Opposing Function of the Proprotein Convertases Furin and PACE4 on Breast Cancer Cells’ Malignant Phenotypes: Role of Tissue Inhibitors of Metalloproteinase-1

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Abstract
Proteolytic cleavage of various cancer-related substrates by the proprotein convertases (PC) was reported to be important in the processes of neoplasia. These enzymes are inhibited by their naturally occurring inhibitors, the prosegments (ppPC), and by the engineered general PC inhibitor, the serpin variant α1-PDX. In the present study, we sought to compare the effect of these PC inhibitors on malignant phenotypes of breast cancer cells. Overexpression in a stable manner of α1-PDX and the prosegment ppPACE4 in MDA-MB-231 breast cancer cells resulted in increased matrix metalloproteinase (MMP)-9 (but not MMP-2) activity and a reduced secretion of tissue inhibitor of metalloproteinase 1 (TIMP-1). This was associated with significant enhancement in cell motility, migration, and invasion of collagen in vitro. In contrast, ppFurin expression in these cells decreased MMP-9 activity and diminished these biological functions, but had no significant effect on TIMP-1 secretion. Taken together, these data showed the specific and opposing roles of Furin and PACE4 in the regulation of MMP-9/TIMP-1-mediated cell motility and invasion. [Cancer Res 2007;67(19):9030–4]

Introduction
The proprotein convertases (PC) are subtilisin-related proteases that include Furin, PC1, PC2, PC4, PACE4, PC5, and PC7 (1). These enzymes cleave precursor proteins at basic residues within the motif (K/R)-(X)n-(K/R), where n = 0, 2, 4, or 6 and X is any amino acid except Cys (1). Since the discovery of Furin, the growing evidence of PCs implication in various pathologic processes, including cancer, made these enzymes important potential therapeutic targets (2). Accordingly, many attempts have been made to develop specific PC inhibitors, including the use of either the PC-prosegments or engineered serpins (3, 4). To date, the PC-prosegments (ppPC) stoichiometrically bind the cognate convertase and remain attached to it until its zymogen autoactivation (4). Overexpression of ppPCs leads to potent inhibition of PC-mediated precursor processing (4–8). On the other hand, a variant of their naturally occurring inhibitors, the prosegments (ppPC), is important in the processes of neoplasia. These enzymes are inhibited by their naturally occurring inhibitors, the prosegments (ppPC), and by the engineered general PC inhibitor, the serpin variant α1-PDX. In the present study, we sought to compare the effect of these PC inhibitors on malignant phenotypes of breast cancer cells. Overexpression in a stable manner of α1-PDX and the prosegment ppPACE4 in MDA-MB-231 breast cancer cells resulted in increased matrix metalloproteinase (MMP)-9 (but not MMP-2) activity and a reduced secretion of tissue inhibitor of metalloproteinase 1 (TIMP-1). This was associated with significant enhancement in cell motility, migration, and invasion of collagen in vitro. In contrast, ppFurin expression in these cells decreased MMP-9 activity and diminished these biological functions, but had no significant effect on TIMP-1 secretion. Taken together, these data showed the specific and opposing roles of Furin and PACE4 in the regulation of MMP-9/TIMP-1-mediated cell motility and invasion. [Cancer Res 2007;67(19):9030–4]

Materials and Methods
Transfections and cell culture. The human MDA-MB-231 cells were stably transfected with pRES2–enhanced green fluorescent protein (EGFP) empty vector or cDNAs expressing Furin, ppPACE4, or α1-PDX (12, 13). Stably transfected cells were selected using G418 resistance. To generate pools of MDA-MB-231 cells expressing PC inhibitors, cells were cultured in the presence of Pseudomonas exotoxin A (8). All transfections were carried out using LipofectAMINE 2000 reagent (Invitrogen). In some experiments, MDA-MB-435 and T47D cells were transfected with α1-PDX and the MDA-MB-231 cells expressing α1-PDX, ppFurin, or ppPACE4 were transiently transfected with pRES2/EGFP/platelet-derived growth factor A (PDGF-A)/V5 construct (5).

Reverse transcription-PCR analysis. Total RNA was extracted using Trizol and reverse transcription-PCR was done as previously described (6) using the oligonucleotides 5′-GCAAACCTGTTGATCAGA-3′ and 5′-TCTGCAGTATGATCTGTTGG-3′ for Furin, 5′-GTACCTCAACTTGCGGC-3′ and 5′-TCTGATCTGCGTAGGAAC-3′ for PACE4, and 5′-TGGAAATCCCATCACCATCT-3′ and 5′-GTTCTCTGGTGCCAGT-3′ for glyceraldehyde-3-phosphate dehydrogenase. The presence of empty vectors (pRES2-EGFP) or plasmids containing α1-PDX, ppFurin, or ppPACE4 in transfected tumor cells was analyzed by PCR following DNA extraction by QiAamp DNA mini kit (Qiagen) using the oligonucleotides 5′-GCTAACCTGTTAGTA-3′ and 5′-CTTATTCCAGGGCTCTG-3′ for pRES2-EGFP and pRES2-α1-PDX.

Immunoblotting. Cell lysates or conditioned media were subjected to SDS-PAGE and proteins were blotted onto nitrocellulose membranes. The primary antibodies used were anti-V5 (Invitrogen), anti–green fluorescent protein, and anti-tubulin (Sigma). The secondary antibodies were anti-rabbit or anti-mouse (Amersham). Blots were visualized by enhanced chemiluminescence (Amersham). Ponceau S staining was used as a loading control. The reaction mixtures which contain all the reagents necessary for amplification but exclude the template DNAs were used as negative controls.

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protein (Santa Cruz), anti-insulin-like growth factor I receptor (Santa Cruz), or anti-TIMP-1 (Chemicon). Primary antibodies were revealed by horseradish peroxidase–conjugated secondary antibodies and enhanced chemiluminescence (Amersham).

**Gelatin zymography.** Zymography analysis was done on serum-free conditioned medium derived from control cells or stably transfected with the PC inhibitors. SDS-PAGE gels were copolymerized with gelatin and samples were loaded onto gels without boiling. The gels were washed at room temperature in renaturing solution and in 10 mmol/L of Tris-HCl (pH 8). For the enzymatic reaction to take place, the gels were incubated at 37°C in a solution of 50 mmol/L of Tris-HCl (pH 8) containing 10 mmol/L of CaCl₂. The gels were then stained in Coomassie blue R250 solution and regions without staining were indicative of gelatin lysis.

**Cell migration and collagen invasion assay.** Cell migration was determined using 24-well microchemotaxis chamber alone or precoated with collagen type IV (Becton Dickinson Labware). Control MDA-MB-231 cells or cells stably transfected with the PC inhibitors were resuspended in serum-free media and loaded into the upper chamber of each well. Cells were incubated at 37°C for 24 h, after which, the filters were fixed and stained with Diff-Quik (Baxter Scientific). Cell migration and invasion were quantified by the determination of the number of cells that migrated directly through the membrane towards the medium containing FCS, which was used as a chemoattractant. All cells in each well were counted and the results were represented as the percentage of the migrating cells. Student’s t test was used for statistical analyses.

**Wound healing assay.** Confluent cells were wounded by applying a razor blade to the dish and scraping perpendicularly to the plane of the blade. After being washed, the cells were incubated in DMEM for 6, 24, 30, and 48 h. The cells were then observed under a microscope (Axiovert 10, Carl Zeiss, Inc.) and the images of the wounded areas were collected using MetaMorph software. The motility of the cells was evaluated by the determination of the repaired area percentage.

**Results**

**Expression of Furin and PACE4 in control and α1-PDX–ppFurin-, or ppPACE4-transfected MDA-MB-231 cells.** To investigate the effect of Furin and PACE4 inhibition on malignant phenotypes of breast cancer, MDA-MB-231 cells were stably transfected with vectors that contained ppFurin (MDA/ppFurin) or ppPACE4 (MDA/ppPACE4). For comparison, MDA-MB-231 cells were also transfected with α1-PDX (MDA/PDX). Using reverse transcription-PCR analysis, we found that the MDA-MB-231 cells expressed Furin and PACE4 (Fig. 1A). Similarly, PCR analysis confirmed the presence of empty pIRES2-EGFP vectors or PC inhibitors in the transfected cells (Fig. 1A). No amplified products which indicated possible contamination during the reverse transcription-PCR or PCR reactions were detected. Using specific antibodies, Western blot analysis revealed the presence of coexpressed green fluorescent protein reporters in the transfected cells (Fig. 1A) confirming the presence of the indicated PC inhibitors in these cells. In MDA/PDX cells, the mRNA level of Furin was reduced and PACE4 mRNA apparently increased. In MDA/ppFurin, the levels of Furin were not affected significantly and PACE4 levels were reduced. In MDA/ppPACE4 cells, the Furin and PACE4 mRNA levels remained unchanged (Fig. 1A).

**PC activity in MDA/PDX, MDA/ppFurin, and MDA/ppPACE4 cells.** To assess the ability of α1-PDX, ppFurin, and ppPACE4 to inhibit PCs activity in MDA-MB-231 cells, we evaluated the maturation of two PC substrates: PDGF-A tagged with the V5 epitope (5) and insulin-like growth factor I receptor (8). As can be seen in Fig. 1B, transfection of MDA/control cells with PDGF-A cDNA revealed the ability of these cells to process this precursor. In contrast, transfection with PDGF-A cDNA of MDA/ppFurin, MDA/ppPACE4, or MDA/PDX cells resulted in pro–PDGF-A processing blockade (Fig. 1B). Similarly, analysis of insulin-like growth factor I receptor processing in these cells revealed that MDA/ppFurin, MDA/ppPACE4, and MDA/PDX failed to adequately process insulin-like growth factor I receptor (Fig. 1B).

**Regulation of MDA-MB-231 cell motility by α1-PDX, ppFurin, and ppPACE4.** To test whether Furin and PACE4 activity were required for cell motility, we examined confluent monolayers of MDA/PDX, MDA/ppFurin, and MDA/ppPACE4 cells. At 6, 24, 30, and 48 h following a scratch wound of these monolayers, it was apparent that wounds in MDA/PDX > MDA/ppPACE4 were rapidly repaired as compared with MDA/controls. In MDA/ppFurin cell monolayers, it was delayed (Fig. 2).

**Effect of α1-PDX, ppFurin, and ppPACE4 on MDA-MB-231 cell migration and invasion of collagen.** To evaluate the importance of Furin and PACE4 on MDA-MB-231 cell migration and invasion of collagen, we did Boyden chamber assays on MDA/control, MDA/PDX, MDA/ppFurin, and MDA/ppPACE4 cells. The latter were incubated for 24 h in a microchemotaxis chamber alone (Fig. 3A) or precoated with collagen IV (Fig. 3B). As illustrated, the expression of α1-PDX > ppPACE4 in these cells increased the ability of MDA-MB-231 cells to migrate and invade collagen. In contrast,
the expression of ppFurin significantly inhibited these processes (Fig. 3).

**Effect of α1-PDX, ppFurin, and ppPACE4 on MDA-MB-231 cells’ gelatinase enzymatic activity and TIMP-1 secretion.** Following 48 h of incubation of cells in serum-free conditions, media were collected and analyzed for gelatinase enzymatic activity. As illustrated in Fig. 4A, media derived from MDA/control cells presented high MMP-9 and low MMP-2 activity. Expression of PC inhibitors resulted in an increased MMP-9 activity in media derived from MDA/PDX and MDA/ppPACE4 cells. In contrast, the latter was reduced in medium from MDA/ppFurin cells. In all media analyzed, the activity of MMP-2 didn’t change significantly. We next analyzed the expression of TIMP-1, in an attempt to rationalize the observed altered activity of MMP-9 in MDA/PDX, MDA/ppFurin, and MDA/ppPACE4 cells. Using Western blot analysis, we found an important decrease in TIMP-1 secretion in MDA/PDX and MDA/ppPACE4 cells, and much less so in MDA/ppFurin cells as compared with control MDA-MB-231 cells (Fig. 4B). In parallel, expression of α1-PDX in the MDA-MB-435 and T47D breast cancer cells induced a similar effect on TIMP-1 secretion (Fig. 4B). Quantitation of the ratio of (MMP-9 activity) / (secreted TIMP-1 levels) indicates that compared with control cells, this ratio increased 26.35- and 4.14-fold in MDA/PDX and MDA/ppPACE4 cells, respectively, but decreased 2.80-fold in MDA/ppFurin cells (Fig. 4C).

**Discussion**

The critical role of the PCs in the proteolytic maturation of multiple precursor substrates implicated in neoplasia make them attractive targets for the development of potent and selective inhibitors (3, 4). In the present study, we investigated the importance of Furin and PACE4, reported to be differentially

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**Figure 2.** Regulation of MDA-MB-231 cell motility by α1-PDX, ppFurin, and ppPACE4. A scratch wound was induced in MDA/control, MDA/PDX, MDA/ppFurin, and MDA/ppPACE4 confluent cell monolayer. Following indicated time periods of incubation, the motility of cells was evaluated by the determination of the percentage of the repaired area.

**Figure 3.** Effect of α1-PDX, ppFurin, and ppPACE4 on MDA-MB-231 cell migration and invasion of collagen. Boyden chamber assays were done on MDA/control, MDA/PDX, MDA/ppFurin, and MDA/ppPACE4 cells to analyze their migration (A) and invasion of collagen (B). Three independents experiments were done (n = 3) and the results are represented as the percentage of the migrating cells. Student’s t test was used for statistical analysis. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
expressed in various cancers (10, 14–17). Earlier reports showed that α1-PDX overexpression in different tumor cell systems resulted in a reduced invasiveness and \textit{in vivo} tumorigenicity in nude mice (2, 8, 16). In other studies, this inhibitor was found to increase cell migration and accelerate the process of metastasis (17). Accordingly, overexpression of Furin has been established in various types of cancers (2, 10, 14) and has been linked to aggressive behavior in some model systems (14). Using ppFurin cells, Lopez de Cicco et al. showed that overexpression of this inhibitor in various head and neck squamous cells induced significant reductions in cell proliferation, tumorigenicity, and invasiveness (18). These changes were directly related to the inhibition of Furin-mediated activation of crucial cancer-related substrates (18).

The metalloprotease MMP-9 is required for penetration of the basement membrane during intravasation as well as extravasation and colonization of various metastatic cells (11). MMP-9 has also been shown to play a role in the initiation of cell movement on extracellular matrix and to be able to degrade most extracellular matrix proteins, particularly type IV collagen, the main component of basement membranes (11). In the present study, we found that ppFurin inhibits cell migration and invasion (Fig. 3), and prevents adequate repair of scratch wounds (Fig. 2). These alterations were associated with reduced MMP-9 activity (Fig. 4). Contrary to MDA/ppFurin cells, overexpression of α1-PDX or ppPACE4 increased MDA-MB-231 invasion and motility which correlated with high MMP-9 activity and reduced TIMP-1 secretion, a naturally occurring inhibitor of MMP-9 (11), suggesting that the loss of TIMP-1 resulted in the increased MMP-9 activity observed in these cells. Similarly, using other breast cancer cell lines, we found that overexpression of α1-PDX was also associated with a reduction in TIMP-1 secretion (Fig. 4B).

Interestingly, overexpression of ppFurin seems to decrease MMP-9 activity and TIMP-1 secretion, albeit affecting the latter to a lesser extent than α1-PDX or ppPACE4 (Fig. 4). Thus, inhibition of MMP-9 activity by ppFurin was not correlated with TIMP-1 levels, which actually decreased, suggesting that other proteins regulated by Furin are implicated in this balance. This agrees with a previous report which showed overexpression of Furin increases MMP-9 activity in head and neck small cell carcinoma cells, likely by an indirect, unknown activation mechanism (19). In our model, we found that PCs could also regulate their own expression, probably through activation/inhibition of different substrates and/or downstream effectors. Indeed, analysis of Furin and PACE4 expression in PC inhibitor-transfected cells revealed that α1-PDX reduced and increased Furin and PACE4 expressions, respectively. In contrast, in MDA/ppFurin cells, PACE4 expression was reduced, and in MDA/ppPACE4 cells, the level of Furin and PACE4 remained unchanged (Fig. 1A).

There are opposing views in the literature arguing the relevance of PACE4 expression in carcinogenesis. Indeed, some investigations showed that overexpression of PACE4 in immortalized nontumorigenic or papilloma-derived keratinocytes increased their invasiveness (20); whereas other studies linked the absence or reduced PACE4 expression levels to ovarian cancer (15). These conflicting data show that PC expression and/or function in cancer cells varies in a tumor-specific fashion, and raises the

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\caption{Regulation of MMP-9 activity and TIMP-1 secretion by α1-PDX, ppFurin, and ppPACE4 in MDA-MB-231 cells. 
A, serum-free media derived from the indicated cells were collected and analyzed for gelatinase enzymatic activity as described in Materials and Methods. 
B, media collected from the indicated cells were subjected to Western blot analyses using specific antibodies against TIMP-1. 
C, quantitation of the ratio of (MMP-9 activity) / (secreted TIMP-1 levels) in MDA/Control, MDA/PDX, MDA/ppFurin, and MDA/ppPACE4.}
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effects (Figs. 2–4). In conclusion, the identification of Furin and motility of tumor cells, whereas ppFurin decreases these effects. Taken together, our results strongly suggest that regulation of the MMP-9/TIMP-1 ratio by PCs in breast cancer cells may be one of the major factors that control their invasive and metastatic abilities. Both α1-PDX and ppPACE4 increase this ratio and result in increased invasiveness and motility of tumor cells, whereas ppFurin decreases these effects (Figs. 2–4). In conclusion, the identification of Furin and PACE4-regulated MMP-9/TIMP-1 ratio revealed a potential new layer in our understanding of the PC-mediated acquisition of malignant phenotype. It will be of interest to determine exactly how Furin and PACE4 contribute to the regulation of MMP-9 and TIMP-1 activity and/or secretion. Elucidating these mechanisms might provide new opportunities for cancer therapeutic intervention.

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