The Hyaluronan Receptors CD44 and Rhamm (CD168) Form Complexes with ERK1,2 That Sustain High Basal Motility in Breast Cancer Cells

CD44 is an integral hyaluronan receptor that can promote or inhibit motogenic signaling in tumor cells. Rhamm is a non-integral cell surface hyaluronan receptor (CD168) and intracellular protein that promotes cell motility in culture. Here we describe an autocrine mechanism utilizing cell surface Rhamm-CD44 interactions to sustain rapid basal motility in invasive breast cancer cell lines that requires endogenous hyaluronan synthesis and the formation of Rhamm-CD44-ERK1,2 complexes. Motile/invasive MDA-MB-231 and Ras-MCF10A cells produce more endogenous hyaluronan, cell surface CD44 and Rhamm, an oncogenic Rhamm isoform, and exhibit more elevated basal activation of ERK1,2 than less invasive MCF7 and MCF10A breast cancer cells. Furthermore, CD44, Rhamm, and ERK1,2 uniquely co-immunoprecipitate and co-localize in MDA-MB-231 and Ras-MCF10A cells. Combinations of anti-CD44, anti-Rhamm antibodies, and a MEK1 inhibitor (PD98059) had less-than-additive blocking effects, suggesting the action of all three proteins on a common motogenic signaling pathway. Collectively, these results show that cell surface Rhamm and CD44 act together in a hyaluronan-dependent autocrine mechanism to coordinate sustained signaling through ERK1,2, leading to high basal motility of invasive breast cancer cells. Therefore, an effect of CD44 on tumor cell motility may depend in part on its ability to partner with additional proteins, such as cell surface Rhamm.

Breast cancer invasion and progression involves a motile cell phenotype, which is under complex regulation by growth factors/cytokines and extracellular matrix (ECM) components within the tumor microenvironment (1, 2). Motogenic signaling in tumor cells can be stimulated by both paracrine and autocrine factors; the latter decrease the requirement of invasive carcinomas for stromal support and are often associated with tumor progression (3–6). Hyaluronan (HA) (an anionic polymer of repeating units of glucuronic acid and N-acetylgalactosamine) is one stromal ECM component that is associated with breast cancer progression (7, 8). In culture, HA stimulates breast cancer cell motility (9–11), pointing to a possible important role of this glycosaminoglycan in breast cancer cell invasion in vivo.

CD44 is a broadly expressed, type I, integral cell surface membrane glycoprotein that participates in cell-cell and cell-matrix adhesions and, in particular, binds to HA (12, 13). It is encoded by a single gene but exists as multiple isoforms that are generated by alternative splicing of 10 variable exons, as well as through post-translational modifications (12). The most commonly expressed CD44 isoform (the standard form or CD44std) is an 85-kDa protein that contains none of the variable exons. Originally described as the principal cell surface receptor for HA (14), CD44 has since been shown to bind multiple ligands including fibronectin (15) and osteopontin (16); in general, the ligands for many of the CD44 variants are not yet known. However, a role for CD44std as a mediator of HA-promoted motility in breast cancer cell lines is well established on tissue culture plastic (two-dimensional cultures) (7, 17–19). CD44 isoforms containing variable exons v6 and v9 are also involved in HA-mediated signaling (e.g. in activated T cells (20)), and expression of isoforms containing variable exons v4–v7 enhances HA binding of a rat pancreatic adenocarcinoma cell line (21). These results suggest that in addition to

4 The abbreviations used are: ECM, extracellular matrix; aa, amino acid(s); Ab, antibody; BSA, bovine serum albumin; CD44std, CD44 standard form; CD44v, CD44 variant; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FACS, fluorescence-activated cell sorting; FCS, fetal calf serum; GST, glutathione S-transferase; HA, hyaluronan; HBSS, Hanks’ buffered saline solution; MAP, mitogen-activated protein; MEK, MAP/ERK kinase; PBS, phosphate-buffered saline; Rhamm, receptor for hyaluronic acid-mediated motility; TBS, Tris-buffered saline.
Rhamm and CD44 Coordinate in Breast Cancer Motility

CD44std, at least some variants of CD44 isoforms can bind HA. CD44 binds to HA via an extracellular domain, whereas the cytoplasmic tail activates intracellular signaling pathways that regulate the association of signaling complexes with the cortical actin cytoskeleton (13, 17, 22, 23).

Evidence suggesting that CD44 has motogenic/invasive functions in two-dimensional cultures has motivated numerous histopathological evaluations of CD44 expression in breast cancer. Although several groups report that CD44std expression positively correlates with disease-related survival, whereas expression of CD44 variants correlates with poor prognosis (50), other studies contradict these results (24–27). Furthermore, analysis of breast cancer progression in a CD44−/− mouse background (where there is an absence of all CD44 isoforms) indicates that loss rather than gain of CD44 expression is associated with increased metastasis (13, 27). These observations predict a potential for CD44 to act as both as a tumor progression enhancer and a tumor suppressor (e.g. Refs. 28 and 29). The basis for an association of CD44 with different outcomes in breast cancer patients or in animal models of this disease is not well understood. One possibility is that differential expression/function of CD44 isoforms in tumor cell subsets, including progenitors, may affect clinical outcome (30–32). However, CD44 is also known to associate with, and facilitate, signaling through such tumor cell-associated proteins/receptors as matrix metalloproteinases (MMPs) (33, 34), c-Met, and EGF receptor (35, 36). Therefore, the consequences of CD44 expression to tumor cell behavior and its signaling properties may be modified by the proteins it associates with and vice versa. For example, co-expression of CD44v4 with one of its cell surface binding partners (MMP-9) correlates with node positivity in breast cancer patients, whereas the expression of CD44v4 alone does not (34). Furthermore, hyperexpression of other CD44 binding partners, including specific hyaluronan synthases and hyaluronidases (e.g. HAS-2 and hyal-2), together with CD44 (but not CD44 alone), correlates with the degree of invasiveness of human breast cancer cell lines (10).

Cell surface Rhamm (CD168) is an HA-binding protein/receptor that is not highly expressed in normal tissues but is commonly overexpressed in many advanced cancers (18, 19), including breast cancer (37, 38). Rhamm was first identified as an HA-dependent motility cell surface receptor that can transform fibroblasts when overexpressed (39, 40). Rhamm is also a cytoplasmic and nuclear protein that interacts with interphase microtubules, centrosomes, and the mitotic spindle, suggesting that it performs multiple functions in a number of cell compartments (41–43). Importantly, spindle-associated functions of Rhamm are blocked by the breast/ovarian tumor suppressor gene BRCA1 (43). This functional link between Rhamm and BRCA1, a factor in hereditary forms of breast cancer, as well as evidence that hyperexpression of Rhamm predicts poor clinical outcome and increased risk of sporadic breast cancer metastasis (37), suggests that Rhamm may be influential in both inherited and noninherited forms of breast cancer. However, the relevance of its multiple intracellular versus extracellular functions to human breast cancer aggressiveness has not been fully established yet. We recently showed that the motility defects of Rhamm−/− wound fibroblasts could be rescued by soluble recombinant Rhamm protein linked to Sepharose beads. Rescue required a concomitant surface display of CD44 (42) but did not require expression of intracellular Rhamm forms. These results suggest that at least some of the functions regulated by intracellular versus extracellular Rhamm are distinct.

As a consequence of its ability to bind to HA, cell surface Rhamm activates multiple motogenic signaling pathways that have been implicated in breast cancer progression. These include Ras (40), pp60-c-Src (44), and ERK1,2 (37). Cell surface Rhamm is required for sustained activation and intracellular targeting of ERK1,2 in dermal wound fibroblasts (45), suggesting that the extracellular Rhamm form could potentially function in tumor progression to increase the intensity and duration of signaling pathways associated with tumor invasion/motility. Importantly, cell surface Rhamm can additionally perform motogenic/invasive functions similar to CD44 and can even replace CD44 (46). These observations have raised the possibility that cell surface Rhamm may partner with CD44 to “unleash” its motogenic potential (45, 46).

Although cell-autonomous tumor progression events can clearly contribute to the aggressiveness of breast cancer cells, such cells still remain sensitive to some exogenous factors in their microenvironment (for review see Ref. 47), including cytokines/growth factors and extracellular matrix components such as HA (48, 49). Indeed, the accumulation of HA within breast tumors or peritumor stroma is an indicator of poor prognosis in breast cancer patients (50). ECM factors such as HA act coordinately with activating mutations in critical signal transduction pathways to modify tumor cell behavior (51). ECM-mediated activation of the Ras/Raf/MEK1,2/ERK1,2 cascade (52–54) is one motogenic pathway associated with breast tumor progression. Invasive breast cancer cell lines such as MDA-MB-231 have higher basal ERK1,2 activity than less invasive cell lines (including MCF7), and sustained ERK1,2 activity is required for the increased motility/invasion of the aggressive breast cancer cell lines (54). The molecular mechanisms driving sustained ERK1,2 activation and the consequent effects on tumor motility/invasion are poorly understood (55–58).

In this study we show that invasive breast cancer cells (MDA-MB-231 and Ras-MCF10A) sustain elevated levels of ERK1,2 activation upon growth factor/motogenic stimulation when cell surface CD44 and Rhamm are co-expressed and co-associate with each other. In contrast, less invasive (MCF7) and non-malignant (MCF10A) cell lines express lower levels of either HA receptor at the cell surface, despite the fact that Rhamm is expressed as a cytoplasmic protein and can only transiently activate ERK1,2. We demonstrate also that CD44 and Rhamm co-associate with ERK1,2 as complexes in aggressive breast tumor cell lines. Finally, we show that these CD44-Rhamm-ERK1,2 complexes are required for rapid basal motility of the more invasive cell lines. These results are consistent with a model in which HA, CD44, Rhamm, and activated ERK1,2 are physically and functionally linked in a biological complex to establish an autocrine mechanism for promoting motility in breast cancer cells.
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EXPERIMENTAL PROCEDURES

Reagents (Antibodies, Growth Factors, Hyaluronan and Peptide Inhibitors)—Medical grade HA prepared from bacterial fermentation (provided by Hyal Pharmaceutical Co., Mississauga, Ontario, Canada) was free of detectable proteins, DNA, or endotoxins. The molecular mass range was 250–300 kDa. The following primary antibodies were obtained commercially or as gifts: ERK1 and nonimmune IgG (Santa Cruz Biotechnology), phospho-ERK1,2 (Cell Signaling), p21Ras (Oncogene Science, Cambridge, MA), CD44 (IM7, Pharmingen), CD44 (Hermes-3, kind gift of Dr. Sirpa Jalkanen, University of Kuopio, Finland). Anti-CD44 antibodies used were raised against sequence in the CD44std form and therefore could detect all CD44 isoforms. Polyclonal Rhamm antibodies used in this study were prepared (Zymed Laboratories Inc., San Diego, CA) against the following human Rhamm sequences: antibody-1 (Ab-1): KSKFSENGNQKN (aa 150–162), antibody-2 (Ab-2): VSIEKEKIDEKS (aa 217–229), and antibody-3 (Ab-3): QLRRQDDEFR (aa 543–553) (59, 60). Specificities of Rhamm and CD44 antibodies were determined using Rhamm and CD44 lysates, respectively, and with Rhamm peptide competition. The following secondary antibodies were purchased: for Western blot detection, horseradish peroxidase-conjugated anti-mouse (Bio-Rad Laboratories), anti-rabbit (Amersham Biosciences), and anti-rat (Santa Cruz Biotechnology); for immunofluorescence analysis, anti-rabbit Alexa 555 and anti-rat Alexa 433 (Molecular Probes). The MEK1 inhibitor PD098059 (2-[2-amino-3-methoxyphenyl]-oxanaphthalene-4-one) was purchased from Calbiochem. An HA-binding peptide, YLKQKKVKKHIV, was synthesized (61). A scrambled peptide, YKQKIKHVVKLQ, which blocks macrophage migration, was used as a control.

Cell Culture—Human breast carcinoma cell lines MDA-MB-231 and MCF7 were obtained from American Type Culture Collection (Manassas, VA) and were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and 10 mM HEPES (Sigma) at pH 7.2. The immortalized human breast epithelial cell line MCF10A, transfected with the empty pH106 plasmid containing the neomycin resistance gene, and MCF10A cells, transfected with the human mutant H-ras oncogene (mutated at G12-V12), were a kind gift of Dr. Channing Der (North Carolina and were grown as described previously (62, 63). Briefly, the cells were grown in DMEM/F-12 (1:1) supplemented with 5% equine serum, 0.1 μg/ml cholaris toxin, 10 μg/ml insulin (Invitrogen), 0.5 μg/ml hydrocortisone (Sigma), and 0.02 μg/ml epidermal growth factor (Collaborative Research Inc., Palo Alto, CA). All cultures were incubated in a humidified atmosphere of 5% CO2 at 37 °C.

Western Immunoblotting—Cells plated at 50% confluency for 12 h were washed with ice-cold phosphate-buffered saline (PBS) and lysed in ice-cold radiommune precipitation assay buffer (25 mM Tris-HCl, pH 7.2, 0.1% SDS, 1% Triton-X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA, and 50 mM HEPES, pH 7.3) containing the protease and phosphatase inhibitors leupeptin (1 μg/ml), phenylmethylsulfonyl fluoride (2 mM), peptstatin A (1 g/ml), aprotinin (0.2 trypsin inhibitory units/ml) and 3,4-dichloroisocoumarin (200 μM), sodium orthovanadate, and 1 mM NaF (Sigma). Cell lysates were then microcentrifuged at 13,000 × g for 20 min at 4 °C (Heraeus Biofuge 13, Baxter Diagnostics, Mississauga, Ontario, Canada) after standing for 20 min on ice. Protein concentrations of the supernatants were determined using the DC protein assay (Bio-Rad). 10 μg of total protein from each cell lysate was loaded and separated by electrophoresis on a 10% SDS-polyacrylamide gel together with prestained molecular weight standards (Invitrogen). Following electrophoresis, proteins were transferred to nitrocellulose membranes (Bio-Rad) in a buffer containing 25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol using electrophoretic transfer cells (Bio-Rad) at 100 V for 1.5 h at 4 °C. Additional protein binding sites on the membrane were blocked with 5% defatted milk in TBST (10 mM Tris base (pH 7.4), 150 mM NaCl, and 0.1% Tween 20 (Sigma)). The membranes were incubated with the primary antibodies for Rhamm, CD44, Ras, or ERK1,2 (all diluted at 1:1000 or 1 μg/ml in 1% defatted milk in TBST) for 2 h at room temperature. The membranes were washed three times at 15-min intervals with 1% defatted milk in TBST. Immunodetection was performed using secondary antibodies conjugated to horseradish peroxidase (diluted 1:5000 or 1 mg/ml in 1% defatted milk in TBST for 1 h at room temperature followed by three washes with TBST. Blotting was visualized by the enhanced chemiluminescence Western blotting detection system (ECL, Amersham Biosciences) according to the manufacturer’s instructions. Quantification of optical densities of the reactive protein bands was performed on a Bio-Rad video densitometer. To account for variations in loading, parallel SDS gels were carried out with each experiment in which equal amounts of the protein lysates were run. These gels were then stained with Coomassie Blue dye to confirm equal loading. The densitometric results were presented as a mean of three experiments ± S.D. and are presented as a percentage of the protein of interest normalized to a dominant 60-kDa marker band on the Coomassie-stained gels.

Analysis of EGF-stimulated ERK1,2 Activation—5 × 104 MCF7 and MDA-MB-231 cells were plated in complete growth medium (DMEM, 10% FCS) on 6-cm cell culture plates and allowed to attach for 4 h. The growth medium was replaced by defined medium (DMEM, 4 μg/ml insulin, 8 μg/ml transferrin). After overnight culture, cells were stimulated with 20 ng/ml EGF (Sigma) in defined medium. For antibody blocking experiments, cell surface Rhamm and/or CD44 function was blocked by preincubating cells for 30 min in the presence of anti-CD44 antibody (IM7, 10 μg/ml), anti-Rhamm antibody (10 μg/ml), IgG (10 μg/ml), or a combination of anti-CD44 and anti-Rhamm antibodies prior to EGF stimulation. EGF stimulation, protein isolation, and SDS-PAGE were performed as described above. The densitometric results are presented as a mean of triplicate samples ± S.D. Levels of phospho-ERK1,2 protein were normalized to total ERK1,2 protein and presented as -fold changes.

Measurement of HA Production—Cells were plated at subconfluence in DMEM plus 10% FCS for 12 h followed by serum-free medium replacement for another 24−48 h. HA released into the medium was collected and assayed using an enzyme-
linked immunosorbent assay (Amersham Biosciences) as per the manufacturer’s instructions.

**Fluorescence-activated Cell Sorting (FACS)—**Cells were grown to 50% subconfluence on 15-cm culture plates in growth medium and rinsed with Ca²⁺/Mg²⁺-free Hanks’ buffered saline solution (HBSS)/20 mM HEPES (pH 7.3) 12 h later. Cells were harvested with non-enzymatic HBSS-based cell dissociation solution (Sigma), resuspended in 5 ml of cold PBS, and centrifuged at 1200 rpm for 3 min. Cells were washed in another 5 ml of cold PBS and then blocked in cold 10% FCS/HBSS/HEPES (FACS buffer) for 30 min. The viability of released cells was between 85 and 95% by Trypan blue exclusion. For detection of cell surface Rhamm (64), an aliquot of 2 × 10⁶ cells was incubated with anti-Rhamm antibody (1:100 or 10 μg/ml) in a total volume of 200 μl of FACS buffer for 30 min on ice and then washed three times in cold FACS buffer. Rabbit IgG (1:100 or 10 μg/ml) was used as a negative control for each cell line. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (1:30 dilution, Sigma) in FACS buffer was then added and incubated for 30 min in the dark on ice. The cells were washed again and examined with a flow cytometer (Beckman Coulter) using FACS Calibur with Cell Quest acquisition and analysis software (BD Biosciences). For detection of cell surface CD44 (65, 66), 1 × 10⁶ cells were incubated with 1 μg of anti-CD44 antibody (clone IM7, Pharmingen) or 1 μg of rat IgG antibody (Santa Cruz Biotechnology) in PBS, 2% BSA for 1 h on ice after which they were washed with cold PBS, 2% BSA. Cells were then incubated with rabbit-anti-rat Alexa 488 (diluted 1:100, Molecular Probes) in PBS, 2% BSA for 1 h on ice. Cells were washed in cold PBS, 2% BSA. Cells were resuspended in 1 ml of fresh, cold PBS, 2% paraformaldehyde (Sigma) and were stored overnight at 4 °C. Before flow cytometric analysis, samples were filtered (cell strainer caps, BD Biosciences). Data were collected using a Beckman Coulter flow cytometer. Viable cells were gated based on forward and side scatter to eliminate dead aggregates and debris, and then the distribution of fluorescence intensity was calculated.

**Immunoprecipitation Assays—**Co-immunoprecipitation analyses were performed using 400 μg of protein from each cell lysate mixed with 5 μg of anti-Rhamm, anti-CD44, anti-ERK1, anti-rabbit IgG, anti-mouse IgG antibodies, or anti-rat IgG antibodies. After 12 h of incubation at 4 °C on a rotator, 25 μl of a 50% suspension of protein A/G-Sepharose beads (Invitrogen) was added to each tube, and the samples were mixed end-over-end for another 4 h at 4 °C. The beads were pelleted by brief centrifugation at 7000 × g and washed three times with cold 0.5% Triton X-100/PBS. Bound proteins were released from the beads by heating the samples in 25 μl of 2× Laemmli buffer for 5 min. Protein samples were subjected to 12% SDS-PAGE and immunoblotted as described above.

**Pulldown Binding Assays—**In vitro pulldown binding assays were performed using recombinant Rhamm protein (63- and
43-kDa isoforms) that was purified as a GST fusion protein as described previously (67). Briefly, 1 mg of cellular lysate was incubated with recombinant Rhamm-GST or recombinant GST protein on glutathione-Sepharose beads (Amersham Biosciences) overnight at 4 °C. Beads were then pelleted by centrifugation and washed five times with 1 ml of cold lysis buffer. Bound proteins were released from the beads by heating the samples in 25 μl of 2× Laemmli buffer for 5 min. Protein samples were subjected to 10% SDS-PAGE and immunoblotted as described above.

**Time Lapse Cinemicrography**—Motility analyses were performed to quantify the effect of function blocking Rhamm and CD44 antibodies, HA-binding peptide, hyaluronan, and the MEK1 inhibitor PD098059 on cell motility. PD098059 MEK1 inhibitor was used to test the involvement of the MAP kinase pathway in the motility of these cells. Cells were seeded on T-25 flasks (Costar, Cambridge, MA) at 1 × 10⁵ cells/flask. Cells were incubated with anti-Rhamm antibody (30 μg/ml), anti-CD44 antibody (30 μg/ml), a mixture of anti-Rhamm (30 μg/ml) and anti-CD44 (30 μg/ml) antibodies, HA-binding peptide (1 μg/ml) or its scrambled control (1 μg/ml), and/or 50 μM PD098059 for 30 min prior to filming. Alternatively, cells were stimulated with 50 μg/ml HA immediately prior to filming. As a control, a mixture of mouse or rat and rabbit IgG (30 μg/ml each), Me₂SO (for PD098059), or PBS (for HA) was used. Cell locomotion was monitored for a period of 6 h using a 10× modulation objective (Zeiss, Germany) attached to a Zeiss Axiovert 100 inverted microscope equipped with Hoffman modulation contrast optical filters (Greenvale, NY) and a 37 °C heated stage. Cell images were captured with a charge-coupled device (CCD) video camera module attached to a Hamamatsu CCD camera controller. Motility was assessed using Northern Exposure 2.9 image analysis software (Empix Imaging, Mississauga, Ontario, Canada). Nuclear displacement of 20–30 cells was measured, and data were subjected to statistical analysis (see below). Each experiment was repeated at least three times. The results of motility analyses were expressed as means (μm/4 h) ± S.D. unless otherwise indicated.

**Confocal Microscopy**—MCF7 and MDA-MB-231 cells were plated sparsely (~5000 cells/well) on coverslips in a 24-well dish. The cells were incubated overnight in DMEM supplemented with 10% FCS. Cells were rinsed briefly with 1% BSA/TBS and were then fixed in fresh 3.7% paraformaldehyde in TBS for 10 min at room temperature. Cells were rinsed with 1% BSA/TBS and were then permeabilized with 0.5% Triton X-100 in 1% BSA/TBS for 15 min at room temperature. Cells were then again rinsed in 1% BSA/TBS and blocked in 5% FCS in 1% BSA/TBS for 1 h at room temperature. For the phospho-ERK1,2/CD44 double staining, cells were incubated with anti-phosphop44/p42 MAP kinase (Thr202/Tyr204) and anti-CD44 (IM7) antibodies, each diluted 1/100 in 1% BSA/TBS for 1 h at room temperature. For the Rhamm/CD44 double staining, cells were first incubated with an anti-Rhamm (59, 60) antibody diluted 1/100 in 1% BSA/TBS overnight at 4 °C. After the overnight incubation, cells were further incubated with the anti-CD44 antibody (IM7) diluted 1/100 in 1% BSA/TBS for 1 h at room temperature. After incubation with primary antibodies, cells were rinsed four times for 10 min each in 1% BSA/TBS. Primary antibodies were then visualized by incubating cells with anti-rabbit Alexa 555 and anti-rat Alexa 488 diluted 1/150 in 1% BSA/TBS for 1 h at room temperature. After incubation with secondary antibodies, cells were rinsed four times for 10 min each in 1% BSA/TBS. Cells were briefly incubated with 4’,6-diamidino-2-phenylindole diluted in 1% BSA/TBS and then mounted (immunofluorescence mounting medium, Dako) on slides. A Zeiss LSM510 Meta Multiphoton confocal microscope equipped with LSM 5 imaging software was used to visualize the cells (Department of Anatomy and Cell Biology, University of Western Ontario). Green and red images were captured individually and were merged using the LSM 5 Imaging software.

**Image Analysis**—Co-localization was identified using the co-localization finder plug-in of Image J software. This software allows identification of co-localization based on a correlation diagram of two (red and green) images. Pixels that contain information from both channels are considered co-localization and should not be considered quantitative. The results of co-localization were expressed as % co-localization ± S.E., n = 3 experiments. Percent expression was determined by normalizing the densitometric value of CD44std protein to that of a 60-kDa dominant marker protein on parallel Coomassie-stained gels.
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A. Rhamm Protein Isoforms expressed by MDA-MB-231 and MCF7 Cells

![Diagram showing Rhamm protein isoforms]

**FIGURE 3.** Rhamm isoforms (63 and 85 kDa) are more highly expressed in the MDA-MB-231 than in the MCF7 breast cancer cell line. A. diagram showing the location of the sequences to which the three anti-Rhamm antibodies were raised (Ab-1, Ab-2, and Ab-3). Three protein isoforms are expressed in MDA-MB-231 cells, and their sequences are predicted based upon reactivity with these anti-peptide antibodies in Western blots. Full-length Rhamm isoform (85 kDa) reacts with all three antibodies, whereas shorter Rhamm forms (43 and 63 kDa) react only with Ab-2 and Ab-3 (data not shown), suggesting that the latter are N-terminal truncations of the full-length protein. Detectable Rhamm isoforms expressed in MCF7 cells are the 85-kDa (full-length) and 43-kDa isoforms (Ab-2). B. quantification of Rhamm protein expression was determined by calculating the densitometric ratios of each of the protein isoforms/total Rhamm protein (obtained by totaling the densitometric values of each Rhamm immunoreactive band recognized by Ab-2). Values represent the mean ± S.E., n = 3 experiments.

**Statistical Analysis**—Statistically significant (p < 0.05) differences between means were assessed by the unpaired, two-tailed, Student’s t test method. Cell motility was based on means of at least 30 cells/experiment, and Western blot quantification was based on replicate samples unless otherwise indicated. Heparan sulfate production was reported as a mean of 10 separate cultures.

**RESULTS**

**Invasive Breast Tumor Cell Lines Produce Endogenous HA That Sustains Rapid Motility**—HA, a motogenic factor that is synthesized in large amounts by aggressive breast cancers and that correlates with the clinical progression of breast cancer (10), promotes both ERK1,2 activation and breast tumor cell motility/invasion (7, 10, 17, 18). Consistent with these previous reports, HA levels in the medium of cultured cells were significantly greater in the aggressive MDA-MB-231 cells than in less aggressive MCF7 cells (Fig. 1A); similar differences were observed also between Ras-MCF10A and MCF10A cells in culture (data not shown). We therefore quantified the reliance of breast tumor cell lines on endogenous HA production for maintaining motility in culture using an HA-binding peptide that blocks cell-HA interactions (61). The HA-binding peptide (YKQKIKHVVKLK) significantly reduced motility of MDA-MB-231 cells but had no effect on the MCF7 cell line (Fig. 1B).

A scrambled control peptide (YLQKQKKVKKHV), which does not bind to HA or affect HA-mediated macrophage motility (61), did not block motility of either cell line. A motogenic response of breast cancer cell lines to exogenous HA was also quantified. Cell cultures were first serum-starved for 24–48 h to reduce endogenous levels of HA production (<1 ng/ml; Fig. 1C); then rescue of motility, with the addition of 50 μg/ml exogenous HA, was quantified. Using these conditions, the exogenous HA significantly stimulated motility of MDA-MB-231 but not MCF7 cells (Fig. 1C). Collectively, these results show that invasive breast tumor cell lines such as MDA-MB-231 establish an HA-dependent autocrine mechanism in the presence of growth factors and/or serum that is required for rapid rates of motility. By contrast, poorly invasive breast cancer cells (e.g. MCF7) lack this mechanism, because they produce low levels of HA and do not respond to exogenous HA provided in their microenvironment.

**Invasive Breast Tumor Cells Display Both Cell Surface CD44 and Rhamm**—Because the invasive breast cancer cell lines can utilize HA to promote motility, we sought to determine whether HA receptors such as CD44 and Rhamm are expressed on these cells. CD44 and Rhamm protein expression was quantified by Western blot, flow cytometry, and fluorescent confocal analysis. Western blot and flow cytometry analysis indicated that levels of total CD44std (Fig. 2A) and cell surface CD44 (Fig. 2B) were greater in MDA-MB-231 than in MCF7 cells. Confocal analysis confirmed high CD44 expression in MDA-MB-231 cells and lower expression in MCF7 cells (Fig. 5A, panels b and f; see below). Similar differences in CD44 were observed also between Ras-MCF10A cells and their parental, nontransformed counterparts (supplemental Fig. 1a and data not shown).

Like CD44, Rhamm has been reported to exist as several protein isoforms (18, 68); these include N-terminal truncations of full-length Rhamm (40), which transform fibroblasts. To characterize the expression of protein isoforms in breast cancer cell lines, peptide-specific polyclonal antibodies were generated against sequence in the N terminus (aa 150–162, Ab-1) and C terminus (aa 542–553, Ab-3) as well as an internal region (aa 217–229, Ab-2) of the human full-length Rhamm sequence (Fig. 3A). An 85-kDa protein corresponding to full-length Rhamm was expressed to a greater extent in MDA-MB-231 than in MCF7 cells and, as expected, was detected by all three antibodies (Fig. 3A, and data not shown). Shorter Rhamm forms (64 and 43 kDa) expressed by the breast cancer cell lines
were detected by Ab-2 (Fig. 3A) and Ab-3 (data not shown) but not Ab-1, indicating that they are N-terminal truncations of the full-length Rhamm protein. In particular, the 63-kDa Rhamm protein was abundant in MDA-MB-231 cells and corresponds to the oncogenic Rhamm isoform that is expressed in aggressive human tumors (18, 69, 70). This isoform can also transform fibroblasts when expressed (40). Intriguingly, this molecular mass also corresponds to a cell surface form of Rhamm expressed by macrophages (68). FACS analysis showed that MDA-MB-231 cells expressed more cell surface Rhamm than MCF7 cells (Fig. 4), although both cell lines expressed intracellular Rhamm as detected by confocal microscopy (Fig. 5A, panels a and e). Intracellular Rhamm occurred in the cytoplasm and on cytoskeletal structures in MCF7 cells (most likely the interphase microtubules, Fig. 5A, panel a). In contrast, Rhamm was primarily detected in cell processes and the perinuclear region of MDA-MB-231 cells (Fig. 5A, panel e). Similar quantitative and qualitative differences in Rhamm expression were observed also when Ras-MCF10A and parental MCF10A cells were compared (supplemental Fig. 1b and data not shown). Thus, aggressive breast cancer cell lines are characterized by high levels of both cell surface CD44 and Rhamm, whereas less aggressive cell lines express only low levels of these two proteins at the cell surface.

Confocal analyses were done next to look at the distribution of CD44 and Rhamm in the MDA-MB-231 and MCF7 cells. Fig. 5A shows Rhamm (panels a (MCF7) and e (MDA-MB-231)) and CD44 staining in both cell lines (panels b (MCF7) and f (MDA-MB-231)), as well as the overlay of Rhamm and CD44 staining (panels c (MCF7) and g (MDA-MB-231)). IgG was used as control (Fig. 5A, panels d (MCF7) and h (MDA-MB-231)). MCF10A cells (Fig. 5B). In reciprocal immunoprecipitation assays (e.g., detection of Rhamm proteins in CD44 immunoprecipitates), CD44 antibody co-immunoprecipitated the 85-kDa full-length Rhamm protein in all breast cell lines (Fig. 5C). The 63-kDa N-terminal truncated form was co-immunoprecipitated in all of the cancer cell lines (MDA-MB-231, Ras-MCF10A, and MCF7) but not the nontransformed MCF10A cells. Greater amounts of both the 85- and 63-kDa Rhamm proteins were co-immunoprecipitated with CD44 in the invasive (MDA-MB-231 and Ras-MCF10A) versus noninvasive (MCF7) breast tumor cell lines (Fig. 5C). The 43-kDa Rhamm protein was not detected in the immunoprecipitation assays. An association of 63-kDa Rhamm protein, which resembles the transforming and cell surface isoform, with CD44std and the 116-kDa isoform was confirmed in MDA-MB-231 cells by pulldown assays using a 63-kDa recombinant Rhamm protein as bait (Fig. 5D). In contrast, no CD44 isoforms were pulled down from the MCF7 lysates (data not shown). These results suggest that cell surface display and co-association of both CD44 and Rhamm are linked to HA responsiveness and an aggressive tumorigenic phenotype.

To be invasive, tumor cells must acquire the ability to migrate (1, 2). CD44 was shown previously to promote motility of breast cancer cell lines, and Rhamm was shown to promote motility of fibroblasts and immune cells (9, 45, 65, 71, 72). Therefore, we compared the relative roles played by these HA receptors in the motility of the fibroblast-like MDA-MB-231 and Ras-MCF10A tumor cells with the epithelial MCF7 and MCF10A cells, using function-blocking antibodies specific to each of these receptors.
CD44 and Rhamm Are Necessary for Motility of Invasive but Not Noninvasive Breast Tumor Cell Lines—We confirmed that the MDA-MB-231 and Ras-MCF10A aggressive breast cancer cell lines are more motile than either MCF7 or MCF10A cells as reported previously (63, 73) (Fig. 6A). To determine the extent to which motility is coordinated through HA receptor interaction, we blocked receptor function with inhibitory antibodies against CD44 and Rhamm, added alone or in combination. The motility of MDA-MB-231 and Ras-MCF10A cells was significantly inhibited by either anti-CD44 or anti-Rhamm antibodies (Fig. 6B). The addition of both antibodies together had no additive inhibitory effect on motility (Fig. 6B). Antibodies had only minor effects on the motility of the noninvasive MCF7 and MCF10A cell lines (data not shown). These results indicate that both cell surface CD44 and Rhamm contribute to the rapid motility rates of the invasive breast cancer cell lines and that they appear to act coordinately on the same motogenic pathway. However, these receptors are much less important for the motility of poorly invasive breast tumor cell lines.

Sustained activation of ERK1,2 motogenic pathways has been reported previously as an important factor in promoting the invasive and metastatic behavior of aggressive breast cancer cell lines (52–54). We and others have shown that both CD44 and cell surface Rhamm regulate ERK1,2 activity (60, 74). We then determined their role in coordinating ERK1,2 motogenic signaling.

CD44 and Rhamm Complex with ERK1,2, and These Complexes Are Required for Motility in Invasive Breast Cancer Cell Lines—Both MDA-MB-231 and Ras-MCF10A cells expressed more total ERK1,2 protein than MCF7 and MCF10A cells (Fig. 7A), consistent with previous reports (63, 75). Under standard culture conditions, MDA-MB-231 and Ras-MCF10A cells exhibited significantly higher constitutively active (phospho)
ERK1,2 than MCF7 or MCF10A cells, consistent with the expression of mutant active Ras (H-Ras) in the invasive cell lines (data not shown). MDA-MB-231 and MCF7 tumor cells also differed in their ability to activate ERK1,2 in response to EGF, a growth factor linked to breast cancer progression. MDA-MB-231 cells, which have been reported to express high endogenous levels of EGF (76), maintained significantly greater levels of ERK1,2 activity than MCF7 cells (Fig. 7B, time 0). As expected from the endogenous EGF production, the addition of EGF did not increase ERK1,2 activity further in MDA-MB-231 cells, whereas it transiently increased the levels in MCF7 cells, which always maintained significantly less active ERK1,2 in any case (Fig. 7B). These differences correlated with distinct patterns of CD44-Rhamm-ERK1,2 co-localization in the two cell types, as observed using confocal analyses (Fig. 8A). Fig. 8A shows CD44 (panels a (MCF7) and e (MDA-MB-231)) and phospho-ERK1,2 staining in both cell lines (panels b (MCF7) and f (MDA-MB-231)), as well as the overlay of Rhamm and phospho-ERK1,2 staining (panels c (MCF7) and g (MDA-MB-231)). IgG was used as control (Fig. 8A, panels d (MCF7) and h (MDA-MB-231)). These images show, for example, that active ERK1,2 and CD44 co-localized in MDA-MB-231 cells. The insets in Fig. 8A, panels c and g, have been “enhanced” (see “Experimental Procedures”) to show all red/green co-localization as white. These images reveal extensive co-localization of ERK1,2 with CD44 in MDA-MB-231 (Fig. 8A, panel g) but not in MCF7 (panel c) cells. These results suggest that the majority of CD44-Rhamm-activated ERK1,2 complexes occur as vesicles near the nucleus of MDA-MB-231 cells. In contrast, levels of both active ERK1,2 and CD44 were low, and limited co-localization was observed in MCF7 cells.

To confirm the association between ERK1,2 and Rhamm, we co-immunoprecipitated ERK1,2 in all cell lines using anti-Rhamm antibodies. As expected, there were greater amounts of ERK1,2 in immunoprecipitates from MDA-MB-231 and Ras-MCF10A than in MCF7 and MCF10A cells (Fig. 8B). The converse experiment, using anti-ERK1,2 antibodies, also co-immunoprecipitated larger amounts of Rhamm proteins in the aggressive cell lines (Fig. 8C). Intriguingly, the 85-kDa (full-length) Rhamm protein form was not detected in any of the ERK1,2 immunoprecipitates (Fig. 8C); the 63-kDa protein was the predominant Rhamm form present. We confirmed the ability of both the 63- and 43-kDa Rhamm proteins to associate with ERK1,2 in pulldown assays using 63- or 43-kDa N-terminally truncated recombinant Rhamm, which correspond to the endogenous Rhamm isoforms, as bait (Fig. 8D). ERK1,2 was present in both pulldowns confirming that, unlike the full-length Rhamm form, the smaller Rhamm protein forms can associate with ERK1,2. Because the 63-kDa Rhamm expression, in particular, was greater in the invasive breast tumor cell lines, it may be responsible for the high ERK1,2 activity observed in the invasive breast cancer cell lines.

Rhamm proteins that are smaller than the full-length form have been reported to be predominant at the cell surface (68). We determined the role of cell surface Rhamm, as well as CD44, in activating ERK1,2 in MDA-MB-231 cells after exposure to anti-Rhamm, anti-CD44, or both antibodies (Fig. 9A). Exposure to isotype-matched nonimmune IgG served as a control. ERK1,2 activity was significantly reduced by anti-Rhamm antibodies (Fig. 9A). Anti-CD44 antibodies also appeared to reduce ERK1,2 activity, although the effect did not reach a significance level of p < 0.05. The addition of both inhibitory antibodies together had no greater inhibitory effect on ERK1,2 activation than either antibody alone (Fig. 9A).
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A. Total ERK1,2 Protein Expression in Breast Cancer Cell Lines

![Graph showing total ERK1,2 protein expression in breast cancer cell lines.](image)

ERK1,2 associates with Rhamm-CD44 complexes and that both HA receptors are required for activation of these MAP kinases. Because ERK1,2 activity is essential for motility and invasion of MDA-MB-231 cells, we asked whether these HA receptors mediate motility also via an ERK1,2-dependent pathway. The addition of PD098059, a MEK1 inhibitor (Fig. 9B), or anti-Rhamm antibodies (data not shown) significantly reduced the basal motility rate of MDA-MB-231 cells. Again, the addition of these two reagents together had no additive inhibitory effect (Fig. 9B). Furthermore, neither the MEK1 inhibitor nor the anti-Rhamm antibodies, individually or in combination, had a significant inhibitory effect on MCF7 cell motility (Fig. 9B). Similar results were observed when using anti-CD44 antibodies alone or in combination with the MEK1 inhibitor (data not shown). These combined results suggest that Rhamm, CD44, and ERK1,2 activity are required for rapid basal motility of the aggressive cell lines and that they act collectively on the same motogenic pathway. Results suggest further that although Rhamm can associate with both ERK1,2 and CD44, even in the less aggressive breast cancer cell lines, the subcellular localization of these complexes and the consequent effects on the kinetics of ERK1,2 activity as well as motogenic signaling are limited in the cell lines that express little to no cell surface CD44 or Rhamm.

**DISCUSSION**

We have identified an autocrine motility mechanism by which aggressive breast cancer cell lines maintain rapid basal rates of motility. This mechanism requires HA production, ERK1,2 activity, and cell surface display of CD44 and Rhamm (CD168). It is associated with the formation of signaling complexes composed of cell surface CD44, Rhamm, and active ERK1,2, which are most abundant in the aggressive, highly motile breast cancer cell lines. Although previous reports have demonstrated that either CD44 or cell surface Rhamm is required for HA-mediated motility of tumor cells including MDA-MB-231 cells, this is the first report documenting both a functional and physical interaction between these two HA receptors. It is also the first to demonstrate coupling of this complex to a motogenic signaling pathway through ERK1,2 in aggressive tumor cells. Our results raise the possibility that the association of cell surface Rhamm with CD44, and their subsequent association with ERK1,2, may modify tumor suppression by CD44 to favor its latent tumor promoter functions. This is consistent with the strong association among elevated HA accumulation, ERK1,2 activity, Rhamm expression, and aggressive forms of breast carcinoma (31, 37).

Hyaluronan was originally proposed to be an autocrine motility factor for mesenchymal cells (77). Indeed, high endogenous production of HA has since been shown to provide autocrine motility signals in embryonic cells (78, 79), hematopoietic progenitor cells (80), and a variety of other human tumor cells (17, 18). The possibility that HA is an autocrine motility factor that is produced by and required for the motility of aggressive breast cancer cells is attractive given the close relationship of HA production and hyaluronan synthase expression (7, 17, 81) and co-localization of HA with CD44 in later stages of breast and other cancers (27). Our data also fit well with the prognostic value of elevated HA in peritumor stroma or tumors themselves as a marker of poor outcome in this disease (82).

HA has consistently been demonstrated to activate motogenic signaling through Ras (40, 78, 79) and to require activation of ERK1,2 and phosphatidylinositol 3-kinase/akt for this function (17, 49, 83). Although the HA receptors, CD44
and cell surface Rhamm, have been shown to mediate HA regulation of a Ras-controlled motogenic pathway, the majority of studies have focused on the exclusive role of CD44. An overwhelming number of these support a major role for CD44 in promoting aggressive breast cancer behavior, including cell motility in culture and in experimental tumor models as demonstrated by the use of CD44 antibodies, blocking soluble CD44 recombinant protein, or small HA fragments, and genetic deletion or knockdown of this HA receptor (17, 81, 84). Nevertheless, reports also have documented that increased motility or invasion of breast and other tumor cells is associated with shedding of CD44 (e.g. Ref. 85) or genetic loss or blocking of CD44 functions (83, 86). These experimental discrepancies, which reveal the capacity of CD44 to both promote and inhibit motility and invasion, mirror the dual relationship of CD44 expression to clinical outcome of breast cancer patients. In breast cancers, overexpression of the standard or specific variant forms has not consistently been demonstrated to relate to outcome parameters (25, 28, 29, 87). One conclusion from these studies is that it is CD44 variant forms, which are expressed at different stages of breast tumor progression (13, 27), that perform distinct functions from CD44std in tumor progression. For example, different CD44 protein forms may act as tumor suppressors during early stages of breast cancer; CD44std expression correlates positively with disease-related survival in node-negative invasive breast carcinoma (50) but acts as an enhancer of metastasis during later stages (88). Our results support a role for CD44 in aggressive functions of breast tumor
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A. Effect of Anti-Rhamm and / or Anti-CD44 antibodies on ERK1,2 Activation in Breast Cancer Cell Lines

![Graph showing the effect of Anti-Rhamm and / or Anti-CD44 antibodies on ERK1,2 Activation in Breast Cancer Cell Lines.]

B. Effect of PD098059 and / or Anti-Rhamm Antibodies on Breast Cancer Cell Motility

![Graph showing the effect of PD098059 and / or Anti-Rhamm Antibodies on Breast Cancer Cell Motility.]

cells when it is partnered with other motogenic proteins such as cell surface Rhamm (45).

Rhamm is structurally unrelated to CD44, yet cell surface Rhamm can perform similar functions to CD44 including mediating motogenic signaling by HA (46). Because cell surface Rhamm is not an integral protein, it must partner with other receptors that are able to take over these functions of CD44 in its absence. To our knowledge, the identities of such additional proteins have not yet been reported.

In addition to its location at the cell surface, Rhamm also occurs in several intracellular compartments. In contrast to cell surface Rhamm, intracellular forms affect centrosome and mitotic spindle formation/integrity (41, 43) and are not likely involved in motogenic functions. In support of this conclusion, we have shown that cell surface Rhamm is sufficient to rescue ERK1,2-dependent motility in Rhamm−/− fibroblasts that do not express intracellular Rhamm forms (45). Previous studies have suggested that the presence of cell surface Rhamm is required for HA-mediated activation of ERK1,2 in endothelial cells (89) and fibroblasts (60) and for the motility and invasion of endothelial cells (90) when CD44 is co-expressed. Also, we have shown recently that genetic deletion of Rhamm blunts both ERK1,2 activity and motility of dermal fibroblasts even though they express CD44 protein (45). This study further shows that one function of cell surface Rhamm is to promote cell surface display of CD44; in the absence of cell surface Rhamm, CD44 is retained within intracellular vesicles (40). In contrast, in breast cancer cells (e.g. MCF7), the low levels of cell surface CD44 appear to result from its reduced transcription (data not shown) rather than from defects in a Rhamm-regulated display mechanism.

ERK1,2 kinases are ubiquitous and homologous MAP kinases that mediate proliferation, differentiation, and motility via growth factor and ECM receptor activation.
Overexpression and elevated activation of these kinases are common in human tumors. The importance of increased ERK1,2 activity in breast cancer is demonstrated by the anti-tumor effects of a specific inhibitor of the upstream kinase activators MEK1,2 (PD184352) in breast cancer patients (91). Although these MAP kinases are among the most common effectors in growth factor and ECM-regulated signaling pathways, a variety of temporal, spatial, and quantitative cues confer a specificity of functional outcome resulting from their activation (55, 92, 93). For example, sustained activation of ERK1,2 is required to initiate the motility of breast cancer cells (94). The duration of ERK1,2 activity is determined by many factors, including concentration of the stimulus, receptor dimerization, and the expression/compartmentalization of intracellular scaffolding/accessory proteins (92, 93). The mechanisms by which CD44 and Rhamm sustain elevated basal ERK1,2 activity remains unclear, but confocal analysis suggests that these HA receptors co-localize with phospho-ERK1,2, predominantly in the perinuclear area of cells where these proteins appear as vesicles. Although internalization of some receptor-ERK1,2 complexes terminate ERK1,2 activity, internalization of ERK1,2 with other receptors (i.e. protease-activated receptor-2 (PAR-2)) results in sustained ERK1,2 activation (95). We propose that internalization and trafficking of CD44-Rhamm-ERK1,2 complexes to the perinuclear area promotes sustained ERK1,2 activity in the cytoplasm. By this type of mechanism, active ERK1,2 would be available to traffic to key cytoplasmic compartments such as focal adhesions or the nucleus, sites where its activity is required for cell motility/invasion (52, 54). Further experimentation is needed to assess this possibility.

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