Cell, Vol. 120, 303–313, February 11, 2005, Copyright ©2005 by Elsevier Inc. DOI 10.1016/j.cell.2004.12.018

PAR1 Is a Matrix Metalloprotease-1 Receptor that Promotes Invasion and Tumorigenesis of Breast Cancer Cells

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Summary

Protease-activated receptors (PARs) are a unique class of G protein-coupled receptors that play critical roles in thrombosis, inflammation, and vascular biology. PAR1 is proposed to be involved in the invasive and metastatic processes of various cancers. However, the protease responsible for activating the proinvasive functions of PAR1 remains to be identified. Here, we show that expression of PAR1 is both required and sufficient to promote growth and invasion of breast carcinoma cells in a xenograft model. Further, we show that the matrix metalloprotease, MMP-1, functions as a protease agonist of PAR1 cleaving the receptor at the proper site to generate PAR1-dependent Ca^{2+} signals and migration. MMP-1 activity is derived from fibroblasts and is absent from the breast cancer cells. These results demonstrate that MMP-1 in the stromal-tumor microenvironment can alter the behavior of cancer cells through PAR1 to promote cell migration and invasion.

Introduction

Protease-activated receptors (PARs) are tethered-ligand receptors that are activated by proteolytic cleavage of their extracellular domains. Four different PARs have been identified: PAR1, PAR2, PAR3, and PAR4 (Coughlin, 2000). PAR1, the prototypic member of the PAR family, has been shown to respond to a highly select group of serine proteases that include thrombin (Vu et al., 1991), plasmin (Kuliopulos et al., 1999), Xa (Riewald et al., 2001), and activated protein C (Riewald et al., 2002). Proteolytic cleavage exposes a new N terminus that binds to the body of the receptor to induce transmembrane signaling to internally located G proteins (Seeley et al., 2003). The activated G proteins in turn trigger a cascade of downstream events, leading to diverse cellular outcomes such as calcium signaling, engagement of integrins, cell adhesion and migration, gene transcription, and mitogenesis.

An emerging common theme is that the PARs act as high-gain sensors of extracellular protease gradients and allow the cell to react to the proteolytically-altered environment. This unique ability to sense proteases can be utilized both for migration toward proteases and for detection of a changing microenvironment (Kamath et al., 2001). More recently, the PARs have been shown to be critically involved in the tissue remodeling processes necessary for normal development including angiogenesis and trophoblastic invasion (Even-Ram et al., 1998; Tsopanoglou and Maragoudakis, 1999; Griffin et al., 2001; Even-Ram et al., 2003; Caunt et al., 2003). Stimulation of PAR1 in atherosclerotic plaques has been implicated in smooth muscle cell proliferation and restenosis and in the response and repair processes of a variety of acute and chronic inflammatory conditions (Nelken et al., 1992).

Beyond its roles in vascular biology and tissue remodeling, PAR1 was identified as an oncogene by its ability to transform NIH-3T3 cells (Whitehead et al., 1995) via Rho pathways (Martin et al., 2001). Ectopic expression of PAR1 in mammary gland epithelia exhibits an oncogenic phenotype of enhanced ductal complexity in mice (Yin et al., 2003). PAR1 has long been proposed to be involved in the invasive and metastatic processes of cancers of the breast, colon, lung, pancreas, prostate, and melanoma (Nierodzik et al., 1992, 1998; Fischer et al., 1995; Even-Ram et al., 1998, 2001; Zain et al., 2000). Even-Ram et al. (1998) demonstrated that PAR1 expression levels were directly correlated with degree of invasiveness in both primary breast tissue specimens and established cancer cell lines. High levels of PAR1 mRNA were found in infiltrating ductal carcinoma and undetectable levels in normal and premalignant hyperplasia. Recent studies by our group (Kamath et al., 2001) showed that the invasive MDA-MB-231 breast cancer cell line expresses very high levels of functional PAR1, PAR2, and PAR4, whereas minimally invasive MCF-7 cells have no PAR1 and low levels of PAR2 and PAR4. Despite the necessity for the presence of functional PAR1 on the cell surface (Even-Ram et al., 1998), neither thrombin nor other serine proteases were shown to be involved in PAR1-dependent breast cancer cell motility, and at high concentrations, thrombin could actually inhibit breast cancer cell invasion (Kamath et al., 2001). Moreover, Zain et al. (2000) had previously shown that thrombin has a bimodal effect on PAR1-dependent growth of melanoma, colon, and prostate carcinomas. Low thrombin concentrations enhance the growth of these cancer cells, whereas high thrombin impairs growth and induces apoptosis. Therefore, a critical issue to be addressed is whether a nonserine protease found in the tumor environment is responsible for the proinvasive properties of PAR1 in the cancer cells.

The most abundant class of nonserine proteases present in invasive and metastatic tumors are the zinc-dependent matrix metalloproteases (MMPs). MMPs are used by invasive cancer cells to hydrolyze the structural proteins that comprise the extracellular matrix such as collagen, elastin, laminin, fibronectin, vitronectin, and fibrinogen (Sternlicht and Werb, 2001). Host

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stromal cells also respond to nearby tumor cells by the induction of MMPs (Nelson et al., 2000). Immunohistological examination of human tumor sections revealed that MMPs are largely expressed by the stromal fibroblast and inflammatory cells recruited to the tumor (Heppner et al., 1996).

Here, we show that expression of PAR1 in breast carcinoma cells is sufficient to promote growth and invasion of xenografts in nude mice. We demonstrate that the presence of functional PAR1 accounts for the majority of the invasive signal in breast cancer cells. We identify the matrix metalloprotease, MMP-1, to be a novel protease agonist that cleaves PAR1 at the proper site for receptor activation and generates PAR1-dependent Ca^{2+} signals and migration. Targeting PAR1 with cell-penetrating pepducins can block the pathway downstream of MMP-1 and receptor, inhibiting angiogenesis and cancer growth and invasion.

Results

The Presence of Functional PAR1 Confers Increased Invasion in Breast Cancer Cells

Previous studies by our group (Kamath et al., 2001) and others (Even-Ram et al., 1998) established a correlation between PAR1 expression levels and basal migration of breast cancer cell lines toward conditioned media from NIH-3T3 fibroblasts. We first tested whether the presence of PAR1 is sufficient to confer migration and invasion of breast cancer cells that are normally noninvasive. A PAR1 null breast carcinoma cell line, MCF-7, which has a very low migration index (Kamath et al., 2001), was transfected with either vector alone (pcDEF3) or pcDEF3-PAR1. MCF-7 cells transiently expressing PAR1 exhibited a 3.6-fold increase in migration (Figure 1A) and a 4.3-fold increase in invasion (Figure 1B) through matrigel as compared to their pcDEF3-transfected counterparts. Because simple overexpression of GPCRs can promote constitutive activation of G protein signaling pathways in the absence of agonist, we tested whether a proteolytically dead PAR1 mutant receptor could also confer promigratory behavior to the MCF-7 cells. PAR1 was rendered nonactivatable upon proteolytic cleavage by mutating the tethered ligand residue Phe-43 to alanine. The F43A PAR1 mutant does not transduce a signal to internally located G protein following proteolytic cleavage of the R^{41}-S^{42} bond but fully activates G protein upon addition of exogenous peptide ligand such as SFLLRN or TFLLRN. As shown in Figures 1A and 1B, transfection of MCF-7 cells with F43A PAR1 promoted neither cell migration nor invasion, supporting the hypothesis that PAR1 must be able to be activated for PAR1-dependent migration and invasion to occur.

PAR1 Promotes Tumor Growth and Invasion in Nude Mice

Next, we tested whether the proinvasive phenotype conferred by PAR1 in MCF-7 cells under in vitro conditions could be extended to an in vivo tumor model system. MCF-7 cells are thought to represent an early breast cancer phenotype because they are estrogen-sensitive and require transfection with oncogenes or invasogenic factors to become tumorigenic in nude mice. PAR1 was stably transfected into MCF-7 cells and assessed for the ability to confer tumor growth and invasion in an orthotopic mammary fat pad model in nude mice. Stable MCF7-PAR1 clones were isolated and tested for PAR1 surface expression by FACS. Clone N55 was found to express high levels of PAR1, similar to MDA-MB-231 cells (Kamath et al., 2001), and migrated 16-fold and invaded 30-fold faster through matrigel relative to the parental MCF-7 cells (data not shown). MCF7-PAR1/N55 and the MCF-7 control (PAR1 null) parental cell line were injected into the left and right mammary fat pads, respectively, of female 6- to 7-week-old athymic NCR nu/nu mice (Figure 1C). Tumors developed only in mice injected with MCF7-PAR1/N55 (9/9), and there was no detectable tumor formation with MCF-7 cells (0/10) within 8 weeks (Figure 1D). MCF7-PAR1/N55 tumors grew exponentially in size ranging between 117–268 mm^3 at 6 weeks after injection.

Tumors were excised from the mammary pads 4–10 weeks postinoculation, and hematoxylin and eosin staining of the MCF7-PAR1/N55 breast tumors revealed extensive replacement of normal mammary tissue (see Supplemental Figure S1A in the supplemental data available with this article online). MCF7-PAR/N55 cells formed tumors that were tightly cohesive with poorly differentiated, pleomorphic ductal carcinoma morphology and no recognizable glandular pattern. By 4 weeks, the MCF7-PAR/N55 tumor cells had attached to the abdominal muscle layer. At the 6 week time period, MCF7-PAR1/N55 cells had invaded the adjacent structures including the mammary fat tissue and at 10 weeks had invaded throughout the peritoneal cavity and into the diaphragm (Supplemental Figure S1A). In contrast, there was no detectable tumor formation by the PAR1 null MCF-7 cells, and mammary pads had normal morphology consisting of adipocytes and breast ductules. These data clearly demonstrate that PAR1 can promote tumor growth and invasion in nude mice.

The highly tumorigenic MDA-MB-231 cell line (Kang et al., 2003), which constitutively expresses high levels of PAR1 (Kamath et al., 2001), was also injected into the murine mammary fat pads. Contrary to MCF-7, the MDA-MB-231 cells are estrogen receptor negative and provide a model for more aggressive, tamoxifen-insensitive, breast cancers. MDA-MB-231 cells also have enhanced expression of many genes that are associated with poor outcome in breast cancer patients (van't Veer et al., 2002) and are more likely to form metastases in nude mice models (Kang et al., 2003). Indeed, xenografts formed by MDA-MB-231 (10/10) grew more than 2-fold faster relative to the N55 cells and had already begun to invade into the abdominal muscle wall by week 6 (Figure 1D and Supplemental Figure S1A). We then tested whether knockdown of PAR1 gene expression would affect the mobility of the more invasive MDA-MB-231 breast cancer cells. MDA-MB-231 cells were treated for 48 hr with PAR1 siRNA, then PAR1 surface expression was assessed by FACS. As shown in Figure 1E, treatment of MDA-MB-231 cells with PAR1 siRNA caused an 80% decrease in median surface expression of PAR1 relative to control (luciferase) siRNA-treated cells. The PAR1 siRNA treatment did not perturb surface expression of the PAR4 receptor present

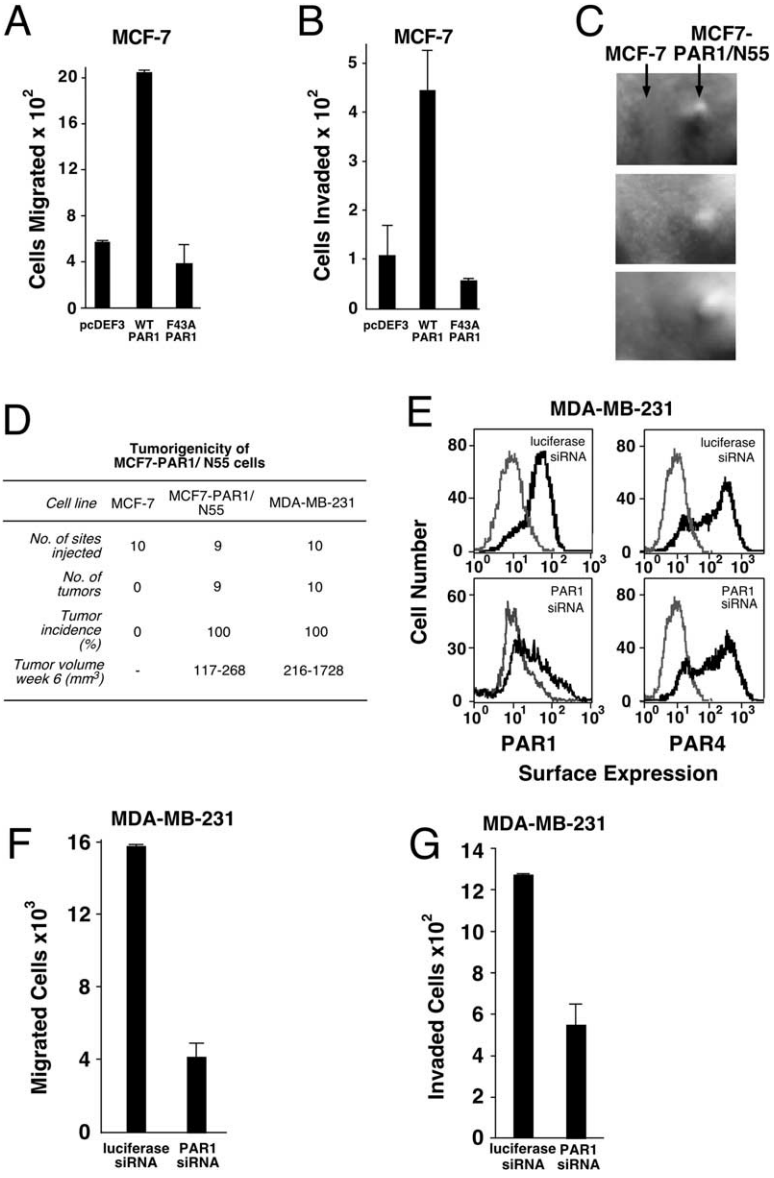

Figure 1. PAR1 Is an Invasogenic Factor in Breast Cancer Cells

(A) Migration of MCF-7 cells transiently transfected with either wt PAR1, a PAR1 mutant (F43A) with a deficient tethered ligand, or vector alone (pcDEF3). MCF-7 cells (50,000) were seeded onto an 8 μm pore membrane in the upper well of a transwell apparatus and allowed to migrate for 5 hr toward conditioned media from NIH-3T3 fibroblasts. Cells that migrated to the lower side of the membrane were counted as described in the Experimental Procedures.

(B) Invasion of transiently transfected MCF-7 cells through 10 μg matrigel per well using NIH-3T3 medium as the chemoattractant source in the bottom well.

(C) Tumor formation of MCF-7 cells (PAR1 null) versus MCF-7 cells stably expressing PAR1 (MCF7-PAR1/N55) inoculated into mammary fat pads of NCR nu/nu mice. Representative tumors shown are from the fourth week postinoculation.

(D) Tumorigenicity of MCF7-PAR1/N55 and MDA-MB-231 breast cancer xenografts.

(E) Silencing of PAR1 expression in MDA-MB-231 cells with PAR1 siRNA versus firefly luciferase siRNA control. Surface expression of PAR1 and PAR4 after 48 hr treatment with siRNA was determined by flow cytometry. Gray traces: secondary antibody alone, Black traces: primary plus secondary antibodies.

(F) Effect of PAR1 siRNA transfection (48 hr) on migration of MDA-MB-231 cells (50,000) toward NIH-3T3 media.

(G) Effect of PAR1 siRNA transfection (48 hr) on invasion of MDA-MB-231 cells (25,000) through matrigel.

on MDA-MB-231 breast cancer cells. Next, we determined the effect of loss of PAR1 expression on the ability of the MDA-MB-231 cells to migrate and invade toward NIH-3T3 medium. As shown in Figures 1F and 1G, the PAR1-siRNA-treated MDA-MB-231 cells migrated 70% less and invaded 60% less through matrigel relative to the control siRNA-transfected counterparts. This 60%–70% loss in migration and invasion as a consequence of the 80% inhibition of PAR1 surface expression suggests that PAR1 may be responsible for the majority of the migratory ability of the MDA-MB-231 cells toward NIH-3T3 media. To provide evidence that the decreased migration response following PAR1 gene silencing is specific to PAR1-mediated events, we tested the effect of PAR1 siRNA treatment on migration toward IL-8, a chemotactic ligand for the CXCR1/2 receptors on MDA-MB-231 cells (Muller et al., 2001). As expected, migration of the MDA-MB-231 cells toward IL-8 was unaffected by the siRNA knockdown of PAR1 relative to untreated control cells (Supplemental Figure S1B).

Matrix Metalloproteases from Fibroblast Media Are Responsible for the Majority of Migration of MDA-MB-231 Breast Cancer Cells

We next sought to determine whether a protease was in fact activating PAR1 and if so, determine the origin of the protease. To address these questions, conditioned medium was prepared from MDA-MB-231 breast cancer cells, NIH-3T3 (murine) immortalized fibroblasts, or CRL-2076 (human) primary fibroblasts. Interestingly, MDA-MB-231 cells migrated about half as well toward their own media as compared to media from the fibroblasts (Figure 2). A panel of protease inhibitors was then assessed for ability to block migration of the MDA-MB-231 cells. Hirudin, a specific thrombin inhibitor, did

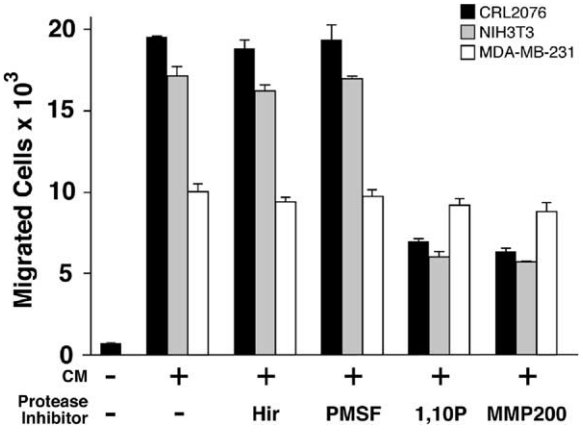

Figure 2. Fibroblast-Derived MMPs Induce Breast Cancer Cell Migration

Migration of MDA-MB-231 breast cancer cells toward either RPMI-1640 (–) or conditioned medium (CM) prepared from primary human CRL-2076 fibroblasts, NIH-3T3 fibroblasts, or MDA-MB-231 cells. The CM was supplemented with the protease inhibitors hirudin (0.015 U), PMSF (100 μM), 1,10-phenanthroline (100 μM + 250 μM $CaCl_2$), MMP-200 (200 nM), or vehicle alone (0.2% DMSO).

not inhibit migration toward any of the media, consistent with previous observations (Kamath et al., 2001). Likewise, addition of the serine protease inhibitor, PMSF, to the media in the bottom well of the chemotactic chambers had no effect on mobility of the MDA-MB-231 cells. These results militate against the direct or indirect participation of serine proteases or thrombin in the migration of the breast cancer cells toward fibroblast or MDA-MB-231 media.

We then turned our attention to another class of proteases which are highly abundant in tumors—namely the Zn^{2+}-dependent matrix metalloproteases. Two broad-spectrum inhibitors of MMPs were employed: 1,10 phenanthroline, a divalent cation-chelating agent, and MMP-200, a hydroxyamate inhibitor of MMPs. Strikingly, the MMP inhibitors reduced the migration of MDA-MB-231 cells toward the fibroblast-derived media by two-thirds (Figure 2). In contrast, the MMP inhibitors did not significantly reduce the migration of MDA-MB-231 cells toward their own medium. Therefore, our data indicate that the MMPs derived from the MDA-MB-231 cancer cells themselves are not sufficient for cell migration. In these in vitro assays, PAR1-dependent migration does not require proteolysis of extracellular matrix; therefore, MMP involvement cannot be attributed to matrix digestion but can be assigned to induction of promigratory signaling pathways in the cells. We hypothesized that MMPs secreted from the fibroblasts were responsible for PAR1 activation and breast cancer cell migration.

PAR1 Activation Induces Promigratory Signaling in MDA-MB-231 Breast Cancer Cells

The MDA-MB-231 cells were treated with a panel of PAR1 antagonists in order to correlate the effects of PAR1 siRNA knockdown with pharmacologic blockade of PAR1. The BMS-200261 and RWJ-56110 compounds are small molecule ligand-based antagonists (Bernatowicz et al., 1996; Andrade-Gordon et al., 1999) that block intramolecular activation of PAR1 by proteolytically generated tethered ligand (Seeley et al., 2003). As shown in Figure 3A, the RWJ-56110 and BMS-200261 compounds blocked up to 57% of the migration of MDA-MB-231 cells toward fibroblast media.

We also determined whether cell-penetrating pepducin antagonists of PAR1 (Covic et al., 2002a; Covic et al., 2002b) could inhibit migration of the MDA-MB-231 cells. The P1pal-12 pepducin is a palmitoylated peptide based on the N-terminal portion of the third intracellular loop (i3) of PAR1 that can interdict signaling between PAR1 and intracellular G proteins. In addition, we tested a C-terminal i3 loop pepducin, P1pal-7, which is a full antagonist of PAR1-G protein signaling (Supplemental Figure S2). As shown in Figure 3A, the P1pal-12 and P1pal-7 pepducins gave similar inhibitory effects as the extracellular PAR1 antagonists RWJ-56110 and BMS-200261 and blocked approximately half of the migration of MDA-MB-231 cells toward fibroblast media.

To further demonstrate that proteolytic activation of PAR1 is capable of stimulating migration of the MDA-MB-231 breast cancer cell line, we inhibited the endogenous MMPs present in the media with MMP-200 and added low concentrations of exogenous thrombin. If it is true that proteolytic activation of PAR1 by an MMP present in the media induces migration, then preventing MMP cleavage of PAR1 should allow activation of PAR1 by exogenous thrombin and recovery of full migration. Indeed, we showed that low concentrations of exogenous thrombin (10–500 pM) can induce full migration of the MDA-MB-231 breast cancer cells. Thrombin induction of MDA-MB-231 cell motility was completely blocked by the BMS-200261 PAR1 antagonist (Figure 3B), thereby demonstrating that the effect of thrombin on the MDA-MB-231 cells was PAR1 dependent. Together, these data suggest that PAR1 activation induced by MMP(s) present in the fibroblast media can be mimicked by exogenous thrombin to cause a major increase in migration of breast cancer cells.

Ability of Individual MMPs to Affect Migration of MDA-MB-231 Breast Cancer Cells

Pure MMPs were tested individually to determine their effects on migration of MDA-MB-231 breast cancer cells. Commercially available pro-MMPs were first activated with aminophenylmercuric acetate (APMA) and then dialyzed to remove the cytotoxic mercury compound. The active form of the MMP was then added to MDA-MB-231 medium placed in the lower well of the chemotactic chamber. Of the MMPs that were tested (MMP-1, -2, -3, -7, and -9), only MMP-1 was able to induce an increase in migration that was nearly equivalent to full migration toward fibroblast-derived media (Figure 4A). Addition of 5 nM MMP-1 to RPMI-1640 serum-free media in the bottom well also induced a significant increase in migration which was completely blocked by the PAR1 antagonist BMS-200261 (Figure 4B).

We knocked down gene expression of PAR1 with siRNA and confirmed that MMP-1 could not act as a chemotactic agent for MDA-MB-231 cells in the ab-

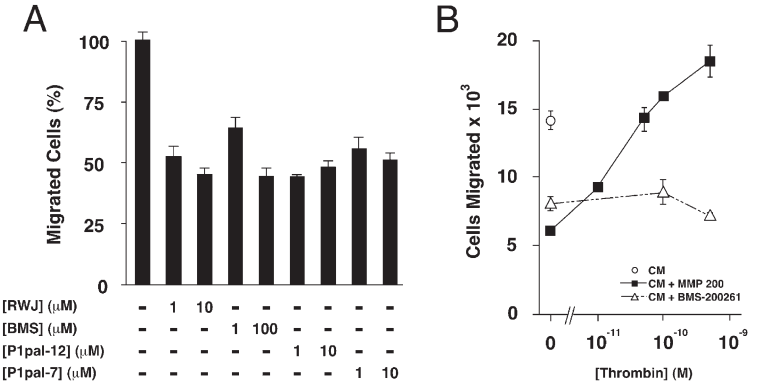

Figure 3. Effect of Inhibition or Stimulation of PAR1 on Migration of MDA-MB-231 Cells

(A) MDA-MB-231 cells migration toward NIH-3T3 CM supplemented with PAR1 antagonists based on either the extracellular ligand (RWJ-56110, BMS-200261) or cell-penetrating pepducins derived from the third intracellular loop of PAR1 (P1pal-12, P1pal-7).

(B) MDA-MB-231 cells migration toward NIH-3T3 CM supplemented with 0–500 pM thrombin in the presence of either MMP-200 (200 nM), BMS-200261 (100 μM), or 0.2% DMSO vehicle (open circle).

sence of PAR1. As shown in Figure 4C, pretreatment of MDA-MB-231 cells with PAR1 siRNA completely blocked MMP1-induced chemotaxis of MDA-MB-231 cells, independently demonstrating that the chemotactic effects of MMP-1 require PAR1. In the converse experiment, we immunodepleted individual MMPs from human CRL-2076 fibroblast media with the corresponding anti-human MMP antibodies coupled to beads. As shown in Figure 4D, immunodepletion of MMP-1 from CRL-2076 media caused a 45% drop in cell migration which accounted for all of the MMP-200-sensitive activity. Treatment of the CRL-2076 media with antibodies directed against MMP-2 or MMP-9 gave slight drops in cell migration. Simultaneous immunodepletion of MMP-1 and MMP-2 gave the same drop in chemotaxis as immunodepletion of MMP-1 alone. No effects were seen upon treatment with anti-MMP-3 or -7 antibodies.

The effect of selective pharmacologic blockade of MMP-1 on cell migration toward NIH-3T3 media was also tested. Three different MMP inhibitors with varying selectivity to MMP-1, MMP-2, and MMP-3 were added to the media in the bottom well. The tetrapeptidyl hydroxamic acid, MMP Inh-1 (FN-439), which targets MMP-1 but not MMP-2 or MMP-3 (Odake et al., 1994), blocked 75% of migration. In contrast, MMP2-Inh (blocks MMP-2) and MMP2/3-Inh (blocks MMP-2 and MMP-3) had no effect on migration of MDA-MB-231 toward NIH-3T3 media. Together, these data demonstrate

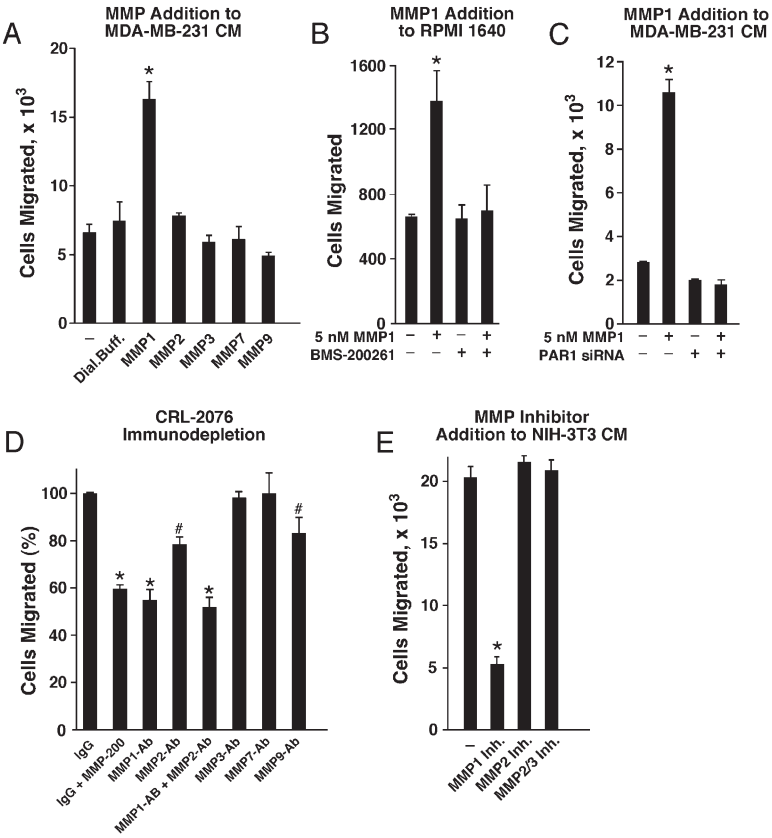

Figure 4. Fibroblast MMP-1 Mediates PAR1-Dependent Migration of Breast Cancer Cells

(A) MDA-MB-231 cell migration toward MDA-MB-231 CM in the presence of 5 nM of APMA-activated MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, or dialysis buffer alone.

(B) MDA-MB-231 migration toward RPMI-1640/0.1% BSA in the presence or absence of MMP-1 and/or the PAR1 antagonist BMS-200261.

(C) MDA-MB-231 cells were treated for 48 hr with PAR1 siRNA or luciferase siRNA control (–) and allowed to migrate toward MDA-MB-231 CM in the presence or absence of MMP-1.

(D) MDA-MB-231 cell migration toward CRL-2076 CM that was immunodepleted for individual MMPs. Monoclonal antibodies against the indicated MMPs or IgG control were coupled to Protein A Sepharose beads and used to immunodeplete CRL-2076 CM. The immunodepleted media served as chemoattractant in a standard migration assay.

(E) MDA-MB-231 cell migration toward NIH-3T3 CM that was supplemented with selective MMP inhibitors used at concentrations $\geq$3-fold above relevant IC_{50}: 3 μM MMP Inh-1 (IC_{50} = 1 μM [MMP-1], 30 μM [MMP-3], 1 μM [MMP-8], 150 μM [MMP-9]), 5 μM MMP2-Inh (IC_{50} = 1.7 μM [MMP-2]), 50 μM MMP2/3-Inh (IC_{50} = 17 μM [MMP-2], 150 nM [MMP-3]). # $p < 0.05$, * $p < 0.01$.

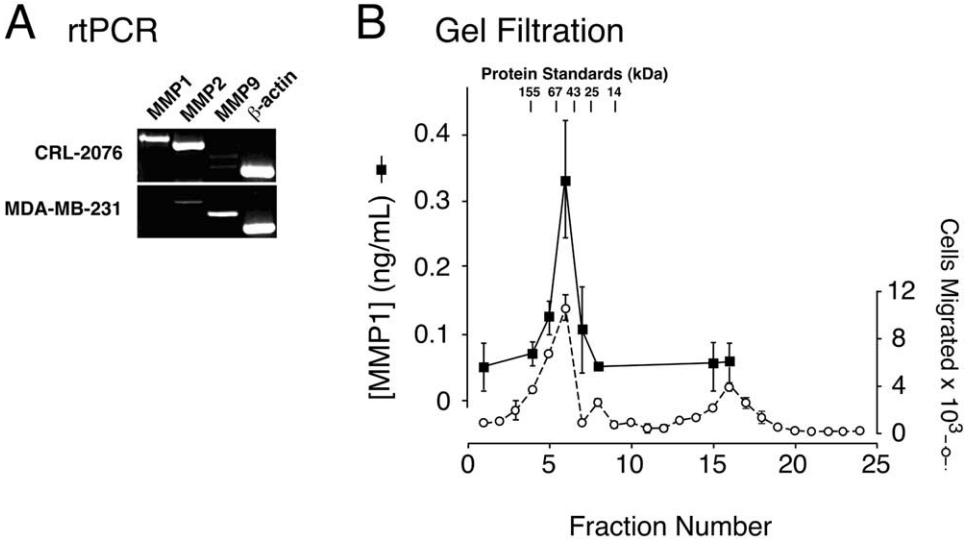

Figure 5. Primary Human CRL-2076 Fibroblasts Secrete MMP-1 into Their Media

(A) CRL-2076 fibroblasts but not MDA-MB-231 breast cancer cells express *MMP-1* mRNA. Total RNA was extracted from CRL-2076 or MDA-MB-231 cells. cDNA was prepared and portions of the cDNA corresponding to *MMP-1*, *MMP-2*, *MMP-9* or *β-actin* messages were amplified by reverse transcriptase PCR and separated on a 1.5% agarose gel.

(B) Serum-free CRL-2076 CM was concentrated 20-fold and 200 μl applied onto a Superose-12 column equilibrated with TBS at 4°C. MMP-1 concentration (mean ± 1 SD; n = 3) was determined in 1 ml fractions by Quantikine ELISA (R&D Systems, Minneapolis). Chemotactic activity of each fraction (dashed line) was determined in duplicate using a standard MDA-MB-231 cell migration assay. This fractionation experiment was repeated three times and gave similar results.

that MMP-1 is capable of inducing PAR1-dependent migration of MDA-MB-231 breast cancer cells and may be responsible for most of the MMP chemotactic activity in the fibroblast media.

Identification of MMP-1 in Media from CRL-2076 Fibroblasts

Using RT-PCR, we confirmed that CRL-2076 fibroblasts express mRNA encoding MMP-1 (Figure 5A). CRL-2076 cells also express mRNA for MMP-2 and very low levels of MMP-9 mRNA. ELISA and collagenase analyses indicated that MMP-1 levels were 34 ± 8 ng/ml (0.6 ± 0.2 nM) and MMP-2 levels were 22 ± 9 ng/ml (0.3 ± 0.1 nM) in the CRL-2076 fibroblast media. Similarly, NIH-3T3 media contained 0.6–1.0 nM MMP-1 collagenase activity. As expected, MDA-MB-231 cells do not have mRNA encoding MMP-1 but do express low levels of MMP-2 mRNA and higher levels of MMP-9 mRNA. The MDA-MB-231 cells did not secrete detectable levels (<0.1 ng/ml) of pro- or active forms of MMP-1 or MMP-2. Together, these data are consistent with other reports which show that MDA-MB-231 cells do not normally express MMP-1 or MMP-2 (Kang et al., 2003).

Extensive studies have shown that breast cancer cells can also migrate toward a variety of small chemokines (0.5–10 kDa) which are abundant in tumor specimens (Youngs et al., 1997; Muller et al., 2001). We next sought to separate the MMP chemotactic activity from the other chemokines potentially secreted by the fibroblasts. Conditioned medium containing fetal bovine serum proved to be exceedingly difficult to fractionate due to the high concentration of albumin. Therefore, we prepared medium from the CRL-2076 fibroblasts under serum-free culture conditions. This serum-free media possessed 85% of the chemoattractive activity of the original CRL-2076 medium for MDA-MB-231 cells. The serum-free CRL-2076 medium was fractionated by size over a Superose-12 gel-filtration column. Individual fractions were assayed for ability to promote migration of the MDA-MB-231 cells. We found that the promigratory activity separated into a major peak (fractions 3–6) and a minor peak (fractions 13–18) (Figure 5B). The chemotactic activity of fractions 3–6 peaked at a relative molecular mass of 55 kDa and together accounted for 55% of the total chemoattractive activity of the loaded material and was blocked by the MMP inhibitor MMP-200 (data not shown). ELISA assays were conducted in order to confirm that MMP-1 was present in the higher molecular mass peak of chemotactic activity. As shown in Figure 5B, MMP-1 eluted at fraction 6, which contained the highest level of chemotactic activity. The small-molecular-mass (<10 kDa) fractions 13–18 contributed to 34% of the total chemotactic activity—in close agreement with the residual chemotactic activity left after treatment of CRL-2076 media with MMP-200 (Figure 2). Thus, the chemotactic activity in fractions 13–18 likely represents small chemokines secreted by the fibroblasts, though this remains to be determined.

MMP-1 Cleaves PAR1 at the Proper Site for Receptor Activation and Generates PAR1-Dependent Ca^{2+} Signals in Breast Cancer Cells

PAR1 is activated by proteolytic cleavage between residues R^{41} and S^{42} which creates a new N terminus, S^{42}FLLRN, that acts as a tethered ligand (Vu et al., 1991; Seeley et al., 2003). To demonstrate that MMP-1 or other MMPs can directly cleave PAR1 at this site, we placed a T7 epitope to the N-terminal side of the

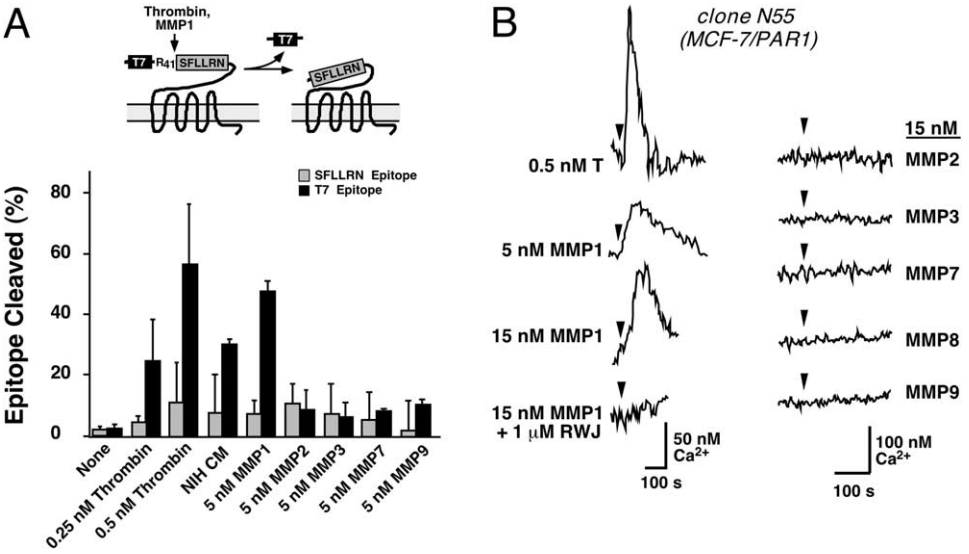

Figure 6. MMP-1 Directly Cleaves PAR1 and Generates PAR1-Dependent Ca^{2+} Signals in Breast Cancer Cells

(A) COS7 cells were transiently transfected with T7-tagged PAR1 incubated with thrombin (T), NIH-3T3 media, or the indicated matrix metalloproteases for 30 min at 37°C. Loss of T7 and SFLLRN epitopes over time (mean ± 1 SD; n = 3) were analyzed by flow cytometry as described (Kuliopulos et al., 1999).

(B) Calcium-flux measurements of MCF7-PAR1/N55 cells following challenge with MMPs or thrombin (T) were performed at 25°C with emission recorded at 510 nm with dual excitation at 340 and 380 nm as described (Kuliopulos et al., 1999).

R^{41}-S^{42} site and expressed the T7-tagged PAR1 in COS7 cells. Cleavage of PAR1 at the R^{41}-S^{42} peptide bond by the MMP would result in loss of the T7 epitope but retention of the SFLLRN epitope (Figure 6A). Indeed, we demonstrated that treatment of the COS7 cells with 5 nM MMP-1 resulted in 50% loss of the T7-epitope while leaving the SFLLRN epitope intact. Similarly, treatment of the T7-PAR1 COS7 cells with 0.5 nM thrombin caused 55% loss of the T7 epitope with retention of the SFLLRN epitope. Treatment with NIH-3T3 media caused 30% loss of the T7 epitope, similar to 0.25 nM thrombin. In contrast, 5 nM concentrations of MMP-2, MMP-3, MMP-7, and MMP-9 gave little cleavage of COS7-expressed T7-PAR1 either to the N- or C-terminal side of the SFLLRN epitope (Figure 6A). These cleavage experiments demonstrate that PAR1 is cleaved at the appropriate site by MMP-1 but is not readily cleaved by MMP-2, -3, -7, or -9.

To directly elicit a functional MMP-1-dependent PAR1 response on the surface of breast cancer cells, we conducted Ca^{2+} flux measurements of the MCF7-PAR1/N55 cells. Addition of 5 nM MMP-1 to N55 cells gave 50% of the Ca^{2+} peak height relative to 0.5 nM thrombin but gave a similar total integrated (area) Ca^{2+} response (Figure 6B). Challenge of the N55 cells with 15 nM MMP-1 gave a robust Ca^{2+} response that was completely blocked by the PAR1-selective antagonist RWJ-56110. None of the other MMPs that were tested (15 nM MMP-2, MMP-3, MMP-7, MMP-8, or MMP-9) were able to elicit a Ca^{2+} signal from the N55 cells.

The Therapeutic Potential of Targeting PAR1 versus MMP-1 on the Growth and Angiogenesis of Breast Tumors

We have demonstrated that the presence of PAR1 promotes tumor growth and invasion; however, we have not yet shown that MMP-1 is also necessary for the tumorigenicity of PAR1-expressing breast cancer cells in vivo. To examine the contribution of MMP-1 to the tumorigenicity of the MCF7-PAR1/N55 cells, we first determined whether the MMP-1 levels were elevated in the N55 tumors relative to control mammary pads. N55 tumors were harvested from untreated mice at week 6 and examined for *MMP-1* mRNA expression and collagenase activity. As shown in Figure 7A, murine *MMP-1* mRNA was induced 2-fold relative to control mammary fat pads. Collagenase activity was enhanced 4-fold in the N55 tumors relative to mammary fat pads (Figure 7B). The collagenase activity in the N55 tumors was inhibited by 50% upon addition of the selective MMP-1 antagonist, MMP Inh-1 (Odake et al., 1994), with no effect on the collagenase activity in the control fat pads, which demonstrates that *MMP-1* expression and MMP-1 collagenase activity are enhanced in the PAR1-expressing N55 breast tumors.

To provide direct evidence that MMP-1 was essential for the tumorigenicity of the PAR1-dependent tumors, we compared the effects of pharmacologic blockade of PAR1 versus MMP-1 on the growth of the N55 breast cancer xenografts. To block PAR1, we used the cell-penetrating i3 loop PAR1 pepducin antagonist, P1pal-7. MCF7-PAR1/N55 cells were injected into the mammary fat pads and the mice treated two days later (to allow implantation) with either vehicle or P1pal-7 (10 mg/kg s.c. every other day) for 6 weeks. As shown Figure 7C, by the 6 week time point, P1pal-7 significantly inhibited (62%) the growth of the MCF7-PAR1/N55 xenografts. The P1pal-7 pepducin treatment was well tolerated by the mice with no observable toxicity. We then treated the mice with MMP Inh-1 for 6 weeks and measured tumor growth. Quite strikingly, MMP Inh-1 treatment of the N55 mice gave 82% inhibition of tumor growth (Fig-

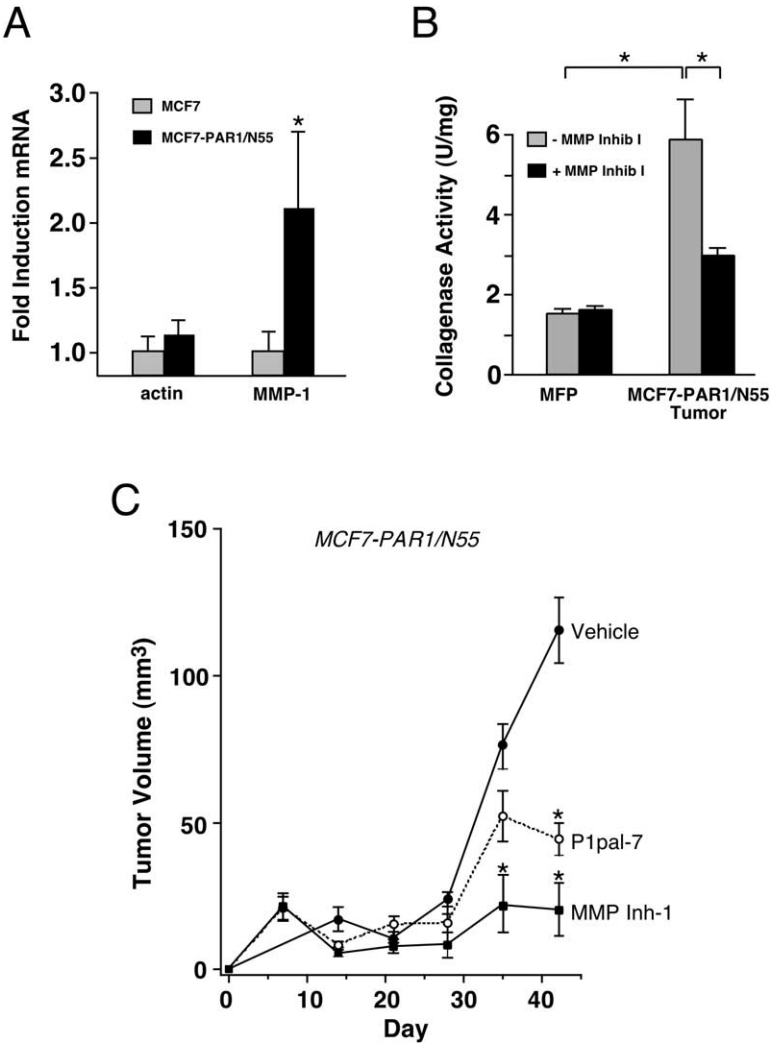

Figure 7. Treatment of Nude Mice with a Cell-Penetrating Pepducin Targeted against PAR1 or an MMP-1 Antagonist Inhibits Growth of Breast Cancer Xenografts

(A) MCF7-PAR1/N55 or MCF-7 cells (4×10^6) were inoculated in the mammary fat pads of NCR nu/nu mice and harvested at 6 weeks. N55 breast tumors or MCF-7 control mammary fat pads were flash frozen in liquid N_2 and tissue disrupted as described in the Experimental Procedures. PCR primers were used to amplify the murine *MMP1A* (Mcol-A) transcript or *actin* control (mean ± 2 SEM; n = 3).

(B) Collagenase activity of the MCF7-PAR1/N55 xenografts or control mammary fat pads (MFP) harvested at 6 weeks was conducted as described in the Experimental Procedures. Collagenase activity was determined in the presence or absence of 5 μM MMP Inh-1 (mean ± 2 SEM; n = 3).

(C) MCF7-PAR1/N55 cells (4×10^6) were inoculated into the mammary fat pads of NCR nu/nu mice. Two days later, treatments (100 μl subcutaneously, every other day) were initiated: (1) vehicle alone (20% DMSO), (2) 10 mg/kg P1pal-7, or (3) 5 mg/kg MMP Inh-1. Tumor volume was measured once weekly using a digital caliper. The median tumor size ± 1 SD, n = 10 breast tumors, is indicated for each data point. * $p < 0.01$.

ure 7C) as compared to untreated control. In in vitro assays, MMP Inh-1 did not nonspecifically block thrombin activity (data not shown). Therefore, these in vivo data provide functional support for the involvement of MMP-1 in the tumorigenesis of human breast cancer cells that express PAR1. These data also indicate that thrombin does not dominate the system and is not able to complement the defect of MMP-1 in the MMP Inh1-treated mice. It still remains possible that MMP-1 plays other roles beyond cleavage and activation of PAR1 in these tumors.

Lastly, we compared the morphology, invasive edge, and degree of vascularization of the untreated (vehicle) versus P1pal-7- and MMP Inh-1-treated N55 breast tumors harvested at the end of the experiment of Figure 7C. Hematoxylin and eosin staining showed that the morphology of the N55 tumors from all three groups was similar to poorly differentiated invasive ductal carcinoma of human breast specimens. The xenografts from the P1pal-7 and MMP Inh-1 treatment groups gave a very similar infiltrating pattern of tumor growth into the adjacent skeletal muscle (Supplemental Figure S3). Immunohistochemical staining for endothelial cells revealed prominent von Willebrand Factor-positive vascular structures within the central tumor mass of the untreated group. In contrast, significantly less vascularity was present in the Plpal-7- and MMP Inh-1-treated groups (Supplemental Figure S3). Therefore, P1pal-7 and MMP Inh-1 treatment have the same effects on tumor morphology, invasion, and vascularity. Furthermore, these results are consistent with the proposed role of PAR1 in angiogenesis (Griffin et al., 2001; Yin et al., 2003; Caunt et al., 2003) and provide evidence that MMP-1 may also promote angiogenesis by activating PAR1.

Discussion

The present study made the unanticipated discovery that PAR1 can be directly activated by the interstitial collagenase MMP-1. Although several serine proteases have been identified as PAR agonists, this is the first report that a PAR can be activated by a member of the zinc-dependent matrix metalloprotease family. PAR1 activation by MMP-1 provides a link between extracellular proteolytic activity important for remodeling of the

extracellular matrix with signaling leading to cell migration and invasion.

We also showed that PAR1 is a potent tumorigenic and invasogenic factor for human MCF-7 breast cancer cells in an orthotopic nude mouse model. PAR1 expression has been directly correlated with degree of invasiveness in both primary breast tissue specimens (Even-Ram et al., 1998) and in established cancer cell lines from humans (Nierodzik et al., 1998; Ross et al., 2000). Similarly, MMP-1 has been shown to be a marker of poor prognosis in breast, colorectal, and esophageal tumors (Murray et al., 1998; van't Veer et al., 2002).

As a new example of tumor-host interdependence, we showed that the MMP-1 that was cleaving PAR1 was not produced by the breast cancer cells themselves but instead was secreted by the fibroblasts. Indeed, overwhelming evidence suggests that MMPs necessary for the invasion of cancer cells originate mainly from stromal fibroblasts, myofibroblasts, inflammatory cells, and endothelium (Egeblad and Werb, 2002). Immunohistological examination of human tumor sections revealed that MMP-1 and other MMPs are expressed by the stromal cells recruited to the tumor (Heppner et al., 1996; Nelson et al., 2000). The tumor cells secrete several factors including interleukins, cytokines, and angiogenesis factors, to induce the host stromal cells to produce MMPs (Zucker et al., 1998; Nelson et al., 2000, Sternlicht and Werb, 2001). Conversion of fibroblast-derived pro-MMP-1 to active MMP-1 by membrane-tethered or membrane-associated MMPs may present high local concentrations of MMP-1 to PAR1 on the same tumor cell surface.

We showed that the endogenous MMP-1 activity generated in situ from the fibroblast media cleaves PAR1 and is sufficient to cause robust migration and invasion of breast cancer cells via PAR1. Indeed, MMP-1 cleavage of PAR1 compares favorably with factor Xa (Camerer et al., 2000), plasmin (Kuliopulos et al., 1999), and activated protein C (APC) (Riewald et al., 2002) activation of PAR1 relative to thrombin. To our knowledge, we have provided the first demonstration of direct activation of a G protein-coupled receptor by an MMP.

A biological rationale for our results is that the breast cancer cells first utilize fibroblast-derived MMP-1 to activate PAR-1 which promotes cell migration toward the stroma and MMP-1 source. MMPs derived from the cancer cell itself or adherent to the cancer cell may help to catalyze activation of pro-MMP-1 and digest the ECM at the invasion front and together with the MMP1-PAR1 chemotactic signal allow the cell to invade into the surrounding stromal tissues. During the later vascular invasive and metastatic stage when the cancer cells acquire the ability to enter the blood stream, PAR1 may be activated by the TF-thrombin system and help mediate adhesion to the endothelium and underlying matrix at distant metastatic sites, perhaps with the assistance of adherent platelets (Nierodzik et al., 1992; Fischer et al., 1995). It is possible that MMP-1 may also play a role in the later metastatic phase of breast cancer progression. Interestingly, Kang et al. (2003) showed that bone metastases originating from MDA-MB-231 fat pad xenografts have enhanced MMP-1 expression. Therefore, depending on the relative local concentrations of MMP-1 and thrombin and the stage of tumor progression, either protease could activate PAR1-dependent signaling to cause the cancer cell to migrate, invade, or adhere.

Another area where MMPs and PARs have both been shown to play important roles is in inflammation and atherosclerosis (Nelken et al., 1992; Libby and Aikawa, 2002). Increased expression of MMP-1 and other MMPs occurs in the vulnerable shoulder and cap regions of atherosclerotic plaques (Libby and Aikawa, 2002). MMP-1, which is expressed in the macrophages, vascular smooth muscle cells, and endothelium of atheromatous plaques, is the most abundant MMP present that can cleave the native collagen types I and III which comprise the major tensile components of the fibrous plaque cap (Libby and Aikawa, 2002). Therefore, it has been suggested that MMP-1 may be partly responsible for plaque rupture. Stimulation of PAR1 in atherosclerotic plaques has also been implicated in smooth muscle cell proliferation and restenosis and exaggerated vasoconstrictory response (Nelken et al., 1992; Ku and Dai, 1997). It has been assumed that thrombin is the agonist that is activating PAR1-dependent proinflammatory and proliferative signaling within the atherosclerotic plaque; perhaps MMP-1 may contribute to PAR1 activation in the inflammatory lesion.

Finally, our results, together with the foregoing studies, suggest that therapeutics that block MMP-1 may prove beneficial in the treatment of a variety of invasive, proliferative, and inflammatory conditions. However, numerous phase III clinical trials with broad-spectrum MMP inhibitors for treatment of diverse cancers have suffered from dose-limiting joint toxicity thought to be due to inhibition of MMP-1 (Nelson et al., 2000; Yamamoto et al., 2002). Hence, PAR1, a new receptor for MMP-1, becomes an attractive target as a novel therapeutic approach, as exemplified by the PAR1 pepducin P1pal-7, for blocking the progression and angiogenesis of invasive and metastatic cancers and in inflammatory conditions when it is not possible to directly inhibit MMP-1.

Experimental Procedures

Reagents

Recombinant hirudin and purified human MMPs were obtained through EMD Biosciences (San Diego, California). MMP-200 was from Enzyme Systems Products (Livermore, California). MMP Inh-I, MMP2-Inh, and MMP2/3-Inh were from Calbiochem (La Jolla, California). BMS-200261 was synthesized by AnaSpec (San Jose, California). N-palmitoylated peptides P1pal-12 and P1pal-7 were synthesized by standard fmoc solid phase synthetic methods with C-terminal amides as before (Covic et al., 2002a). Rabbit polyclonal PAR1-Ab and PAR4-Ab were purified by peptide affinity chromatography as described previously (Kuliopulos et al., 1999).

Molecular Biology

Construction of the genes encoding human PAR1 have been described (Kuliopulos et al., 1999; Jacques and Kuliopulos, 2003). A PAR1 mutant with a deficient tethered ligand, F43A, was constructed as described (Chen et al., 1994). All PAR1 constructs were inserted into the mammalian expression vector pcDEF3, which places PAR1 under the control of a human EF-1a promoter and carries a neomycin selectable marker. Small interfering (si)-RNA directed against PAR1 (5′-AAGGCUACUAUGCCUACUACU-3′), and firefly luciferase (5′-CGTACGCGGAATACTTCGA-3′) were synthesized by Dharmacon (Lafayette, Colorado). For rt-PCR, total RNA was extracted from MDA-MB-231 and CRL-2076 cells with the

Rneasy mini kit (Qiagen, Valencia, California). cDNA was prepared with 5 μg of total RNA using MMLV reverse transcriptase. The desired products were amplified with Taq polymerase and the following primers: hMMP-1 sense 5′-CGACTCTAGAAACACAAGA GCAAGA-3′, antisense 5′-AAGGTTAGCTTACTGTCACA CGCTT-3′; hMMP-2 sense 5′-GTGCTGAAGGACACACTAAAGAAGA-3′, antisense 5′-TTGCCATCCTTCTCAAAGTTGTAGG-3′; hMMP-9 sense 5′-CACTGTCCACCCCTCAGAGC-3′, antisense 5′-GCCACTTGTCG GCGATAAGG-3′; mMMP-1 (Mcol-A) sense 5′-TCTTTATGGTCCA GGCGATGAA-3′, antisense 5′-CCTCTTCTATGAGGCGGGGATA-3′.

Cell Culture

MDA-MB-231 and MCF-7 cells (human breast carcinomas) were obtained from the NCI (Frederick, Maryland). NIH-3T3 and CRL-2076 fibroblasts were from the ATCC (Manassas, Virginia). MCF-7 cells were transfected using LipofectAMINE, and 20 μg of DNA were used for each 100 mm tissue culture dish. MCF-7 cells stably expressing wt PAR1 such as clone N55 (MCF7-PAR1/N55) were first selected with 750 μg/ml geneticin for one week and then retrieved with magnetic Dynabeads (M450) coupled with the PAR1-Ab. MDA-MB-231 cells were transfected with OligofectAMINE using 20 μM siRNA oligo per 100 mm plate.

Conditioned Media Preparation and Migration and Invasion Assays

MDA-MB-231, NIH-3T3, or CRL-2076 cells were growth to 50% confluency. The media was then changed to fresh growth media and cells were incubated for an additional 48 hr. The conditioned media was drawn off the cells and centrifuged at 25°C for 5 min at 200 × g to remove debris and stored at −20°C. Invasion of cells through matrigel was conducted using a Transwell apparatus (Corning Costar) as described previously (Kamath et al., 2001). Data is expressed as mean number of cells migrating ±1 SEM (n = 2–4).

Orthotopic Mammary Fat Pad Model in Nude Mice

These experiments were conducted in full compliance with the Tufts-NEMC Institutional Animal Care and Use Committee. Groups of five NCR nu/nu mice (Taconic Farms) each received two mammary fat pad inoculations consisting of 4×10^6 MCF-7 and 4×10^6 MCF7-PAR1/N55 human breast cancer cells. Tumors and surrounding tissue were excised from sacrificed mice, fixed in 10% formalin/PBS, and imbedded in paraffin. Tissue sections (5 μm) were prepared and stained with hematoxylin-eosin.

MMP-1 Expression and Collagenase Activity

Breast tumor xenografts or control mammary fat pads were excised from nude mice, and tissue samples were lysed by freezing the tissue in liquid nitrogen, grinding the frozen tissue to a powder, and adding the powder to lysis buffer. The lysis buffer consisted of RNeasy lysis buffer (Qiagen) for RT-PCR and 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% Triton X-100, 100 μM PMSF, 100 mM dithiothreitol, and 10 μg/ml leupeptin for the collagenase assay. Collagenase activity was assayed in lysed tumor samples and in fibroblast media by measuring the cleavage of fluorescein conjugated DQ collagen (Molecular Probes). Collagenase assays contained 10 μg DQ collagen in 50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM $CaCl_2$, and 0.2 mM NaN_3, and cleavage was monitored continuously over time by a fluorescence microplate reader at 538 nm (excitation at 485 nM) at 25°C. One unit of collagenase activity was defined as the cleavage of 1 μg collagen per minute at 25°C.

Activation of pro-MMPs

Stock solutions of pro-MMPs containing 400 nM pro-MMPs were activated with 2 mM APMA in 50 mM Tris (pH 7.7), 5 mM $CaCl_2$, 0.2 M NaCl, and 50 μM $ZnCl_2$ at 37°C for 30 min and then transferred to an ice bath prior to use. For migration assays, the APMA was removed by overnight dialysis in 10 kDa MWCO Mini Slide-A-Lyzers (Pierce, Rockford, Illinois) at 4°C.

Immunodepletion of MMPs

CRL-2076-conditioned medium was diluted 1:3 with 20 mM potassium phosphate buffer (pH 7.5), 150 mM NaCl (PBS) (1.3 ml final volume), and 5 μg MMP-Ab or IgG control was added and the mixture and gently rocked at 4°C for 2 hr. Antibody-antigen complexes were bound to Protein A beads (Pharmacia, Piscataway, New Jersey) (130:5 v/v) by incubating for an additional 2 hr. The Protein A-antibody/antigen beads were removed from the medium by centrifugation (300 × g for 5 min, 4°C).

Supplemental Data

Supplemental Data include three figures and can be found with this article online at http://www.cell.com/cgi/content/full/120/3/303/DC1/.

Acknowledgments

This work was funded in part by a predoctoral fellowship from the Susan G. Komen Breast Cancer Foundation (A.B.) and by NIH grant HL64701 (A.K.). We are especially grateful for the expert advice and support of John Erban. We thank Claudia Derian and Patricia Andrade-Gordon of Johnson and Johnson Pharmaceuticals for providing RWJ-56110 and Nga Nguyen for conducting the RT-PCR analyses and Richard Wong for conducting the IL-8 migration assays.

Received: April 15, 2004
Revised: November 9, 2004
Accepted: December 6, 2004
Published: February 10, 2005

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