Protease-Activated Receptor-2 Is Essential for Factor VIIa and Xa–Induced Signaling, Migration, and Invasion of Breast Cancer Cells

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Abstract
Protease-activated receptors (PAR) are G protein–coupled receptors that function as cell-surface sensors for coagulant proteases, as well as other proteases associated with the tumor microenvironment. PAR1 is activated by thrombin whereas the upstream coagulant protease VIIa bound to tissue factor and Xa can activate both PAR1 and PAR2. PAR1 has been implicated in tumor cell growth, migration, and invasion whereas the function of PAR2 in these processes is largely unknown. Towards defining the functional importance of PAR2 in cancer cells, we used small interfering RNAs to deplete highly invasive breast cancer cells of endogenous PAR proteins. Our findings strongly suggest that PAR2 is critical for MDA-MB-231 and BT549 breast cancer cell migration and invasion towards NIH 3T3 fibroblast conditioned medium. To define the relative importance of PAR1 versus PAR2 in mediating factor VIIa and Xa responses, we assessed signaling in cancer cells lacking either endogenous PAR1 or PAR2 proteins. Strikingly, in MDA-MB-231 cells depleted of PAR2, we observed a marked inhibition of VIIa and Xa signaling to phosphoinositide hydrolysis and extracellular signal–regulated kinase 1/2 activation whereas signaling by VIIa and Xa remained intact in PAR1-deficient cells. Factor VIIa and Xa–induced cellular migration was also impaired in MDA-MB-231 cells deficient in PAR2 but not in cells lacking PAR1. Together, these studies reveal the novel findings that PAR2, a second protease-activated G protein–coupled receptor, has a critical role in breast cancer cell migration and invasion and functions as the endogenous receptor for coagulant proteases VIIa and Xa in these cells. (Cancer Res 2006; 66(1): 307-14)

Introduction
A link between malignant cancer and hyperactivation of the coagulation system is well documented in the literature (1–4). However, the mechanism by which coagulant activity contributes to tumor cell invasion and metastasis remains poorly understood. The expression of tissue factor, the principal cellular activator of the coagulation cascade, on cancer cells leads to generation of active coagulant proteases on the cell surface (5). Thrombin, the main effector protease of the coagulation cascade, is thought to promote metastasis, at least in part, by facilitating tumor cell interactions with host vascular cells including platelets and endothelial cells lining blood vessels (6–8). Thrombin and upstream coagulant proteases are also generated in the tumor microenvironment independent of blood coagulation (1) and potentially contribute to cancer cell migration and invasion by acting directly on tumor cells. Thus, coagulant proteases are likely to facilitate tumor cell metastasis by modulating a number of host vascular cell responses as well as by acting directly on tumor cells themselves.

Thrombin is formed during a series of zymogen conversions that are triggered when coagulant proteases contact tissue factor. Tissue factor, a single spanning integral membrane protein, complexed with VIIa, cleaves factor X to generate the active protease Xa. The formation of Xa is localized to the cell surface and triggers proteolytic conversion of prothrombin to thrombin. In addition to cleaving fibrinogen, thrombin activates cells through at least three G protein–coupled protease-activated receptors (PAR): PAR1, PAR3, and PAR4. In contrast, PAR2 is activated by multiple tryptsin-like serine proteases including the upstream coagulant proteases VIIa and Xa but not by thrombin. Thrombin is the main physiologic activator of PAR1; however, activation of PAR1 is not restricted to this protease. PAR1 can be cleaved and activated by factors VIIa and Xa (9, 10), plasmin (11), activated protein C (12), and metalloproteases (13). Clearly, multiple proteases can cleave and activate these receptors; thus, the particular PAR and protease that function in a specific cellular setting remain largely undefined.

PAR1 has been implicated in tumor cell growth, invasion, and metastasis of several types of human malignant cancers, including breast cancer. PAR1 expression has been directly correlated with the degree of invasiveness exhibited by primary human breast tissue specimens and established cancer cell lines whereas PAR1 expression is minimal or absent in benign and normal breast tissue and noninvasive carcinoma (14–17). Ectopic expression of PAR1 in mouse mammary gland epithelia induces an oncogenic phenotype (18) and PAR1 also has the capacity to transform NIH 3T3 fibroblasts, further suggesting a role for this receptor in oncogenesis (19). Moreover, expression of PAR1 in noninvasive breast carcinoma is sufficient to promote growth and invasion of breast cancer cells in a xenograft nude mouse model (13). PAR2 is also expressed in malignant breast carcinoma and in highly invasive breast carcinoma cell lines (15, 20). However, in contrast to PAR1, the functional importance of PAR2 in breast cancer cells is largely unknown. Activation of PAR1 by thrombin or matrix metalloprotease 1 (MMP1) occurs on breast carcinoma (13, 16, 21), whereas the actual protease responsible for PAR2 activation remains to be determined. Interestingly, the upstream coagulant proteases VIIa and Xa have also been reported to stimulate signaling and

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migration of certain breast carcinoma cells (22, 23); however, whether this involves direct activation of PAR1 and/or PAR2 has not been clearly defined.

We hypothesize that besides thrombin, other tumor–generated proteases activate PAR1, and perhaps PAR2, to promote breast cancer cell invasion and metastasis. Tissue factor is highly overexpressed in invasive tumor cells and is responsible for generation of active coagulant proteases VIIa and Xa. Factors VIIa and Xa potentially make important contributions to cancer cell invasion and metastasis via activation of PAR1 and/or PAR2. In the present study, we used small interfering RNAs (siRNA) to knock down endogenous PAR2 protein in an effort to define the importance of this receptor in breast cancer cells. Our findings strongly suggest that PAR2 is an important mediator of breast cancer cell migration and invasion. Our studies further show an essential role for PAR2 in mediating coagulant protease VIIa and Xa–induced signaling and migration of MDA-MB-231 breast cancer cells. These findings are the first to show a critical role for PAR2 in mediating cancer cell migration and invasion and also define a new role for PAR2 as the endogenous receptor for factors VIIa and Xa in invasive breast cancer cells. Moreover, these studies are the first to show the relative contribution of PAR1 versus PAR2 in mediating coagulant protease effects on breast cancer cells and provide important information for the design and use of protease inhibitors in breast cancer disease.

Materials and Methods

Reagents and antibodies. Human plasma–derived factor VIIa, factor Xa, and α-thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). α-Trypsin, treated with tosylamide-2-phenylthiochloromethyl ketone, lanamin, and collagen type IV were from Sigma–Aldrich (St. Louis, MO). Agonist peptides TFFLRNPKD (PAR1 specific) and SLIKGV (PAR2 specific) were synthesized as the carboxyl amide and purified by reverse-phase high-pressure liquid chromatography (University of North Carolina Peptide Facility, Chapel Hill, NC).

Monoclonal anti–tissue factor antibody was from Enzyme Research Laboratories. Goat anti-mouse immunoglobulin G (IgG) was from Pierce (Rockford, IL). A rabbit polyclonal anti-PAR1 antibody was generated against the amino-terminal hirudin-like domain peptide sequence YEP-WEDEKANESLGTEYC as previously described (24). A monoclonal anti-PAR2 antibody (AM1) was generously provided by L. Brass (University of Pennsylvania, Philadelphia, PA) and a polyclonal anti-PAR2 rabbit antibody was raised against an amino-terminal peptide sequence SLIKGVDTGSHVTGKGVC. Monoclonal anti-phospho-p44/42 mitogen-activated protein kinase (MAPK; extracellular signal–regulated kinase (ERK) 1/2) antibody and polyclonal anti–p44/p42 MAPK (ERK1/2) antibody were from Cell Signaling Technology, Inc. (Beverly, MA). Anti-actin antibody was purchased from Sigma–Aldrich. Horseradish peroxidase (HRP)–conjugated goat anti-mouse and anti-rabbit antibodies were from Bio-Rad (Hercules, CA).

Cell lines. MDA-MB-231 and BT549 cells were obtained from American Type Culture Collection (Manassas, VA). MDA-MB-231 cells were grown in Leibovitz’s L-15 medium supplemented with 10% fetal bovine serum (FBS). BT549 cells were grown in RPMI 1640 supplemented with 10% FBS and 10 μg/mL insulin.

Phosphoinositide hydrolysis. MDA-MB-231 cells were plated at 1.6 × 10⁶ per well in 24-well dishes and labeled with 2 μCi/mL of myo-[3H]inositol in serum- and inositol-free medium containing 1 mg/mL bovine serum albumin (BSA) overnight. Cells were washed and then incubated in the absence or presence of agonists diluted in medium containing 1 mg/mL BSA and 20 mM/L lithium chloride for various times at 37°C. Cell incubation medium was removed and total cellular [3H]inositol phosphates were extracted, isolated, and quantified as described (25).

siRNA electroporation. Semiconfluent monolayers of MDA-MB-231 or BT549 cells were removed from flasks with an enzyme-free cell dissociation solution (Specialty Media, Phillipsburg, NJ). Cells (1 × 10⁶) were then electroporated with 600 nM/L of either PAR1–specific, PAR2–specific, or nonspecific control siRNA using a system developed by Amaxa, Inc (Gaithersburg, MD). Cells were then plated in normal growth media. PAR-specific siRNAs were synthesized by Dharmaco, Inc. (Lafayette, CO) and used to target the following mRNA sequences: PAR1 siRNA, 5'-AGAU-UAGCUCCUCAUCUUAA-3'; PAR2 siRNA, 5'-GGAAAGAGCUUUGAAGU-3'. The nonspecific siRNA, 5'-GGCUACUGCCAGGACCC-3', was used as a negative control.

Immunoblotting for PAR expression. To assess PAR1 or PAR2 protein expression, MDA-MB-231 cells were plated at 1 × 10⁵ per well on a six-well plate and grown for 24, 48, or 72 hours after siRNA electroporation (Amaxa). Cells were lysed in 1% Triton X-100 lysis buffer containing 50 mM/L Tris-HCl (pH 7.4), 100 mM/L NaCl, 5 mM/L EDTA, 50 mM/L NaF, and 10 mM/L sodium pyrophosphate with protease inhibitors. Equivalent amounts of protein lysates (~30 μg) were resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with anti-PAR1 or anti-PAR2 antibodies. Blots were then stripped and reprobed with a monoclonal anti-actin antibody. Immunoblots were developed with enhanced chemiluminescence plus (Amersham Biosciences, Inc., Piscataway, NJ) and imaged by autoradiography.

Cell-surface ELISA. MDA-MB-231 cells electroporated with siRNAs were plated on fibronectin-coated 24-well dishes at 1.6 × 10⁶ per well. After 72 hours, cells were incubated with anti-PAR1 antibody, anti-PAR2 antibody, or preimmune serum for 1 hour at 4°C. Cells were washed to remove unbound antibody, fixed with 4% paraformaldehyde, and then incubated with HRP-conjugated goat anti-rabbit secondary antibody for 1 hour at 25°C. Cells were then washed and incubated with HRP substrate, One-Step ABTS (2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid, Pierce), and the absorbance was determined at 405 nm using a Molecular Devices (Sunnyvale, CA) SpectraMax Plus microplate reader.

ERK1/2 activation. MDA-MB-231 cells were plated in 24-well dishes at a density of 1.6 × 10⁶ per well. Serum-starved cells were incubated in the absence or presence of agonists for various times at 37°C and then lysed in 2× SDS-gel loading buffer [100 mM/L Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.02% bromophenol blue]. Cell lysates were resolved by SDS-PAGE, transferred to membranes, and immunoblotted with an anti-phospho-p44/42 MAPK (ERK1/2) antibody. To detect total p44/42 MAPK (ERK1/2), membranes were stripped and reprobed with an anti–p44/p42 MAPK (ERK1/2) antibody. Immunoblots were developed, imaged, and quantitated using a Bio-Rad Fluor-S MultiImager.

Cell migration. Cell migration was assessed using a 24-well format containing a transwell insert (8-μm pore size polycarbonate filter; BD Biosciences, Bedford, MA) that was coated with 7.5 μg/mL collagen type IV for MDA-MB-231 cells or with 10 μg/mL laminin for BT549 cells. After 72 hours of electroporation with siRNAs, serum-deprived MDA-MB-231 and BT549 cells (5 × 10⁵) were diluted in 100 μL of DMEM containing 0.1% BSA and 10 mM/L HEPES (pH 7.4) and added to the upper chamber of the transwell. Cells were then allowed to migrate in response to the addition of 600 μL of either serum-free media, NIH 3T3 fibroblast conditioned medium, or various agonists added to the lower chamber for 3 hours at 37°C. Nonmigrated cells in the upper chamber were removed by wiping with a cotton swab while the attached migrated cells were fixed and stained with 0.1% crystal violet diluted in 0.1 mol/L borate (pH 9.0) and 2% ethanol. Three different fields of the cells were photographed with IX81 Olympus microscope fitted with a 20× objective and the number of migrated cells in each field was counted.

Cell invasion. After 72 hours of electroporation with siRNAs, serum-starved MDA-MB-231 cells (1 × 10⁵) or BT549 cells (5 × 10⁵) diluted in 100 μL of DMEM (without phenol red) containing 0.1% BSA, 10 mM/L HEPES (pH 7.4) were added to the upper well of a 24-transwell insert coated with matrigel (BD Biosciences). The lower well contained 600 μL of NIH 3T3 fibroblast conditioned medium prepared as previously described (26). Cells were then incubated for 24 hours at 37°C. Cells remaining in the upper chamber were removed and invaded cells were fixed, stained, and quantified as described above.

Data analysis. Data were analyzed using Prism 3.0 software and statistical significance was determined using InStat 3.0 (GraphPad, San Diego, CA).
Results

Protease-activated receptors are capable of mediating tumor cell responses to a variety of extracellular proteases associated with the tumor microenvironment including coagulant proteases. Towards defining the relative importance of the individual PARs in mediating extracellular protease responses in cancer cells, we generated PAR-specific siRNAs to deplete highly invasive breast cancer cells of endogenous PAR proteins. Characterization of PAR family members expressed in MDA-MB-231 breast carcinoma using reverse transcription-PCR revealed the presence of PAR1 and PAR2 mRNA transcripts (data not shown), consistent with the high level of receptor protein expression previously reported (14, 20). We first determined whether siRNAs targeted against PAR1 and PAR2 mRNA transcripts were effective at reducing expression of these proteins. MDA-MB-231 cells were electroporated with PAR-specific siRNAs and after 24, 48, and 72 hours of siRNA incubation, cell lysates were prepared and the amounts of endogenous PAR1 and PAR2 protein remaining were determined by immunoblotting. A significant decrease in both PAR1 and PAR2 protein expression was observed after 72 hours of siRNA incubation (Fig. 1A). siRNAs directed against specific PAR mRNA sequences also caused a significant and selective loss of cell-surface expression of the targeted receptor after 72 hours of incubation (Fig. 1B). These findings suggest that siRNAs targeted to specific PAR mRNA transcripts are effective and selective at causing loss of receptor protein.

We then determined whether siRNA-mediated depletion of specific PAR proteins resulted in loss of functional responses by examining agonist-induced phosphoinositide hydrolysis. PAR1 and PAR2 stimulate Gqα-mediated phosphoinositide hydrolysis primarily through the activation of phospholipase C-β (27, 28). In control nonspecific siRNA–treated cells, thrombin and TFLLRNPNDK induced an ~2-fold increase in [3H]inositol phosphate accumulation following 60 minutes of agonist exposure at 37°C (Fig. 1C). In contrast, activated PAR1-stimulated phosphoinositide hydrolysis was markedly impaired in cells depleted of PAR1 by siRNA whereas signaling by trypsin in the same cells was unaffected (Fig. 1C). Similarly, activation of PAR2 with trypsin or SLIGKV caused an ~2-fold increase in signaling in control siRNA–treated cells, which was virtually abolished in cells depleted of PAR2 by siRNA (Fig. 1C). Thrombin signaling remained intact in PAR2-deficient cells (Fig. 1C). These results suggest that depletion of either PAR1 or PAR2 protein by siRNA is sufficient to ablate receptor-stimulated signaling to phosphoinositide hydrolysis in highly invasive breast cancer cells.

We next evaluated whether siRNA-mediated knockdown of specific PAR proteins affected agonist-induced ERK1/2 activation, an important mitogenic signaling pathway. Activation of PAR1 with TFLLRNPNDK caused a rapid and sustained ~3-fold increase in ERK1/2 activity in control siRNA–treated cells (Fig. 2A, top), consistent with the magnitude and duration of thrombin-induced ERK1/2 activation typically observed in these cells (16). In contrast, TFLLRNPNDK-mediated activation of ERK1/2 was virtually abolished in cells in which PAR1 protein was depleted by siRNA (Fig. 2A, bottom). However, the PAR2-specific agonist peptide SLIGKV retained the capacity to induce ERK1/2 activation in PAR1-depleted cells, indicating that siRNAs specifically targeted PAR1 (Fig. 2C, lanes 4–6). Activation of PAR2 with SLIGKV also caused a prolonged ~3-fold increase in ERK1/2 activity, which was completely ablated in cells treated with PAR2-specific siRNAs (Fig. 2B). In contrast to loss of PAR2 signaling, however, activation of ERK1/2 by PAR1-specific agonist peptide TFLLRNPNDK remained intact in PAR2 siRNA knockdown cells (Fig. 2C, lanes 7–9). Together, these findings strongly suggest that siRNAs targeting specific PAR mRNA sequences are effective and selective at depleting highly invasive breast carcinoma cells of endogenous PAR protein and corresponding functional responses.

Figure 1. PAR-specific siRNAs cause selective and effective loss of receptor-stimulated signaling. A, MDA-MB-231 cells were electroporated with siRNAs targeted to either PAR1, PAR2 mRNA sequences, or nonspecific siRNA control and after 24, 48, or 72 hours, cell lysates were prepared. Equivalent amounts of protein lysates (~30 μg) were resolved by SDS-PAGE, transferred, and immunoblotted with anti-PAR1 or anti-PAR2 antibodies. The membrane was stripped and reprobed with anti-actin antibody to control for loading. B, MDA-MB-231 cells electroporated with either nonspecific siRNA and PAR-specific siRNAs were incubated with either anti-PAR1 antibody or anti-PAR2 antibody (Ab), fixed, and the amount of antibody bound to the cell surface was determined by ELISA. Columns, mean (n = 3); bars, SD. Representative of at least three separate experiments. Control, MDA-MB-231 cells were electroporated with nonspecific siRNA or PAR-specific siRNAs and after 72 hours of electroporation, cells labeled with myo-[3H]inositol were incubated in the absence or presence of PAR1-specific agonists (10 nmol/L u-thrombin or 100 μmol/L TFLLRNPNDK) or PAR2 agonists (10 nmol/L trypsin or 100 μmol/L SLIGKV) for 60 minutes at 37°C and amounts of [3H]inositol phosphates generated were measured. Columns, mean (n = 3); bars, SD. Representative of three independent experiments. **, P < 0.01, trypsin-induced signaling in PAR1 siRNA–treated cells was significantly different compared with untreated control cells (unpaired Student’s t test). **, P < 0.01, thrombin signaling was significantly different compared with control PAR2-siRNA cells (unpaired Student’s t test).
The functional importance of PAR2 in mediating breast cancer cell migration and invasion has not been previously determined. We initially assessed the effect of loss of PAR2 protein on the ability of highly invasive MDA-MB-231 and BT549 breast cancer cells to migrate towards NIH 3T3 fibroblast conditioned medium. BT549 cells express endogenous PAR1 and PAR2 proteins and PAR-specific siRNAs were effective and selective at reducing endogenous receptor function in these cells (data not shown). Control siRNA–treated MDA-MB-231 and BT549 breast cancer cells exhibited a high degree of basal migration; however, the addition of NIH 3T3 fibroblast conditioned medium caused an even greater ~2-fold increase in migratory responses (Fig. 3A and B). In striking contrast, cells lacking PAR2 protein showed a substantial ~50% inhibition of migration towards conditioned medium comparable to that observed in cells depleted of PAR1 whereas basal migration was modestly reduced (Fig. 3A and B). These findings suggest that PAR2 is an important mediator of MDA-MB-231 and BT549 cell migration induced by NIH 3T3 cell conditioned medium.

To determine a role for PAR2 in cellular invasion, we evaluated the ability of MDA-MB-231 and BT549 cells to migrate and invade towards NIH 3T3 fibroblast conditioned medium. Knockdown of PAR1 expression by siRNA resulted in significant inhibition of breast cancer cell invasion; only ~37% of MDA-MB-231 cells invaded compared with control siRNA–treated cells (Fig. 3C). Depletion of endogenous PAR1 protein by siRNA in BT549 cells caused a similar reduction of migration and invasion (Fig. 3D). These findings are consistent with a recent study showing loss of MDA-MB-231 cell invasion towards NIH 3T3 cell conditioned medium after treatment with PAR1-specific siRNAs (13) and strongly suggest that PAR1 is an important mediator of breast cancer cell invasion. Strikingly, in cells depleted of PAR2, we also observed a significant loss of MDA-MB-231 and BT549 cell invasion, with only ~50% of cells exhibiting an ability to invade through matrigel in response to NIH 3T3 cell conditioned medium (Fig. 3C and D). The simultaneous knockdown of both PAR1 and PAR2 proteins also resulted in significant inhibition of MDA-MB-231 and BT549 cellular invasion (Fig. 3C and D). These findings are the first to show a critical role for PAR2 in mediating breast cancer cell migration and invasion.

Tissue factor is expressed in many invasive carcinomas including MDA-MB-231 cells, but not in BT549 breast cancer cells, and is critical for the formation and activity of upstream coagulant proteases VIIa and Xa. Tissue factor–bound VIIa and Xa can activate PAR1 and PAR2, and hence the mechanism by which these upstream coagulant proteases contribute to breast cancer cell migration and invasion remains poorly defined. Towards understanding the function of factors VIIa and Xa in breast cancer cell migration and invasion, we first determined whether these proteases induce signaling responses in MDA-MB-231 cells by evaluating phosphoinositide hydrolysis. The concentration-effect curves for factors VIIa and Xa are within the range previously reported for VIIa- and Xa-elicited signaling responses observed in fibroblasts and endothelial cells. We next used an anti–tissue factor neutralizing antibody to stimulate half-maximal response (EC50) at 30 minutes were ~7 and ~70 nmol/L, respectively (Fig. 4A). These EC50 values are within the range previously reported for VIIa- and Xa-elicited signaling responses observed in fibroblasts and endothelial cells (10, 29).
to determine whether tissue factor is essential for VIIa and Xa signaling in MDA-MB-231 cells. In cells preincubated with control IgG or medium only, VIIa and Xa induced a robust \(3\)- to \(4\)-fold increase in signaling (Fig. 4B). Interestingly, anti–tissue factor antibody virtually ablated signaling by VIIa whereas Xa signaling remained intact (Fig. 4B). Thus, tissue factor is critical for VIIa-induced signaling in MDA-MB-231 breast cancer cells whereas Xa can signal independent of tissue factor in these cells.

The functional importance of PAR1 versus PAR2 in mediating responses to the coagulant proteases VIIa and Xa in highly invasive breast cancer cells has not been previously defined. To determine which PAR is essential for VIIa and Xa cellular effects, we assessed phosphoinositide hydrolysis in MDA-MB-231 cells deficient in either endogenous PAR1 or PAR2 proteins. In cells treated with control siRNAs, both VIIa and Xa caused a significant \(1.5\)-fold increase in \([3H]\)inositol phosphate accumulation after 30 minutes of agonist exposure at 37°C whereas the addition of VIIa and Xa together resulted in a slightly greater \(2\)-fold increase in phosphoinositide hydrolysis (Fig. 5A). In cells depleted of PAR1 protein by siRNA, factors VIIa and Xa induced an \(1.5\)- to \(2\)-fold increase in phosphoinositide hydrolysis, similar to that observed in control siRNA–treated cells (Fig. 5A). These findings strongly suggest that PAR1 is not essential for VIIa- or Xa-stimulated signaling in breast cancer cells. Interestingly, however, factor VIIa and Xa–stimulated phosphoinositide hydrolysis was virtually abolished in cells lacking endogenous PAR2 (Fig. 5A). At face value, these findings suggest that PAR2 is the predominant mediator of coagulant protease VIIa and Xa–induced signaling in breast cancer cells.

To determine which PAR mediates coagulant protease signaling to ERK1/2, we examined the ability of factors VIIa and Xa to increase ERK1/2 activity in cells depleted of either endogenous PAR1 or PAR2 protein. In siRNA control cells, factor Xa induced a rapid and sustained \(4\)-fold increase in ERK1/2 activity (Fig. 5B, top), similar to that observed with direct activation of either PAR1 or PAR2 (Fig. 2). Hirudin, a thrombin inhibitor, blocked ERK1/2 activation to thrombin but not to Xa, suggesting that Xa effects are not due to thrombin generation (data not shown). Moreover, factor Xa signaling remained intact in cells depleted of endogenous PAR1 protein, suggesting that Xa can signal independent of PAR1 in breast cancer cells (Fig. 5B, middle). In contrast, Xa-induced ERK1/2 activation was abolished in cells lacking PAR2 protein (Fig. 5B, bottom). Similarly, factor VIIa stimulated an increase in ERK1/2 activity at 30 minutes, which was completely inhibited in cells deficient in PAR2, whereas VIIa-induced increase in ERK1/2 activity was not affected in cells lacking PAR1 (Fig. 5C). Together, these findings strongly suggest that PAR2 is the endogenous receptor for upstream coagulant protease Xa and VIIa signaling in breast cancer cells.

We next assessed the effect of PAR1 versus PAR2 knockdown on coagulant protease–induced breast cancer cell migration. We initially examined the ability of MDA-MB-231 cells to migrate in response to various concentrations of factor Xa. MDA-MB-231 cells exhibited a high level of migration in the absence of agonist and a submaximal 52 nmol/L concentration of factor Xa was most effective at stimulating cell migration (Fig. 6A). The highly migratory MDA-MB-231 cells were then electroporated with PAR-specific or nonspecific siRNAs and allowed to migrate in response to low concentrations of VIIa, Xa, or VIIa and Xa together for 3 hours at 37°C. In cells depleted of PAR1, \(80\%\) to \(90\%\) of cells retained the capacity to migrate in response to factors VIIa and/or Xa compared with control siRNA–treated cells (Fig. 6B). In contrast, cells lacking PAR2 showed a substantially greater inhibition of cell migration with only \(70\%\) of cells exhibiting migratory responses to coagulant proteases (Fig. 6B). These findings indicate that the majority of MDA-MB-231 migratory responses occur independent of coagulant protease stimulation. However, activation of PAR2 by factors VIIa and Xa is capable of enhancing migration of a subpopulation of MDA-MB-231 breast cancer cells. The MDA-MB-231 cell line is heterogeneous with different subpopulations of cells displaying distinct growth and metastatic abilities due to discrete variations in gene expression patterns (30). Together, these studies strongly suggest a critical
function for PAR2 in mediating breast cancer cell migration and invasion and reveal a new role for PAR2 as the endogenous receptor for coagulant proteases VIIa and Xa in highly invasive breast cancer cells.

Discussion

A link between coagulation and several types of human malignant cancers is well established. Tissue factor and coagulant proteases are likely to promote cancer cell invasion and metastasis by modulating host vascular cell responses, as well as by acting directly on tumor cells themselves. Thrombin is the major effector for PAR1 whereas tissue factor-bound VIIa and Xa can activate both PAR1 and PAR2. PAR1 promotes breast cancer cell migration and invasion (13, 16, 21) whereas the function of PAR2 in these processes remains poorly defined. Towards understanding the mechanism by which coagulant proteases and PARs promote cancer cell invasion and metastasis, we sought to determine the functional importance of PAR2 in breast cancer cells. Depletion of PAR2 protein significantly reduced MDA-MB-231 and BT549 cell migration and invasion towards NIH 3T3 fibroblast conditioned medium. These results strongly suggest that PAR2 is a critical mediator of breast cancer cell migration and invasion. Moreover, our findings strongly suggest that PAR2 is the endogenous receptor for upstream coagulant proteases VIIa and Xa whereas thrombin

Figure 4. MDA-MB-231 cells respond to tissue factor–dependent VIIa and Xa signaling. A, the concentration-effect curves of VIIa and Xa were determined in MDA-MB-231 cells labeled with myo-[3H]inositol after 30 minutes of agonist incubation at 37°C. Points, mean from one experiment (n = 3); bars, SD. Representative of at least three independent experiments. B, MDA-MB-231 cells labeled with myo-[3H]inositol were preincubated with anti–tissue factor (TF) antibody, IgG control antibody, or media only for 1 hour, washed, and then incubated in the absence or presence of VIIa (10 nmol/L), Xa (174 nmol/L), or both for 60 minutes at 37°C and the amounts of [3H]inositol phosphates formed were then measured. Columns, mean (n = 3); bars, SD. Representative of three different experiments.

Figure 5. PAR2 is essential for factor VIIa and Xa signaling in MDA-MB-231 cells. MDA-MB-231 cells were electroporated with PAR-specific or nonspecific siRNA control and after 72 hours, VIIa and Xa signalings were assessed. A, cells labeled with myo-[3H]inositol were incubated in the absence or presence of VIIa (10 nmol/L), Xa (174 nmol/L), or both for 30 minutes at 37°C. The amounts of accumulated [3H]inositol phosphates were then measured. Columns, mean fold increase in [3H]inositol phosphate accumulation over basal cpm from one experiment (n = 3); bars, SD. Representative of three separate experiments. *** P < 0.005, significant difference in VIIa and Xa–induced phosphoinositide hydrolysis compared with untreated control cells (unpaired Student’s t test). B, serum-deprived MDA-MB-231 cells were incubated with 174 nmol/L factor Xa for various times at 37°C. An equivalent amount of cell lysates was then resolved by SDS-PAGE, transferred, and immunoblotted with anti–phospho-p44/42 (MAPK) ERK1/2 antibody. The membranes were reprobed with anti–p44/42 (MAPK) ERK1/2 antibody to control for equal loading. The time course of factor Xa–induced endogenous ERK1/2 activation shown is a representative experiment. Points, mean fold increase over basal from at least three independent experiments; bars, SE. C, serum-starved MDA-MB-231 cells were treated in the absence or presence of 10 nmol/L VIIa for 30 minutes at 37°C and ERK1/2 activation was assessed as described above. Representative experiment.
acts mainly at PAR1 in breast cancer cells. Coagulant protease VIIa and Xa–stimulated increases in phosphoinositide hydrolysis were virtually abolished in MDA-MB-231 cells deficient in PAR2 expression. Consistent with a role for PAR2 in mediating VIIa and Xa responses, a concomitant inhibition of agonist-triggered ERK1/2 activation was also observed in cells lacking PAR2. In contrast, factor VIIa and Xa signaling to phosphoinositide hydrolysis and ERK1/2 activation remained intact in PAR1-depleted cells. Factor VIIa and Xa–stimulated MDA-MB-231 cell migration was also diminished in cells lacking PAR2 but was not affected in PAR1-depleted cells. Thus, our study reveals the relative importance of PAR1 versus PAR2 in mediating breast cancer cell responses to coagulant proteases VIIa and Xa and provides important information for the design and use of protease inhibitors in breast cancer disease.

In addition to PAR1, we show for the first time that PAR2 makes equally important contributions to breast cancer cell migration and invasion, as a reduction of PAR2 protein by siRNA caused significant decreases in cellular migration and invasion towards NIH 3T3 cell conditioned medium comparable to that observed with loss of PAR1. The depletion of PARs by siRNA provides an opportunity to rigorously define the role of these receptors in breast cancer cell migration and invasion. Previous studies have relied on the use of blocking antibodies, cross-desensitization experiments, and antagonists that lack PAR selectivity. Our siRNA approaches have proved to be highly effective and selective in targeting PAR degradation resulting in loss of receptor-specific functional responses in breast cancer cells (Figs. 1 and 2). The transwell invasion assays used in our experiments provide a reliable measure of in vitro cellular invasiveness. However, tumor cell metastasis involves complex interactions between blood cells, endothelial cells, mesenchymal cells, and extracellular matrix, which cannot be accurately reproduced ex vivo. Thus, future studies are necessary to definitively determine the importance of PAR2 in mediating breast cancer cell invasion and metastasis in vivo.

Our studies strongly suggest that PAR2 is the endogenous receptor for factor VIIa and Xa signaling in invasive breast cancer cells. Whether tumor cells or surrounding stromal cells generate endogenous factors VIIa and Xa has not been determined. Tissue factor VIIa alone seems to be sufficient to stimulate PAR2 signaling; however, tissue factor VIIa–generated endogenous Xa might also contribute to signaling. In contrast, Xa activation of PAR2 occurs independent of tissue factor, indicating that formation of a ternary tissue factor VIIa-Xa complex is not essential for signaling in breast cancer cells. Consistent with our findings, PAR2 is the major effector for Xa-elicted responses in endothelial cells (29). However, in fibroblasts, PAR1 is responsible for virtually all Xa-induced signaling responses (10, 29). The ability of factors VIIa and Xa to preferentially cleave and activate PAR2, as opposed to PAR1, is likely due to surface-expressed cofactors that localize and regulate coagulant protease activity and specificity. Indeed, substrate recognition and cleavage by coagulant proteases is facilitated by cofactors, which function to properly position the substrate scissile bond to the relative membrane-associated protease (31). Clearly, tissue factor serves this function for VIIa; however, the cofactor and/or mechanism that confers preferential recognition and cleavage of PAR2 versus PAR1 by Xa in cancer cells remains to be determined. The amino-terminal cleavage and activation domain of PAR2 lacks obvious protease recognition features besides an adjoining positively charged arginine residue essential for serine protease cleavage of the receptor. However, the receptor is not cleaved by thrombin, indicating that the scissile bond may confer some specificity. The tumor microenvironment is replete with serine proteases as well as zinc-dependent metalloproteases, raising the possibility that PAR2 is likely to be cleaved and activated by other proteases released by tumor, activated stromal, infiltrating immune, or endothelial cells.

The mechanism by which PAR2 promotes cancer cell migration and invasion is poorly understood. Activated PAR2 stimulates phosphoinositide hydrolysis and mobilization of Ca²⁺ in a variety of cell types, suggesting that the receptor is capable of coupling to Gq and/or Gs signaling pathways (28, 32). However, whether PAR2 regulates important effectors of malignant progression, such as Ras- and Rho-GTPases, in cancer cells has not been determined. In addition, activation of PAR2 leads to prolonged increases in ERK1/2 activity, an important mitogenic signaling pathway implicated in cancer progression. The early transient increase in ERK1/2 activity induced by PAR2 is likely mediated by a G protein–dependent pathway whereas the slower and more
persistent ERK1/2 activation involves receptor interaction with arrestins on endocytic vesicles (28, 33). Arrestins are multifunctional proteins that act as scaffolds and transducers of MAPK signaling cascades. Indeed, PAR2-mediated ERK1/2 activation and cell migration are significantly inhibited in MDA-MB-231 cells lacking arrestins (34). However, whether the spatial and/or temporal activation of ERK1/2 by PAR2 is particularly dysregulated in cancer cells has not been determined. Moreover, PAR2 simulates secretion of angiogenic factors and is capable of regulating the angiogenic process (23, 35). Thus, PAR2 may facilitate tumor cell invasion and metastasis, at least in part, by contributing to tumor angiogenesis.

In conclusion, there is increasing evidence for the role of aberrant PAR activation in cancer cell invasion and metastasis. PARs seem to be the target of a variety of extracellular proteases associated with the tumor microenvironment, including serine proteases as well as metalloproteases. Previous studies have identified thrombin and MMP1 as effectors of PAR1 activation on tumor cells (13, 16, 21). In this study, we report that PAR2, a second protease-activated G protein-coupled receptor, has important functions in mediating cancer cell migration and invasion towards NIH 3T3 fibroblast conditioned medium. The actual proteases responsible for PAR2 cleavage and activation present in conditioned medium remain to be defined. However, our studies also reveal a new role for PAR2 as the endogenous receptor for factors VIIa and Xa in breast cancer cells. Whether or not dysregulated PAR2 activation and signaling promotes breast cancer cell migration and invasion is important to be determined.

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