Metastasis Suppression by Breast Cancer Metastasis Suppressor 1 Involves Reduction of Phosphoinositide Signaling in MDA-MB-435 Breast Carcinoma Cells

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Abstract

Several molecules that suppress metastasis without suppressing tumorigenicity have been identified, but their mechanisms of action have not yet been determined. Many block growth at the secondary site, suggesting involvement in how cells respond to signals from the extracellular milieu. Breast cancer metastasis suppressor 1 (BRMS1)–transfected MDA-MB-435 cells were examined for modifications of phosphoinositide signaling as a potential mechanism for metastasis suppression. 435/BRMS1 cells expressed <10% of phosphatidylinositol-4,5-bisphosphate compared with parental cells, whereas levels of the PtdIns(4)P and phosphatidylinositol-3-phosphate were unchanged. Inositol (1,4,5)-trisphosphate [Ins(1,4,5)P3] were decreased in 435/BRMS1 cells by ~50%. Phosphatidylinositol-3,4,5-trisphosphate levels were undetectable in 435/BRMS1 cells, even when stimulated by exogenous insulin or platelet-derived growth factor. Immunofluorescence microscopy to examine cellular distribution confirmed that phosphatidylinositol-4,5-bisphosphate distribution with cells was unchanged but was uniformly decreased throughout the cell. Although the gross morphology of 435/BRMS1 cells is similar to the parent, filamentous actin was more readily apparent in 435/BRMS1. Intracellular calcium, measured using Fluo-3 and Fura-2 fluorescent calcium indicator dyes, was somewhat lower, but not statistically different in 435/BRMS1 compared with parental cell. However, when stimulated with platelet-derived growth factor, MDA-MB-435 cells, but not 435/BRMS1 cells mobilized intracellular calcium. Taken together, these results implicate signaling through phosphoinositides in the regulation of breast cancer metastasis, specifically metastasis that can be suppressed by BRMS1.

Introduction

Metastasis suppressors are a relatively new class of proteins defined by their ability to suppress formation of secondary tumor masses without blocking growth of neoplastic cells at orthotopic or subcutaneous sites. To date, 14 metastasis suppressors have been discovered (1), but their mechanisms of action remain largely unknown. Breast cancer metastasis suppressor 1 (BRMS1) was discovered using differential display comparing mRNA expression levels between metastasis-competent MDA-MB-435 and metastasis-suppressed, human breast carcinoma cell lines that had received an intact copy of human chromosome 11 by microcell-mediated chromosome transfer. Constitutive expression of BRMS1 in human breast (MDA-MB-435 and MDA-MB-231), murine mammary (4T1 and 66cl4), and human melanoma (C8161 and MelJuSo) cells results in significant suppression of metastasis without blocking tumor formation. Rare metastases that developed had lost expression of BRMS1 (2).

BRMS1-transfected cells are equally invasive to their metastatic parents and have been found in the vasculature of mice bearing orthotopic tumors, suggesting that BRMS1, like many metastasis suppressors, affects late steps in the metastatic cascade, particularly a cells ability to proliferate at secondary sites. BRMS1 protein localizes predominantly (>90%) to the nucleus, restores gap junctional intercellular communication and is a component of the mSin3a family of histone deacetylase complexes (3). This study was initiated in order to test the hypothesis that BRMS1 expression could regulate second messengers that, in turn, could affect growth and other cellular processes.

Because metastasis involves multiple cell-cell and cell-matrix interactions, a multitude of signaling changes have been implicated in various steps of the metastatic cascade. For the current study, the focus was on phosphoinositides because modulation of intracellular calcium, a downstream effector of phosphoinositide signaling, can affect invasion through Matrigel-coated membranes and metastasis in vivo (4). Because phosphoinositides and inositol phosphates are key regulators of cellular calcium signaling, we tested whether changes in BRMS1 expression alter phosphoinositide levels and/or localization. We found that BRMS1 expression led to a dramatic, and relatively specific, reduction of phosphatidylinositol-4,5-bisphosphate [PtdIns(4,5)P2] levels, suggesting that metastasis suppression by BRMS1 may be due to regulation of phosphoinositide signaling.

Materials and Methods

Cell Lines and Cell Culture. MDA-MB-435 and MDA-MB-231 human breast carcinoma cells were cultured in a 1:1 mixture of DMEM and Ham's F12 medium supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 1% nonessential amino acids, and 1 mmol sodium pyruvate. BRMS1-transfected cells also received 500 μg/mL G-418 (Geneticin; Invitrogen, San Diego, CA). Cells were cultured on 100-mm Corning tissue culture dishes at 37°C with 5% CO2 in a humidified atmosphere.

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Requests for reprints: Danny R. Welch, Department of Pathology, University of Alabama at Birmingham, 1670 University Drive, Volkler Hall, Room G019A, Birmingham, AL 35294-0019. Phone: 205-934-2956; Fax: 205-934-2957; E-mail: dwelch@b Family of Histone Deacetylase Complexes (3). This study was initiated in order to test the hypothesis that BRMS1 expression could regulate second messengers that, in turn, could affect growth and other cellular processes.

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Cells were passaged at 80% to 90% confluence using a 2 mmol EDTA solution in Ca2+/Mg2+-free Dulbecco's PBS. BRMS1 was cloned into the
constitutive mammalian expression vector pcDNA3 (Invitrogen) under control of the cytomegalovirus promoter. No antibiotics or antimitotics were used. All cell lines were negative for Mycoplasma sp. contamination using a PCR-based assay (TaKaRa, Madison, WI).

**Radiochemicals.** myo-[2-3H]inositol (10-25 Ci/mmol) was purchased from Perkin-Elmer Life Sciences (Boston, MA). For high-performance liquid chromatography standards, [32P]PtdIns(3)P and PtdIns(4)P were from in vitro phosphorylation reactions with yeast phosphatidylinositol 3-kinase or phosphotidylinositol 4-kinase and [32P]ATP, followed by separation and extraction by thin-layer chromatography as previously described (5). PtdIns(4,5)P_2 (inositol-2,4,5-tri-1H) and Ins(1,4,5)P_3 (inositol-1,3,4-1H) were prepared from myo-inositol-3-phosphate [PtdIns(3,4,5)P_3] by in vitro phosphorylation of PtdIns(4)P and PtdIns(4,5)P_2, respectively, by mammalian phosphatidylinositol 3-kinase with [γ-32P]ATP, followed by thin-layer chromatography purification (6).

**Radioabeling Cells and Lipid Analysis.** MDA-MB-435, MDA-MB-231, 231/BRMS1 and 435/BRMS1 cells were grown to 60% to 80% confluence in 75 cm² tissue culture flasks, washed with Ca²⁺/Mg²⁺-free Dulbecco’s PBS and labeled with myo-[2-3H]inositol (20 μCi/mL; 24 hours) in inositol-free DMEM containing 10% calf serum. After 24 hours, the medium was replaced. For experiments where growth factor stimulation [platelet-derived growth factor (PDGF)] was included, cells were serum-deprived for 2 hours in inositol-free and serum-free DMEM containing 0.2% bovine serum albumin and 10 μCi/mL of myo-[2-3H]inositol, followed by PDGF (50 ng/mL) stimulation and harvest. Ice-cold trichloroacetate was added to the flasks to a final concentration of 10% and incubated on ice for 1 hour before scraping and placing the liquid in 15 mL conical screw-cap centrifuge tubes. Cells were collected by centrifugation (5 minutes, 20,000 × g) before resuspension in 5 mL 5% trichloroacetic acid to remove contaminating carrier inositol. Cells were deacetylated as described (6) with minor modifications. Deacetylated lipids were suspended in 0.5 mL n-butanol/petroleum ether/ethanol formate (20:1 v/v/v) for separation by reversed-phase HPLC. After determination of the radioactivity by liquid scintillation counting. For preparation of loading samples for HPLC standardization was done using the ³H counts, which approximates phosphatidylinositol content.

**Phosphoinositides.** Lipids were extracted from the cell pellet by resuspending cells in 0.75 mL chloroform/methanol/HCl (40:30:1 v/v/v) and vortexing vigorously every 15 seconds for 15 minutes. Then, 0.25 mL of chloroform and 0.45 mL of 0.1 mol/L HCl were added to the cells and they were vortexed for 2 minutes, centrifuged (17,500 × g for 2 minutes), and the bottom, organic layer was transferred to another tube for continued processing. Ammonia (50 μL of a 1 mol/L solution) was added and the solutions in the tubes were dried. Lipids were decylated as described (6) with minor modifications. Dried lipids were resuspended in 0.5 mL of methylamine (42.8% of 25% methylamine, 45.7% of methanol, 11.4% of n-butanol) before resination, incubated at 53°C for 50 minutes, and dried under reduced pressure. Deacylated lipids were suspended in 0.75 mL H₂O by sonication and extracted thrice with 0.5 mL n-butanol/petroleum ether/ethanol formate (20:1 v/v/v). The aqueous phase was dried under reduced pressure and extracted thrice with 0.5 mL of 10 mol/L, 60 mL of a linear gradient, 10 mmol to 0.8 mol/L, 2 mL of a linear gradient, 0.8 to 1 mol/L, 3 mL of 1 mol/L, respectively. Fractions (0.5 mL) were collected every 20 seconds, mixed with 5 mL of water/miscible scintillation cocktail, and counted in a liquid scintillation counter.

**Immunolocalization of Phosphoinositides.** Cover slips and slides were purchased from Fisher Scientific (Pittsburgh, PA). Formaldehyde and slides were obtained from Ted Pella, Inc. (Redding, CA). Purified RC6F8 anti-PtdIns(3,4,5)P_3 IgM and 2C11 anti-PtdIns(3,4,5)P_3 IgM were purchased from Echelon Biosciences, Inc. (Salt Lake City, UT). Fluorophore-tagged (Texas red) anti-mouse IgM secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cells analyzed during the logarithmic stage on cover slips were washed with cold TBS and fixed with 2% formaldehyde, then permeabilized with 0.5% Triton X-100 in TBS. After blocking with 10% goat serum in TBS, either R6G8S monoclonal antibody ascites (1:50 dilution) or 10F8 (1:5,000 dilution) was added and incubated for 1 hour at room temperature. After washing thrice with blocking solution, fluorophore-labeled anti-mouse IgM (1:2,000 dilution) was added and incubated at room temperature for 1 hour. After washing the cells thrice with deionized water, cells were observed using a confocal microscope (model FBFCC1024; Bio-Rad, Hercules, CA).

**Calcium Imaging.** MDA-MB-435 or MDA-MB-231/BRMS1 cells grown on cover slips were incubated in media containing 5 μmol/L Fura-2 AM (Fura-2-acetoxymethyl ester; Molecular Probes, Eugene, OR) from a 5 mmol DMSO stock solution for 90 minutes. At the end of the incubation period, slides were washed twice and transferred to a glass plate with an 8 mm hole and sealed with vacuum grease forming a well to which 40 μL of media was added. The plate was placed on Nikon Diaphot TE200 inverted microscope using a 40× Plan Fluor lens. The cells were illuminated with a Lambda DG-4 (Sutter Instruments, Novato, CA) and images were collected with a CoolSnap HQ (Roper Scientific, Duluth, GA) camera controlled by MetaFluor (Universal Imaging, Downingtown, PA). Multiple cells within the field of view were tracked as a separate data set; one image was selected per second and ratio values were saved. Excitation wavelengths were 340 and 380 nm and collected at 510nm; the ratio was the result of the 340/380 image.

For collection of ratiometric data from growth factor–stimulated cells, the experiments were carried out as above except that cells grown on cover slips were incubated in serum-free media for 2 to 4 hours prior to loading with Fura-2. PDGF (50 ng/mL) was added to the media in the well and collection of data was begun. Ratiometric data was collected for 10 minutes to confirm a stable baseline. Then PDGF was added to the media and collection of data was continued. Calcium ionophore (A23187, Calbiochem, San Diego, CA) was added at the end of the experiment to show that all cells were still able to mobilize calcium.

For detection of calcium mobilization using Fluor-3, cells were cultured on cover slips as described above, and cell-permeant Fluor-3 AM in DMSO was added to 20 μL of medium in the well. The cells were maintained at 22°C for 15 minutes in the Fluor-3 solution. Then, the Fluor-3-containing medium was removed and 20 μL of fresh medium was placed in the well. Optical sections were collected at 30-second intervals for ~5 minutes prior to the treatment, confirming that fluorescence intensity was stable, after which imaging was done for 60 minutes.

**Results and Discussion.** Although millions of cells per gram of tumor may enter the bloodstream daily, vanishingly small percentages successfully colonize secondary tissues (7–9). Every step of the metastatic cascade is rate-limiting in that any tumor cell incapable of completing any step is effectively nonmetastatic. Recently, several laboratories have shown that a relatively high proportion of cells entering the vasculature are able to arrive at secondary sites (7). Yet, relatively few respond to the local microenvironment by proliferating. Controlling growth at secondary sites represents a potentiallylatable step in the metastatic cascade for therapeutic intervention (10). However, developing agents that block growth at the secondary site requires better biochemical and molecular definition of tumor cell growth (11).

Metastasis suppressors, by definition, block the growth of tumor cells at secondary sites, although still allowing proliferation at the site of tumor local control. By inference, many metastasis suppressors are hypothesized to control cellular responses to exogenous signals. This study was undertaken to assess whether signaling pathways implicated in calcium mobilization might be involved in cancer metastasis, specifically cellular suppression of metastasis due to the re-expression of BRMS1. Several laboratories, including ours, previously showed that perturbation of intracellular
calcium levels could modify tumor cell invasion and/or metastasis (4, 12, 13). Because phosphoinositides have been implicated in numerous cellular processes involved in tumorigenesis and metastasis, and are key regulators of intracellular calcium (14), we examined phosphoinositide levels in metastatic MDA-MB-435 cells compared with metastasis-suppressed 435/BRMS1 cells.

Phosphoinositide levels were determined by metabolically labeling cells with [3H]myo-inositol, extracting lipids, deacylating the phosphoinositides, and analyzing the resulting glycerophosphoinositols by anion exchange HPLC. Glycerophosphoinositols from MDA-MB-435 cells were detected in decreasing order of abundance: gPI(4)P > gPI(4,5)P2 > gPI(3)P > gPI(3,5)P2 (Fig. 1C; Table 1). gPI(5)P, gPI(3,4)P2, and gPI(3,4,5)P3 were not detected. Although most of the glycerophosphoinositols were similar, a striking difference of gPI(4,5)P2 levels was observed. Figure 1 shows qualitative visual representation comparing MDA-MB-435 and 435/BRMS1 cells. Radioactive counts (cpm) from each gPI(4,5)P2 peak were also measured (Table 1). gPI(4,5)P2 levels in 435/BRMS1 cells averaged between 5% and 10% of those measured for parental MDA-MB-435. Data from Table 1 and Fig. 1 are from the same experiment; however, the data are representative of at least six independent assays.

Table 1. BRMS1 expression alters PtdIns(4,5)P2 levels in 435/BRMS1 cells

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Total gPis</th>
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<tbody>
<tr>
<td></td>
<td>gPI (%)</td>
</tr>
<tr>
<td>MDA-MB-435</td>
<td>96.7</td>
</tr>
<tr>
<td>435/BRMS1</td>
<td>98.5</td>
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NOTE: Total glycerophosphoinositol cpm was calculated by adding cumulative cpm found in each glycerophosphoinositol peak. The percentage of each glycerophosphoinositol was determined by dividing total glycerophosphoinositol cpm into cpm for individual glycerophosphoinositols.

The dramatic difference in gPI(4,5)P2 concentrations between MDA-MB-435 and 435/BRMS1 cells could represent PtdIns(4,5)P2 changes at the whole cell level or changes in specific cell structures like the plasma membrane or organelle concentrations. To examine distribution of PtdIns(4,5)P2 in cells, immunofluorescence confocal microscopy with an anti-PtdIns(4,5)P2 monoclonal antibody was done (Fig. 2). Immunofluorescence indicated that PtdIns(4,5)P2 was clearly localized in the plasma membrane, Golgi-like perinuclear structures, and filopodia-like cellular extensions in MDA-MB-435 cells. Nuclear fluorescence was detectable but significantly lower than in other regions of the cells. Although gross morphologies of MDA-MB-435 and 435/BRMS1 are similar, the intensity of anti-PtdIns(4,5)P2 staining was much lower in 435/BRMS1 cells. PtdIns(4,5)P2 was detected only in the perinuclear regions of the 435/BRMS1 cells. No PtdIns(4,5)P2 was observed in cellular extensions, but this may be a consequence of overall reduced staining. Although an extensive panel of assays have not yet been completed for MDA-MB-231 and 231/BRMS1 human breast carcinoma cells, the trends for levels of PtdIns(4,5)P2 and other phosphoinositides are similar (data not shown).

To begin elucidating the mechanisms by which modulation of PtdIns(4,5)P2 levels might explain BRMS1 suppression of metastasis, the roles of phosphoinositides were considered. The concentrations of Ins(1,4,5)P3 were measured because Ins(1,4,5)P3 is released from PtdIns(4,5)P2 as a result of phospholipase C activity. Metastatic MDA-MB-435 cells contained ~2-fold higher concentrations of Ins(1,4,5)P3 than metastasis-suppressed 435/BRMS1 cells (Fig. 1). The difference, although consistent and biologically significant in many cellular contexts, does not entirely reflect the differences in precursor levels.

Phosphoinositide signaling is considered especially critical in the regulation of intracellular calcium following growth factor stimulation. In response to exogenous signals (e.g., PDGF and insulin), calcium is released from cellular organelles. The calcium acts as a second messenger, mediating a variety of signals. Because we previously found that manipulation of intracellular calcium by chelation with BAPTA-AM (15) could significantly suppress C8161 melanoma cell invasion, motility and metastasis following i.v. inoculation directly into the lateral tail vein of mice,5 intracellular
calcium signaling was measured using ratiometric fluorescence with the calcium indicator dye, FURA-2. Monitoring of individual cells revealed generally higher basal activities in MDA-MB-435 cells compared with 435/BRMS1 (Fig. 3); however, the differences were variable and not statistically significant. The differences were pronounced in MDA-MB-435 cells treated with exogenous PDGF (50 ng/mL), a known activator of phosphoinositide signaling; whereas, 435/BRMS1 cell response to PDGF was muted. Control measurements using the calcium ionophore, A23187/ionomycin, showed that intracellular calcium could still be mobilized in 435/BRMS1 cells. It is not known whether PDGF is a key signal for metastatic cells in the lungs in vivo, but our observations highlight the principle that BRMS1 may be mediating one or more signaling pathways from the extracellular milieu to the nucleus. More extensive analyses of suspected growth factors and pathways will be required to ascertain what would be the most relevant to the process of metastatic colonization.

PtdIns(4,5)P2 can directly serve as a regulator of vesicle-mediated protein trafficking (16) and as a modulator of cytoskeletal architecture in mammalian cells (5) by binding to effector proteins. Despite the similarity of gross morphology between them, we questioned whether BRMS1-transfected cells might exhibit changes in cytoskeletal architecture. Using fluorophore-tagged phalloidin, consistent changes in filamentous actin levels and changes in cytoskeletal architecture. Using fluorophore-tagged we questioned whether BRMS1-transfected cells might exhibit changes in cytoskeletal architecture. Using fluorophore-tagged phalloidin, consistent changes in filamentous actin levels and organization were observed. But the differences were relatively minor and nondescript.

PtdIns(4,5)P2 plays a pivotal role as a precursor for signaling phosphoinositides. Both precursors of PtdIns(4,5)P2 [PtdIns(4)P, and PtdIns(5)P] and a downstream phosphoinositide [PtdIns(3,4,5)P3] were measured by HPLC. Similar levels were observed in both MDA-MB-435 and 435/BRMS1. Although interexperimental variation was observed, there were no consistent differences noted in detectable phosphoinositides. Because metabolism of the phosphoinositides involves tight regulation of kinase and phosphatase activities, levels of the other molecules may reflect compensatory actions of the enzymes. Direct testing of this hypothesis is not yet possible because the identities of all of the enzymes have not yet been discovered in mammalian cells.

Previous reports have implicated phosphoinositide 3-kinase in metastasis based primarily on experiments using phosphoinositide 3-kinase inhibitors like wortmannin and LY294002, which inhibited invasion in vitro (17). Likewise, loss of the PTEN phosphatase or PTEN function have been correlated with acquisition of metastatic potential (18). Phosphoinositide 3-kinase phosphorylates PtdIns(4,5)P2 to make PtdIns(3,4,5)P3 which, in turn, feeds into the Akt/PKB pathways regulating, among other important phenotypes, apoptosis. Conversely, PTEN acts by removing the 3-phosphate group from PtdIns(3,4,5)P3 to make PtdIns(4,5)P2. Taken together, these observations suggest that BRMS1 might alter metastasis by manipulating PtdIns(3,4,5)P3 via modulation of PtdIns(4,5)P2. Despite numerous attempts to directly test this possibility, PtdIns(3,4,5)P3 could not be routinely detected in either MDA-MB-435 or 435/BRMS1, which is typical of low levels found in cells not stimulated with exogenously added growth factors. Additional experiments are underway to elucidate PtdIns(3,4,5)P3 induction in BRMS1-transfected cells treated with growth factors; however, the data to date do not definitively support or refute a role for PtdIns(3,4,5)P3 in BRMS1-mediated suppression of breast cancer metastasis.

Despite significant improvements in the understanding of cellular growth control and the perturbations involved in developing tumors, the mechanisms that regulate tumor cells' abilities to grow in tissues other than those from which they originated (i.e., metastasis) are still largely unknown. The discovery of metastasis suppressors, the demonstration that many metastasis suppressors seem to block metastasis by

6 D.B. DeWald and D.R. Welch, unpublished observations.
interfering with sustained cell proliferation at the secondary site, and the availability of matched metastatic and nonmetastatic cell lines now enable researchers to design experiments that will aid in the understanding of the important biological phenomenon of metastatic colonization. The dissemination of cancer cells from the primary tumor is apparently not infrequent. However, the colonization of tissues by disseminated cells fortunately is rare. If the mechanisms of action for metastasis suppressors, like BRMS1, can be understood at a molecular level, then it may be possible to intervene and reduce morbidity and mortality due to cancer metastasis. The findings of this report suggest that BRMS1 suppresses metastasis by modulation of phosphoinositide signaling, specifically by controlling the levels of PtdIns(4,5)P2.

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References

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