times with cold phosphate-buffered saline, the pellet was resuspended in 30 µl of 2× Laemmli sample buffer (33), incubated for 3 min at 90 °C, and subjected to SDS-PAGE and Western blot analysis. We also used three mAbs (numbers 30, 154, and 159) raised against rat HARE (9) that cross-react with both the 315- and 190-kDa HARE (12). HARE was immunoprecipitated, as described above, using mAb 30 alone or mAbs 30, 154, and 159 attached to CNBr-activated Sepharose (8, 12, 16, 17). ECL and film was utilized for detection.

HA Endocytosis Assays—Cells were grown to ~90% confluence, washed, and incubated for 1 h at 37 °C in Dulbecco's modified Eagle's medium with 0.5% BSA to allow clearance of serum-derived GAGs bound to cell surface HARE. The cells were washed and then incubated with 1.5 µg/ml ¹²⁵I-HA with and without a 100-fold excess of unlabeled HA (to assess specific binding) for 4 h. The cells were then chilled, washed, lysed, and radioactivity and protein were determined (16, 17).

Statistical Analysis—Data from experimental groups (usually *n* = 3) were compared by the unpaired Student's *t* test using SigmaPlot version 10 (Systat Software, Inc., Point Richmond, CA) and are presented as the mean ± S.E. Values of *p* ≤ 0.05 were considered statistically significant.

Results

HA Treatment Induces HARE Phosphorylation—First, we wanted to determine whether HARE is phosphorylated on Tyr, because many endocytic, recycling receptors contain Tyr(P) (35, 36), and it is unlikely that HARE would be an exception. In addition, the cytoplasmic domain of HARE has 4 Tyr that could be phosphorylated (*e.g.* as assessed by NetPhos 2.0). To test this, we used anti-Tyr(P) mAb to purify Tyr(P)-containing proteins and then Western blot analysis with anti-V5 Ab to detect co-immunoprecipitated recombinant HARE (Figure 1A). The 315-kDa HARE was detected among Tyr(P)-containing proteins from 315-HARE cells and 190-kDa HARE was detected in cells expressing 190-HARE alone (Figure 1A). Some experiments showed the minor 190-kDa band present in 315-HARE cells. Neither HARE band was present in cells containing empty vector (EV). Also, no immunoreactive bands were observed at the HARE band positions in control samples using non-immune mouse IgG (not shown).

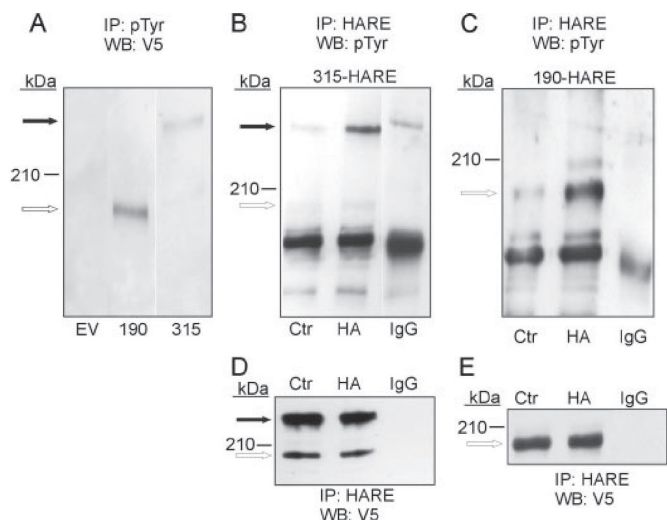


Figure 1. HA induces Tyr phosphorylation of HARE. *A*, cells expressing 315-HARE, 190-HARE, or EV alone were cultured in T-25 flasks until confluent, washed, scraped, and lysed. Cell lysates (100 μ g of protein) were immunoprecipitated (IP) with anti-Tyr(P) mAb and the immune complexes were subjected to 5% SDS-PAGE and Western blot (WB) analysis using anti-V5 pAb as described under "Experimental Procedures." Cells expressing 315-HARE (*B*) or 190-HARE (*C*) cDNA were grown until confluent, washed, incubated for 1 h in medium without serum, and then incubated with (HA) or without (Ctrl) 5 μ g/ml HA for 20 min. The cells were lysed and 200 μ g of total lysate protein was immunoprecipitated with anti-HARE mAb-30. Immune complexes were collected and subjected to 5% SDS-PAGE and Western analysis using anti-Tyr(P) mAb as described under "Experimental Procedures." The same blots in *B* and *C* were reprobed with anti-V5 Ab (*D* and *E*, respectively). Immunoprecipitation using non-immune mouse IgG served as another control in *B–E*. The solid and open arrows indicate the positions of the 315-HARE and 190-HARE, respectively.

We then tested whether HA treatment induced HARE phosphorylation in cells expressing 315- or 190-HARE by immunoprecipitating HARE with mAb 30 and probing blots with anti-Tyr(P) Ab (Figure 1, *B* and *C*). Cells were incubated for 1 h in serum-free medium, incubated with or without 5 μ g/ml of HA for 20 min, and HARE was then immunoprecipitated. Exposing cells to HA increased the amount of Tyr(P) in HARE almost 5-fold compared with untreated cells. This occurred in 315-HARE cells containing both the 315- and 190-kDa HARE (Figure 1*B*) or in 190-HARE cells expressing just the 190-kDa HARE (Figure 1*C*). When these latter two immunoblots were stripped and reprobed using Ab against the C-terminal V5 epitope (Figure 1, *D* and *E*), no differences in the amount of receptor expression were detected, confirming that exposure to HA enhanced the Tyr phosphorylation of HARE.

Basal Phosphorylation of MAP Kinases Increases in 315-HARE or 190-HARE Cells—We addressed the possibility that MAP kinases, which are Ser/Thr protein kinases, could be phosphorylated in cells expressing HARE. The basal phosphorylation level of MAP kinases in Flp-In 293 cells stably expressing the full-length 315- and 190-kDa HARE or the 190-kDa HARE alone were determined by Western blot analysis using three phospho-specific anti-MAP kinase Abs that detect the phosphorylated (active) forms of ERK1 (44 kDa) and ERK2 (42 kDa) proteins (Figure 2*A*), JNK1 (54 kDa) and JNK2 (46 kDa) proteins (Figure 2*B*), or p38 (38 kDa) MAP kinase (Figure 2*C*). Cells expressing either 315-HARE or 190-HARE showed 3–4-fold increases in phosphorylated ERK1

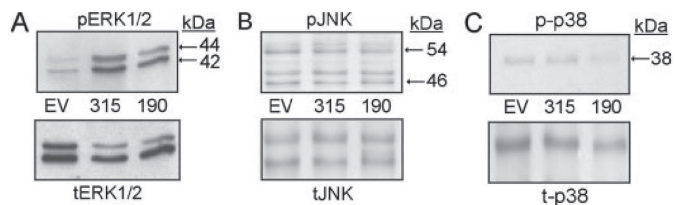


Figure 2. Expression of MAP kinases in 315-HARE and 190-HARE cells. Cells expressing 315-HARE, 190-HARE, or EV alone were grown to confluence, washed, scraped, and lysed as described under "Experimental Procedures." Lysate samples were analyzed by 10% SDS-PAGE and Western blot analysis using anti-pERK (*A*), anti-pJNK (*B*), or anti-p-p38 (*C*) Ab, all of which detect activated (phosphorylated) forms of ERK1/2 (*pERK1/2*), JNK (*pJNK*), and p38 (*p-p38*) MAP kinases. The same blots were stripped and reprobed with anti-ERK1/2 or anti-t-p38 Ab, all of which detect total protein.

and ERK2 compared with cells transfected with the EV. In contrast, we did not find increases in JNK or p38 phosphorylation in cells expressing 190- or 315-HARE. We detected no differences in the protein levels of the three MAP kinases when immunoblots were reprobed using Abs that recognize total ERK, JNK, or p38 proteins.

HARE and ERK1/2 Form Complexes—We examined whether there is a physical interaction between HARE and ERK1 or ERK2 in cells expressing 315- and 190 kDa-HARE or 190-kDa HARE alone by immunoprecipitating either ERK or HARE protein from cell lysates. The associated proteins were then cross-probed in Western blots. The 315- and 190-kDa HARE were both detected in blots of anti-ERK immunoprecipitates (Figure 3*A*). Consistent with this result, ERK1 and ERK2 were detected in anti-HARE immunoprecipitates probed with anti-ERK Ab (Figure 3*B*). Cells transfected with EV did not show bands (Figure 3, *A* and *B*) at the position of either HARE, indicating that the observed complexes were specific and dependent on the presence of both HARE and ERK. Also, no HARE-containing immunoreactive material was observed in control samples when mouse IgG was used for immunoprecipitation or immunoblotting (not shown). Stimulation of 190-HARE cells with HA did not increase the level of HARE-ERK complexes, as assessed by co-immunoprecipitation using Ab against tERK, which recognizes active pERK plus inactive non-phosphorylated ERK (Figure 3*C*). Taken together, these results clearly show that HARE and ERK1 and ERK2 are associated with each other as a stable complex in cells expressing either the full-length HARE or the 190-kDa isoform.

HARE and JNK or p38 MAP Kinase Also Form Complexes—We determined whether there is also an association between HARE and the other MAP kinases, JNK and p38. We performed immunoprecipitations with anti-JNK or anti-p38 Ab followed by immunoblotting with anti-V5 Ab using 315- or 190-HARE cells. Association of HARE with JNK (Figure 4*A*) and p38 (Figure 4*B*) was detected in cells expressing either the full-length HARE or 190-kDa HARE alone. No similar MAP kinase complexes were found in cells transfected with EV. These results demonstrate that both JNK and p38 physically interact with HARE.

HA Stimulates ERK Phosphorylation in a Time-dependent Way—To examine the activation of ERK1 and ERK2, cells expressing 315- and 190-kDa HARE, 190-kDa HARE only, or EV were incubated for 1 h at 37 $^{\circ}$ C without serum and then with 10 μ g/ml HA for various times. Extracts were assessed

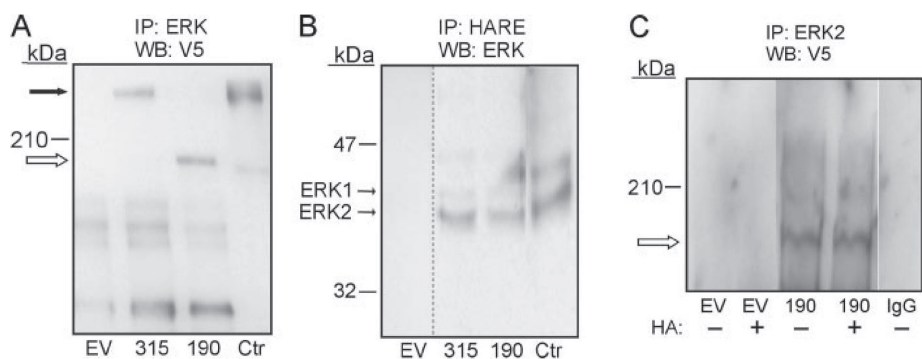


Figure 3. HARE and ERK form complexes in 315-HARE and 190-HARE cells. Cells expressing 315-HARE, 190-HARE, or EV were grown to confluence, washed, scraped, lysed, and centrifuged, and samples were subjected to immunoprecipitation (IP) with anti-TERK (A) Ab or anti-HARE (B) mAbs 30, 154, and 159. Immune complexes were collected and subjected to SDS-PAGE and Western blot (WB) analysis using anti-V5 or anti-TERK Ab, as indicated. Lysates from 315-HARE cells served as a positive control (Ctr) to identify the positions of 315- and 190-kDa HARE (A), or pERK1 and pERK2 (B). The *solid* and *open* arrows in A indicate the positions of 315-HARE and 190-HARE, respectively. C, cells expressing 190-HARE or EV were incubated for 1 h in serum-free medium, and then incubated as indicated in the presence or absence of 5 μ g/ml HA for 20 min at 37 °C. Cell lysates were immunoprecipitated with anti-ERK2 Ab conjugated to agarose, and analyzed as above. As a negative control, release of interfering Ab was estimated by treating rabbit IgG-agarose with Laemmli buffer (33).

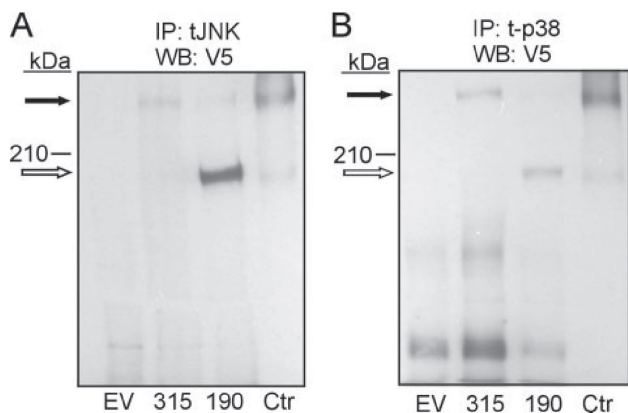


Figure 4. Detection of HARE-JNK and HARE-p38 MAP kinase complexes. Confluent cultures of 315-HARE, 190-HARE, or EV cells were washed and scraped, and lysates were immunoprecipitated (IP) with anti-tJNK (A) or anti-t-p38 MAP kinase Ab (B). Immune complexes were collected and subjected to SDS-PAGE and Western blot (WB) analysis using anti-V5 Ab. Lysates from 315-HARE cells (Ctr) served to identify 315- and 190-kDa HARE. The *solid* and *open* arrows indicate the positions of 315-HARE and 190-HARE, respectively.

by Western blot analysis for HA-induced changes in ERK activation (*i.e.* phosphorylation). Other results (not shown) indicated that the 1-h incubation in serum-free medium was optimal for detection of subsequent HA-induced protein phosphorylation. Cells expressing 315- or 190-kDa HARE that were exposed to HA showed time-dependent changes in pERK1 and pERK2. After 30 min, pERK1 and pERK2 in 315-HARE (Figure 5, A and D) and 190-HARE (Figure 5, B and E) cells reached a maximum that was almost 4- and 8-fold greater, respectively, than the time 0 values. Over the next 30 min, pERK1/2 declined to baseline levels, due to dephosphorylation of the proteins. Elevated pERK levels in 315- and 190-HARE cells were statistically significant at 5 ($p = 0.02$, $p = 0.01$, respectively), 15 ($p = 0.02$, $p = 0.07$, respectively), and 30 min ($p = 0.01$, $p = 0.05$, respectively). Control cells transfected with EV, not expressing HARE, did not show increased pERK1/2 levels (Figure 5, C and F).

HARE-mediated Stimulation of ERK Phosphorylation by HA Is Dose-dependent

—We used increasing HA concentrations to assess the concentration dependence of ERK activation in 190-kDa HARE cells (Figure 6). As HA increased between 0.1 and 5 μ g/ml, pERK1 and pERK2 increased until reaching a maximum at 5 μ g/ml (Figure 6, A and C). The response then declined at 10 or 20 μ g/ml HA. Total ERK1 and ERK2 protein levels remained the same. In contrast, cells transfected with EV showed no activation of ERK1/2 in the presence of HA (Figure 6, B and D). Densitometric analyses of 190-HARE cell samples (Figure 6C) revealed a significant ($p = 0.03$) 2.5-fold increase in pERK1/2 at the lowest HA dose (0.1 μ g/ml) used. Compared with untreated 190-HARE cells, the pERK to tERK ratio was increased about 3-fold ($p = 0.001$) at 1 μ g/ml HA, 4-fold ($p = 0.006$) at 5 μ g/ml, and 2-fold ($p = 0.05$) at 10 μ g/ml (Figure 6C). A similar dose response with a maximum increase of pERK1/2 at 5 μ g/ml was observed in cells expressing full-length 315-kDa HARE cDNA (not shown).

HA Has No Effect on Phosphorylation of JNK and p38 MAP Kinase—Next, we determined if there was also activation of JNK and p38 MAP kinase, or if there was a differential activation of ERK kinases, compared with the other two MAP kinases. In contrast to the ERK1/2 results, no time- (Figure 7, A and C) or dose-dependent (Figure 7, B and D) increases in phosphorylation were observed for either JNK or p38 MAP kinase in 190-HARE or 315-HARE (not shown) cells treated with HA. These results indicate that HA binding to HARE mediates a specific response to activate ERK in cells expressing the full-length 315-HARE or 190-HARE alone.

HA-HARE-mediated ERK Phosphorylation Is Blocked by a MEK Inhibitor—We tested the effect of PD 98059, a specific inhibitor of MEK, which is the upstream kinase activator of ERK, on HA-induced phosphorylation of ERK in 190- and 315-HARE cells. MEK inhibition decreased HA-stimulated pERK levels, without affecting the levels of the two ERK or the two HARE proteins (Figure 8A). HA-induced ERK phosphorylation was greatly inhibited by 50 μ M PD 98059 and the pERK to tERK ratio was lower than that observed in the absence of inhibitor (Figure 8B). Quantification of results from two dose-response experiments showed that PD 98059 at 0.1 and 1 μ M had no or little effect on ERK phosphorylation, whereas 10 μ M caused a ~30% reduction of ERK activation (Figure 8C). HA-induced phosphorylation of ERK was ~70% inhibited by 50 μ M PD 98059, reaching essentially the basal level of pERK1/2 in untreated cells. The results support the conclusion that HA binding to HARE stimulates pERK production by MEK.

HARE-mediated ERK Activation Requires Its HA-binding Activity—To confirm that HA binding to HARE is responsible for the intracellular signaling events that activate ERK1/2, we assessed the effect of HA on cells expressing a 190-HARE mutant lacking the 93-aa Link domain (Figure 9). Compared with cells with WT HARE, cells expressing HARE(Δ Link) were 90% inhibited in their ability to bind and endocytose

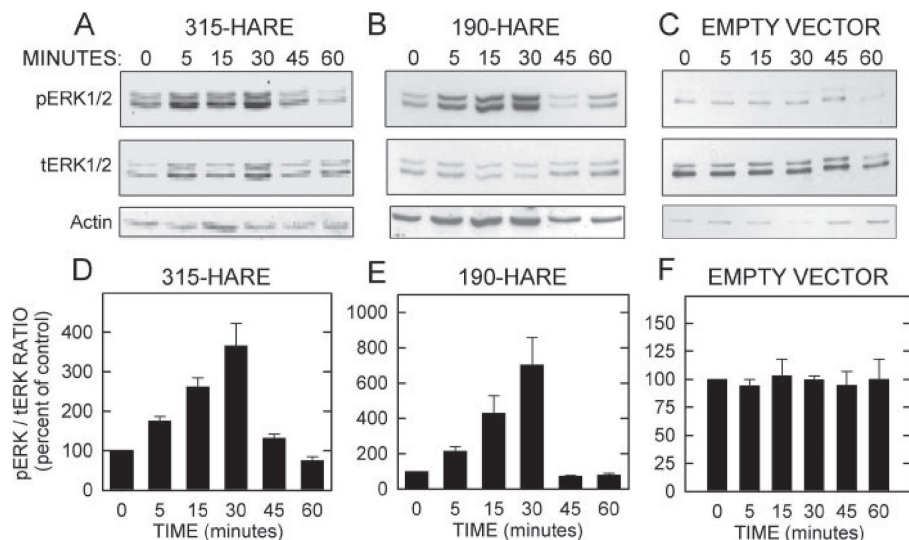


Figure 5. HA induces time-dependent changes in ERK phosphorylation in 315- and 190-HARE cells. Cells expressing 315-HARE (A), 190-HARE (B), or EV (C) were grown to confluence and washed. After a 1-h incubation in serum-free medium at 37 °C, the cells were incubated in the presence or absence of 10 $\mu\text{g/ml}$ HA for 0–30 min as indicated. Lysate samples were then subjected to 10% SDS-PAGE and Western analysis with Ab against pERK1/2 (top panels). The same blots were stripped and reprobed with Ab against tERK1/2 protein (middle panels), and then anti-actin (bottom panels). Blots from independent experiments were scanned, densitometry analyses performed, and the normalized results are presented in D (315-HARE, $n = 3$), E (190-HARE, $n = 3$), and F (EV, $n = 2$) as the mean \pm S.E. of the pERK to tERK ratio (compared with untreated cells at zero time, as 100%).

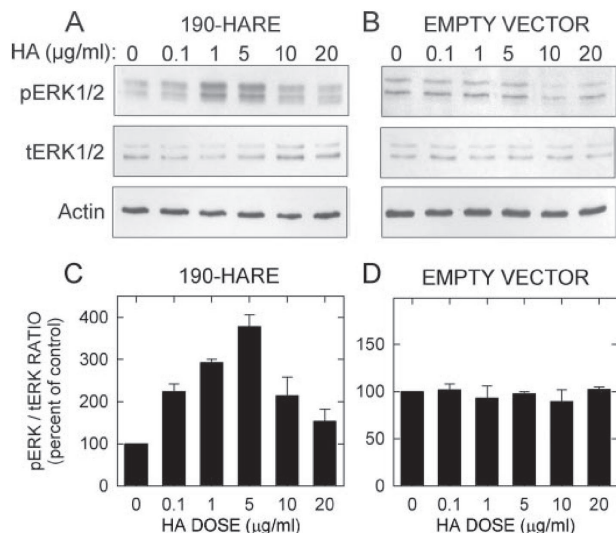


Figure 6. HA induces dose-dependent changes in ERK phosphorylation in 190-HARE cells. Cells stably expressing 190-HARE (A and C) or EV (B and D) were grown to confluence, washed, and incubated for 1 h in serum-free medium at 37 °C. The cells were then incubated for 20 min at 37 °C with the indicated concentrations of HA, chilled, lysed, and samples were analyzed by 10% SDS-PAGE and Western blotting with Ab against pERK1/2. The same blots were successively reprobed with anti-tERK1/2 Ab to detect total protein and then anti-actin Ab, as indicated. The blots from independent experiments were scanned, densitometry analysis performed, and the normalized results are presented as the mean \pm S.E. of the pERK to tERK ratio (compared with untreated cells, as 100%) for 190-HARE (C, $n = 3$) and EV (D, $n = 2$) cells.

^{125}I -HA (Figure 9A). In experiments to assess ERK1/2 activation in response to HA (as in Figures 5 and 6), the HARE(Δ Link) cells showed no detectable ERK phosphorylation response, even up to 20 $\mu\text{g/ml}$ HA (Figure 9B). Thus, HA binding to HARE initiates the intracellular ERK activation

and signaling processes. The signaling response is not due to a contaminant in the HA preparations, nor is it due to different ligand-binding regions of HARE that interact with other endogenous ligands (e.g. acetylated-LDL) on the cell surface or in the medium.

Discussion

The results presented here show for the first time that HA binding to HARE stimulates intracellular signal transduction pathways associated with cellular responses to ECM components. The Link domains in many HA-binding proteins and receptors are directly responsible for their HA binding activity (37–39). Deleting the Link domain of HARE almost completely eliminated its ability to mediate HA binding and endocytosis, and to stimulate intracellular ERK activation in stably transfected cells.

HA binding induces a time- and dose-dependent increase in the phosphorylation of ERK1 and ERK2 in cells stably expressing either the 315- and 190-HARE or the 190-HARE alone. After incubation with HA for 30 min, phosphorylated ERK1 and ERK2 reached maximum levels in both cell types. A maximum increase of pERK1/2 occurred at 5 $\mu\text{g/ml}$ HA. In contrast to ERK activation, HA (up to 20 $\mu\text{g/ml}$ for 60 min) did not stimulate phosphorylation of two other MAP kinases, JNK and p38. In the absence of added HA, both HARE isoforms are found in stable complexes with all three MAP kinases. HA binding to 190-HARE cells did not increase the amount of ERK-HARE complexes, indicating that HA binding to HARE might induce activation of ERK1/2 in pre-existing complexes. HA binding to HARE also resulted in increased phosphorylation of HARE on Tyr. HA-induced ERK phosphorylation was also diminished \sim 70% by the MEK inhibitor PD 98059 (50 μM), which prevents activation (*i.e.* phosphorylation) of ERK by MEK in other cell types (40, 41).

HARE was first characterized as an endocytic clearance receptor that mediates the systematic removal of HA and CSs from the circulatory and lymphatic systems (1, 42). HARE is highly expressed in the sinusoidal cells of lymph nodes, which are the initial and primary sites for the clearance of high molar mass HA ($>10^6$ Da) and CS; about 85% of the HA entering nodes is degraded. A second HA/CS clearance site utilizes HARE in the sinusoidal endothelial cells of liver, accounting for degradation of the remaining (\sim 15%) smaller HA that leaves lymph nodes and enters the vascular system (43, 44). HARE is also highly expressed in spleen, where it may play an as yet unknown role in the immune system, and to a lesser extent in other tissues, as noted under Introduction. In addition to HA and multiple CS types, HARE/Stabilin-2 specifically recognizes advanced glycation end products (45), and we recently discovered that the 190-HARE and 315-HARE are also endocytic receptors for heparin (E. N. Harris, J. A. Wei-

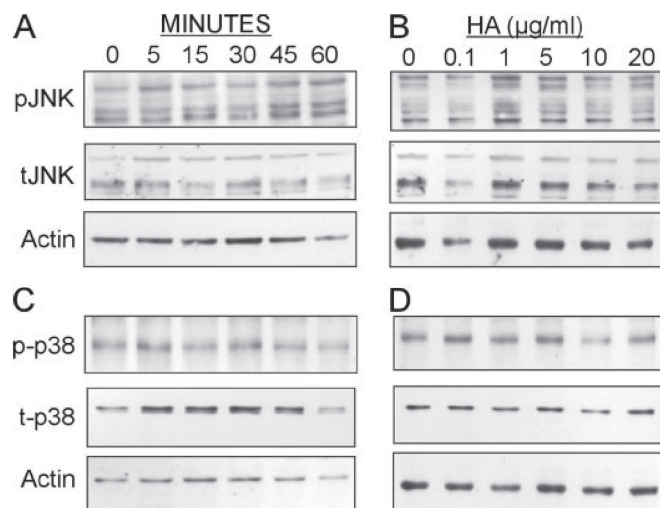


Figure 7. HA does not induce time- or dose-dependent changes in the phosphorylation of JNK or p38 MAP kinase. Cells stably expressing 190-HARE were grown to confluence, washed, incubated in serum-free medium for 1 h at 37 °C, and then incubated either with 10 µg/ml HA for the indicated times (A and C) or with the indicated amounts of HA for 1 h (B and D). The cells were then lysed and samples were analyzed by SDS-PAGE and Western blotting using Ab against either pJNK (A and B) or p-p38 MAP kinase (C and D). The membranes were stained with anti-tJNK, successively stripped, and probed with anti-t-p38 and then anti-actin Ab, as indicated.

gel, and P. H. Weigel *J. Biol. Chem.* [April 22, 2008] doi: 10.1074/jbc.M710360200).

Thus, HARE can bind and internalize four different classes of ligands. Additionally, ongoing studies indicate that the cytoplasmic domain, although small, contains at least three redundant motifs for targeting HA-HARE complexes to coated pits (M. S. Pandey, E. N. Harris, J. A. Weigel, and P. H. Weigel, unpublished data). Deletion of any of these three motifs only partially decreases the rate of endocytosis, indicating that HARE complexes might have multiple intracellular routing pathways. Because HARE is expressed in tissues that are not involved in the systemic clearance of HA or CS, we have suspected that HARE may have functions other than simply ligand clearance. This study supports the possibility that the HARE isoforms allow cells to sense and respond to a variety of different ECM molecules.

The HA field has been energized in recent years by the elucidation of HA-dependent signaling pathways, providing novel insights into the biological functions of this ECM molecule (23, 46–48). Binding of HA to CD44 or RHAMM stimulates intracellular signal transduction, by activating protein phosphorylation, and modifies cell-cell and cell-matrix interactions (22, 23). HA activates several protein Tyr and Ser/Thr kinases (including MAP kinases, protein kinase C, focal adhesion kinase, and Src protein Tyr kinase) and thereby promotes the expression of specific proteins involved in ECM remodeling and cell differentiation (23). The biology of HA now encompasses a wider array of cellular behaviors, in addition to its important and well established ECM structural roles.

Our experiments used non-animal-derived HA with a weight-average molar mass of 580 kDa for several reasons. Using HA made by bacterial fermentation avoids artifacts due to contaminating mammalian (*e.g.* umbilical cord-derived) or other eukaryotic (*e.g.* chicken-derived) proteins

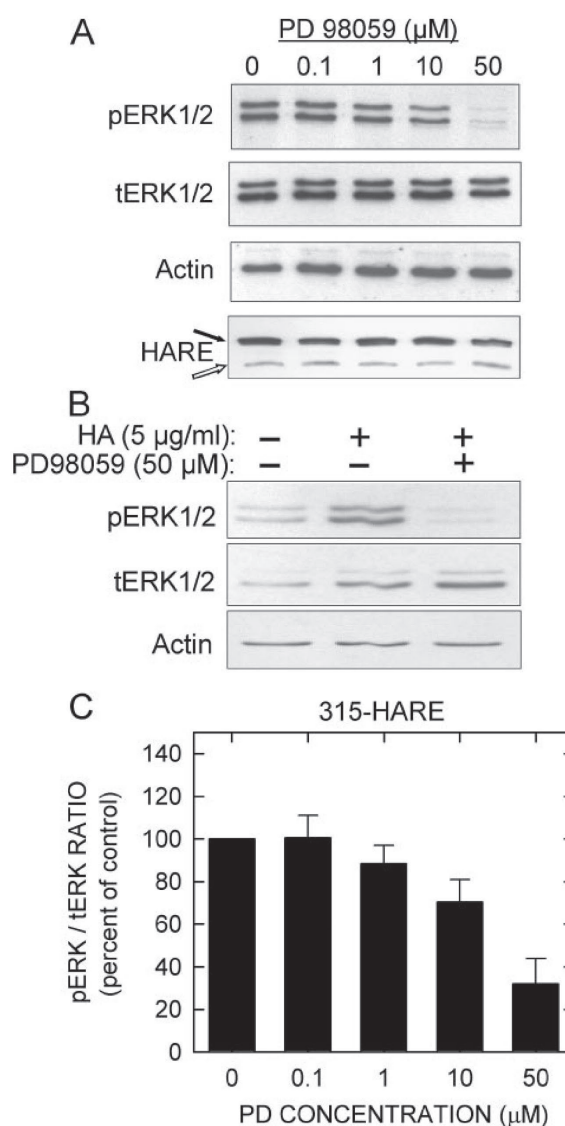


Figure 8. The MEK inhibitor PD 98059 inhibits HA- and HARE-mediated ERK phosphorylation. Cells stably expressing 315-HARE (A and C) or 190-HARE (B) were grown to confluence, washed, and incubated in serum-free medium for 1 h at 37 °C. A, the 315-HARE cells were incubated with different amounts of PD 98059 for 30 min, as indicated, and then treated for 20 min with 10 µg/ml HA. The cells were chilled, lysed, and samples subjected to 10% SDS-PAGE and Western blot analysis using anti-pERK1/2. The same blot was successively stripped and reprobed with anti-tERK and then anti-actin Ab, as indicated. A parallel 5% SDS-PAGE was run with the same amount of protein and Western blot analysis was performed using anti-V5 Ab to identify the 315-HARE (solid arrow) and 190-HARE (open arrow) proteins. B, 190-HARE cells were grown to confluence, washed, incubated in serum-free medium for 1 h at 37 °C and then incubated, as indicated, with or without PD 98059 for 30 min and then without or with 5 µg/ml HA for 20 min. Cell lysates were subjected to SDS-PAGE and Western blot analysis using anti-pERK1/2, and the blot was stripped and reprobed with anti-tERK and then anti-actin Abs, as indicated. C, two sets of blots from independent experiments using 315-HARE cells were scanned, analyzed by densitometry, and the normalized results are expressed as the mean ± S.E. ratio of pERK to tERK; the value with no inhibitor present is set to 100%.

bound to the HA. We used the same size HA in previous endocytosis studies of the recombinant human 190- and 315-HARE and know it is bound with high affinity and efficiently

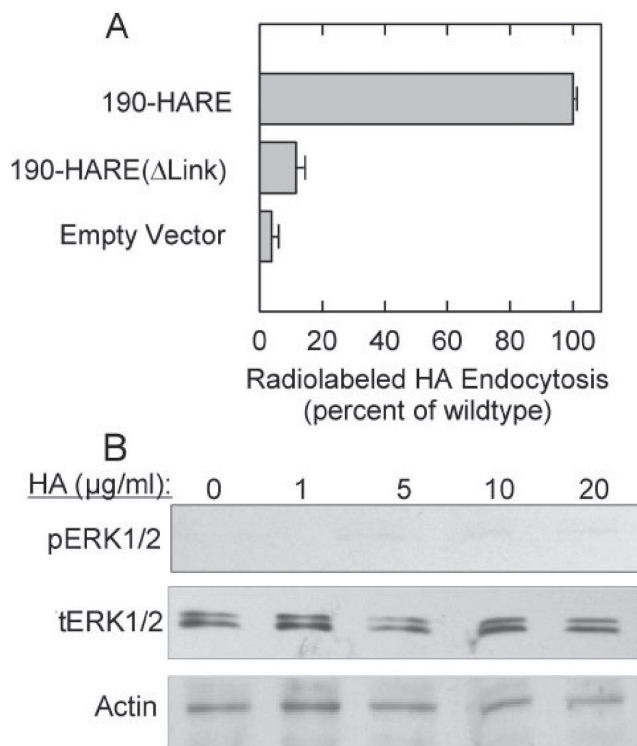


Figure 9. HA endocytosis and ERK activation are greatly decreased in a 190-HARE(Δ Link) mutant. Cells expressing WT or (Δ Link) mutant 190-HARE or EV were grown and preincubated as described in the legend to Figure 6. *A*, the cells were incubated with 125 I-HA for 4 h at 37°C to assess specific endocytosis. Values are the mean \pm S.E. ($n = 9$ –12) percent of endocytosis compared with WT (100%), from four independent experiments. *B*, ERK phosphorylation was assessed as described in the legend to Figure 6 after incubation with the indicated concentrations of HA for 20 min. Lysate samples were then subjected to 10% SDS-PAGE and Western analysis with Ab against pERK1/2 (*top panel*). The same blot was stripped and reprobed with Ab against tERK1/2 protein (*middle panel*), and anti-actin (*bottom panel*).

internalized (16, 17). Finally, it makes sense that cells may need to detect and respond to smaller HA fragments with more urgency than native high mass HA. Under pathological conditions lower molar mass HA can accumulate and affect inflammation, angiogenesis, wound healing and cancer (49). For example, HA of molar masses up to 950 kDa induces Tyr phosphorylation in NCI-H82 cells stably transfected with human CD44E cDNA (50).

MAP kinases are a family of evolutionary well conserved proteins that are expressed in all eukaryotic cells. They are components of signaling pathways that relay, amplify, and integrate signals from a variety of extracellular stimuli, thus controlling the genomic and physiological response of a cell to its environment (24, 28). The three major groups of MAP kinases, ERK, JNK, and p38, are Ser/Thr protein kinases. Distinct MAP kinase cascades occur in selective activation responses to different extracellular stimuli, leading to a wide variety of cellular function changes. In this study we found that basal pERK1/2 levels in cells stably expressing the full-length HARE or 190-kDa HARE were >3 times higher than in EV cells. In contrast, there was no difference in the basal phosphorylation status of JNK or p38 MAP kinase. Furthermore, co-immunoprecipitation results demonstrated an as-

sociation of HARE not only with ERK but also with the two other members of the MAP kinase family. Formation of intracellular multiprotein complexes is a mechanism by which cells integrate and transmit different signals. We observed basal levels of HARE-ERK, HARE-JNK, and HARE-p38 complexes, although only ERK1/2 were activated upon HARE binding of HA. We do not know if all three MAP kinases simultaneously associate with HARE to create quaternary complexes, but this may be likely. RHAMM also associates with ERK, but not with JNK or p38 (51).

Protein phosphorylation, specifically on Ser, Thr, and Tyr residues, is one of the most important and widespread post-translational modifications. Protein phosphorylation regulates many metabolic pathways and also regulates the activation of signal transduction cascades that in turn regulate gene expression and cellular functions (52). To understand the molecular basis of these regulatory mechanisms, it is necessary to identify the key protein phosphorylation sites. The cytoplasmic domain of the 190- and 315-HARE (Tyr²⁴⁸⁰-Leu²⁵⁵¹) contains four Tyr, seven Ser, one His, and five Thr residues (10, 12), although only residues Ser²⁴⁹⁷, Ser²⁵³⁷, Thr²⁵²³, Tyr²⁵¹⁹, and Tyr²⁵³¹ are predicted (by NetPhos 2.0) to be phosphorylated. As expected, the cytoplasmic domain contains several candidate Tyr residues whose phosphorylation may be HA-mediated. To identify key HARE residues in the HARE cytoplasmic domain required for formation of HARE-ERK complexes and to mediate the HA-dependent activation of ERK-dependent signal transduction pathways, we are creating stable cell lines expressing 190-HARE variants with defined aa substitutions or deletions.

In summary, we present evidence that HA binding to hHARE induces Tyr phosphorylation of HARE and selectively induces the phosphorylation of ERK1 and ERK2 in a dose- and time-dependent manner. In contrast, HA binding to HARE does not induce the phosphorylation of JNK or p38 MAP kinase. The conclusion that HA-induced ERK phosphorylation is mediated by MEK, an upstream ERK kinase, is supported by the inhibitory effect of the MEK inhibitor PD 98059. Our results indicate that HA binding to hHARE regulates intracellular Tyr and Ser/Thr protein kinases. This study uncovers a novel and previously unknown role for hHARE in cell signaling, particularly in activating the ERK signaling pathway.

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References

- Laurent, T. C., and Fraser, J. R. E. (1992) *FASEB J.* 6, 2397–2404
- Laurent, T. C., Laurent, U. B., and Fraser, J. R. E. (1996) *Ann. Med.* 28, 241–253
- Toole, B. P. (2001) *Semin. Cell Dev. Biol.* 12, 79–87
- Hascall, V. C., Majors, A. K., De la Motte, C. A., Evanko, S. P., Wang, A., Drazba, J. A., Strong, S. A., and Wight, T. N. (2004) *Biochim. Biophys. Acta* 1673, 3–12
- Termeer, C., Sleeman, J. P., and Simon, J. C. (2003) *Trends Immunol.* 24, 112–114

6. Bertrand, P., Girard, N., Delpech, B., Duval, C., Danjou, J., and Dauce, J. P. (1992) *Int. J. Cancer* 52, 1-6
7. Anttila, M. A., Tammi, R. H., Tammi, M. I., Syrjänen, K. J., Saarikoski, S. V., and Kosma, V. M. (2000) *Cancer Res.* 60, 150-155
8. Zhou, B., Oka, J. A., Singh, A., and Weigel, P. H. (1999) *J. Biol. Chem.* 274, 33831-33834
9. Zhou, B., Weigel, J. A., Fauss, L. A., and Weigel, P. H. (2000) *J. Biol. Chem.* 275, 37733-37741
10. Politz, O., Gratchev, A., McCourt, P. A. G., Schledzewski, K., Guillot, P., Johansson, S., Svineng, G., Franke, P., Kannicht, C., Kzhyshkowska, J., Longati, P., Velten, F. W., and Goerdts, S. (2002) *Biochem. J.* 362, 155-164
11. Adachi, H., and Tsujimoto, M. (2002) *J. Biol. Chem.* 277, 34264-34270
12. Zhou, B., McGary, C. T., Weigel, J. A., Saxena, A., and Weigel, P. H. (2003) *Glycobiology* 13, 339-349
13. Zhou, B., Weigel, J. A., Saxena, A., and Weigel, P. H. (2002) *Mol. Biol. Cell* 13, 2853-2868
14. Falkowski, M., Schledzewski, K., Hansen, B., and Goerdts, S. (2003) *Histochem. Cell Biol.* 120, 361-369
15. Ulbrich, S. E., Schoenfelder, M., Thoene, S., and Einspanier, R. (2004) *Mol. Cell. Endocrinol.* 214, 9-18
16. Harris, E. N., Weigel, J. A., and Weigel, P. H. (2004) *J. Biol. Chem.* 279, 36201-36209
17. Harris, E. N., Kyosseva, S. V., Weigel, J. A., and Weigel, P. H. (2007) *J. Biol. Chem.* 282, 2785-2797
18. McGary, C. T., Raja, R. H., and Weigel, P. H. (1989) *Biochem. J.* 257, 875-884
19. Smedsrod, B., Malmgren, M., Ericsson, J., and Laurent, T. C. (1988) *Cell Tissue Res.* 253, 39-45
20. Pierce, K. L., Maudsley, S., Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 1489-1494
21. McPherson, P. S., Kay, B. K., and Hussain, N. K. (2001) *Traffic* 2, 375-384
22. Entwistle, J., Hall, C. L., and Turley, E. A. (1996) *J. Cell. Biochem.* 61, 569-577
23. Turley, E. A., Noble, P. W., and Bourguignon, L. Y. W. (2002) *J. Biol. Chem.* 277, 4589-4592
24. Kyosseva, S. V. (2004) *Int. Rev. Neurobiol.* 59, 201-220
25. Khaldoyanidi, S., Moll, J., Karakhanova, S., Herrlich, P., and Ponta, H. (1999) *Blood* 94, 940-949
26. Hamerman, D., Todaro, J., and Green, H. (1965) *Biochim. Biophys. Acta* 101, 343-351
27. Ohno, S., Im, H. J., Knudson, C. B., and Knudson, W. (2006) *J. Biol. Chem.* 281, 17952-17960
28. Wang, C., Thor, A. D., Moore, D. H., Zhao, Y., Kerschmann, R., Stern, R., Watson, P. H., and Turley, E. A. (1998) *Clin. Cancer Res.* 4, 567-576
29. Hamilton, S. R., Fard, S. F., Paiwand, F. F., Tolg, C., Veiseh, M., Wang, C., McCarthy, J. B., Bissell, M. J., Koropatnick, J., and Turley, E. A. (2007) *J. Biol. Chem.* 282, 16667-16680
30. Bourguignon, L. Y. W., Gilad, E., Rothman, K., and Peyrollier, K. (2005) *J. Biol. Chem.* 280, 11961-11972
31. Raja, R. H., LeBoeuf, R. D., Stone, G. W., and Weigel, P. H. (1984) *Anal. Biochem.* 139, 168-177
32. Bradford, M. M. (1976) *Anal. Biochem.* 72, 248-254
33. Laemmli, U. K. (1970) *Nature* 227, 680-685
34. Burnette, W. N. (1981) *Anal. Biochem.* 112, 195-203
35. Haynes, P. H., Oka, J. A., and Weigel, P. H. (1994) *J. Biol. Chem.* 269, 33146-33151
36. Mellman, I. (1996) *Annu. Rev. Cell Dev. Biol.* 12, 575-625
37. Toole, B. P. (1990) *Curr. Opin. Cell Biol.* 2, 839-844
38. Seyfried, N. T., McVey, G. F., Almond, A., Mahoney, D. J., Dudhia, J., and Day, A. J. (2005) *J. Biol. Chem.* 280, 5435-5448
39. Day, A. J., and Prestwich, G. D. (2002) *J. Biol. Chem.* 277, 4585-4588
40. Davies, S. P., Reddy, H., Ciavano, M., and Cohen, P. (2000) *Biochem. J.* 351, 95-105
41. Bastow, E. R., Lamb, K. J., Lewthwaite, J. C., Osborne, A. C., Kavanagh, E., Wheeler-Jones, C. P., and Pitsillides, A. A. (2005) *J. Biol. Chem.* 280, 11749-11758
42. Weigel, P. H., and Yik, J. H. N. (2002) *Biochim. Biophys. Acta* 1572, 341-363
43. Fraser, J. R. E., Appelgren, L.-E., and Laurent, T. C. (1983) *Cell Tissue Res.* 233, 285-293
44. Weigel, J. A., Raymond, R. C., McGary, C. T., Singh, A., and Weigel, P. H. (2003) *J. Biol. Chem.* 278, 9808-9812
45. Hansen, B., Longati, P., Elvevold, K., Nedredal, G.-I., Schledzewski, K., Olsen, R., Falkowski, M., Kzhyshkowska, J., Carlsson, F., Johansson, S., Smedsrod, B., Goerdts, S., Johansson, S., and McCourt, P. (2005) *Exp. Cell Res.* 303, 160-173
46. Toole, B. P. (2004) *Nature Rev.* 4, 528-539
47. Bourguignon, L. Y., Gilad, E., and Peyrollier, K. (2007) *J. Biol. Chem.* 282, 19426-19441
48. Lee, J. Y., and Spicer, A. P. (2000) *Curr. Opin. Cell Biol.* 12, 581-586
49. Stern, R., Asari, A. A., and Sugahara, K. N. (2006) *Eur. J. Cell Biol.* 85, 699-715
50. Fujita, Y., Kitagawa, M., Nakamura, S., Azuma, K., Ishii, G., Higashi, M., Kishi, H., Hiwasa, T., Koda, K., Nakajima, N., and Harigaya, K. (2002) *FEBS Lett.* 528, 101-108
51. Zhang, S., Chang, M. C., Zylka, D., Turley, S., Harrison, R., and Turley, E. A. (1998) *J. Biol. Chem.* 273, 11342-11348
52. Kim, S. K., and Novak, R. F. (2007) *Pharmacol. Ther.* 113, 88-120