Hyaluronan-mediated CD44 Interaction with RhoGEF and Rho Kinase Promotes Grb2-associated Binder-1 Phosphorylation and Phosphatidylinositol 3-Kinase Signaling Leading to Cytokine (Macrophage-Colony Stimulating Factor) Production and Breast Tumor Progression*

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In this study we have examined CD44 (a hyaluronan (HA) receptor) interaction with a RhoA-specific guanine nucleotide exchange factor (p115RhoGEF) in human metastatic breast tumor cells (MDA-MB-231 cell line). Immunoprecipitation and immunoblot analyses indicate that both CD44 and p115RhoGEF are expressed in MDA-MB-231 cells and that these two proteins are physically associated as a complex in vivo. The binding of HA to MDA-MB-231 cells stimulates p115RhoGEF-mediated RhoA signaling and Rho kinase (ROK) activity, which, in turn, increases serine/threonine phosphorylation of the adaptor protein, Gab-1 (Grb2-associated binder-1). Phosphorylated Gab-1 promotes PI 3-kinase recruitment to CD44v3. Subsequently, PI 3-kinase is activated (in particular, α, β, γ forms but not the δ form of the p110 catalytic subunit), AKT signaling occurs, the cytokine (macrophage-colony stimulating factor (M-CSF)) is produced, and tumor cell-specific phenotypes (e.g. tumor cell growth, survival and invasion) are up-regulated. Our results also demonstrate that HA/CD44-mediated oncogenic events (e.g. AKT activation, M-CSF production and breast tumor cell-specific phenotypes) can be effectively blocked by a PI 3-kinase inhibitor (LY294002). Finally, we have found that overexpression of a dominant-negative form of ROK (by transfection of MDA-MB-231 cells with the Rho-binding domain cDNA of ROK) not only inhibits HA/CD44-mediated RhoA-ROK activation and Gab-1 phosphorylation but also downregulates oncogenic signaling events (e.g. Gab-1-PI 3-kinase-CD44v3 association, PI 3-kinase-mediated AKT activation, and M-CSF production) and tumor cell behaviors (e.g. cell growth, survival, and invasion). Taken together, these findings strongly suggest that CD44 interaction with p115RhoGEF and ROK plays a pivotal role in promoting Gab-1 phosphorylation leading to Gab-1-PI 3-kinase membrane localization, AKT signaling, and cytokine (M-CSF) production during HA-mediated breast cancer progression.

CD44 (an hyaluronan (HA) receptor) belongs to a family of multifunctional transmembrane glycoproteins expressed in a number of tissues and cells, including breast tissues and cells (1–6). It is encoded by a single gene that contains 19 exons (7). Of the 19 exons, 12 exons can be alternatively spliced (7). Most often, breast tumor cells and tissues express several different CD44 spliced variant (CD44v) isoforms in addition to CD44s (the standard form) and CD44E (the epithelial form) (7). Because various CD44 isoforms mediate different functions, much attention has been paid to the changes in CD44v isoform production during malignant transformation (1–6). Specifically, there is accumulating evidence that the induction and overexpression of CD44v isoforms are associated with the invasion and progression of breast carcinomas and tumor cell lines (1–6). These CD44v isoforms appear to confer the malignant properties of abnormal adhesion, growth, migration, and invasion (1–6). Furthermore, CD44 has been shown to interact with extracellular matrix components (e.g. hyaluronan (HA) at the N terminus of the extracellular domain) (8–10) and to contain specific binding sites for the cytoskeletal proteins (e.g. ankyrin and ERM) within the 70-amino acid C terminus of its cytoplasmic domain (11–16).

Several mechanisms for the regulation of HA/CD44-mediated function have been suggested. These include modifications by an additional exon-coded structure (via an alternative splicing process) (1–6), variable N/O-linked glycosylation on the extracellular domain of the CD44 (17, 18), and selective interactions of the cytoplasmic domain of the CD44 with ankyrin (11, 15) and various signaling molecules (e.g. the Src family tyrosine kinases (14, 19), p185HER2 (20, 21), Rho kinase (ROK) (22), transforming growth factor-β receptor kinases (23), and the guanine nucleotide exchange factors Tiam1 (24) and Vav2 (21)). In addition, CD44 has been shown to be involved in the production of cytokines (e.g. interleukin 8 (1) and fibroblast

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The abbreviations used are: HA, hyaluronan; ROK, Rho kinase, also called Rho-associated kinase; PI, phosphatidylinositol; aa, amino acid(s); Gab-1, Grb2-associated binder-1; IRS, insulin receptor substrate; GFP, green fluorescent protein; GTP-γ-S, guanosine 5'-3-O-(thiotriphosphate; GST, glutathione S-transferase; M-CSF, macrophage-colony stimulating factor; DMEM, Dulbecco’s modified Eagle’s medium; CHAPS, 3-[3-Cholamidopropyl]dimethylammonio]-1-propanesulfonate acid; PIP3, phosphatidylinositol 1,4,5-triphosphate; PIP2, phosphatidylinositol 1,4-bisphosphate; PIP1, phosphatidylinositol 1,4,5-trisphosphate; PtdIns, phosphatidylinositol; EGFr, epidermal growth factor; GEF, guanine nucleotide exchange factor; RGS, regulator of G-protein signaling domain; DH, dbl homology domain; PH, pleckstrin homology domain; RB, Rho-binding domain; RANKL, receptor activator of nuclear factor κB ligand; RANK, RANKL receptor; ERM, ezrin/radixin/moesin.

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growth factor-2 (13)) and hormones (e.g. parathyroid hormone-related protein (23)) in breast tumor cells. These findings clearly indicate that CD44 plays a pivotal role in activating oncogenic signaling and HA-mediated breast tumor cell function.

Members of the Rho subclass of the Ras superfamily (small molecular weight GTPases, e.g. RhoA, Rac1, and Cdc42) are known to transduce signals regulating many cellular processes (25). The rationale for our focusing on RhoGTPase is based on previous reports suggesting that CD44-associated cytoskeletal proteins (e.g. ankyrin and ERM) and that tumor cell-specific phenotypes are dependent on RhoGTPase signaling events (16, 22, 26). Overexpression of certain RhoGTPases in human tumors often correlates with poor prognosis (28, 29). In particular, coordinated RhoGTPase signaling is considered to be a possible mechanism underlying cell proliferation and motility, an obvious prerequisite for metastasis (16, 25, 26, 28–31).

Presently, very little information is available regarding how they are activated by cell surface receptors. To date, at least 30 different guanine nucleotide exchange factors (GEFs) have been identified (32). Our recent results indicate that the interaction between CD44v3 isoform and two GEFs, such as Tiam1 (24) and Vav2 (21), up-regulates Rac1 signaling and cytoskeleton-mediated metastatic tumor progression. As part of our continued effort to identify CD44 isoform-linked GEFs, which correlate with certain metastatic behaviors, a new candidate molecule, named p115RhoGEF, has been identified. p115RhoGEF, which is the human homolog of the mouse protein Lsc, is involved in RhoA activation (33); structurally, it contains numerous functional domains and structural motifs found in signal transduction proteins and oncoproteins. These motifs include N-terminal RGS (regulator of G protein signaling) domain, a dbl homology domain (DH), and a pleckstrin homology domain (PH) (33, 34). In particular, the sequence between amino acids (aa) 421 and aa 635 contains significant sequence homology to the dbl homology domain (DH) of many proteins that exhibit GDP/GTP exchange activity for specific members of the Ras superfamily of GTP-binding proteins (32). The p115RhoGEF has been clearly shown to act as a GDP/GTP exchange protein for the Rho subfamily of GTPases, including RhoA (33). In addition, p115RhoGEF (similar to PDZ-RhoGEF) contains an RGS domain, which interacts with Gα/i subunits and regulates the ability of DH to carry out GDP/GTP exchange activity for RhoA (33, 34). In the C-terminal region, p115RhoGEF contains one PH domain that is commonly detected in signaling molecules and cytoskeletal proteins (35, 36). Activation of RhoGEF pathways such as RhoA has been shown to produce specific structural changes in the plasma membrane associated with actin filament bundling, stress fiber formation, and acto-myosin-based cytoskeletal function (25).

Several different enzymes have been identified as possible downstream targets for RhoA signaling. One such enzyme is Rho kinase (ROK, also called Rho-associated kinase), which is a serine-threonine kinase known to interact with Rho in a GTP-dependent manner (37–39). ROK is composed of four functional domains, including a kinase domain (catalytic site), a coiled-coil domain, a Rho-binding (RB) domain, and a PDZ domain (40). This enzyme has been shown to regulate cytoskeletal function by phosphorylating several important cytoskeletal regulators, including myosin-binding subunit of myosin phosphatase (41), calponin (42), adducin (43), and Lin 11/Isl-1/Mec-3 (LIM) kinase (44). ROK is also involved in the “cross-talk” between Ras and Rho signaling leading to cellular transformation (30). We have demonstrated that ROK phosphorylates the cytoplasmic domain of the CD44v3, 8–10 isoform and up-regulates the interaction between the CD44v3, 8–10 isoform and the cytoskeletal protein, ankyrin, during HA/CD44-regulated tumor cell migration (22). Most recently, the PH domain of ROK is found to be involved in the direct binding to CD44- and HA-mediated Ca2+ signaling in endothelial cell function (45). Thus, ROK is clearly one of the important signaling molecules required for membrane-cytoskeleton interaction, Ca2+ regulation, and HA/CD44-mediated cell function (22, 45).

In the pathogenesis of cancer, Grb-2-associated binder-1 (Gab-1) and phosphatidylinositol 3-kinase (PI 3-kinase) are key mediators in regulating oncogenesis (for review see Ref. 46). Specifically, Gab-1 (a member of the insulin receptor substrate (IRS) family) functions as one of the major adapter molecules downstream of growth factor signaling (47, 48). Gab-1 also possesses multiple phosphorylation sites that could act as docking sites for PI 3-kinase known to consist of a catalytic subunit p110 (α, β, and δ) and regulatory subunit p85 (α, β, and p55γ) or the catalytic subunit p110γ and the regulatory subunit p101 (for reviews see Refs. 46, 49–51). One recent study found a positive link between HA-CD44 interaction and Gab-1-associated PI 3-kinase activation during the stimulation of cellular transformation by a Met-hepatocyte growth factor oncoprotein (Tpr-Met) (52). HA and CD44 also promote PI 3-kinase signaling in a tumor cell-specific manner. For example, PI 3-kinase participates in CD44-mediated survival pathway in colon carcinoma cells (53). HA activates PI 3-kinase-akt pathways leading to cell motility and cell survival-signaling pathways (54). The active mutant of p110 subunit of PI 3-kinase exerts its action on the cleavage of CD44 during cancer cell migration (55). These findings suggest PI 3-kinase activation is closely coupled with HA-mediated CD44 signaling.

Because both Rho signaling and PI 3-kinase activation play an important role in regulating breast tumor progression, we have focused in this study on the relationship between these two signaling pathways during HA/CD44-mediated breast tumor progression. A unique mechanism is described concerning CD44 interaction with p115RhoGEF and ROK that stimulates Gab-1 phosphorylation/membrane localization and PI 3-kinase-AKT activation leading to HA/CD44-regulated cytokine production and tumor cell behaviors required for breast cancer progression.

MATERIALS AND METHODS

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

Antibodies and Reagents—Monoclonal rat anti-human CD44 antibody (clone: 1290; Iso type: IgG1; obtained from CMB-Tech, Inc., San Francisco, CA) used in this study recognizes a common determinant of the CD44 class of glycoproteins. Both rabbit anti-CD44v3 antibody and rabbit anti-Rho kinase (ROK) were prepared according to the procedures described previously (22). For the preparation of polyclonal rabbit anti-p115RhoGEF antibody, specific synthetic peptides (<15–17 amino acids unique for the ROK or p115RhoGEF sequence) were prepared by the Peptide Laboratories using an Advanced Chemtech automatic synthesizer (model ACT350). All polyclonal antibodies were prepared using conventional DEAE-cellulose chromatography and tested to be monospecific (by immunoblot assays). Mouse monoclonal anti-green fluorescent protein (GFP) and mouse monoclonal anti-FLAG (M2) were purchased from BD Pharmingen and Sigma, respectively. Rabbit anti-phospho-threonine antibody and rabbit anti-phospho-serine antibody were obtained from Zymed Laboratories Inc.. Monoclonal mouse anti-p110α, mouse anti-p110β, mouse anti-p110γ and mouse anti-p105β were purchased from Santa Cruz Biotechnology. Several other immunoreagents, including mouse anti-AKT-1 (protein kinase B), rabbit anti-AKT-3 (threonine 308), and rabbit anti-Gab-1, were purchased from Upstate Biotechnology, Inc. The specific inhibitor of PI 3-kinase (LY294002) was obtained from Calbiochem. Rooster comb hyaluronan (HA) was purchased from Sigma. High molecular mass HA polymers (~10^6 Da) were purified by gel filtration column chromatography using
a Sephacryl S1000 column as described previously (18). 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.

Cloning, Expression, and Purification of CD44 Cytoplasmic Domain (CD44ccty) from E. coli—The cytoplasmic domain of human CD44 (CD44cty) was cloned into pFLAG-AST using the PCR-based cloning strategy (15). Using human CD44 cDNA as template, one PCR reaction pair (left, FLAG-EcoRI; right, FLAG-XbaI) was designed to amplify complete CD44 cytoplasmic domain. The amplified DNA fragments were one-step-cloned into a pCR2.1 vector and sequenced. Then, the DNA fragments were cut out by double digestion with EcoRI and XbaI and subcloned into EcoRI/XbaI double-digested pFLAG-AST (Eastman Kodak, NY) to generate a PCR-ROK/CD44ccty fusion construct. The nucleotide sequence of FLAG-CD44ccty junction was confirmed by sequencing. The recombinant plasmids were transformed to BL21-DE3 to produce a FLAG-CD44cty fusion protein. The FLAG-CD44cty fusion protein was further purified by using an anti-FLAG M2 affinity gel column (Eastman Kodak-IBI). The nucleotide sequence of primers used in this cloning protocol is as follows: FLAG-EcoRI, 5′-GAGAATTCTCA-ACAGTCGAAGAGGTGTCCTTAAGG-3′; FLAG-XbaI, 5′-AGCTT-AGATTACACCCCAATCTTCAT-3′.

Cloning and Expression of the PH Domain of Gab-1—The Gab-1 cDNA fragment containing the PH domain was constructed according to the protocol described previously (19). The specific primers linked with an enzyme (HindIII) was designed to amplify 226–362 bp of Gab-1 PH domain (22). The amplified DNA fragments were cut out by double digestion with HindIII and XhoI and ligated into the pEGFP-C1 vector (Clontech) digested with HindIII and XhoI. The sequence was confirmed by nucleotide sequencing analyses. This vector (pEGFP-C1) containing the PH domain of Gab-1 was used for a transient expression in MDA-MB-231 cells with transient transfection (e.g. LipofectAMINE 2000). The transfected cells were then developed using ECL chemiluminescence reagent according to the manufacturer’s instructions. During these immunological analyses, an equal amount of cellular protein (50 μg) immunoprecipitated with the antibody was applied to SDS-PAGE followed by immunoblotting with various reagents (e.g. anti-p115RhoGEP, anti-p115RhoGEF-free serum, or anti-p115RhoGEF antibody pre-absorbed by an excess amount of p115RhoGEF-mediated immunoblot or immunoprecipitated with anti-p115RhoGEF antibody followed by anti-CD44v3 antibody or anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of CD44v3-mediated immunoblot or immunoprecipitated with anti-CD44 antibody) followed by immunoblotting with various immunoreagents (e.g. anti-His, anti-Gab-1, or anti-PI-3-kinase antibodies such as anti-p110α, anti-p110β, anti-p110γ, or anti-p110δ). In some experiments, P1D10-40-solubilized cell lysate isolated from transfectants (MDA-MB-231 cells) was treated (treated with or without LY294020) or transfected with GFP-tagged ROK-RBcDNA or vector alone. Then, Gab-1 PH domain (CD44-Gab-1-PH binding assay was conducted according to the manufacturer’s instructions. During these immunological analyses, an equal amount of cellular protein (50 μg) immunoprecipitated with the antibody was applied to SDS-PAGE followed by immunoblotting with various reagents (e.g. anti-p115RhoGEF, anti-p115RhoGEF-free serum, or anti-p115RhoGEF antibody pre-absorbed by an excess amount of p115RhoGEF-mediated immunoblot or immunoprecipitated with anti-p115RhoGEF antibody followed by anti-CD44v3 antibody or anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of CD44v3-mediated immunoblot or immunoprecipitated with anti-CD44 antibody) followed by immunoblotting with various immunoreagents (e.g. anti-His, anti-Gab-1, or anti-PI-3-kinase antibodies such as anti-p110α, anti-p110β, anti-p110γ, or anti-p110δ). In some experiments, P1D10-40-solubilized cell lysate isolated from transfectants (MDA-MB-231 cells) was treated (treated with or without LY294020) or transfected with GFP-tagged ROK-RBcDNA or vector alone. Then, Gab-1 PH domain (CD44-Gab-1-PH binding assay was conducted according to the manufacturer’s instructions. During these immunological analyses, an equal amount of cellular protein (50 μg) immunoprecipitated with the antibody was applied to SDS-PAGE followed by immunoblotting with various reagents (e.g. anti-p115RhoGEF, anti-p115RhoGEF-free serum, or anti-p115RhoGEF antibody pre-absorbed by an excess amount of p115RhoGEF-mediated immunoblot or immunoprecipitated with anti-p115RhoGEF antibody followed by anti-CD44v3 antibody or anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of CD44v3-mediated immunoblot or immunoprecipitated with anti-CD44 antibody) followed by immunoblotting with various immunoreagents (e.g. anti-His, anti-Gab-1, or anti-PI-3-kinase antibodies such as anti-p110α, anti-p110β, anti-p110γ, or anti-p110δ).
231 cells preincubated with monoclonal rat anti-CD44 antibody (or normal rat IgG) followed by HA treatment or fibronectin treatment (as no treatment). Data represent an average of triplicates from three to five experiments. The standard deviation was less than 5%.

**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 μg/ml leupeptin, 100 μM ATP, 1 mM γ-32P-ATP, 10 μM p110γ or no p110γ (100 ng of MDA-MB-231 cells’ ROK or no ROK) and 1 μg of Gab-1 (obtained from anti-Gab-1-associated beads) in the presence or absence of GTPγS-GST-RhoA fusion protein (1 μM) or GST-RhoA alone (1 μM). After an incubation for various time intervals (e.g., 0, 10, 20, 30, and 60 min) at 30 °C, the reaction mixtures were boiled in SDS-sample buffer and subjected to SDS-PAGE. The protein bands were revealed by antiphospho-serine/anti-phospho-threonine-mediated immunoblot and anti-Gab-1-mediated immunoblot, respectively.

**Measurement of PI 3-Kinase Activity**—MDA-MB-231 cells (untransfected (treated with or without LY294002) or transfected with ROK-RbCDNA or vector alone) were treated with HA (50 μg/ml) (or pre-treated with rat anti-CD44 followed by HA treatment (50 μg/ml) or treated with no HA). Cell membranes of these cells were solubilized in buffer A (1.0% Nonidet P-40, 1.0% Na3VO4, 1.0% phenylmethylsulfonyl fluoride, okadaic acid (25 ng/ml) in 0.1 m phosphate-saline (pH 7.4) and Complete™ protease inhibitors (Roche Applied Science)) at 4 °C for 1 h followed by immunoprecipitation with anti-PI 3-kinase (e.g., anti-p110α, and anti-p110β or anti-p110γ or anti-p110δ) conjugated beads. To measure PI 3-kinase activity, anti-p110α (or anti-p110β or anti-p110γ or anti-p110δ)-conjugated beads were incubated in 50 μl of buffer A containing 20 μg of sonicated PIP2 (Sigma Co.) and 10 μCi of [γ-32P]ATP for 30 min at 32 °C. Subsequently, the reaction was terminated by an addition of acidified chloroform:methanol (1:1, v/v). Extracted lipids were then spotted onto a silica TLC G-60 plate (20 × 20 cm, Whatman, precoated with potassium oxalate) and developed using a solvent system containing chloroform/acetonemethanol/acetic acid/water (80:30:26:24:14). The TLC plates were dried and, radioactively labeled PIP2 spots were visualized by autoradiography.

**Measurement of M-CSF Production**—MDA-MB-231 cells (untransfected (treated with or without LY294002) or transfected with ROK-RbCDNA or vector alone) were washed three times with serum-free DMEM and incubated in 3 ml of serum-free DMEM containing 20% FCS, 100 ng of human M-CSF, 100 μM okadaic acid (25 ng/ml) (or pre-treated with rat anti-CD44 followed by HA treatment (50 μg/ml) or treated with no HA). M-CSF concentrations in the conditioned media were measured using the Quantikine M-CSF immunoassay. Subsequently, M-CSF concentrations in the conditioned media were determined using the Quantikine M-CSF immunoblot assay according to the procedures provided by Promega (2). Subsequently, M-CSF concentrations in the conditioned media and cells were determined using the Quantikine M-CSF immunoblot assay (R&D Systems). Statistical analysis was done using the Student t test. All data were expressed as the mean ± S.D.

**In Vitro Tumor Cell Growth Assays**—MDA-MB-231 cells (untransfected (treated with or without LY294002) or transfected with ROK-RbCDNA or vector alone) were washed three times with serum-free DMEM and incubated in 3 ml of serum-free DMEM containing various reagents (e.g., HA (50 μg/ml) or anti-CD44 antibody plus HA (50 μg/ml) or without HA treatment) for 24 h at 37 °C in a 5% CO2 humidified chamber. Subsequently, M-CSF concentrations in the conditioned media and cells were determined using the Quantikine M-CSF immunoblot assay (R&D Systems). Statistical analysis was done using the Student t test. All data were expressed as the mean ± S.D.

**RESULTS**

**Interaction of CD44v3 and p115RhoGEF in Breast Tumor Cells (MDA-MB-231 Cells).**—The level of CD44v3 isoform expression increases as the histological grade of the breast tumor progresses. In fact, there is a direct correlation between CD44v3 isoform expression and increased histological grade of the malignancy (22). Using anti-CD44v3 antibody and immunoblot analysis, we have confirmed the presence of the 85-kDa CD44v3 protein in MDA-MB-231 cells (Fig. 1, lane 1). A number of studies have shown that CD44v3 and RhoGTPases (in particular, RhoA) are structurally and functionally coupled in breast tumor cells (22). Although several RhoA-specific guanine nucleotide exchange factors (RhoGEFs) have been shown to be involved in RhoA signaling (33, 34, 58), identification of the specific RhoGEF protein that plays a direct role in regulating CD44v3-specific metastatic behaviors in breast tumor cells is addressed in this study.

Immunoblotting with anti-p115RhoGEF antibody indicates that a single polypeptide (molecular mass, ~115 kDa) is expressed in MDA-MD-231 cells (Fig. 1, lane 2). In addition, we have carried out anti-p115RhoGEF-mediated or anti-CD44v3-mediated immunoprecipitation followed by anti-CD44v3 immunoblot (Fig. 1, lane 3) or anti-p115RhoGEF immunoblot (Fig. 1, lane 4), respectively. Our results clearly indicate that the CD44v3 is present in anti-p115RhoGEF-immunoprecipitated materials (Fig. 1, lane 3). Conversely, the p115RhoGEF band is also detected in the anti-CD44v3-immunoprecipitated materials (Fig. 1, lane 4). The results with control samples confirm the specificity of these immunological techniques. For example, little or no p115RhoGEF is detected in anti-CD44v3-mediated immunoprecipitated materials blotted by anti-p115RhoGEF-free serum (anti-p115RhoGEF antibody pre-absorbed by an excess amount of p115RhoGEF) (Fig. 1, lane 5). Similarly, no CD44v3 is observed in anti-p115RhoGEF-mediated immunoprecipitated materials blotted by an anti-CD44v3-free serum (anti-CD44v3 antibody pre-absorbed by an excess amount of CD44v3) (Fig. 1, lane 6). These findings indicate that...
CD4v3α and p115RhoGEF are physically associated in breast tumor cells.

Analyses of p115RhoGEF-catalyzed RhoA Activation in MDA-MB-231 Cells—It has been reported that p115RhoGEF functions as an exchange factor for Rho-like GTPases such as RhoA in G-protein-coupled receptor activation (22, 33, 34). To confirm that the p115RhoGEF-like molecule in this study functions as a GDP/GTP exchange factor (or a GDP-dissociation stimulator protein) for RhoGTPases in breast tumor cells, we first isolated p115RhoGEF from MDA-MB-231 cells using anti-p115RhoGEF-conjugated Sepharose beads. As indicated in Fig. 2, the purified p115RhoGEF activates GDP/GTP exchange on GST-tagged RhoA (Fig. 2). The initial onset of the exchange reaction on GST-tagged RhoA occurs within 0.5–2 min after the addition of p115RhoGEF, and the reaction reaches its maximum ~6 min after p115RhoGEF addition (Fig. 2). Most importantly, we have observed that the addition of HA to CD4v3-containing MDA-MB-231 cells stimulates both the rate of p115RhoGEF-mediated GDP/GTP exchange reaction and the maximal amount of bound [35S]GTP·S to GST-RhoA (at least a 1.75-fold increase) (Fig. 2, line a) as compared with the p115RhoGEF isolated from MDA-MB-231 cells without HA treatment (Fig. 2, line b). In control samples, a low level of [35S]GTP·S-bound material was detected in GST alone under same GDP/GTP exchange reaction using p115RhoGEF isolated from MDA-MB-231 cells in the presence (Fig. 2, line c) or absence (Fig. 2, line d) of HA treatment. Therefore, we conclude that p115RhoGEF in MDA-MB-231 cells functions as a GDP/GTP exchange factor for Rho-like GTPases such as RhoAGTase.

Identification of Gab-1 as a New Cellular Substrate for ROK—Several studies indicate that Rho kinase (ROK, also called Rho-associated kinase-a serine-threonine kinase) interacts with RhoA in a GTP-dependent manner during HA/CD44-mediated cell activation (22, 45). Using a ROK-specific antibody, we have confirmed the presence of ROK (~160-kDa polypeptide) in MDA-MB-231 cells (Fig. 3, lane 2). No ROK-containing material was observed in control samples when pre-immune rabbit serum was used in these experiments (Fig. 3, lane 1). This RhoA-activated ROK has been shown to induce phosphorylation of a number of cytoskeletal proteins and signaling molecules (22, 41–45). A recent study indicates that overexpression of an active form of RhoA (RhoA(V14)) promotes ROK-mediated serine phosphorylation of insulin-receptor substrate-1 (IRS-1) during insulin signaling (59). In this study we have found that a member of the IRS family, Gab-1, is expressed in MDA-MB-231 cells (Fig. 4A, panel c). Furthermore, our data indicate that ROK isolated from MDA-MB-231 cells is capable of phosphorylating the Gab-1 at both serine (Fig. 4A, panel a, lane 3) and threonine residues (Fig. 4A, panel b, lane 3) (as detected by anti-phospho-serine and anti-phospho-threonine-mediated immunoblot, respectively, followed by blotting with anti-Gab-1 antibody (Fig. 4A, panel c, lane 3) in the presence of activated GTP·S-RhoA (Fig. 4A, panels a–c, lane 3). The level of serine/threonine phosphorylation of Gab-1 appears to be relatively low if ROK was incubated with unactivated RhoA (RhoA without GTP·S bound) (Fig. 4A, panels a–c, lane 2). In the absence of ROK, no serine/threonine phosphorylation of Gab-1 was detected (Fig. 4A, panels a–c, lane 1). These results indicate that Gab-1 serves as one of the cellular substrates for the RhoA-dependent kinases such as ROK in vitro.

Furthermore, we have examined the effect of ROK-mediated Gab-1 phosphorylation on CD44 binding. Specifically, the highly phosphorylated form of Gab-1 (by ROK plus activated RhoA [RhoA with GTP·S bound, GTP·S·RhoA]; as shown in Fig. 4A, lane 3) or the minimally phosphorylated form of Gab-1 (using ROK plus unactivated RhoA [e.g. RhoA without GTP·S bound]; as shown in Fig. 4A, lane 2) was incubated with various concentrations of 125I-labeled CD44 cytoplasmic domain (FLAG-CD44cyt) under equilibrium binding conditions. Our results indicate that CD44 binds to either highly phosphorylated Gab-1 or minimally phosphorylated Gab-1 at a single site. Importantly, the highly phosphorylated Gab-1 displays at least 3-fold higher CD44 binding affinity (with an apparent Kd of ~0.18 nM) (Fig. 4B, panel a) than the minimally phosphorylated Gab-1 (with an apparent Kd of ~0.58 nM) (Fig. 4B, panel b). These results clearly support the notion that Gab-1 phosphorylation by RhoA-activated ROK enhances its binding interaction with CD44, which may be required for Gab-1-mediated recruitment of signaling components into CD44-associated membrane during HA activation. Other approaches, such as mutational analyses of the appropriate serine/threonine residues on Gab-1 (activated by RhoA-activated ROK) required for CD44 binding, are under investigation in our laboratory.

Previous studies determined that HA-CD44 interaction promotes ROK activation in breast tumor cells (22). Several cellular proteins, including the cytoplasmic domain of CD44 and IP3 receptors, have been identified as ROK-specific cellular sub-
incubated with the reaction mixture in the absence of ROK (lane 1) or in the presence of ROK plus unactivated RhoA (RhoA without GTP) (lane 1) or ROK plus activated RhoA (RhoA with GTP) (lane 2). a, anti-phospho-serine-mediated immunoblot of Gab-1 (obtained from anti-Gab-1-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 GTP) (lane 1) or ROK plus activated RhoA (RhoA with GTP) (lane 2). b, anti-phospho-threonine-mediated immunoblot of Gab-1 (obtained from anti-Gab-1-associated beads) incubated with the reaction mixture in the absence of ROK (lane 1) or in the presence of ROK plus unactivated RhoA (e.g., RhoA without GTP) (lane 1) or ROK plus activated RhoA (RhoA with GTP) (lane 2). c, anti-Gab-1-mediated immunoblot of Gab-1 (obtained from anti-Gab-1-associated beads) incubated with the reaction mixture in the absence of ROK (lane 1) or in the presence of ROK plus unactivated RhoA (e.g., RhoA without GTP) (lane 1) or ROK plus activated RhoA (RhoA with GTP) (lane 2). 125I-labeled CD44cyt (the cytoplasmic domain of CD44 fusion protein) was incubated with Sepharose-beads containing Gab-1 phosphorylated by ROK plus activated RhoA (RhoA with GTP) (lane 3). a, anti-Gab-1-mediated immunoprecipitation followed by immunoblotting with anti-serine (Fig. 5B, panel a) or anti-threonine (Fig. 5B, panel b) or anti-Gab-1 (Fig. 5B, panel c), respectively) is greatly enhanced in vector-transfected cells treated with HA (Fig. 5B, panel a, lane 2; panel b, lane 2). In contrast, Gab-1 serine and threonine phosphorylation is relatively low in vector-transfected cells without any HA treatment (Fig. 5B, panel a, lane 1; panel b, lane 1) or those vector-transfected cells pre-treated with anti-CD44 followed by HA treatment (Fig. 5B, panel a, lanes 1–3) treated with HA or no HA (or pretreated with anti-CD44 during HA-CD44 signaling) (45). Overexpression of a dominant-negative form of ROK (by transfecting cells with the RB domain of ROK (ROK-RB) cDNA) is capable of inhibiting endogenous ROK activity leading to down-regulation of HA/CD44 signaling and tumor-specific phenotypes (22). To demonstrate ROK-mediated phosphorylation of Gab-1 in vivo, a dominant-negative form of ROK (ROK-RB) cDNA was cloned into a GPC1 vector followed by a transient transfection of GFP-tagged expression vector (pEF-GPC1 vector) by HA-activated ROK and/or the dominant-negative form of ROK (by transfecting cells with the RB domain of ROK (ROK-RB overexpression) in breast tumor cells is the focus of this study.

The results of our analyses indicate that the level of serine and threonine phosphorylation of Gab-1 (as detected by anti-Gab-1-mediated immunoprecipitation followed by immunoblotting with anti-serine (Fig. 5B, panel a) or anti-threonine (Fig. 5B, panel b) or anti-Gab-1 (Fig. 5B, panel c), respectively) is greatly enhanced in vector-transfected cells treated with HA (Fig. 5B, panel a, lane 2; panel b, lane 2). In contrast, Gab-1 serine and threonine phosphorylation is relatively low in vector-transfected cells without any HA treatment (Fig. 5B, panel a, lane 1; panel b, lane 1) or those vector-transfected cells pre-treated with anti-CD44 followed by HA treatment (Fig. 5B, panel a, lane 3; panel b, lane 3). These observations strongly support the conclusion that HA-mediated Gab-1 phosphorylation is CD44-dependent. It is also noted that transfection of MDA-MB-231 cells with RbcDNA of ROK does not alter the basal level of Gab-1 serine and threonine phosphorylation in

strates during HA-CD44 signaling (45). Overexpression of a dominant-negative form of ROK (by transfecting cells with the RB domain of ROK (ROK-RB) cDNA) is capable of inhibiting endogenous ROK activity leading to down-regulation of HA/CD44 signaling and tumor-specific phenotypes (22). To demonstrate ROK-mediated phosphorylation of Gab-1 in vivo, a dominant-negative form of ROK (ROK-RB) cDNA was cloned into a green fluorescent protein (GFP)-tagged expression vector (pEF-GPC1 vector) followed by a transient transfection of GFP-RbcDNA into MDA-MB-231 cells. Anti-GFP-mediated immunoblot analysis indicates that the RB fragment of ROK is expressed in MDA-MB-231 cells transfected with the RbcDNA of ROK (Fig. 5A, panel a, lanes 4–6) but not in those cells transfected with vector alone (Fig. 5A, panel a, lanes 1–3). Using anti-ROK-mediated immunoblot, we have detected no significant difference in endogenous ROK expression between the ROK RbcDNA-transfected cells (Fig. 5A, panel a, lanes 4–6) and vector-transfected cells (Fig. 5A, panel a, lanes 1–3) treated with HA or no HA (or pretreated with anti-CD44 followed by HA treatment). The question of whether Gab-1 phosphorylation and Gab-1-mediated signaling events are affected by HA/CD44-activated ROK and/or the dominant-negative form of ROK (ROK-RB overexpression) in breast tumor cells is the focus of this study.

The results of our analyses indicate that the level of serine and threonine phosphorylation of Gab-1 (as detected by anti-Gab-1-mediated immunoprecipitation followed by immunoblotting with anti-serine (Fig. 5B, panel a) or anti-threonine (Fig. 5B, panel b) or anti-Gab-1 (Fig. 5B, panel c), respectively) is greatly enhanced in vector-transfected cells treated with HA (Fig. 5B, panel a, lane 2; panel b, lane 2). In contrast, Gab-1 serine and threonine phosphorylation is relatively low in vector-transfected cells without any HA treatment (Fig. 5B, panel a, lane 1; panel b, lane 1) or those vector-transfected cells pre-treated with anti-CD44 followed by HA treatment (Fig. 5B, panel a, lane 3; panel b, lane 3). These observations strongly support the conclusion that HA-mediated Gab-1 phosphorylation is CD44-dependent. It is also noted that transfection of MDA-MB-231 cells with RbcDNA of ROK does not alter the basal level of Gab-1 serine and threonine phosphorylation in
transfectants without HA treatment (Fig. 5B, panel a, lane 4; panel b, lane 4) or transfectants pre-treated with anti-CD44 followed by HA treatment (Fig. 5B, panel a, lane 6; panel b, lane 6). However, overexpression of the RB of ROK appears to greatly reduce the ability of Gab-1 to respond to HA-mediated serine (Fig. 5B, panel a, lane 5) and threonine phosphorylation (Fig. 5B, panel b, lane 5). These findings suggest that ROK is closely involved in the regulation of HA/CD44-mediated Gab-1 phosphorylation in vivo.

HA-CD44-mediated Activation of Gab-1-Linked PI3 Kinase Signaling—Gab-1 is known to play an important role as a linker molecule that interacts with several signaling molecules, including PI 3-kinase (46–48). Gab-1 also acts to potentiate and diversify signals downstream from receptors by virtue of its ability to assemble multiprotein complexes (47, 48). In addition, phosphorylation of Gab-1 has been shown to play an important role in membrane localization and activation of PI 3-kinase (46–48). We next examined the potential impact of ROK-mediated Gab-1 phosphorylation on the regulation of PI 3-kinase in MDA-MB-231 cells transfected with the RBcDNA of ROK or vector alone. Using anti-Gab-1-mediated immunoprecipitation of MDA-MB-231 cell lysates (isolated from the RBcDNA of ROK or vector-transfected cells) followed by immunoblotting with various PI 3-kinase antibodies (e.g. anti-PI 3-kinase (α, β, γ, and δ form of p110 catalytic subunit antibody)), we have found that all four PI 3-kinases (such as p110α (Fig. 6, panel a, lanes 1–4), p110β (Fig. 6, panel b, lanes 1–4), p110γ (Fig. 6, panel c, lanes 1–4), and p110δ (Fig. 6, panel d, lanes 1–4)) are equally co-precipitated with Gab-1 in these transfectants either with (Fig. 6, panels a–d, lanes 2 and 4) or without any HA treatment (Fig. 6, panels a–d, lanes 1 and 3). Thus, we believe that both the highly phosphorylated (Fig. 5B, panel a, lane 2; panel b, lane 2) and minimally phosphorylated forms of Gab-1 (Fig. 5B, panel a, lane 1, 3–6; panel b, lane 1, 3–6) are capable of binding to PI 3-kinases (e.g. α, β, γ, and δ form of p110 catalytic subunit of PI 3-kinase) in vivo (Fig. 6).

Many reports have shown that certain adaptor molecules such as Gab-1 are involved in bringing p110 to the plasma membrane during cellular signaling (46–51). Gab-1 contains several functional domains, including the pleckstrin homology (PH) domain (Fig. 7A, panel a), which may mediate association with the submembrane region of the cell via protein-protein or protein-lipid interactions (35, 36). To determine whether the PH domain of Gab-1 is involved in a direct interaction with CD44, we have prepared a purified recombinant Gab-1-PH fragment (conjugated to Sepharose beads) and FLAG-tagged cytoplasmic domain of CD44 (FLAG-CD44 cyt) fusion protein for in vitro binding analysis. Our results show that FLGCD44 cyt is tightly associated with Gab-1-PH-His fragment-conjugated beads (Fig. 7A, panel b, lane 2) but not with His-conjugated beads (Fig. 7A, panel b, lane 1). These observations establish the fact that the PH domain of Gab-1 may be responsible for the recognition of CD44 in vitro.
Fig. 7. Interaction between the cytoplasmic domain of CD44 (CD44cyst) and Gab-1-PH fragment in vitro (A) and detection of CD44v3-Gab-1-PH 3-kinase complex in Gab-1-PH cDNA-transfected/vector-transfected cells (B). In A: panel a, illustration of Gab-1 full-length and Gab-1-PH fragment cDNA construct. The full-length Gab-1 contains PH domain (aa 6–115) and the Met-binding domain (MBD, a proline-rich domain) (aa 450–531). The Gab-1-PH fragment construct encodes the sequence between aa 5–117. Panel b, characterization of various recombinant proteins (FLAG-CD44cyst and Gab-1-PH-V5/His-bound beads) used in the in vitro binding assay. Lane 1, anti-FLAG-mediated immunoblot of FLAG-CD44cyst associated with V5/His-bound beads. Lane 2, anti-FLAG-mediated immunoblot of FLAG-CD44cyst associated with Gab-1-PH-V5/His-bound beads. In B: Analyses of CD44v3-Gab-1-PH 3-kinase complex formation in MDA-MB-231 transfectants. MDA-MB-231 cells (transfected with Gab-1-PH cDNA or vector alone) treated with HA or no HA were solubilized by 1% Nonidet P-40 buffer. Cell lysates were then used for anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with anti-CD44v3 antibody or anti-Gab-1 or anti-PI 3-kinase antibody, respectively, as described under “Materials and Methods.” Panel a, anti-p110α-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel b, anti-p110β-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel c, anti-p110γ-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel d, anti-p110δ-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel e, anti-Gab-1-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel f, anti-His-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3).

absence of HA) (Fig. 7B, lane 3 and 4). No detectable cellular protein (Fig. 7B, panel f, lanes 1 and 2) was found in anti-CD44v3-mediated immunoprecipitated/immunoblotted materials (Fig. 7B, panel g, lanes 1 and 2) isolated from vector-transfected cells treated with HA or no HA (Fig. 7B, lanes 1 and 2). These results indicate that the PH domain of Gab-1 is closely expressed with CD44v3 in vivo.

Moreover, we have demonstrated that HA is capable of promoting the recruitment of endogenous Gab-1 (Fig. 7B, panel e, lanes 1 and 2) together with various PI 3-kinases (e.g. p110α, p110β, p110γ, and p110δ (Fig. 7B, panels a–d, lanes 1 and 2) into a complex with CD44v3 (Fig. 7B, panel g, lanes 1 and 2) in vector-transfected cells (Fig. 7B, lanes 1 and 2). In contrast, transfection of MDA-MB-231 cells with the PH cDNA of Gab-1 not only causes significant reduction in endogenous Gab-1 (Fig. 7B, panel e, lanes 3 and 4) association with CD44v3 (Fig. 7B, panel g, lanes 3 and 4) but also exhibits a marked inhibition in HA-mediated recruitment of Gab-1-linked PI 3-kinases (Fig. 7B, panels a–d, lanes 3 and 4) to CD44v3 (Fig. 7B, panel g, lanes 3 and 4). These findings suggest that the Gab-1 fragment

or

immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel b, characterization of various recombinant proteins (FLAG-CD44cyst and Gab-1-PH-V5/His-bound beads) used in the in vitro binding assay. Lane 1, anti-FLAG-mediated immunoblot of FLAG-CD44cyst associated with V5/His-bound beads. Lane 2, anti-FLAG-mediated immunoblot of FLAG-CD44cyst associated with Gab-1-PH-V5/His-bound beads. In B: Analyses of CD44v3-Gab-1-PH 3-kinase complex formation in MDA-MB-231 transfectants. MDA-MB-231 cells (transfected with Gab-1-PH cDNA or vector alone) treated with HA or no HA were solubilized by 1% Nonidet P-40 buffer. Cell lysates were then used for anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with anti-CD44v3 antibody or anti-Gab-1 or anti-PI 3-kinase antibody, respectively, as described under “Materials and Methods.” Panel a, anti-p110α-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel b, anti-p110β-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel c, anti-p110γ-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel d, anti-p110δ-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel e, anti-Gab-1-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel f, anti-His-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with Gab-1-PH cDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3).
containing the PH domain acts as a potent competitive inhibitor for endogenous intact Gab-1 binding to CD44v3 in vivo; it also functions as a strong dominant-negative mutant for blocking CD44-Gab-1-mediated PI 3-kinase membrane localization.

In addition, we have investigated the effects of ROK-RB overexpression (by transfecting cells with the RBcDNA of ROK) on CD44v3 interaction with Gab-1 and PI 3-kinase. Using anti-CD44v3 immunoprecipitation of MDA-MB-231 cells followed by immunoblotting with anti-Gab-1, we have confirmed that both CD44v3 and Gab-1 are physically linked as a complex in vector-transfected cells without HA treatment (Fig. 8A, panels e and f, lane 1). However, HA treatment of vector-transfected cells appears to promote a significant recruitment of Gab-1 into CD44v3 (Fig. 8A, panels e and f, lane 2). Moreover, we have used anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with various anti-PI 3-kinase antibodies (e.g. anti-α, β, γ, and δ forms of p110 catalytic subunit of PI 3-kinase). Our results show that a relatively low level of some PI 3-kinase (e.g. p110α and p110β) is co-precipitated with CD44v3 in vector-transfected cells without HA treatment (Fig. 8A, panels a–f, lane 1). However, HA treatment of vector-transfected cells appears to enhance the recruitment of PI 3-kinase (including p110α, p110β, p110γ, and p110δ) into CD44v3 (Fig. 8A, panels a–f, lane 2). We have also determined that the basal level of Gab-1-PI 3-kinase (p110γ and p110δ) association with CD44v3 is greatly reduced in the RBcDNA of ROK-transfected cells (without HA treatment) (Fig. 8A, panels a–f, lane 3). Most noticeably, overexpression of ROK-RB in MDA-MB-231 transfectants abolishes the ability of HA to recruit Gab-1-linked PI 3-kinase (Fig. 8A, panels a–f, lane 4). It is therefore possible that HA/CD44-activated ROK plays an important role in the recruitment of Gab-1-PI 3-kinase complexes to certain plasma membrane proteins such as CD44v3 in breast tumor cells.

In addition, we have examined Gab-1-linked PI 3-kinase activity in anti-p110α, p110β, p110γ, and p110δ form-specific immunoprecipitates (obtained from membrane fraction of vector-transfected or the RBcDNA of ROK-transfected cells treated with HA or without any HA) (Fig. 8B). Our results indicate that

FIG. 8. Detection of CD44v3-Gab-1-PI 3-kinase complex in ROK-RB-transfected/vector-transfected cells (A) and measurement of PI 3-kinase activity (B). In A: analyses of CD44v3-Gab-1-PI 3-kinase complex formation in MDA-MB-231 transfectants: MDA-MB-231 cells (transfected with ROK-RBcDNA or vector alone) treated with HA or no HA were solubilized by 1% Nonidet P-40 buffer. Cell lysates were then used for anti-CD44v3-mediated immunoprecipitation followed by immunoblotting with anti-CD44v3 antibody or anti-Gab-1 or anti-PI 3-kinase antibody, respectively, as described under "Materials and Methods." Panel a, anti-p110α-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel b, anti-p110β-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel c, anti-p110γ-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel d, anti-p110δ-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel e, anti-Gab-1-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel f, anti-CD44v3-mediated immunoblot of anti-CD44v3-mediated immunoprecipitated materials isolated from MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). In B: measurement of PI 3-kinase activity. MDA-MB-231 cells (transfected with ROK-RBcDNA or vector alone) treated with HA or no HA. Membranes of these transfectants were used for anti-PI 3-kinase-mediated immunoprecipitation. Precipitated proteins were incubated with [γ-32P]ATP, followed by extraction of lipids and separation by TLC. The TLC plate was then exposed on an x-ray film. PIP3, phosphatidylinositol (3,4,5)P3. Panel a, PI 3-kinase activity detected by anti-p110α-mediated immunoprecipitated materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel b, PI 3-kinase activity detected by anti-p110β-mediated immunoprecipitated materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel c, PI 3-kinase activity detected by anti-p110γ-mediated immunoprecipitated materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3). Panel d, PI 3-kinase activity detected by anti-p110δ-mediated immunoprecipitated materials isolated from membrane fraction of MDA-MB-231 cells transfected with ROK-RBcDNA (lanes 3 or 4) or vector alone (lanes 1 and 2) treated with HA (lanes 2 and 4) or no HA (lanes 1 and 3).
activation of PI 3-kinase (in particular, p110α (Fig. 8B, panel a, lanes 1 and 2), p110β (Fig. 8B, panel b, lane 1 and 2), and p110γ (Fig. 8B, panel c, lanes 1 and 2)) but not p110δ (Fig. 8B, panel d, lanes 1 and 2)) occurs as early as 5 min after HA addition to vector-transfected cells, resulting in an increase in the level of PtdIns(3,4,5)P3 formation. However, HA-induced increase in PtdIns(3,4,5)P3 by Gab-1-associated PI 3-kinases (in particular, p110α (Fig. 8B, panel a, lanes 3 and 4) and p110γ (Fig. 8B, panel b, lanes 3 and 4)) appears to be significantly inhibited in the RBCDNA of ROK-transfected cells. Thus, it is likely that activation of both p110α and p110β are HA/CD44- and ROK-dependent. Although HA is capable of up-regulating p110γ activity (Fig. 8B, panel c, lanes 1 and 2), the failure of blocking HA-activated p110γ activity in the RBCDNA of ROK-transfected cells (Fig. 8B, panel c, lanes 3 and 4) supports the possibility that p110γ stimulated by HA is ROK-independent. Finally, we have noted that HA fails to activate p110δ activity in both vector-transfected (Fig. 8B, panel d, lanes 1 and 2) and the RBCDNA of ROK-transfected cells (Fig. 8B, panel d, lanes 3 and 4), suggesting that p110δ activity is not regulated by HA/CD44-mediated ROK signaling pathway in breast tumor cells.

Effects of RhoA-activated ROK and Gab-1-linked PI3 Kinase on AKT Activation, Cytokine (M-CSF) Production, and Breast Tumor Cell Growth and Invasion—Activation of AKT by growth factors is known to be mediated by PI3-kinase (60–62). Most importantly, AKT acts as an important mediator of many cell survival-signaling pathways and is often overexpressed/amplified in tumor progression (60–62). In this study we have investigated the possible role of ROK and PI 3-kinase in regulating AKT-1 activation in breast tumor cells during HA-CD44 signaling. Specifically, we have determined that AKT-1 kinase is significantly stimulated by HA treatment in vector-transfected cells (detected by anti-phospho-AKT-1 (Fig. 9, panel a, lane 1) followed by reblotting with anti-AKT-1 antibody) (Fig. 9, panel b, lane 2) as compared with the level of AKT-1 phosphorylation in vector-transfected cells without any HA treatment (Fig. 9, panels a and b, lane 1). Moreover, treatment of vector-transfected cells with a PI 3-kinase inhibitor (LY294002) results in down-regulation of AKT-1 activation during the response to HA treatment (Fig. 9, panels a and b, lanes 3 and 4). Furthermore, HA-activated AKT-1 phosphorylation is also greatly reduced in the RBCDNA of ROK-transfected cells (Fig. 9, panels a and b, lanes 5 and 6) comparing with AKT-1 phosphorylation in vector-transfected cells treated with HA treatment (Fig. 9, panels a and b, lanes 1 and 2). These observations strongly support the conclusion that both PI 3-kinase signaling and ROK activation are important upstream activators for AKT signaling required for HA/CD44-mediated tumor cell survival.

As part of our effort to identify new effector functions influenced by HA/CD44-mediated ROK and PI 3-kinase signaling in breast tumor cells, we decided to examine the production of M-CSF, which is a homodimeric cytokine in the colony-stimulating factor family (63). In breast cancers, M-CSF serum levels correlate with prognosis, activity, and invasiveness of the disease (64). Here, we have found that HA promotes M-CSF production in untransfected MDA-MB-231 cells (Table I). It has also noted that the amount of M-CSF production detected in HA/CD44- and ROK-dependent. Treatment of MDA-MB-231 cells transfected with vector alone (not anti-CD44 (but not normal rat IgG) followed by HA treatment was significantly reduced (Table I). These results support the notion that the stimulation of M-CSF production by HA is CD44-dependent. Furthermore, treatment of untransfected cells with a PI 3-kinase inhibitor, LY294002, blocked HA-mediated M-CSF production (Table II). Moreover, our data show that HA/CD44- and PI 3-kinase-regulated M-CSF production are significantly inhibited in MDA-MB-231 cells transfected with the RBCDNA of ROK as compared with that detected in vector-transfected cells (Table I). Together, these findings suggest that both RhoA-ROK and PI 3-kinase activation are closely involved in HA/CD44-mediated M-CSF production in breast tumor cells.

Furthermore, using in vitro growth and invasion assays, we have found that CD44v3-containing MDA-MB-231 cells undergo active cell growth (Table IIA) and invasion (Table IIB). HA activates both breast tumor cell growth (Table IIA) and invasion (Table IIB). In contrast, MDA-MB-231 cells pretreated with rat anti-CD44 (but not normal rat IgG) followed by HA addition greatly reduced their ability to undergo growth and invasion (data not shown). These observations suggest that HA-mediated tumor cell growth and invasion is also CD44-dependent. Treatment of MDA-MB-231 cells with various agents such as the PI3 inhibitor (LY294002) causes a significant inhibition of HA-mediated tumor cell growth (Table IIA) and invasion (Table IIB). Importantly, transfection of MDA-MB-231 cells with the dominant-negative form of ROK (ROK-RB) cDNA (but not vector alone) also effectively blocked HA/CD44-mediated breast tumor cell growth (Table IIA) and invasion (Table IIB). Together, these findings support the notion that HA-mediated CD44 signaling is functionally linked to RhoA-ROK and Gab-1-linked PI 3-kinase activation required for metastatic breast tumor progression.

**DISCUSSION**

CD44 belongs to a family of transmembrane glycoproteins (e.g. CD44s (standard form), CD44E (epithelial form), and CD44v (variant isoforms)), which are often overexpressed in a variety of human solid neoplasms, including breast cancers (1–6). In particular, the CD44v3-containing isoforms are expressed preferentially in highly malignant breast carcinoma tissue samples (1–6). In breast tumor cells, CD44v3 has a heparin sulfate addition site in the membrane-proximal extracellular domain of the molecule that confers the ability to bind vascular epithelial growth factor, but not fibroblast growth factor-2 (13). The attachment of vascular epithelial growth factor to the heparin sulfate sites on CD44v3 may be respon-
MDA-MB-231 cells (untransfected or treated with or without LY294002) or transfected with ROK-RBcDNA or vector alone) were washed three times with serum free (SF)-DMEM and incubated in 3 ml of serum free-DMEM containing various reagents (e.g. HA (50 μg/ml) or anti-CD44 antibody plus HA (50 μg/ml) or without HA treatment) for 24 h at 37 °C in a 5% CO2 humidified chamber. Subsequently, M-CSF concentrations in the conditioned medium and cells were determined using the Quantikine M-CSF immunoassay (R&D Systems). Statistical analysis was done using the Student t test. All data are expressed as the mean ± S.D.

Table I

| Treatments                                                                 | Amount of M-CSF production (pg/ml) |
|---------------------------------------------------------------------------|-----------------------------------|
| A) Measurement of M-CSF production in untransfected cells                 |                                    |
| No IgG treatment (control)                                               | 75 ± 1.0 (100%)                   |
| Normal Rat IgG                                                            | 77 ± 2.3 (102%)                   |
| Rat anti-CD44 IgG                                                        | 74 ± 2.2 (99%)                    |
| B) Measurement of M-CSF production in untransfected cells treated with a PI 3-kinase inhibitor (LY294002) |                                    |
| No drug treatment (control)                                              | 74 ± 2.2 (100%)                   |
| LY294002 treatment                                                       | 55 ± 1.0 (74%)                    |
| C) Measurement of M-CSF production in vector and ROK's RB-transfected cells |                                    |
| Vector-transfected (control)                                             | 75 ± 1.4 (100%)                   |
| ROK-RBcDNA-transfected                                                   | 61 ± 0.6 (81%)                    |

Table II

| Cells                                                                 | Tumor cell growth % of control |
|---------------------------------------------------------------------|--------------------------------|
| A) Analysis of tumor cell growth                                     |                                |
| Untransfected cells (control)                                        | 100                            |
| LY294002-treated untransfected cells                                 | 79                             |
| ROK-RBcDNA-transfected cells                                         | 62                             |
| B) Analysis of tumor cells invasion                                  |                                |
| Untransfected cells (control)                                        | 100                            |
| LY294002-treated untransfected cells                                 | 80                             |
| ROK-RBcDNA-transfected cells                                         | 54                             |

The invasive phenotype of breast tumor cells, characterized by HA-CD44-mediated "invadopodia" formation (57), matrix metalloproteinase-9 activation (57), and tumor cell migration and invasion (21–24, 56, 57), has been linked to cytoskeletal function, a process in which the small GTP-binding proteins such as RhoA are known to play important roles (22). The activities of members of the RhoGTPases (including RhoA) are often regulated by guanine nucleotide exchange factors (GEFs) that contain a dbl homology (DH) domain (33, 34). In recent years, a RhoA-specific GEF (p115RhoGEF) molecule containing several functional domains (an N-terminal RGS domain, a dbl homology (DH) domain, and a PH domain) have been identified and characterized (33, 34). In particular, the DH domain of p115RhoGEF by itself exhibits GDP/GTP exchange activity for RhoA and plays an important role in RhoGTPase signaling (33, 34). Unique to p115RhoGEF, compared with other GEFs for small GTPases, is the presence of an RGS domain. A number of laboratories have reported that the N-terminal RGS domain interacts with the a subunit (α12/13) of the G-protein (33, 34). In fact, the RGS sequence has been shown to play an important role in regulating p115RhoGEF-Gα12/13 interactions during G-protein-coupled receptor signaling (33, 34). Our laboratory has demonstrated that p115RhoGEF is expressed in MDA-MB-231 cells (Fig. 1). In addition, we have presented new evidence for a close, physical interaction between p115RhoGEF and the transmembrane glycoproteins, CD44v3 isoform (Fig. 1). Most importantly, our results show that the binding of HA to CD44v3 promotes p115RhoGEF-catalyzed RhoA activation (Fig. 2). Therefore, it is likely that CD44v3 serves as an activator of p115RhoGEF function. Clearly, these two proteins, CD44v3 and p115RhoGEF, are not only structurally linked, but also functionally coupled. It is also noted that two other RGS-containing RhoGEFs (e.g. PDZ-RhoGEF and/or leukemia-associated RhoGEF) have been shown to activate RhoA signaling (34, 58). The relationship between PDZ-RhoGEF and/or leukemia-associated RhoGEF in HA/CD44-mediated cellular events remains to be elucidated.

Rho kinase (ROK) stimulated by activated RhoA (GTP-bound form of RhoA) appears to play a pivotal role in promoting cytoskeletal function and a variety of other cellular activities (22, 40–45). In a previous study (22) we demonstrated that ROK phosphorylates the cytoplasmic domain of CD44v3, 8,10 isoform and up-regulates the interaction between the CD44v3, 8,10 isoform and the cytoskeletal protein, ankyrin, during HA/CD44-regulated tumor cell migration. More recently, we have shown that the PH domain of ROK is the primary binding site for CD44 (45). Most importantly, our results indicate that CD44 interaction with ROK plays a piv-
otal role in the phosphorylation of inositol trisphosphate receptor (IP$_3$ receptor) leading to an increase of IP$_3$ binding followed by a rapid stimulation of Ca$^{2+}$ mobilization during HA-mediated cell migration (45). Therefore, we believe that ROK plays an important role in HA/CD44-mediated functions (45). Structurally, ROK is composed of catalytic, coiled-coil, RB, and PH domains (37–40). A number of substrates, including myosin-binding subunit of myosin phosphatase (41), calponin (42), adducin (43), Lin 11/Isl-1/Mec-3 (LIM) kinase (44), IP$_3$ receptors (45), and CD44 (22), have been shown to be phosphorylated by ROK. In MDA-MB-231 cells, we have identified a 160-kDa protein as a ROK molecule (Fig. 3). In an effort to identify possible new cellular substrates for ROK, we have focused on the multiple docking protein, Gab-1, that is often tyrosine-phosphorylated by receptor tyrosine kinases such as hepatocyte growth factor receptor, c-Met (65), and epidermal growth factor receptors (66). In this study we have demonstrated that ROK (by binding to activated RhoA) is capable of inducing marked serine and threonine phosphorylation of Gab-1 (isolated from MDA-MB-231 cells) in vitro (Fig. 4). The ability of ROK to phosphorylate Gab-1 in the presence of unactivated RhoA appears to be greatly reduced (Fig. 4). These results clearly indicate that ROK acts as one of the downstream effectors of RhoA signaling and utilizes Gab-1 as one of its cellular targets in vitro. Overexpression of the RB domain of ROK has been found to function as a dominant-negative form of ROK, which inhibits ROK activity, ROK-mediated protein phosphorylation, and HA/CD44-mediated breast tumor progression (22). This information has prompted us to examine whether ROK plays a role in regulating Gab-1 phosphorylation and Gab-1-mediated cell signaling in HA/CD44-mediated breast tumor cell activation in vivo. Transfection of MDA-MB-231 cells with the RB domain cDNA of ROK (Fig. 5), which effectively competes for endogenous activated RhoA binding to ROK and inhibits the ability of ROK to phosphorylate Gab-1 during HA-CD44 signaling (Fig. 5), strongly suggests that RB of ROK is a potent inhibitor of ROK-mediated Gab-1 phosphorylation in vivo.

A number of studies have shown that tyrosine-phosphorylated Gab-1 is involved in PI 3-kinase binding, membrane translocation, and activation (47, 48, 67, 68). The question of whether HA/CD44-activated RhoA-ROK serine/threonine phosphorylation of Gab-1 plays a role in regulating PI 3-kinase signaling is addressed in this study. Previous reports have indicated that co-expression of multiple PI 3-kinase isoforms (e.g., α, β, γ, and δ forms of p110) occurs in many different cells (46, 49–51). The p110α, p110β, and p110γ forms are expressed in all tissue, whereas p110δ is preferentially expressed in leukocytes (46, 49–51). The α, β, and δ forms of the p110 catalytic subunit belong to the class IA PI 3-kinase known to transmit signals from tyrosine kinase-coupled receptors (66, 67). These class IA PI 3-kinases (e.g. p110α, p110β, and p110δ) are often associated with certain regulatory units (e.g. p85, p55, or p50) that bind to specific phosphorylated tyrosine residues in receptor proteins or other adaptor molecules such as Gab-1 (66, 67). The class IB PI 3-kinase (the γ form of p110 catalytic subunit) can be activated by G-protein-coupled receptors (46, 49–51, 67). This p110γ form interacts with a unique regulatory protein, p101 (but not p85, p55, or p50) (69). Here, we have found that multiple isoforms of PI 3-kinase (as detected by immunoblot with specific anti-α, β, γ, and δ forms of the p110 catalytic subunit) are also co-expressed in MDA-MB-231 cells (Fig. 6). Apparently, the status of Gab-1 serine/threonine phosphorylation (either highly phosphorylated or unphosphorylated form) does not affect Gab-1 association with any particular isoform of PI 3-kinase (e.g. p110α, p110β, p110γ, and p110δ). These results suggest that the physical association between Gab-1 and PI 3-kinase is always present and serine/threonine phosphorylation of Gab-1 plays a small role (if any) in influencing Gab-1-PI 3-kinase complex formation (Fig. 6). The question of whether Gab-1 (phosphorylated or unphosphorylated form) directly binds to p110 subunits or selectively interacts with various regulatory domains (e.g. p85, p55, p50, or p101) in HA/CD44-mediated RhoA-ROK signaling awaits further analyses.

In addition, using two recombinant proteins (the PH domain of Gab-1 and the cytoplasmic domain of CD44), we have demonstrated that the PH of Gab-1 is responsible for the recognition of CD44 in vitro (Fig. 7A). We have also provided new evidence that phosphorylation of intact Gab-1 by RhoA-activated ROK enhances its binding to CD44 (Fig. 4B). Furthermore, we have found that transfection of MDA-MB-231 cells with Gab-1-PH cDNA (Fig. 7B) effectively blocks endogenous Gab-1 association with CD44v3 and the subsequent recruitment of signaling molecules (e.g. PI 3-kinase) to CD44v3-containing membrane (Fig. 7B). These findings further support our conclusion that Gab-1-PH acts as a potent competitive inhibitor, which is capable of interfering with endogenous Gab-1-CD44v3 interaction in vivo. These results are consistent with our previous study showing a sequence adjacent to the N-terminal region of PH domain (the PHn-CC-EX domain) of Tiam1 (an Rac1-specific guanine nucleotide exchange factor [GEF]), which is involved in the direct binding to CD44v3 isoform during HA-stimulated Rac1 signaling and cytoskeleton-mediated tumor cell migration (24). Our recent study also shows that the PH domain of ROK binds to CD44v10 in endothelial cells and this ROK-PH interaction with CD44v10 regulates HA-mediated Ca$^{2+}$ signaling and endothelial cell migration (45). It is therefore, apparent that the close interaction between CD44 isoforms and certain PH domain-containing molecules (e.g. Gab-1, Tiam1, and ROK) plays an important role in HA signaling. Moreover, we have observed that the Gab-1 (highly serine/threonine-phosphorylated form of Gab-1) PI 3-kinase complex becomes preferentially recruited into CD44v3-containing membranes in vector-transfected cells treated with HA (Fig. 8A). In contrast, in other situations, the Gab-1 (with a minimal level of serine/threonine phosphorylation) PI 3-kinase complex fails to become membrane-localized with CD44v3 in vector-transfected cells with no HA treatment (Fig. 8A) or in the RbDNA of ROK-transfected cells with or without HA treatment (Fig. 8A). Thus, our results provide evidence for HA/CD44-activated RhoA-ROK to play a role in the membrane translocation of phosphorylated Gab-1-PI 3-kinase complexes into CD44v3. The recruitment of phosphorylated Gab-1-PI 3-kinase into CD44v3-associated membranes may bring the p110 catalytic subunits into close proximity to the inositol lipid substrates and thereby facilitate the onset of enzymatic activity.

Previously, PI 3-kinase has been shown to be activated by guanine-nucleotide-binding proteins (70, 71). PI 3-kinase can also be activated via its binding to the active conformation of Ras during the membrane localization of p110α p85 complex (72, 73). Our analyses indicate that ROK-mediated Gab-1 phosphorylation and Gab-1-PI 3-kinase membrane localization are accompanied by a robust stimulation of PI 3-kinase activity (in particular, α, β, and γ forms, but not the δ form of p110 catalytic subunit) in vector-transfected cells treated with HA (Fig. 8B). Under physiological conditions, hyaluronan often exists as a high molecular mass polymer (>10$^6$ Da). However, generation of hyaluronan fragments may occur during tumor progression. Both HA and HA fragments of certain size (3–25 disaccharide units) have been found to either promote up-
regulation or down-regulation of CD44-specific biological events (18). Because we have used HA of large size (>10^6 Da) for all experiment described in this study, it is possible that large HA fragments preferentially activate p110α, p110β, and p110γ forms but not the p110δ form in MDA-MB-231 cells (Fig. 8B). The question of whether one or more certain small size HA fragments are capable of promoting p110δ and/or the same p110α, p110β, and p110γ forms as the native large molecular weight hyaluronan used in this study is currently undergoing investigation in our laboratory.

Recently, Sawyer et al. (79) have shown that PI 3-kinase (e.g. p110α, p110β, and p110δ) is expressed in breast tumor cells. In particular, p110δ (to a lesser extent p110β) but not p110α is involved in epidermal growth factor (EGF)-mediated motility in vitro (79). PI 3-kinase (such as p110δ) is known to be activated by tyrosine-phosphorylated regulatory subunits (e.g. p85α, p85β, and p55γ) during EGF-mediated EGF receptor tyrosine kinase signaling (46, 49–51). Thus, the migratory property associated with p110δ may be specific for EGF signaling. In this study we have shown that HA promotes recruitment of PI 3-kinase p110δ to CD44v3-containing membrane (Figs. 7 and 8) but does not activate PI 3-kinase p110δ in MDA-MB-231 cells (Fig. 8B). The cytoplasmic domain of CD44 contains no intrinsic tyrosine kinase. Consequently, tyrosine phosphorylation of regulatory subunits (e.g. p85α, p85β, and p55γ) and activation of p110δ fail to occur during HER-CD44 signaling.

Furthermore, our results indicate that the reduction of Gab-1 threonine/serine phosphorylation and the lack of Gab-1-PI 3-kinase membrane localization in the RBCdNA of ROK-transfected cells (Fig. 7B) are closely associated with an inhibition of certain PI 3-kinase activity (e.g. α and β forms but not the γ form of p110 catalytic subunit) during HA treatment (Fig. 8). Apparently, the activation of both α and β forms of p110 catalytic subunits is ROK-dependent, whereas the stimulation of the γ form of p110 catalytic subunit is ROK-independent (Fig. 8). Moreover, our observations, showing that ROK is required for the recruitment of PI 3-kinase p110γ but ROK is not required for HA-mediated activation of this form (in terms of PIP3 production), are noteworthy. Activation of the catalytic subunit p110γ is known to be mediated by the regulatory subunit p101. It is possible that membrane localization of PI 3-kinase p110γ is ROK-dependent, whereas p101-mediated activation of PI 3-kinase p110γ is ROK-independent during HER-CD44 signaling. Characterization of p101-mediated activation of p110γ (in a ROK-independent process) during HER-CD44 signaling awaits further investigation. The fact that HA-mediated PI 3-kinase p110γ recruitment and activation can be readily blocked by anti-CD44 antibody pretreatment of vector and ROK-RBCdNA-transfected cells (data not shown) suggests that CD44 is involved in this HA signaling event. Of course, we cannot preclude the possibility that other HA receptors (e.g. receptor for HA-mediated motility [RHAMM]) are also capable of generating PI 3-kinase signaling in either a ROK-dependent or independent manner. The selective activation of α, β, and γ forms (but not the δ form of p110 catalytic subunit) may significantly influence the biological outcomes of HA-CD44-mediated oncogenic events in breast tumor progression.

One of the well established effector molecules for PI 3-kinase is AKT, which is known as an important mediator of many cell survival-signaling pathways (60–62). Recent evidence has suggested that AKT signaling plays a central role in human malignancy. AKT is a putative oncogene encoding a member of a subfamily of protein-serine/threonine that includes the protein kinase B family (60–62). In humans, at least three AKT genes (designated AKT-1, AKT-2, and AKT-3) have been identified (62). It is still unclear how much functional redundancy exists among these three AKTs. In this study we have found that AKT-1 is expressed in MDA-MB-231 cells (Fig. 9), and HA-CD44 interaction promotes AKT-1 activation (Fig. 9). The facts that overexpression of ROK-RB (by transfecting MDA-MB-231 cells with ROK-RBCdNA) impairs HA-mediated AKT-1 activation and that a PI 3-kinase inhibitor (LY294002) also blocks AKT-1 activation (Fig. 9) suggest that both ROK and PI 3-kinase are located upstream of AKT-1 and are involved in regulating HA/CD44-mediated AKT signaling required for anti-apoptotic processes and tumor cell survival. Our results are consistent with a previous report showing that HA/CD44 interaction is involved in PI 3-kinase/AKT cell survival signaling in human lung carcinoma cells (27). Perturbation of HA/CD44 binding effectively down-regulates PI 3-kinase/AKT activities and inhibits tumor cell growth (27).

A recent study indicates that breast tumor cells (e.g. MDA-MB-231 cells) are capable of inducing osteoclastogenesis by secreting M-CSF, which is a homodimeric cytokine of the colony-stimulating factor family (63). The addition of M-CSF to osteoclast precursors induces the expression of RANK (the receptor for RANKL, the receptor activator of nuclear factor κB ligand), which in turn interacts with RANKL and induces differentiation of cells in the macrophage/osteoclast lineage (74–78). In the presence of M-CSF, the RANK/RANKL/osteoprotegerin axis mediates osteoclast formation and activity and thereby bone resorption (63, 74–78). These findings suggest that the stimulation of osteoclastogenesis by M-CSF-producing breast tumor cells is closely involved in osteolytic metastases. In MDA-MB-231 cells we have found that HA promotes M-CSF production in a CD44-dependent manner (Table I). Inhibition of both ROK and PI 3-kinase results in significant reduction in M-CSF production (Table I). These findings suggest that these two signaling molecules (e.g. ROK and PI 3-kinase) are functionally linked to HA/CD44-mediated M-CSF production during breast tumor progression. We have also demonstrated that overexpression of ROK-RB (by transfecting MDA-MB-231 cells

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FIG. 10. A proposed model for the interaction between CD44v3-mediated Rhoa-ROK activation and Gab-1-linked PI 3-kinase signaling during breast tumor progression. HA-CD44 interaction is tightly coupled with p115RhoGEF in a complex that can up-regulate RhoA signaling and Rho kinase (ROK) activity (Step 1). Activated ROK then phosphorylates certain cellular proteins, including the linker molecule, Gab-1 (Step 2). Most importantly, phosphorylation of Gab-1 by ROK promotes the membrane localization of Gab-1 and PI 3-kinase to CD44 and activates certain isoforms of PI 3-kinase to convert PtdIns(4,5)P2 to PtdIns(3,4,5)P3 (Step 3) leading to AKT activation, cytokine (M-CSF) production, and tumor cell behaviors (e.g. tumor cell growth, survival, and invasion) required for breast tumor progression.
with ROK-RBDcDNA) exhibits a dominant-negative effect on PI 3-kinase (and to a lesser extent the p110 isoforms of PI 3-kinase) leading to an inhibition of HA-mediated M-CSF production in MDA-MB-231 cells (Table I). These findings suggest that both p110α and p110β forms of PI 3-kinase (and to a lesser extent the p110γ or p110δ isoforms of PI 3-kinase) are most likely involved in the regulation of HA-mediated M-CSF production. Of course, we cannot rule out the possibility that other PI 3-kinases (e.g. p110y or p110δ) may also be involved in M-CSF production in an HA-independent manner. The use of small interference RNA to selectively knock down PI 3-kinase (e.g. p110α, p110δ, p110γ, or p110δ) forms and thereby interfere with PI 3-kinase isoform-mediated functions and breast tumor progression is currently being investigated in our laboratory.

Finally, we have found that treatment of MDA-MB-231 cells with the PI 3-kinase inhibitor LY294002 (Table II) or overexpression of the Rho-binding (RB) domain of ROK (by transfecting MDA-MB-231 cells with RBcDNA) (Table II) induces reversion of the Rho-binding (RB) domain of ROK (by transfecting MDA-MB-231 cells with ROK-RBcDNA) exhibits a dominant-negative effect on PI 3-kinase (and to a lesser extent the p110 isoforms of PI 3-kinase) leading to an inhibition of HA-mediated M-CSF production. Of course, we cannot rule out the possibility that other PI 3-kinases (e.g. p110y or p110δ) may also be involved in M-CSF production in an HA-independent manner. The use of small interference RNA to selectively knock down PI 3-kinase (e.g. p110α, p110δ, p110γ, or p110δ) forms and thereby interfere with PI 3-kinase isoform-mediated functions and breast tumor progression is currently being investigated in our laboratory.

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Hyaluronan-mediated CD44 Interaction with RhoGEF and Rho Kinase Promotes Grb2-associated Binder-1 Phosphorylation and Phosphatidylinositol 3-Kinase Signaling Leading to Cytokine (Macrophage-Colony Stimulating Factor) Production and Breast Tumor Progression

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