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Serines in the Intracellular Tail of Podoplanin (PDPN) Regulate Cell Motility

Podoplanin (PDPN) is a transmembrane receptor that affects the activities of Rho, ezrin, and other proteins to promote tumor cell motility, invasion, and metastasis. PDPN is found in many types of cancer and may serve as a tumor biomarker and chemotherapeutic target. The intracellular region of PDPN contains only two serines, and these are conserved in mammals including mice and humans. We generated cells from the embryos of homozygous null Pdpn knock-out mice to investigate the relevance of these serines to cell growth and migration on a clear (PDPN-free) background. We report here that one or both of these serines can be phosphorylated by PKA (protein kinase A). We also report that conversion of these serines to non-phosphorylatable alanine residues enhances cell migration, whereas their conversion to phosphomimetic aspartate residues decreases cell migration. These results indicate that PKA can phosphorylate PDPN to decrease cell migration. In addition, we report that PDPN expression in fibroblasts causes them to facilitate the motility and viability of neighboring melanoma cells in coculture. These findings shed new light on how PDPN promotes cell motility, its role in tumorogenesis, and its utility as a functionally relevant biomarker and chemotherapeutic target.

Experimental Procedures

Kinase Assays—Phosphorylation of the peptide VVMKKIS-GRFSP (synthesized by Proteintech, Chicago, IL), containing the entire intracellular region of mouse PDPN (residues 161–172), was measured using a phosphocellulose paper binding assay (16). Reaction mixtures contained 300 μM peptide in 20 mM Tris-HCl (pH 7.4), 10 mM MgCl2, 1 mg/ml bovine serum albumin, 0.25 mM ATP, and [γ-32P]ATP (100 cpm/pmol). The reactions were initiated by the addition of 25 μg/ml PKA (Promega V516A). Aliquots were withdrawn at various time points and quenched by the addition of 10% trichloroacetic acid. The reactions were spotted onto P81 phosphocellulose filters, washed three times with 0.5% phosphoric acid, and analyzed by scintillation counting to determine the picomoles of phosphate transferred.

Generation of Wild Type and Mutant Pdpn Cell Lines—PdpnKO3 cells were isolated from the trunks of homozygous null Pdpn knock-out mice at day 10 postcoitum (1). Cells were maintained in DMEM supplemented with 10% FBS as

The abbreviations used are: PDPN, podoplanin; 8-Br-cAMP, 8-bromo-cyclic AMP.

3 Throughout this study, the following designations are used: PdpnKO, PDPN knock-out mouse construct; PdpnWT, full-length wild-type mouse PDPN construct; PdpnAA, Pdpn(WT767A/S717A) construct encoding nonphosphorylatable PDPN with both intracellular serines mutated to alanine; PdpnDD, Pdpn(S167D/S171D) construct encoding phosphomimetic PDPN with both intracellular serines mutated to aspartate; PdpnEF4, PDPN empty vector control.

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described previously (15, 17). The cells passed crisis during standard serial passaging, and genotypes were verified by PCR. Pdpn mutant constructs were produced with the QuikChange II XL site-directed mutagenesis kit according to the manufacturer’s protocol (Stratagene 200521). The vector pEF4Pdpn encoding full-length wild type mouse PDPN (PdpnWT) (4, 18) was used as a template. Pdpn(S167A/S171A) encoding non-phosphorylatable PDPN (PdpnAA) was generated with the complementary primer pairs 5'-GTTGATAGAAGATGGATCGTTGAGGCCGC-3', 5'-GGGACATCTTCTCCACAC-3' followed by 5'-TGGGAACCTGCTAAAGGGGCCCTTCGAGG-3', 5'-CTCTGAGGAGGCCCCTTTAGGGCGAACCCTACT-3'. Pdpn(S167D/S171D) encoding phosphomimetic PDPN (PdpnDD) was generated with the complementary primer pairs 5'-GTATAAGAAGATTGATGGATCGTTGAGGCCGC-3', 5'-GCTCATTGGAGGGAACCTGCTAAAGGGGCCCTTCGAGG-3', 5'-CTCTGAGGAGGCCCCTTTAGGGCGAACCCTACT-3'. All constructs were verified by sequencing. PdpnWT, PdpnAA, PdpnDD constructs, and the control vector pEF4/V5HisA (Invitrogen) were cotransfected with pBABEpuro into a single expanded PdpnKo cell clone with Lipofectamine 2000 according to the manufacturer’s protocol (Invitrogen 11668-027). Transfectants were selected with Zeocin (InvivoGen ant-zn-1p) and puromycin (Calbiochem 540411). This double transfection/selection technique produces high levels of transgene expression in about 80% or more of surviving cells (4, 15, 19).

Western Blotting—Western blotting was performed as described previously (4, 18). Protein was resolved by SDS-PAGE, transferred to Immobilon-P membranes (Millipore IH1079562), and incubated with antisera specific for PDPN (University of Iowa Developmental Studies Hybridoma Bank 8.1.1), or GAPDH (Santa Cruz Biotechnology A1978). Primary antiserum was recognized by appropriate secondary antisera conjugated to horseradish peroxidase and detected using enhanced chemiluminescence (Thermo Scientific 32106). After blotting, membranes were stained with India ink to verify equal loading and transfer.

Evaluation of Cell Growth and Migration—Cell growth and migration were evaluated as described previously (4, 15, 18, 19). Briefly, cell growth was measured by plating 10,000 cells/well on standard 24-well tissue culture plates (Falcon 353047) and counting cells at the indicated time points in a Coulter counter. For wound healing migration assays, confluent cell monolayers were scratched, and migration was quantitated as the number of cells that entered a 300-μm area in the center of the wound at 18 h. For some experiments, the PKA modulators 8-Br-cAMP (Calbiochem 203800) or H-89 (Calbiochem 371963) were added immediately after wounding. For Transwell invasion assays, 600,000 cells were added to each chamber of 6-well Transwell membranes with an 8-μm pore size (Transwell-Clear, Costar 3428), incubated for 24 h, released separately from the top and bottom of the membrane, counted in a Coulter Counter, and quantified as the percentage of cells that migrated from the top of the membrane to the bottom of the membrane.

Video Microscopy—To analyze unopposed movement, PdpnKo cell lines were plated on 35-mm culture dishes at 5% confluence and incubated overnight. To analyze the effects of PdpnKo cells on the movement of melanoma cells in coculture, B16F10 melanoma cells were labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethyldilaurindocarbocyanine perchlorate as described (17, 20, 21), plated with unlabeled B16F10 cells or PdpnKo cell lines at a 1:100 ratio on 35-mm culture dishes at 85% confluence, and incubated overnight. Medium was then removed and replaced with fresh medium before dishes were mounted on an INUBG2A-ZILCS with UNIV-35 stage top incubator (Tokai Hit, Shizuoka, Japan) maintained at 37 °C, 5% CO₂, and 100% humidity. Images of cells were captured with AxioCam MRm camera at 5-min intervals for 18 (unopposed movement) or 36 h (cocultured melanoma cells) with a 5× CP-Achromat objective on a Zeiss Axiovert 40 CFL inverted microscope or a 10× EC Plan NEOF objective with a Zeiss Axiovert Observer Z1 inverted microscope, respectively. Distance and displacement traveled from the starting point to the end point were measured by tracking the center of the nuclei of motile cells with AxioVision software (version 4.3, Carl Zeiss). The percentage of morphologically intact cells was used to quantitate the survival of cocultured melanoma cells.

Statistical Analysis—Experiments were repeated with similar results and means, errors, and unpaired two-tailed t tests were calculated from representative data sets with Prism (version 4, GraphPad Software).

RESULTS

Phosphorylation of the PDPN Intracellular Tail by PKA—PDPN activity correlates well between humans and mice (22). For example, PDPN expression is strongly induced in human (22, 23) and mouse (5, 24) skin cancers. There are 2 conserved serines in the intracellular region of mouse and human PDPN. These serine residues could potentially serve as phosphorylation sites for PKC or PKA. Using a peptide that corresponds to the entire cytoplasmic portion of PDPN, we performed in vitro kinase assays to evaluate the ability of these kinases to phosphorylate these sites. As shown in Fig. 1, PKA catalyzed the time-dependent phosphorylation of the PDPN cytoplasmic peptide. In contrast, PKC did not phosphorylate the peptide to any significant extent (data not shown).

Nonphosphorylated PDPN Enhances Cell Migration—We generated homozygous null Pdpn knock-out mouse embryonic fibroblasts (PdpnKo cells) to study the effects of PDPN modification on cell behavior. The absence of endogenous PDPN background of these cells facilitated evaluation of how modification of PDPN intracellular serines affected cell growth and motility. These cells were stably transfected with constructs encoding wild type PDPN (PdpnWT), nonphosphorylatable PDPN with both intracellular serines mutated to alanine (PdpnAA), phosphomimetic PDPN with both intracellular serines mutated to aspartate (PdpnDD), or empty vectors (PdpnEF4) as controls. Expression of these transgenes was verified by Western blotting as shown in Fig. 2a.
Serine Residue Modifications Affect Cell Motility

Although PDPN has been shown to promote cell migration, its effects on cell growth and proliferation are less defined (8–10). A role for PDPN in cell growth seemed to emerge on the clear background of homozygous null knock-out cells. As shown in Fig. 2b, expression of wild type (PdpnWT) or non-phosphorylatable (PdpnAA) PDPN appeared to decrease cell growth rates by about 50% when compared with control transfectants (PdpnEF4). Interestingly, this effect was not seen in cells transfected with the phosphomimetic PDPN (PdpnDD) construct.

As expected, wild type PDPN augmented the migration of PdpnKO cells. PdpnWT cells migrated about 50% more than the control transfectants (PdpnEF4) in wound healing migration assays shown in Fig. 2, c and d. However, although expression of the PdpnAA construct did not exceed that of PdpnWT, PdpnAA cells migrated about 40% more than PdpnWT cells in these assays. These results were confirmed by Transwell invasion assays in which PdpnWT cells moved about 4-fold better through 8-μm pores than PdpnEF4 cells, and PdpnAA cells moved about 50% more than the PdpnWT cells (Fig. 2e).

**Phosphomimetic PDPN Decreases Cell Migration**—Results from wild type (PdpnWT) and nonphosphorylatable (PdpnAA) constructs suggest that PDPN can be phosphorylated to inhibit cell migration. This hypothesis is supported by results from phosphomimetic (PdpnDD) constructs. In contrast to PdpnWT or PdpnAA constructs, phosphomimetic (PdpnDD) Pdpn did not significantly increase cell motility when compared with PdpnEF4 control cells in wound healing migration (Fig. 2, c and d) or Transwell invasion (Fig. 2e) assays. Interestingly, in contrast to wild type or PdpnAA transfectants, PdpnDD transfection did not significantly decrease cell growth when compared with PdpnEF4 controls (Fig. 2b). These results suggest that PDPN signaling may convert some metabolic energy used for cell proliferation into processes needed for motility.

**Live Cell Imaging of Individual Cells Confirms That PDPN Serine Residue Modifications Affect Cell Motility**—PDPN can promote both collective and individual cell migration in the absence or presence of epithelial-mesenchymal transition (7, 25). Thus, in addition to wound healing and Transwell assays, live cell imaging was used to investigate the effects of PDPN serine modifications on the motility of individual cells in sparse cultures. These assays also allowed direct measurements of total displacement and distance traveled by individual cells over time.

As with wound healing and Transwell assays, cells, PDPN transfected cells moved better than control cells, and nonphosphorylatable PDPN increased the motility of these cells more than wild type PDPN. PdpnKO cells transfected with PdpnEF4, PdpnWT, or PdpnAA moved at an average displacement speed of 1.6, 3.7, or 4.2 μm/h (Fig. 2f and supplemental Fig. 1, a–c) to travel an average distance of 3.4, 6.2, or 11 μm/h, respectively. In contrast, the movement of cells transfected with phosphomimetic PDPN (PdpnDD) was not significantly different from controls (PdpnEF4) when measured by displacement (1.3 μm/h) and significantly less than controls when measured by distance (1.9 μm/h) (Fig. 2f and supplemental Fig. 1d). These data indicate that PDPN can be phosphorylated to decrease both collective and individual cell motility.

**Modulators of PKA Activity Affect Migration of Cells Expressing Wild Type PDPN**—Results from kinase assays and site mutants suggest that PKA activation inhibits PDPN-mediated cell migration. As shown in Fig. 3 and supplemental Fig. 2, the PKA agonist 8-Br-cAMP significantly decreased the migration of cells transfected with wild type PDPN. Conversely, treatment of these cells with the PKA blocker H-89 significantly increased their migration. These data are consistent with the hypothesis that PKA phosphorylates PDPN to decrease cell migration.

**PDPN Expression in Fibroblasts Facilitates Neighboring Tumor Cell Migration and Survival**—PDPN expression in cancer-associated fibroblasts has been linked to increased tumor invasive and metastatic potential (26, 27). We utilized PdpnKO cells to investigate mechanisms that may underlie the relationship between cancer cells and their neighboring fibroblasts. Melanoma cells that express PDPN as a functionally relevant biomarker and potential chemotherapeutic target were chosen as subjects for this investigation (18).

Melanoma cells migrated an average distance of about 9.2 μm/h when surrounded by other melanoma cells (Fig. 4, a and b, and supplemental Fig. 3a). The migration of melanoma cells when surrounded by fibroblasts expressing (PdpnWT cells) was not significantly different from their migration when surrounded by melanoma cells (Fig. 4, a and b, and supplemental Fig. 3b). However, melanoma cell migration was decreased by over 50%, to an average distance of only 4.2 μm/h, when surrounded by PdpnEF4 control cells (Fig. 4, a and b). Thus, fibroblasts expressing PDPN appeared to facilitate the migration of neighboring tumor cells better than PDPN-deficient fibroblasts.

In addition to increasing tumor cell motility, PDPN expression in fibroblasts affected the viability of neighboring melanoma cells. As shown in Fig. 4, a and c, about 70% of melanoma cells cocultured with cells lacking Pdpn (PdpnEF4) went through morphological changes indicative of apoptosis (see supplemental Fig. 3c) when compared with about 20% of melanoma cells cocultured with PdpnWT cells or 10% cocultured with other melanoma cells. Also, as shown in Fig. 4, melanoma cells cocultured with fibroblasts expressing phosphomimetic PDPN behaved similarly to those cultured with control trans-
flectants (see supplemental Fig. 3d). Taken together, these data indicate that PDNP expression levels and phosphorylation status in fibroblasts can affect the motility and viability of neighboring tumor cells.

**DISCUSSION**

PDNP expression enhances the motility and invasion of several transformed cell types including mammary carcinoma (7, 8), glioma (28), and squamous carcinoma cells (29–31). PDNP expression...

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**FIGURE 2. Effect of Pdpn expression on cell growth and motility.**

*a*, PDPN and GAPDH were detected by Western blotting of protein (7.5 μg/lane) from homozygous null Pdpn knock-out mouse embryonic fibroblasts transfected with empty parental vector (EF4), wild type Pdpn (WT), Pdpn with both intracellular serines mutated to alanine (AA), or Pdpn with both intracellular serines mutated to aspartate (DD) as indicated. *b*, cell growth was evaluated by plating 10,000 cells/well on 24-well tissue culture plates and counting at the indicated time points. Data are shown as number of cells/well (mean ± S.D., n = 2). *c* and *d*, cell migration was measured by wound healing assays and quantitated as the number of cells that entered a 300 × 200-μm area of in the center of a wound in 18 h (mean ± S.E., n = 12). *Bar* = 100 μm. *e*, Transwell invasion assays were performed and quantitated as the percentage of cells that moved from the top of the membrane to the bottom of the membrane in 24 h (mean ± S.E., n = 3). *f* and *g*, total displacement (*f*) and net distance (*g*) traveled were measured by video microscopy of individual motile cells and presented as micrometers traveled over 18 h (mean ± S.E., n = 20). Single, double, and triple asterisks indicate p < 0.05, p < 0.01, and p < 0.001 when compared with empty vector controls by Student’s t test, respectively.
is found at the invasive front of many tumors, which is consistent with its role in promoting invasion (7, 8). In addition to tumor cells themselves, PDPN is also located on endothelial cells of lymphatic vessels, which can facilitate tumor invasion and metastasis (32, 33). PDPN expression in cancer-associated fibroblasts has also been linked to tumor aggression and poor prognosis of lung cancers (26, 27, 34, 35). Consistent with these findings, our results indicate that PDPN expression in fibroblasts can facilitate migration and survival of neighboring tumor cells in the microenvironment. Further elucidation of events underlying this relationship should lead to a greater understanding of fundamental mechanisms that guide stromatumor interactions and how they may be exploited to combat cancer.

Indeed, PDPN presents great potential as a functionally relevant biomarker and chemotherapeutic target (8–10). For example, reagents that target PDPN and a PDPN-interacting partner (tetraspanin CD9) can inhibit lung metastasis of CHO

FIGURE 3. Effect of PKA modulators on cell migration. Wound healing experiments were performed on confluent monolayers of PdpnWT cells treated with the PKA agonist 8-Br-cAMP or PKA inhibitor H-89 as indicated. Cell migration was quantitated as the number of cells that entered a 300 × 200-μm area of in the center of a wound in 18 h (mean ± S.E., n = 6). Single and triple asterisks indicate p < 0.05 and p < 0.001 when compared with untreated controls by Student’s t test.

FIGURE 4. Effect of Pdpn expression on the motility of neighboring melanoma cells. a, movement of 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate-labeled B16 melanoma cells cocultured with unlabeled B16 cells or PdpnKO cells transfected wild type Pdpn (WT), phosphomimetic Pdpn (DD), or empty parental vector (EF4) was measured by video microscopy for 36 h (bar = 20 μm). b, migration of individual motile cells was quantitated as micrometers traveled over 36 h (mean ± S.E., n = 5–10). c, the percentage of morphologically intact cells was used to quantitate the survival of cocultured melanoma cells in a 600 × 600-μm field (mean ± S.E., n = 4). Double and triple asterisks indicate p < 0.01 and p < 0.001 when compared with B16 cells cultured with B16 cells by Student’s t test, respectively.
cells transfected with PDPN (36, 37), melanoma tumorigenesis (18), and glioma progression (38, 39) in mice.

A variety of signaling agents and cytokines can induce PDPN expression and cell motility (5, 6, 40). For example, the Src tyrosine kinase utilizes the Cas/BCAR1 adaptor protein to augment PDPN expression to promote transformed cell motility required for malignant cells to break out of their microenvironment to become invasive and metastatic (4, 41).

Elucidating how PDPN promotes cell motility is necessary to understand its role in tumorigenesis and its potential as a chemotherapeutic target. Our findings that PDPN can be phosphorylated to inhibit cell migration and that PDPN expression influences the effects of fibroblasts on neighboring tumor cells shed considerable light on this process. For example, modulators of kinases or phosphatases may be employed to enhance the effects of reagents targeting PDPN to treat a variety of cancers (8–10), and possibly other diseases including psoriasis (40).

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