CD44 Interaction with Na\textsuperscript{+}-H\textsuperscript{+} Exchanger (NHE1) Creates Acidic Microenvironments Leading to Hyaluronidase-2 and Cathepsin B Activation and Breast Tumor Cell Invasion\*\

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We have explored CD44 (a hyaluronan (HA) receptor) interaction with a Na\textsuperscript{+}-H\textsuperscript{+} exchanger (NHE1) and hyaluronidase-2 (Hyal-2) during HA-induced cellular signaling in human breast tumor cells (MDA-MB-231 cell line). Immunological analyses demonstrate that CD44s (standard form) and two signaling molecules (NHE1 and Hyal-2) are closely associated in a complex in MDA-MB-231 cells. These three proteins are also significantly enriched in cholesterol and ganglioside-containing lipid rafts, characterized as caveolin and flotillin-rich plasma membrane microdomains. The binding of HA to CD44 activates Na\textsuperscript{+}-H\textsuperscript{+} exchange activity which, in turn, promotes intracellular acidification and creates an acidic extracellular matrix environment. This leads to Hyal-2-mediated HA catabolism, HD modification, and cysteine proteinase (cathepsin B) activation resulting in breast tumor cell invasion. In addition, we have observed the following: (i) HA/CD44-activated Rho kinase (ROK) mediates NHE1 phosphorylation and activity, and (ii) inhibition of ROK or NHE1 activity (by treating cells with a ROK inhibitor, Y27632, or NHE1 blocker, S-(N-ethyl-N-isopropyl)amiloride, respectively) blocks NHE1 phosphorylation/Na\textsuperscript{+}-H\textsuperscript{+} exchange activity, reduces intracellular acidification, eliminates the acidic environment in the extracellular matrix, and suppresses breast tumor-specific behaviors (e.g. Hyal-2-mediated HA modification, cathepsin B activation, and tumor cell invasion). Finally, downregulation of CD44 or Hyal-2 expression (by treating cells with CD44 or Hyal-2-specific small interfering RNAs) not only inhibits HA-mediated CD44 signaling (e.g. ROK-mediated Na\textsuperscript{+}-H\textsuperscript{+} exchanger reaction and cellular pH changes) but also impairs oncogenic events (e.g. Hyal-2 activity, hyaluronan modification, cathepsin B activation, and tumor cell invasion). Taken together, our results suggest that CD44 interaction with a ROK-activated NHE1 (a Na\textsuperscript{+}-H\textsuperscript{+} exchanger) in cholesterol/ganglioside-containing lipid rafts plays a pivotal role in promoting intracellular/extracellular acidification required for Hyal-2 and cysteine proteinase-mediated matrix degradation and breast cancer progression.

CD44 is a multifunctional transmembrane glycoprotein expressed in many cells and tissues including breast tumor cells and carcinoma tissues (1–6). It is often expressed in a number of isoforms, products of a single gene generated by alternative splicing of variant exons inserted into an extracellular membrane-proximal site (7). The expression of certain CD44 variant (CD44v) isoforms is closely associated with breast tumor progression (1–6). The external portion of CD44 binds to extracellular matrix (ECM)\* components, hyaluronan (HA) (8–10), whereas the intracellular domain of CD44 interacts with specific signaling molecules such as Tiam1 (11), Vav2 (12), RhoA-activated ROK (13, 14), c-Src kinase (15, 16), p185\textsuperscript{HER2} (12, 17), and transforming growth factor-\(\beta\) receptors (19). CD44 also binds directly to the cytoskeleton (15, 20–22, 25). The interaction of CD44 with the cytoskeleton and various signaling molecules plays a pivotal role in promoting invasive and metastatic specific tumor phenotypes such as matrix metalloproteinase-mediated matrix degradation, tumor cell growth, migration, and invasion (1, 26).

In many cell types the plasma membrane contains specialized microdomains called lipid rafts that have distinct lipid and protein compositions (27, 28). One type of lipid raft that forms invaginated plasma membrane domains, termed caveolae, is known to consist of caveolin, flotillin, cholesterol, and sphingolipids (27–31). The functional significance of lipid rafts is indicated by the recruitment of key signaling molecules into raft structures during the propagation of signal cascades (27–31). Furthermore, protein acylation is thought to be one of the important mechanisms for recruiting certain proteins into cholesterol-containing lipid rafts (32, 33) or for bringing proteins to the proximal region of lipid rafts (34). The fact that CD44 is fatty acid-acylated (35) and that up to 40% of total cellular CD44 is co-localized with caveolin in lipid rafts (36) suggests that acylation is critical for promoting CD44 accumulation into lipid rafts (33). However, the role of CD44-containing lipid rafts in regulating HA signaling is only now beginning to be elucidated.

Tumor invasion and metastasis are the major causes of treatment failure for cancer patients (37). These processes are often associated with acidification of the tumor microenvironment (38). For example, mammary cancer cells acidify the extracellular environment more effectively than normal mammary...
CD44-NHE1 Signals Activate pH Changes, Hyal-2, and Cathepsin B

cells (40). Moreover, metastatic breast tumor cells (MDA-MB-231 cells) have been observed to develop acidic microenvironments (41). Nevertheless, it is clear that the mechanisms responsible for acidification-related tumor progression are quite complex, involving a number of biochemical and cellular events. It is known that lactate secretion by tumor cells is one of the important factors in the acidification of the immediate microenvironment of primary tumors (42). However, because primary mouse tumors derived from cells lacking lactate dehydrogenase are also capable of acidifying the tumor microenvironment (43), other mechanism(s) must be involved in regulating extracellular acidification during tumor progression.

In this regard, a number of studies have been carried out to identify molecules expressed by tumor cells that correlate with the regulation of pH changes. The Na\(^+\)/H\(^+\) exchanger (NHE) is one of the principal intracellular pH regulatory molecules (44–46). Previous studies (47–49) indicate that NHE1 is one of the principal intracellular pH regulatory molecules in breast tumor cells. NHE1 also appears to be involved in the aberrant regulation of both extracellular pH (pH\(_e\)) and intracellular pH (pH\(_i\)) in human breast tumor cells under nutrient-depleted conditions (50). Some observations on the localization and regulation of certain NHE isoforms suggest that there are connections between NHE activity and cholesterol-enriched lipid rafts (51, 52). Most interesting, NHE1 also serves as one of the cellular substrates for RhoA-activated Rho kinase (ROK) (53). NHE1 phosphorylation by RhoA-activated ROK induces actin stress fiber assembly (53). The question of whether HA/CD44-activated RhoA-ROK signaling plays any role in regulating NHE1 activity and cellular pH changes during breast tumor cell invasion is the focus of this study.

Low pH environment promotes tumor cell-specific behaviors including activation of ECM-degrading enzymes for tumor cell invasion (54–56). In fact, extracellular acidity plays an important role in activating ECM-degrading enzymes including cysteine proteinases such as cathepsin B (54–57). In general, cathepsin B is secreted in a latent form that requires activation at low pH. Malignant tumor progression correlates with increased levels of the activated cathepsin B (54–57). Another acid-active ECM-degrading enzyme is the mammalian hyaluronidases such as hyaluronidase-2 (Hyal-2), which is present in virtually all tissues (58, 59). Hyal-2 belongs to a family of endo-N-acetylated hexosaminidases that hydrolyze HA and to a lesser extent chondroitin sulfates (58–60). It is a cell surface enzyme via glycosylphosphatidylinositol (GPI) linkage to the plasma membrane (60, 61). The enzymatic activity of Hyal-2 is up-regulated in acidic compartments derived from invaginated plasma membrane microdomains such as lipid rafts (60, 61). Activated Hyal-2 hydrolyzes high molecular mass HA into intermediate sized fragments of ~20 kDa corresponding to about 20,000–1,000,000 dalton polymers, which were purified by gel filtration chromatography using Sephacryl S1000 columns. The purity of high molecular weight HA polymers used in our experiments was further verified by anion exchange high performance liquid chromatography. No small HA fragments were detected in these preparations. Y27632 and CA-074-Me were purchased from Calbiochem. EIPA was obtained from Sigma.

**Materials and Methods**

**Cell Culture**—The breast tumor cell line (MDA-MB-231 cells) was obtained from the American Type Culture Collection and grown in Eagle’s minimum essential medium supplemented with Earle’s salt solution, essential and nonessential amino acids, vitamins, and 10% fetal bovine serum.

**Antibodies and Reagents**—Monoclonal rat anti-CD44 antibody (clone, 020; isotype, IgG1, obtained from CMB-TECH, Inc., San Francisco) recognizes a common determinant of the HA binding region of CD44 isoforms including CD44s, CD44E, and CD44 variant species. This rat anti-CD44 was routinely used for HA-related blocking experiments. Polyclonal rabbit anti-CD44s antibody, which was raised against a unique N-0-glycosylated moiety of 85-kDa CD44s, is mono-specific for most of the glycosylated CD44s expressed on the cell surface and displays no cross-reactivity to other CD44 isoforms (e.g., CD44E or CD44 variant species). This reagent was routinely used for immunoblotting experiments. Rabbit anti-Rho kinase (ROK) was prepared according to procedures described previously (13). For the preparation of polyclonal rabbit anti-Hyal-2 antibody, specific synthetic peptides (~15–17 amino acids unique for the Hyal-2 sequence) were prepared by the Peptide Laboratories Inc.(South San Francisco, CA). Monoclonal mouse anti-caveolin, mouse anti-flotillin-1, and mouse anti-flotillin-2 were purchased from BD Biosciences. Other immunoreagents such as rabbit anti-Hyal-2 were purchased from Alpha Diagnostic International (San Antonio, TX). High molecular weight HA polymers (~500,000–1,000,000 dalton polymers) were purified by gel filtration chromatography using Sephacryl S1000 columns. The purity of high molecular weight HA polymers used in our experiments was further verified by anion exchange high performance liquid chromatography. No small HA fragments were detected in these preparations. Y27632 and CA-074-Me were purchased from Calbiochem. EIPA was obtained from Sigma.

**CD44siRNA and Hyal-2si RNA**—Preparations of CD44siRNA and Hyal-2si RNA—Preparations of CD44siRNA and Hyal-2si RNA were carried out in the absence of Superscript II reverse transcriptase or in the presence of compounds included in the PCR mixture. No amplification products were detected in these control samples.

**Preparations of CD44sRNA and Hyal-2si RNA**—The siRNA sequence targeting human CD44 or Hyal-2 (from mRNA sequence, GenBankTM accession number AF251595) corresponds to the coding region relative to the first nucleotide of the start site. Target sequences were
selected using the software developed by Ambion Inc., UK. As recommended by Ambion, CD44 or Hyal-2-specific target regions were selected beginning 50–100 nucleotides downstream from the start codon. Sequences close to 50% G/C content were chosen. Specifically, CD44 target sequence 1 (5'-AAAAATGCTTCGCAAGACCATC-3'), CD44 target sequence 2 (5'-AATAAGCAGCTTGGCCACAACTG-3'), and scrambled sequences (5'-AAGACCGTAGATGGCACCAGT-3') were used. Hyal-2 target sequence 1 (5'-AGAATGGCCTGAGGAAGGCTT-3'), Hyal-2 target sequence 2 (5'-AACGGTGGAGGACATCATC-3'), and scrambled sequences (5'-AAGGGTGTGGAGAAGGCTT-3') were used. CD44 or Hyal-2-specific target sequences were then aligned to the human genome data base in a BLAST search to eliminate sequences with significant homology to other genes. Sense and antisense oligonucleotides were then designed as described previously (68). The CD44 oligonucleotides were provided by the University of California, San Francisco, Biomolecular Research Unit. For construction of the siRNA, a transcription-based kit from Ambion was used (Silencer™ siRNA construction kit). MDAMB-231 cells were then transfected with siRNA using siPORT™ Lipid as transfection reagent (Silencer™ siRNA transfection kit). MDA-MB-231 cells were then transfected with siRNA using siPORT™ Lipid as transfection reagent (Silencer™ siRNA transfection kit; Ambion, TX) according to the protocol provided by Ambion. Cells were incubated with 50 pmol of CD44siRNA or 50 pmol of Hyal-2siRNA (or siRNA containing scrambled sequences or no siRNA) for at least 48 h before biochemical experiments and/or functional assays were conducted as described below.

Measurement of Cholesterol Content and Ganglioside Composition in Lipid Rafts—MDA-MB-231 cells were serum-starved for 24 h followed by incubation with 50 µg/ml HA (or no HA) for 5 min in the presence or absence of a cholesterol-depletion agent, methyl-β-cyclodextrin. These cells were then scraped in phosphate-buffered saline, spun at 2,000 rpm at 4 °C, and lysed with 0.2 ml of TN solution (25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol, mixture of protease inhibitors, 10% sucrose, and 1% Triton X-100) for 30 min on ice. Subsequently, Triton X-100-insoluble materials were mixed with 0.6 ml of cold 60% OptiPREP™ and overlaid with 0.6 ml each of 40, 30, and 20% OptiPREP™ in TN solution. The gradients were centrifuged at 35,000 rpm in an SW 60 rotor for 12 h at 4 °C, and different fractions were collected. Cellular proteins or lipids associated with each fraction were precipitated and analyzed by SDS-PAGE plus immunoblotting analysis as described previously. In some cases, cholesterol content associated with different fractions was also measured using Amplex Red™ cholesterol assay kit (Molecular Probes). Ganglioside compositions associated with lipid rafts were analyzed by high-performance liquid chromatography (HPLC) as described previously (69). The specific activity was measured by Na⁺-dependent pH recovery after an NH₄Cl-induced acid load (69). Specifically, BCECF-labeled cells (treated with no drug or treated with a ROK inhibitor, Y27632, or an NHE1 blocker, EIPA, in the presence or absence of 50 µg/ml HA) were incubated with the HEPES buffer (bicarbonate-free NaHCO₃) containing 20 mM NH₄Cl, followed by replacing the medium with Na⁺-, NH₄⁺-, and lactate-free bicarbonate solution that resulted in acidification of the cytoplasm. The addition of 135 mM Na⁺ produced a rapid rise in pHᵢ, which allowed the initial rate of cellular pH recovery (d(pH)/dt) to be determined. This recovery rate represents a direct measure of the extruding capability of the cell membrane, primarily the activity of the Na⁺/H⁺ exchanger. The rate of pHᵢ recovery (d(pH)/dt) from an acid load was measured by the rapid removal of NH₄Cl was calculated at pHᵢ, intervals of 0.05 units and used as an index of NHE1 activity as described previously (53). Statistical comparisons were made with Student’s t test.

For measuring intravesicular/luminal pH changes, lysosensor yellow/blue (2-(4-pyridyl)-5-((4-(2-dimethylaminoethylarnino-arbamoyl)-methoxy)-phenyl)oxazole (LYB-DND-160, Molecular Probes, Inc., Eugene, OR) was used (70). The emission and excitation spectra of LYB-DND-160 were measured in different fractions and compared with the reaction mixture containing no siRNA (or siRNA containing scrambled sequences or no siRNA). Measurement of Cathepsin B Activity—CD44-NHE1 Signals Activate pH Changes, Hyal-2, and Cathepsin B Activity (71) was monitored by using a spectrophotometer equipped with computer data acquisition. All measurements were made at 23 °C under a 5% CO₂ atmosphere. Fluorescence data were normalized according to the equation: ΔFᵢ/Δt = (Fᵢ - Fᵢ₋₁)/Δt, where Fᵢ is the fluorescence just before pHᵢ of 7.4 and HA addition.

The pHᵢ was measured by phenol-sulfonlfenithalein (phenol red) absorbance according to Gillies et al. (71). Specifically, no cells or HA-treated MDA-MB-231 cells (pretreated with various drugs including 200 nM Y27632, 200 µM EIPA, 10 µM NH₄Cl or transfected with 50 pmol of CD44siRNA or 50 pmol of CD44siRNA containing scrambled sequences or no siRNA) were obtained using a spectrophotometer equipped with computer data acquisition. All measurements were made under a 5% CO₂ atmosphere. Cathepsin B activity in the cell lysate or cellular materials associated with different cellular compartments (e.g. lipid rafts (~20% OptiPREP™ fraction alone), endosomes (66), or lysosomes (67)) were also solubilized by 1.0% Nonidet P-40 followed by immunoblotting with rabbit anti-Hyal-2 antibody. These materials were immunoprecipitated with rat anti-CD44 antibody followed by immunoblotting with anti-CD44s, anti-ROK, anti-NHE1, and anti-phosphothreonine antibodies. In some cases, CD44-NHE1 complexes were then determined by the ECL™ system (Amersham Biosciences). In some experiments, immunoblot analyses of CD44, Hyal-2, ROK, or NHE1 in cells (transfected with siRNA containing scrambled sequences or no siRNA) were also carried out using the procedures described above.

Protein Phosphorylation Assay in Vitro—The kinase reaction was carried out in 50 µl of the reaction mixture containing 40 mM Tris- HCl (pH 7.5), 2 mM EDTA, 1 mM dithiothreitol, 7 mM MgCl₂, 0.1% CHAPS, 0.1 µM calyculin A, 100 µM ATP, purified enzymes (e.g. 100 ng of ROK or no ROK from MDA-MB-231 cells), and 1 µg of NHE1 (obtained from anti-NHE1-associated beads) in the presence or absence of GTP-S•GST-RhoA fusion protein (1 µM) or GST-RhoA alone (1 µM). After incubation for various time intervals, 0, 10, 20, 30, 60, and 120 min, at 30 °C, the reaction mixtures were boiled in SDS sample buffer and subjected to SDS-PAGE. The proteins bands were revealed by anti-phosphoserine/anti-phosphothreonine-mediated immunoblot and anti-NHE1-mediated immunoblot, respectively.
CD44-NHE1 Signals Activate pH Changes, Hya-2, and Cathepsin B

as (arginine/arginine), the creosyl violet leaving group is nonfluorescent (72). Following cathepsin B cleavage at one or both symmetric amide linkage sites, the mono- and nonsubstituted creosyl violet fluorophores generate red fluorescence when excited at 550 nm. Cathepsin B activity was expressed as changes in absorbance at 550 nm wavelength per min due to hydrolysis of cathepsin B fluorogenic substrate. Measurement of cathepsin B activity in the medium of MDA-MB-231 cells was also carried out as follows: a monomer (creosyl violet formed/mg of protein (cell homogenates)) and was determined as

\[ V_{\text{max}} \]

Statistical comparisons were made with Student's t test.

Measurement of Hya-2 Activity—Biotinylated HA covalently bound to Sepharose beads with the aid of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and N-hydroxysulfosuccinimide was incubated with Hya-2 complexed with CD44 or anti-Hya-2-linked Sepharose beads for 5 h at pH conditions (pH 6.5) 

In some experiments, Hya-2 isolated from anti-Hya-2-conjugated beads (using MDA-MB-231 cells transfected with Hya-2siRNA-target sequence 1 and target sequence 2 or Hya-2siRNA-scrubbed sequences) was incubated with biotinylated HA-conjugated beads for 5 h at pH 4.5. The amount of biotinylated HA released from the beads was measured by alkaline phosphatase-conjugated avidin in the presence of p-nitrophenyl phosphate and recorded by a Molecular Devices (Spectra Max) ELISA reader at a wavelength of 405 nm.

In some experiments, Hya-2 isolated from anti-Hya-2-conjugated beads (using MDA-MB-231 cells pretreated with rat anti-CD44 IgG (or normal anti-CD44 IgG) followed by incubating with 50 μg/ml HA) was incubated with purified high molecular weight HA polymers (30,000,000-dalton polymers) for 5 h at pH conditions (pH 7.1 or pH 6.5). These Hya-2-digested HA samples were then analyzed by 5–15% polyacrylamide gels and stained with combined Alcian blue and silver staining according the procedures described by Min and Cowman (39).

HA Size Assays in MDA-MB-231 Cells—The MDA-MB-231 cells (treated with various drugs including 200 nM Y27632, 200 μM EIPA, or 10 μg NH2.CI or transfected with 50 pM of 444siRNA, 50 pM of Hya-2siRNA, or 50 pM of siRNA containing scrambled sequences or no siRNA) were metabolically labeled with 100 μCi/ml [3H]glucosamine (Amersham Biosciences) for 18 h and subsequently rinsed with Hanks' balanced salt solution (HBSS) 2–3 times. The medium and washes were combined and collected. Radioactively labeled cells were then treated with the trypsin/EDTA followed by washing with HBSS for 10 min at 37 °C. Both the trypsin/EDTA solution and cell pellets were collected.

After adding 100 μg/ml unlabeled HA (Healon™, Amersham Biosciences) to various fractions containing medium, trypsin, and cell pellet materials, samples were digested with proteinase K (50 μg/ml) at 60 °C for 4 h, heated at 100 °C for 10 min, and centrifuged at 15,000 × g for 15 min. The supernatants were then precipitated with ethanol, analyzed by distribution using 1 × 30-cm Sephacyl S-300 column chromatography (Amersham Biosciences), and eluted with a solution containing 0.15 m sodium acetate, 0.1% CHAPS (pH 6.8) as described previously (73). Fractions of 0.5 ml were collected. The amount of radioactivity associated with each fraction was determined by scintillation counting. Some fractions were also used to test for their ability to bind to HA-binding protein-coated beads and/or to be digested by PH20 hyaluronidase. Certain size of HA (e.g. the 400,000-dalton 400 kDa) HA isolated from the S-400HR column was also analyzed by 0.5% (w/v) agarose gel electrophoresis and Stains-all stain (23).

Tumor Cell Invasion Assays—Twenty four transwell units were used for monitoring in vitro tumor cell invasion as described previously (74, 75). Specifically, the 5-μm porous polycarbonate filters coated with the reconstituted basement membrane substance Matrigel (Collaborative Research, Lexington, MA) were used for the cell invasion assay (74, 75). MDA-MB-231 cells (1 × 10⁴ cells) well treated with various drugs including 200 nM Y27632, 200 μM EIPA, 10 μg NH2.CI, or 5 nM CA-074-Me, respectively, or transfected with 50 pM of 444siRNA, 50 pM of Hya-2siRNA containing target sequences 1 and 2, or 50 pM of siRNA containing scrambled sequences or no siRNA) were plated in the upper chamber of the transwell unit in the presence or absence of HA.

The growth medium containing high glucose Dulbecco’s modified Eagle’s medium supplemented by 10% fetal bovine serum was placed in the lower chamber of the transwell unit. After 18 h of incubation at 37 °C in a humidified 95% air, 5% CO2 atmosphere, cells on the upper side of the filter were removed by wiping with a cotton tip. Cell invasion processes were determined by measuring the cells that migrated to the lower side of the polycarbonate filters by standard cell number counting methods as described previously (74, 75). The CD44-specific cell invasion was determined by subtracting nonspecific cell invasion (i.e. cells migrate to the lower chamber in the presence of anti-CD44 antibody treatment). Each assay was performed in triplicate and was repeated at least five times. All data were analyzed statistically by Student’s t test, and statistical significance was set at p < 0.01.

RESULTS

Analysis of CD44s-containing Lipid Rafts in MDA-MB-231 Cells

Lipid rafts belong to a specialized plasma membrane microdomain that contains scaffolding proteins such as caveolin and flotillin (27, 28). Specific lipid components such as cholesterol and gangliosides are also present in caveolin/flotillin-containing lipid rafts (27, 28). Furthermore, certain lipid-modified proteins (e.g. GPI-anchored proteins) and some transmembrane proteins such as CD44 are concentrated in lipid rafts (33, 36, 60, 61). In recent years, many investigations have used a Triton X-100 solubility technique to isolate Triton X-100-insoluble materials followed by flotation using OptiPREPTM gradient centrifugation to isolate lipid rafts or caveolin/flotillin-containing microdomains (31, 33).

In order to determine whether CD44 is associated with lipid rafts or caveolin/flotillin-containing microdomains during cellular signaling, we first loaded Triton X-100-insoluble materials (obtained from MDA-MB-231 cells grown in serum) onto a 60% OptiPREPTM gradient layer (i.e. the original loading layer). Following flotation centrifugation, we observed that most of the caveolin-1 (Fig. 1A-a), flotillin-1 (Fig. 1A-b), and flotillin-2 (Fig. 1A-c) migrated from the 60% OptiPREPTM layer to the 20% OptiPREPTM layer. These results are consistent with previous findings indicating that both caveolin-1 and flotillin are found in the 20% OptiPREPTM fraction (31). Furthermore, we have found that ~40–50% of CD44 is also partitioned in the 20% OptiPREPTM layer with the remainder at the 40 and 60% OptiPREPTM layers (Fig. 1A-d). It is noted that some portion of caveolin-1 (but not flotillin-1 or flotillin-2) together with CD44 appears to be retained at the 40 and 60% OptiPREPTM layers (Fig. 1A-a, d) and the amount of CD44 associated with the 40 and 60% OptiPREPTM layer represents ~20 and 10% of the total CD44, respectively. Although the biochemical properties of the 40 and 60% OptiPREPTM fractions are not well characterized, the Triton X-100-insoluble material floating to the low density region (20% OptiPREPTM layers) is well documented as “lipid rafts.” Thus, it is clear that a significant portion of CD44 such as CD44s (the standard form of CD44) in breast tumor cells (MDA-MB-231 cells) is located in lipid rafts.

In order to determine whether HA plays a role in regulating the recruitment of some proteins (e.g. caveolin-1 and CD44) into the lipid raft, MDA-MB-231 cells were serum-starved for 24 h followed by incubation with 50 μg/ml HA (or no HA) for 5 min at 37 °C. It is noted that lipid raft markers, such as caveolin-1 (Fig. 1B-a, lane 1) and a small amount of CD44 (~10–15% of CD44) (Fig. 1B-b, lane 1), are partitioned at the 20% OptiPREPTM layers using Triton X-100-insoluble materials isolated from MDA-MB-231 cells treated with no HA. Most interesting, a significant amount of CD44 (~50% of total CD44) (Fig. 1B-b, lane 2) together with caveolin-1 (Fig. 1B-a, lane 2) is recruited into the 20% OptiPREPTM fraction from the 60% OptiPREPTM layer using Triton X-100-insoluble materials obtained from MDA-MB-231 cells treated with HA. Following cholesterol depletion (by treating MDA-MB-231 cells with methyl-β-cyclodextrin, an agent known to disrupt lipid rafts by depleting cholesterol from the raft membranes), there is a significant reduction of caveolin-1 and CD44 in the lipid raft fractions from MDA-MB-231 cells treated with no HA (Fig. 1, B-a, lane 3, and B-b, lane 3) or with HA (Fig. 1, B-a, lane 4, and B-b, lane 4). The low level of CD44 and caveolin-1 accumulation into the lipid raft (the 20% OptiPREPTM fraction) after cholesterol depletion (either with or without HA treatment) suggests...
that cholesterol plays an important role in stabilizing the CD44 association with caveolin-1 in the lipid raft.

Because both cholesterol and gangliosides have been shown to be important in lipid raft formation and cellular signaling (76), we have also measured the cholesterol concentration (Fig. 1C) and ganglioside content (Fig. 1D) associated with the lipid rafts (the 20–30% OptiPREP™ layer) isolated from untreated MDA-MB-231 cells. Both cholesterol (~3 μg of cholesterol/mg of total protein) (Fig. 1C-a) and the ganglioside, GM3 (a lesser amount of GM1, GM2, and GD1a) (Fig. 1D-a), are present in the lipid rafts isolated from untreated MDA-MB-231 cells. However, treatment of MDA-MB-231 cells with HA greatly increases the cholesterol (~7.5 μg of cholesterol/mg of total protein) (Fig. 1C-b) and GM3 content in the lipid rafts of these cells (Fig. 1D-b). These observations suggest that HA signaling promotes both cholesterol and ganglioside accumulation in CD44 and caveolin-1/flotillin-containing lipid rafts.

HA-induced Recruitment of ROK and NHE1 in CD44-containing Lipid Rafts

Lipid raft structure is known to play an important role in various cellular processes, such as signal transduction (30, 31). In particular, CD44 appears to be involved in the recruitment of key regulatory molecules into raft structures (36, 77). Following density gradient centrifugation of Triton X-100-insoluble material using 20–60% OptiPREP™ layers and immunoblotting with anti-CD44 (a) or anti-CD44 (d)-mediated immunoblotting analyses. B, detection of cellular protein associated with 20% OptiPREP™ layers (from MDA-MB-231 cells serum-starved for 24 h followed by incubation with HA (lanes 2 and 4) or no HA (lanes 1 and 3) in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of a cholesterol-depletion agent, methyl-β-cyclodextrin) using anti-caveolin-1 (a) or anti-CD44 (b)-mediated immunoblotting analyses. C, measurement of cholesterol content in lipid rafts (the 20–30% OptiPREP™ layer) isolated from untreated cells (a) or HA-treated cells (b). D, analysis of the ganglioside composition in lipid rafts (the 20–30% OptiPREP™ layer) isolated from untreated cells (a) or HA-treated cells (b). Gangliosides were separated by high performance (reverse phase-C18) thin layer chromatography and detected by resorcinol using GM3, GM2, GM1, and GD1a as standard markers.
Fig. 2. Detection of NHE1 and ROK in CD44-containing lipid rafts and measurement of ROK-mediated NHE1 phosphorylation. Triton X-100-insoluble materials isolated from MDA-MB-231 cells was mixed with 0.6 ml of cold 60% OptiPREP™ and overlaid with 0.6 ml of each 40, 30, and 20% OptiPREP™. The gradients were centrifuged at 35,000 rpm in an SW 60 rotor for 12 h at 4 °C, and different fractions were collected. The 20% OptiPREP™ layer represents the lipid rafts. Cellular protein associated with each fraction was analyzed as described under “Materials and Methods.” A, detection of cellular protein associated with 20% OptiPREP™ layers isolated from untreated cells (lane 1) or HA-treated cells (lane 2) using anti-NHE1-mediated immunoblotting analyses. B, detection of cellular protein associated with 20% OptiPREP™ layers isolated from untreated cells (lane 1) or HA-treated cells (lane 2) using anti-CD44-mediated immunoprecipitation (IP) followed by immunoblotting in anti-serine (a) or anti-ROK (b) or reblotting with anti-CD44s (c). C, detection of NHE1 phosphorylation by ROK in vitro. The kinase reaction was carried out in the reaction mixture containing purified ROK, NHE1 (obtained from anti-NHE1-associated beads), and ATP in the presence of GTPγS-GST-RhoA or GST-RhoA as described under “Materials and Methods.” a, anti-phosphoserine-mediated immunoblot of NHE1 (obtained from anti-NHE1-associated beads) incubated with the reaction mixture in the absence of ROK (lane 1) or in the presence of ROK plus unactivated RhoA (RhoA without GTPγS bound) (lane 2), ROK plus activated RhoA (RhoA with GTPγS bound) (GTPγS-RhoA) (lane 3), or in the presence of a ROK inhibitor, Y27632 (lane 4), b, anti-phosphothreonine-mediated immunoblot of NHE1 (obtained from anti-NHE1-associated beads) incubated with the reaction mixture in the absence of ROK (lane 1), in the presence of ROK plus unactivated RhoA (e.g., RhoA without GTPγS bound) (lane 2), ROK plus activated RhoA (RhoA with GTPγS bound) (GTPγS-RhoA) (lane 3), or in the presence of a ROK inhibitor, Y27632 (lane 4). D, analyses of HA-induced in vivo phosphorylation of NHE1 in MDA-MB-231 cells. MDA-MB-231 cells were treated with no HA (lane 1) or treated with HA (lane 2). These cells were also pretreated with anti-CD44 (lane 3) or a ROK inhibitor, Y27632 (lane 4), followed by HA treatment. These cells were then solubilized by Nonidet P-40 and immunoprecipitated with anti-NHE1 antibody followed by immunoblotting with anti-phosphoserine (a) or anti-phosphothreonine antibody (b) or reblotting with anti-NHE1 (c).

Intracellular signaling molecules underlying tumor progression (13, 50, 75). In order to determine whether NHE1 and ROK are also present in CD44-associated lipid rafts, we have isolated lipid raft material by 20–60% OptiPREP™ gradient centrifugation, and we then immunoprecipitated samples from untreated (Fig. 2B, lane 1) or HA-treated cells (Fig. 2B, lane 2) with anti-CD44 antibody followed by anti-NHE1 or anti-ROK-mediated immunoblot (or reblotting with anti-CD44s (an antibody recognizes most of the glycosylated CD44s expressed on the cell surface)). Our results indicate that very little NHE1 (Fig. 2B-a, lane 1) or ROK (Fig. 2B-b, lane 1) is detected in the anti-CD44-mediated immunoprecipitated material (Fig. 2B-c, lane 1) by using lipid rafts from untreated cells. However, both NHE1 (Fig. 2B-a, lane 2) and ROK (Fig. 2B-b, lane 2) are found to be complexed with CD44 (Fig. 2B-c, lane 2) in lipid rafts from MDA-MB-231 cells treated with HA. These observations suggest that HA promotes recruitment of signaling complexes, including ROK and NHE1, into lipid rafts of MDA-MB-231 cells.

ROK (also called Rho-associate kinase, a serine-threonine kinase) is known to interact with RhoA in a GTP-dependent manner during HA/CD44-mediated cell activation (13, 14, 75). This enzyme regulates cytoskeleton function by phosphorylating several important signaling regulators during CD44-cytoskeleton interactions (13, 14, 75). NHE1 is also a downstream target of the RhoA-ROK signaling pathway (53). In particular, NHE1 phosphorylation by RhoA-activated ROK is required for the activation of NHE1 (53). Our current data demonstrate that ROK isolated from MDA-MD-231 cells is capable of phosphorylating the NHE1 at both serine (Fig. 2C-a, lane 3) and threonine residues (Fig. 2C-b, lane 3) (as detected by anti-phosphoserine and anti-phosphothreonine-mediated immunoblot, respectively in the presence of activated GTPγS-RhoA).

The level of serine/threonine phosphorylation of NHE1 appears to be relatively low if ROK was incubated with unactivated RhoA (RhoA without GTPγS bound) (Fig. 2, C-a, lane 2, and C-b, lane 2) or with a ROK inhibitor, Y27632 (Fig. 2, C-a, lane 4, and C-b, lane 4). In the absence of ROK, no serine/threonine phosphorylation of NHE1 is detected (Fig. 2, C-a, lane 1, and C-b, lane 1). These results are consistent with a previous finding indicating that NHE1 serves as one of the cellular substrates for RhoA-dependent kinases such as ROK (53).

Further analyses indicate that the level of serine and threonine phosphorylation of NHE1 (as detected by anti-NHE1-mediated immunoprecipitation followed by immunoblotting with anti-serine (Fig. 2D-a, lanes 1–4), anti-threonine (Fig. 2D-b, lanes 1–4), or reblotted with anti-NHE1 (Fig. 2D-c, lanes 1–4)) is greatly enhanced in MDA-MB-231 cells treated with HA (Fig. 2, D-a, lane 1, and D-b, lane 2). In contrast, NHE1 serine and threonine phosphorylation is relatively low in MDA-MB-231 cells without HA treatment (Fig. 2, D-a, lane 1, and D-b, lane 1) or those cells pretreated with anti-CD44 followed by HA treatment (Fig. 2, D-a, lane 3, and D-b, lane 3). These observations strongly support the conclusion that HA-mediated NHE1 phosphorylation is CD44-dependent. It is also noted that treatment of MDA-MB-231 cells with a ROK inhibitor (Y27632) greatly reduces the ability of NHE1 to respond to HA-mediated serine (Fig. 2D-a, lane 4) and threonine phosphorylation (Fig. 2D-b, lanes 1–4). NHE1 serves as one of the cellular substrates for RhoA-dependent kinases such as ROK in vivo (53). These observations suggest that HA not only is involved in the rearrangement of signaling molecules by recruiting ROK and NHE1 into CD44-containing lipid rafts (Fig. 2, A and B) but also is capable of promoting ROK phosphorylation of NHE1 in vivo (Fig. 2D).
Alterations of cellular pH have been found to be closely associated with breast tumor progression (38, 39). Most cells contain a number of membrane transport mechanisms for regulating both pH, and pH$_i$ values (40, 43). In particular, NHE1 has been implicated in cellular pH regulation (44–46). Actually, the level of both intracellular and extracellular pH influences the operation of NHE1 that directly transports H$^+$ either out of the cells or into the endosomes/lysosomes (44–46). To determine whether HA-CD44 signaling influences Na$^+$/H$^+$ exchange activity, we have measured the rate of pH$_i$ recovery (dpH$_i$/dt) after an intracellular acid load produced by the NH$_4$Cl prepulse technique described previously (53, 69). Specifically, MDA-MB-231 cells were first incubated with the fluorescent pH-sensitive probe BCECF. These BCECF-labeled cells were then incubated with the HEPES buffer (bicarbonate-free Na$^+$ medium) containing 20 mM NH$_4$Cl (Fig. 3, A–C, phase I), followed by replacing the medium with Na$^+$-free, N-methylglucamine chloride solution that results in an acidification of the cytoplasm. The addition of Na$^+$-free medium produced a rapid rise in pH$_i$ (Fig. 3, A–C, phase II), which allowed the initial rate of cellular pH$_i$ recovery (dpH$_i$/dt) to be determined (Fig. 3D). This recovery rate (dpH$_i$/dt) represents a direct measure of the acid-extruding capability of the cell membrane, primarily the activity of the Na$^+$/H$^+$ exchanger in these cells. Our results demonstrate that the Na$^+$/H$^+$ exchanger activity, as indicated by the rate of pH$_i$ recovery (dpH$_i$/dt), is significantly inhibited when cells were treated with either the ROK inhibitor Y27632 (Fig. 3, B, phases I and II, and D-b) or the NHE1 blocker EIPA (Fig. 3, C, phases I and II, and D-c) compared with untreated cells (Fig. 3, A, phases I and II, and D-a).

In addition, the rate of pH$_i$ recovery (dpH$_i$/dt) from an NH$_4$Cl-induced acid load of BCECF-labeled cells treated with 50 μg/ml HA (or no HA) in the absence of drug (D-a) or in the presence of Y27632 (D-b) or EIPA (D-c) was also determined at pH$_i$ 6.6 in a HEPES buffer plus 20 mM NH$_4$Cl, followed by replacing the medium with Na$^+$-free, N-methylglucamine chloride solution that results in an acidification of the cytoplasm (phase I). The addition of 135 mM Na$^+$ (phase II) produced a rapid rise in pH$_i$, which allowed the dpH$_i$/dt to be determined in these samples as described under “Materials and Methods.”
inhibit HA-induced Na\(^+\)-H\(^+\) exchanger activity suggests that ROK and NHE1 are not only required for the basal level of Na\(^+\)-H\(^+\) exchange activity but also that this ROK-activated NHE1 function is closely linked to HA-mediated Na\(^+\)-H\(^+\) exchanger activity in MDA-MB-231 cells.

Because both Rho signaling and NHE1 activation play an important role in regulating tumor cell-specific behaviors (13, 50, 75), we have focused this study on the possible involvement of these two signaling molecules in regulating endosomal/lysosomal pH and extracellular pH changes during HA/CD44-mediated breast tumor progression. Specifically, we used the fluorescence indicator LYB (a cell-permeable weak base and acidotropic tracer known to accumulate selectively in acidic endosomes and lysosomes as a result of protonation leading to a pH-dependent increase in fluorescence intensity) (Fig. 4) to measure the pH changes in the endosomal and/or lysosomal compartments after HA binding to MDA-MB-231 cells. First, cells were loaded with the LYB dye and then incubated with unlabeled HA (50 \(\mu\)g/ml) in the cell fluorescence measuring system. The initial fluorescence intensity of the dye is very low in unstimulated cells MDA-MB-231 cells loaded with the LYB probe (Fig. 4A). The low fluorescence level in lysosomes of the unstimulated MDA-MB-231 cells is one of the unique properties of LYB. In order to verify the sensitivity of the LYB dye for detecting intracellular pH changes, we have included epidermal growth factor-induced intracellular pH changes (Fig. 4A-a) as a positive control. It is noted that intra-endosomal/lysosomal H\(^+\) accumulation begins to occur within seconds after the addition of HA (Fig. 4A). These data suggest that the pH changes also occur in both endosomes and lysosomes following HA binding to CD44-expressing MDA-MB-231 cells. The fact that the ROK inhibitor Y27632 (Fig. 4B) or the potent NHE1 blocker EIPA (Fig. 4C) can effectively inhibit HA-induced endo-endosomal/lysosomal pH changes in vivo strongly suggests that ROK-activated NHE1 signaling is involved in HA-mediated endosomal/lysosomal acidification, which also included epidermal growth factor-induced intracellular pH changes (Fig. 4A-a) as a positive control.

Breast tumor cells, such as MDA-MB-231 cells, have been found to cause extracellular acidification by releasing acid into the medium (41). The NHE1 is known to regulate cellular pH (44–46). NHE1 is also involved in the regulation of both pH\(_i\) and pH\(_e\) in human breast tumor cells (50). We have found that the pH regularly drops from pH 7.1 to pH 6.5 in the medium of cells treated with HA (Table 1). In contrast, very little pH change occurs in the medium of cells that are not treated with HA (Table 1). If no cells were added in the medium (in the presence or absence of HA), the change in extracellular pH was also minimal (Table 1). However, when cells were treated with Y27632 (a ROK inhibitor), EIPA (a NHE1 blocker), or NH\(_4\)Cl (a perturbation agent for endosomal/lysosomal acidification), HA-induced extracellular acidification appears to be reduced significantly (Table 1). These observations support the notion that ROK-NHE1 signaling and intra-endosomal/lysosomal pH is functionally coupled with HA-mediated extracellular acidification.

**CD44 and ROK-NHE1 Signaling in Regulating Acid-active Hyal-2 and Cathepsin B and Breast Tumor Progression**

A number of cellular processes are affected by the breast tumor cell acidic intracellular/extracellular pH values, which include the activation of matrix-degrading enzymes (e.g. hyaluronidases (58, 59) and/or cysteine proteinases (e.g. cathepsin B)) (54–57) as well as the invasive behavior of tumors (e.g. tumor cell invasion) (54–63). Here we have placed an emphasis on the involvement of CD44 and ROK-NHE1 signaling in regulating acid-active Hyal-2 and cathepsin B and tumor cell invasion in MDA-MB-231 cells.

**Hyal-2-mediated HA Degradation and Modification**—The GPI-anchored plasma membrane hyaluronidases, Hyal-2, with HA degrading activity participates in oncogenesis (58–63). By using a specific primer pair to amplify Hyal-2 by RT-PCR, we have detected the presence of one Hyal-2 PCR product (~559 bp) (Fig. 5A-a, lane 2) in MDA-MB-231 cells. This Hyal-2-containing PCR product was then “one-step cloned” into the pCRII vector (from Invitrogen) and sequenced. The nucleotide sequence confirms that this band belongs to the Hyal-2 molecule (data not shown). We believe that the RT-PCR is specific because no amplified fragment can be detected in samples incubated without reverse transcriptase (Fig. 5A-a, lane 1). GAPDH-specific primers were also used to verify equal sample loading (Fig. 5A-b, lanes 1 and 2). Our data clearly demonstrate that the Hyal-2 transcript is expressed in the human breast tumor MDA-MB-231 cells. No Hyal-1 (a putative tumor suppressor gene product) transcripts were detected in these cells (data not shown).

Because gene expression at the mRNA level does not always correlate with cellular protein expression, it is important to determine whether Hyal-2 protein expression also occurs in the MDA-MB-231 cells. Immunoblot analysis using anti-Hyal-2 antibody indicates that Hyal-2 (~52-kDa protein) is present in both endosomal (Fig. 5B, lane 1) and lysosomal fractions (Fig. 5B, lane 2). We have confirmed that the protein detected in these two acidic compartments (e.g. endosomes and lysosomes) by anti-Hyal-2-mediated immunoblot is specific, because no protein is detected in these samples incubated with preimmune rabbit IgG (Fig. 5B, lanes 3 and 4). These results clearly establish that Hyal-2 is expressed at both the transcript and protein levels in MDA-MB-231 cells and is located in these acidic organelles.

Recent reports (60, 61) have indicated that GPI-anchored proteins (e.g. Hyal-2) are often located in membrane glycosphingolipid-cholesterol microdomains, the so-called lipid rafts. In this study we have found that Hyal-2 (Fig. 5C-c) together with NHE1 (Fig. 5C-a), ROK (Fig. 5C-b), and CD44 (Fig. 5C-d) are partitioned into the 20% OptiPREPTM layer containing lipid rafts isolated from cells treated with HA (Fig. 5C, lane 2) or without HA (Fig. 5C, lane 1). Specifically, we have immunoprecipitated cellular materials in lipid rafts (the 20% OptiPREPTM layer isolated from untreated (Fig. 5C, lane 1) or HA-treated cells (Fig. 5C, lane 2)) with anti-C44 antibody followed by immunoblotting with various immunoreagents (e.g. anti-NHE1, anti-ROK, anti-Hyal-2). Our results indicate that Hyal-2 association with CD44 in lipid rafts is detectable in the absence of HA (Fig. 5, C-a, lane 1, and C-d, lane 1). The amount of complex formation between Hyal-2 and NHE1/ROK appears to be very low in lipid rafts isolated from cells treated with no HA (Fig. 5, C-a, lane 1). However, Hyal-2 becomes tightly linked to NHE1, ROK, and CD44 as a multimolecular complex in lipid rafts of MDA-MB-231 cells treated with HA (Fig. 5C-c, lane 2). These observations suggest that HA is capable of recruiting signaling molecules (e.g. ROK and NHE1) into CD44-Hyal-2-containing lipid rafts in MDA-MB-231 cells. CD44-associated Hyal-2 activity is also confirmed by using the hyaluronidase assay with HA-coated plates. Specifically, we have observed that Hyal-2 (complexed with CD44) is acid-active (Fig. 6A). Optimal HA degradation occurs when Hyal-2 (complexed with CD44) was incubated in a buffer solution ranging between pH 4.5 and pH 5.5 (Fig. 6A). Transfection of mammalian cells with synthetic siRNAs
(21–23 nucleotides in length) specifically suppresses expression of endogenous and heterologous genes by RNAi in cell culture (78–80). In order to confirm that Hyal-2 is the actual enzyme degrading HA, we have transfected MDA-MB-231 cells with at least two different siRNA sequences targeting human Hyal-2. Our results clearly show that these Hyal-2siRNA target sequences successfully suppress the expression of Hyal-2 in MDA-MB-231 cells (Fig. 6B-a, lanes 2 and 3). In control samples, no Hyal-2 down-regulation is observed in MDA-MB-231 cells treated with transfection reagents containing scrambled sequences (Fig. 6B-a, lane 1). Because other cellular proteins, such as CD44 (Fig. 6B-b, lanes 1–3), are expressed at comparable levels in these two Hyal-2siRNA-treated cells as compared with control cells containing scrambled sequences, we conclude that selective down-regulation of Hyal-2 expression by these two Hyal-2siRNAs is specific. Further analyses indicate that the ability of Hyal-2 (isolated from cells transfected with Hyal-2siRNAs containing both target 1 and target 2 sequences) to degrade HA in vitro is greatly inhibited (Fig. 6C-b) as compared with Hyal-2 activity isolated from cells treated with Hyal-2siRNA with scrambled sequences (Fig. 6C-a). These findings strongly support the notion that Hyal-2 activity is directly involved in HA degradation. In addition, we have observed that Hyal-2 degrades large HA molecules (−500,000–1 × 10^6-dalton polymer) into smaller sizes (−360,000–400,000-dalton polymer) (Fig. 6D, lanes 3 and 4). In Table I, we have
shown that the pH regularly decreases from pH 7.1 to pH 6.5 in cells treated with HA. The drop in pH from 7.1 to 6.5 enhances Hyal-2-mediated HA degradation in vitro (Fig. 6D, lanes 3 and 4). Pretreatment of cells with anti-CD44 antibody blocks the HA/CD44-mediated Hyal-2 activity at both pH 7.1 and pH 6.5 (Fig. 6D, lanes 1 and 2). These findings suggest that HA/CD44-induced cellular acidification is important for the activation of Hyal-2 activity.

HA, one of the longest glycosaminoglycans, is composed of repeating disaccharide units of D-glucuronic acid-N-acetyl-D-glucosamine (81). It is often overexpressed at sites of breast tumor attachment and is involved in CD44-mediated signaling and tumor progression (1, 8). However, the forms of HA that are accumulated inside or outside (in the ECM) of breast tumor cells and whether Hyal-2 and ROK/NHE1 signalings are linked to HA modification and tumor cell behaviors (e.g., tumor cell invasion) are unknown.

To measure HA production, MDA-MB-231 cells were metabolically labeled with $[^{3}H]$glucosamine for 18 h. Hyaluronan was then recovered from both cells and the medium and analyzed using 0.5% (w/v) agarose gel electrophoresis and column chromatography to analyze the distribution of different HA sizes. Since the biosynthetic relationship between the 400,000-dalton HA and the larger HA species (500,000 daltons) awaits further investigation.

Furthermore, our results indicate that externally added HA induces a shift in the polymer size of endogenous HA from 400,000 to 360,000–380,000 daltons (Fig. 6F, (1–3), a and b). These observations suggest that Hyal-2 plays an important role in endogenous HA modification. The biosynthetic relationship between the 400,000-dalton HA and the larger HA species (500,000 daltons) awaits further investigation.

Regulation of Cathepsin B in MDA-MB-231 Cells—Many studies have shown the importance of matrix-degrading enzymes such as cathepsin B in tumor invasion (54–57). Cathepsins such as cathepsin B are activated under acidic pH extracellularly and intracellularly, leading to tumor invasion and metastasis (54–57). Our results indicate that HA induces both intra-endosomal/lysosomal pH changes (Fig. 4A) and extracellular pH acidification (the pH decreases from pH 7.1 to pH 6.5, Table I) followed by cathepsin B activation (Table II). The drop in pH from 7.1 to 6.5 (mimicking HA effects on pH changes in the medium of MDA-MB-231 cells) is sufficient to promote a 2-fold increase in cathepsin B activity (Table II). Furthermore, treatment of cells with various drugs (e.g. the ROK inhibitor Y27632 or the NHE1 blocker EIPA, or NH$_4$Cl (a perturbation agent for endosomal/lysosomal acidification)) effectively blocks HA-activated cathepsin B activity (Table III). These observations suggest that HA/CD44-mediated ROK/NHE1 signaling, intra-endosomal/lysosomal pH changes, and extracellular acidification are closely linked to cathepsin B activation in these breast tumor cells.

### Table I

| Treatments                  | pH*                      |
|-----------------------------|---------------------------|
|                            | No HA addition | HA addition |
| No cells (control)          | 7.20            | 7.21        |
| No drug treatment           | 7.10            | 6.50        |
| Y27632 treatment           | 7.15            | 7.11        |
| EIPA treatment             | 7.17            | 7.20        |
| NH$_4$Cl treatment         | 7.16            | 7.17        |

* The pH was measured by phenol red absorbance. Specifically, no cells or HA-treated MDA-MB-231 cells pretreated with various drugs including 200 nm Y27632 (a ROK inhibitor), 200 μM EIPA (an NHE1 blocker), 10 mM NH$_4$Cl (a perturbation agent for endosomal/lysosomal acidification), or no drug treatment were washed twice with Hank’s balanced salt solution containing 0.03 mM phenol red. Cells were prewarmed at 37°C, equilibrated with CO$_2$, and then incubated with 1.5 ml of the same unbuffered solution without bicarbonate for 2 h at 37°C in a water-saturated atmosphere devoid of CO$_2$. The ratio of 450/490 nm absorbance of phenol red was monitored by a spectrophotometer as described under “Materials and Methods.” The values expressed in this table represent an average of triplicate determinations of 3–5 experiments with an S.D. less than ±5%.
CD44-NHE1 Signals Activate pH Changes, Hyal-2, and Cathepsin B

DISCUSSION

CD44 is expressed in many different types of cells and exists in several different isoforms (1–6). The gene that encodes CD44 contains 19 exons. Alternative splicing of 12 of these exons produces the various CD44 isoforms (7). Most important, a high level of CD44 isoform expression correlates with tumor metastasis (1–6). CD44 acts as a receptor for HA, a major component of the ECM (8–10). HA-CD44 interaction in tumor cells also plays a role in tumor cell aggregation, proliferation, migration, and angiogenesis (8–25). Recently, Al-Hajj and co-workers (82) have shown that CD44 is expressed in tumor stem cells, which have the unique ability to initiate tumor cell-specific properties. In fact, CD44 is suggested as one of the important surface markers for these breast cancer stem cells (82).

Our recent studies (75) indicate that HA-CD44 interaction promotes RhoA signaling in metastatic breast cancer cells. For example, RhoA-activated Rho kinase (also called Rho-binding
kinase or ROK-a serine/threonine kinase) phosphorylates the cytoplasmic domain of the CD44v3 isoform and up-regulates the interaction between CD44v3 and the cytoskeletal protein ankyrin during breast tumor cell migration (13). The binding of HA to the breast tumor cells (MDA-MB-231 cells) also stimulates RhoA-mediated ROK activity which, in turn, increases serine/threonine phosphorylation of the adaptor protein Gab-1 (Grb2-associated binder-1). Phosphorylated Gab-1 promotes
CD44-NHE1 Signals Activate pH Changes, Hyal-2, and Cathepsin B

Fig. 7. Characterization of MDA-MB-231 cells treated with CD44siRNAs. A, expression of CD44, ROK, NHE1, and Hyal-2 in MDA-MB-231 cells treated with CD44siRNAs. Cell lysates isolated from MDA-MB-231 cells (transfected with CD44siRNA-target sequence 1 (lane 2), CD44siRNA-target sequence 2 (lane 3), or CD44siRNA-scrambled sequences (lane 1)) were solubilized by 1% Nonidet P-40 buffer followed by immunoblotting with anti-CD44 antibody (a), anti-ROK antibody (b), anti-NHE1 antibody (c), anti-Hyal-2 antibody (d), or anti-Actin antibody (e) as described under “Materials and Methods.” B, analyses of HA size distribution. MDA-MB-231 cells (untreated or treated with CD44siRNA) were metabolically labeled with [3H]glucosamine. HA was then collected from the medium and analyzed by Sephacryl S-400HR gel filtration as described under “Materials and Methods.”

Table II: Analysis of cathepsin B activity

| Treatments                | Cathepsin B activity* |
|---------------------------|-----------------------|
| pH 7.1 treatment (control)| 4.98 (100%)           |
| pH 6.5 treatment          | 10.96 (220%)          |

* Cathepsin B activity in the medium of MDA-MB-231 cells incubated with pH 7.1 or pH 6.6 buffer or those cells pretreated with various drugs, including 200 nm Y27632 (a ROK inhibitor), 200 μM EIPA (an NHE1 blocker), or 10 mM NH4Cl (a perturbation agent for endosomal/lysosomal acidification), was measured by cathepsin B detection kit (Calbiochem) utilizing the fluorophore cresyl violet. Cathepsin B activity was expressed as (pmol of cresyl violet formed)/min/μg DNA as described under “Materials and Methods.” Statistical comparisons were made with Student’s t test.

Table III: Analysis of cathepsin B activity

| Treatments                | Cathepsin B activity* |
|---------------------------|-----------------------|
| No HA addition            |                       |
| HA addition               |                       |
| No drug treatment (control)| 5.00 (100%)          |
| Y27632 treatment          | 5.20 (104%)           |
| EIPA treatment            | 4.85 (97%)            |
| NH4Cl treatment           | 5.00 (100%)           |

* Cathepsin B activity in the medium of MDA-MB-231 cells incubated with pH 7.1 or pH 6.6 buffer or those cells pretreated with various drugs, including 200 nm Y27632 (a ROK inhibitor), 200 μM EIPA (an NHE1 blocker), or 10 mM NH4Cl (a perturbation agent for endosomal/lysosomal acidification), was measured by cathepsin B detection kit (Calbiochem) utilizing the fluorophore cresyl violet. Cathepsin B activity was expressed as (pmol of cresyl violet formed)/min/μg DNA as described under “Materials and Methods.” Statistical comparisons were made with Student’s t test.

Phosphatidylinositol 3-kinase recruitment to CD44. Subsequently, phosphatidylinositol 3-kinase is activated (in particular, α, β, and γ forms but not the δ form of the p110 catalytic subunit); AKT signaling occurs; the cytokine (macrophage-colony stimulating factor) is produced; and tumor cell-specific phenotypes (e.g., tumor cell growth, survival, and invasion) are up-regulated (75). Thus, RhoA-mediated ROK activation appears to be closely coupled with HA-CD44 signaling in breast tumor progression.

In an effort to identify additional cellular substrates for ROK, we have focused on NHE1 that is often activated by various extracellular stimuli such as growth factors, hormones, and ECM proteins (83–85). In this study we have demonstrated that ROK (by binding to activated RhoA) is capable of inducing serine and threonine phosphorylation of NHE1 (isolated from MDA-MB-231 cells) in vitro (Fig. 2C). The ability of ROK to phosphorylate NHE1 in the presence of unactivated RhoA appears to be greatly reduced (Fig. 2C). These results clearly indicate that ROK acts as one of the downstream effectors of RhoA signaling and utilizes NHE1 as one of its cellular targets in vitro.

The question of whether HA/CD44-activated RhoA-ROK serine/threonine phosphorylation of NHE1 plays a role in regulating cellular signaling such as cellular pH changes is addressed in the present study. Inhibition of ROK and NHE1 activities by treating cells with the ROK inhibitor Y27632 or the NHE1 blocker EIPA not only blocks HA-induced ROK phosphorylation of NHE1 (Fig. 2) but also down-regulates Na’-H’ exchange activity (Fig. 4). Intracellular endosomal/lysosomal pH changes, and extracellular acidification (Fig. 3; Table I). These findings strongly suggest that acidification by the MDA-MB-231 cells is shown.

In order to determine the functional consequences of the
ROK-NHE1-regulated cellular acidification, we first focused on the low pH-dependent cysteine proteases (e.g., cathepsin B) activated during breast tumor invasion (54–57). Cathepsins are characterized as members of the lysosomal cysteine protease family (54–57). Up-regulation of certain cathepsins, such as cathepsin B, has been linked to several different types of cancers including breast cancers (54–57). In order to determine whether conditions exist for MDA-MB-231 cells under which cathepsin B could be activated, we have observed that HA binding to CD44 generates acidic microenvironments (Figs. 3 and 4), stimulates cathepsin B activity (Tables II and III), and promotes tumor cell invasion (Tables IV and V). Our results are consistent with previous findings showing that an acidic pHi environment is closely associated with cysteine proteinase (cathhepsin B)-mediated invasive behaviors of tumor cells (54–57). The fact that both HA/CD44-mediated cathepsin B activation and breast tumor invasion are effectively blocked by Y27632 (the ROK inhibitor), EIPA (a NHE1 blocker), NH4Cl (a perturbation agent for endosomal/lysosomal acidification), and CA-074-Me (a cathepsin B inhibitor) suggests that HA/CD44 and ROK-NHE1-induced intracellular/extracellular acidification is involved in facilitating activation of the secreted cathepsin B required for the degradation of ECM and breast tumor invasion.

Somatic hyaluronidases (acid-active enzymes) have also been found to be increased significantly in breast tumor metastases as compared with primary tumors (86). However, the exact role of hyaluronidase-mediated HA catabolism/modification in breast cancer cells remains unclear. Currently, at least six hyaluronidase-like gene sequence products (e.g., Hyal-1,
The level of Hyal-3 expression increases when fibroblasts undergo chondrocyte differentiation (93). Hyal-4 based on preliminary evidence appears to be a chondroitinase, and PHYAL-1 (a pseudogene) is not commonly translated in humans (91). PH20 (or Spar1), the neutral-active hyaluronidase, is detected primarily in testes and in breast tumors (94, 95). Although the expression of some hyaluronidases has not been thoroughly investigated in MDA-MB-231 cells, our preliminary data indicate that Hyal-1 is not expressed in these cells (data not shown). On the other hand, Hyal-2 appears to be one of the major hyaluronidases expressed in these breast cancer cells (Fig. 5). The activity of Hyal-2 is up-regulated under acidic conditions with a pH optimum of about 4.5 (Fig. 6A). Some Hyal-2 activity can also be detected between pH 6.0 and pH 7.0 (Fig. 6, A and D). These results are consistent with previous findings (58, 63) showing a similar acidic pH optimum of Hyal-2 isolated from C6 glioma cells that also express high levels of CD44.

Lipid rafts have been implicated in a variety of cellular functions including signal transduction (27–31). In particular, caveolin and cholesterol-containing lipid rafts are involved in endosome sorting and vesicular trafficking (96). In particular, HA is endocytosed via its interaction with CD44 and then transported into endosomes and/or lysosomes resulting in hyaluronidase-mediated degradation (73, 97). In the MDA-MB-231 cells, Hyal-2 is localized in acidic compartments (e.g., endosomes and lysosomes (Fig. 5)) and in lipid rafts (containing CD44-associated ROK/NHE1 signaling complexes (Fig. 5)). Most importantly, the binding of HA to MDA-MB-231 cells promotes recruitment of ROK and NHE1 into Hyal-2 and CD44-containing lipid rafts (Figs. 1, 2, and 5). The fact that co-localization of ROK/NHE1 with Hyal-2 and CD44 in lipid rafts (Figs. 1, 2, and 5) is closely coupled with the onset of N\textsuperscript{a}–H\textsuperscript{+} exchange activity (Fig. 4), intracellular endosomal/lysosomal pH changes and extracellular acidification (Fig. 4; Table I) suggest that a localized pH gradient could be critical for the activation of low pH-dependent enzymes such as Hyal-2 (Fig. 6).

Hyaluronan is often accumulated at sites of breast tumor cell attachment and actively participates in CD44-mediated signaling and tumor progression (1, 8, 81). The HA synthase family of enzymes is responsible for the production and extrusion of the growing HA chain through the plasma membrane and into the ECM or onto the cell surface (98). One of the important biological consequences of acid production by breast cancer cells appears to be the modification of the ECM. It has been shown that acid-active Hyal-2 is involved in HA turnover during the early stage of lung injury (99). Because the properties of HA in breast tumor cells are not well characterized, we have focused our study on the analyses of HA size patterns.

A previous study showed that the S-400 column can be used to resolve different HA sizes in keratinocytes (73). The new generation of HiPrep\textsuperscript{TM} Sephacryl\textsuperscript{TM} 400 HR (S-400HR) gel filtration columns (from Amersham Biosciences) used in this study provides good resolution for analyzing molecules between 2 × 10\textsuperscript{4} and 8 × 10\textsuperscript{5} daltons. Based on the elution position of two HA markers (500,000 and 280,000 daltons, Figs. 5E, (1) arrowheads) and three protein markers (232,000, 440,000, and 669,000 daltons, Figs. 6F and 7B, arrows), we are able to calibrate accurately the sizes of HA (e.g., 360, 400, and 500 kDa) isolated from breast tumor cells. Our results demonstrate that the major HA species detected in the medium of MDA-MB-231 cells has an ~360,000–400,000-dalton size (Fig. 6). A recent study indicated that HA fragments in aqueous solution are controlled and sequestered by reversible tertiary structures of HA (100). In fact, NMR evidence provides proof of a tertiary structure of HA, probably a β-sheet-like array, stabilized by...
H-bonds and hydrophobic bonds at ambient temperatures. Therefore, it is possible that transitions between secondary and tertiary structures in the newly identified 360,000-dalton HA fragments (Figs. 6 and 7) under physiological conditions participate in the interaction of HA with CD44, subsequent oncopogenic signaling (Figs. 3 and 4; Tables I–III), and tumor cell invasion (Tables IV and V). When CD44-mediated ROK/NHE1 signaling or cellular acidification is inhibited (by treating cells with Y27632 (the ROK inhibitor), EIPA (a NHE1 blocker), or NH₄Cl (a perturbation agent for endosome/lysosome acidification)) or Hyal-2 is down-regulated (by treating cells with Hyal-2siRNA), the HA size distribution is also altered. The HA polymer size shifts from the 360,000–400,000-dalton size into a larger 500,000-dalton size (Fig. 6). These findings suggest that ROK/NHE1 signaling, acidic pH, and/or the Hyal-2 enzyme are involved in the partial degradation or modification of the 500,000-dalton form of HA into 360,000–400,000-dalton species. Size modification of HA fragments (shifting 360,000–400,000-dalton sizes into 500,000-dalton fragments) caused by treating cells with various signaling perturbation agents (e.g. Y27632 (the ROK inhibitor), EIPA (a NHE1 blocker), NH₄Cl (a perturbation agent for endosome/lysosome acidification), or Hyal-2siRNA/CD44siRNA) (Figs. 6 and 7) could significantly alter the proper HA configuration required for CD44 binding and signal transduction. The fact that the loss of 360,000–400,000-dalton HA fragments correlates well with the reduction of tumor invasive properties (Table IV and V) suggests that this 360,000–400,000-dalton species (and to a lesser extent 500,000 daltons) is critically important for breast tumor invasion. Previous studies have shown that Hyal-2 has the unusual properties of hydrolyzing high molecular mass HA into intermediate sized fragments of ~20,000 daltons (58). The inability of Hyal-2 to efficiently degrade large HA species into 20,000-dalton fragments in MDA-MB-231 cells may be due to the unique physical-chemical properties of HA polysaccharides that could alter the accessibility of the substrate (HA) for Hyal-2 action in these cells. However, we cannot rule out the possibility that some Hyal-2-digested 20,000-dalton HA fragments may have been accumulated in the medium but are not detectable under our experimental conditions.

In summary (Fig. 8), we propose that CD44 is localized in specialized plasma membrane microdomains containing high cholesterol and gangliosides (designated as lipid rafts) in breast tumor cells (Fig. 8, Step 1). The binding of HA to CD44 on MDA-MB-231 cells increases the recruitment of ROK and NHE1 into CD44-containing lipid rafts and stimulates ROK phosphorylation of NHE1 (Step 2). Most important, the phosphorylation of NHE1 by ROK promotes Na⁺/H⁺ exchange activity, intra-endosomal/lysosomal pH changes, and extracellular acidification (Step 3) leading to a concomitant activation of at least two low pH-dependent enzymes, Hyal-2 (located at lipid rafts) and cathepsin B (secreted in the medium) (Step 4), required for ECM degradation, HA modification (Step 5), and tumor cell invasion.

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