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# CUB Domain–Containing Protein 1, a Prognostic Factor for Human Pancreatic Cancers, Promotes Cell Migration and Extracellular Matrix Degradation

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## Abstract

CUB domain–containing protein 1 (CDCP1) is a membrane protein that is highly expressed in several solid cancers. We reported previously that CDCP1 regulates anoikis resistance as well as cancer cell migration and invasion, although the underlying mechanisms have not been elucidated. In this study, we found that expression of CDCP1 in pancreatic cancer tissue was significantly correlated with overall survival and that CDCP1 expression in pancreatic cancer cell lines was relatively high among solid tumor cell lines. Reduction of CDCP1 expression in these cells suppressed extracellular matrix (ECM) degradation by inhibiting matrix metalloproteinase-9 secretion. Using the Y734F mutant of CDCP1, which lacks the tyrosine phosphorylation site, we showed that CDCP1 regulates cell migration, invasion, and ECM degradation in a tyrosine phosphorylation–dependent manner and that these CDCP1-associated characteristics were inhibited by blocking the association of CDCP1 and protein kinase Cδ (PKCδ). CDCP1 modulates the enzymatic activity of PKCδ through the tyrosine phosphorylation of PKCδ by recruiting PKCδ to Src family kinases. Cortactin, which was detected as a CDCP1-dependent binding partner of PKCδ, played a significant role in migration and invasion but not in ECM degradation of pancreatic cells. These results suggest that CDCP1 expression might play a crucial role in poor outcome of pancreatic cancer through promotion of invasion and metastasis and that molecules blocking the expression, phosphorylation, or the PKCδ-binding site of CDCP1 are potential therapeutic candidates.
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## Introduction

CUB domain–containing protein 1 (CDCP1) is a type I transmembrane protein with several tyrosine residues that can be phosphorylated by Src family kinases (SFK; refs. 1–5). CDCP1 was first identified as the product of a gene preferentially expressed in colon cancer cells compared with normal tissue (1). We recently reported that tyrosine-phosphorylated CDCP1 in lung cancer cells plays a novel role in acquiring resistance to anoikis, a type of cell death caused by detachment from extracellular matrix (ECM). CDCP1 was reported to directly bind to protein kinase Cδ (PKCδ) at a unique C2 domain—a novel phosphotyrosine-binding domain—in a phosphorylation-dependent manner (4). The biological meaning of interaction with PKCδ had not been revealed until we recently discovered that tyrosine-phosphorylated CDCP1 regulates the anoikis resistance of lung cancer cells by acting as a physical link between SFKs and PKCδ, which is a putative cell death–associated molecule (5). Using scirrhous gastric cancer cells, we further reported that phosphorylation of CDCP1 promotes cell migration and invasion *in vitro* and peritoneal dissemination *in vivo* in mice (6). Recent studies of lung adenocarcinoma and renal cell carcinoma have shown that CDCP1 expression has important associations with disease progression (7, 8). Despite accumulating evidence showing the significant involvement of CDCP1 in tumor progression, metastasis, and invasion, the role of the CDCP1 signaling pathway during these biological procedures is not yet well understood.

Pancreatic cancer is one of the most malignant tumors, with poor prognosis due to its aggressive behavior and high metastatic potential. Moreover, pancreatic cancer is sometimes accompanied with specific types of invasion, such as perineural invasion, which can cause severe pain and discomfort. Appropriate treatment that inhibits invasion and metastasis of pancreatic cancers is urgently required to improve both the survival and quality of life of patients.

In this study, we found that CDCP1 is expressed in primary pancreatic tumors as well as in sites of invasion and metastasis of pancreatic cancer. We analyzed the expression levels

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of CDCP1 protein in human pancreatic cancer tissues by using immunohistochemistry and discovered that high CDCP1 expression is correlated with poor prognosis. Tyrosine phosphorylation of CDCP1 was also shown to be essential for ECM degradation in pancreatic cancer through the formation of the CDCP1-PKCδ complex and enzymatic activation of PKCδ in highly invasive pancreatic cancer cell lines. Our results suggest that CDCP1 is a promising therapeutic target that modulates metastasis and invasion of several cancer types.

## Materials and Methods

### Cell culture and transfection

Pancreatic (Suit4, Capan1, PANC1, BxPC3, and CFPAC1) and gastric (HSC44As3 and HSC59) cancer cell lines were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) at 37°C with 5% $CO_2$. The fibrosarcoma cell line HT1080 was cultured in DMEM with 10% FBS. For transfection, cells were seeded on a plate at $2.0 \times 10^6$ per 10-cm dish, and transfection was performed after 24 hours. Expression plasmids were transfected with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Transfected cells were selected in the presence of G418 at the concentration of 1,200 μg/mL (BxPC3) or 900 μg/mL (Capan1).

### Short interfering RNA treatment

Two sets of short interfering RNAs (siRNA) of CDCP1 were synthesized as described elsewhere (5). Two sets of siRNAs of PKCδ or cortactin were synthesized as follows: PKCδ siRNA-1, 5′-GGUGCAGAAGAAGCCGACCAUGUAU-3′ (sense) and 5′-AUACAUGGUCGGCUUCUUCUGCACC-3′ (antisense); PKCδ siRNA-2, 5′-CCAAGGUGUUGAUGGUCGGUUCAGUA-3′ (sense) and 5′-UACUGAACCGACCAUCAACACCUUGG-3′ (antisense); cortactin siRNA-1, 5′-CCCAGAAAGACUAUGUGAAAGGGUU-3′ (sense) and 5′-AACCCUUUCACAUAGUCUUUCUGGG-3′ (antisense); cortactin siRNA-2, 5′-GGAGAAGCACGAGUCACAGAGAGAU-3′ (sense) and 5′-AUCUCUCUGUGACUCGUGCUUCUCC-3′ (antisense). The control siRNA was Stealth RNAi Negative Control Medium GC Duplex. All siRNAs were obtained from Invitrogen. siRNAs (40 pmol) were incorporated into cells using Lipofectamine 2000 according to the manufacturer's instructions. Cells were used for further experiments at 72 hours after siRNA treatment.

### Plasmids, antibodies, and reagents

Plasmids of human CDCP1 and of CDCP1 Y734F ($Tyr^{734}$ to Phe) with FLAG tag have already been described (5). CDCP1 rescue mutant, which introduced silent mutations not to be suppressed by CDCP1 siRNA, with COOH terminus FLAG tag was generated by PCR using KOD-Plus-Mutagenesis kit (Toyobo Co. Ltd.). A cDNA fragment of the C2 domain of human PKCδ with hemagglutinin (HA) tag has already been described (5). The antibodies against PKCδ (C-20; 1:2,500), HA (Y-11; 1:2,500), and actin (I-19; 1:200) were purchased from Santa Cruz Biotechnology. The phospho-PKCδ ($Tyr^{311}$; 1:500) and phospho-PKCδ ($Thr^{505}$; 1:500) antibodies were from Cell Signaling. The FLAG M2 (1:5,000) and α-tubulin (1:10,000) antibodies were from Sigma. Polyclonal antibody against CDCP1 (1:500) and tyrosine-phosphorylated CDCP1 ($Tyr^{734}$; 1:1,000) was prepared as described previously (5). The monoclonal antibodies that recognize cortactin (clone 4F11; 1:5,000), matrix metalloproteinase-9 (MMP-9; 1:500), and phosphotyrosine (4G10; 1:2,500) were purchased from Millipore.

### Western blotting and immunoprecipitation

Western blotting and immunoprecipitation were performed as described previously (5). Protein concentration was measured by bicinchoninic acid protein assay kit (Pierce). Supernatant was concentrated by 10% trichloroacetic acid precipitation, and then samples were washed twice with diethyl ether. Polyvinylidene difluoride membrane (Immobilon-P, Millipore) was used for the transfer membrane, and Blocking One (Nakarai Tesque) was used for the blocking of the membrane. For immunoprecipitation, 1,000 μg protein was mixed with 2 μg of each antibody, and then samples were rotated with protein G–Sepharose beads (GE Healthcare).

### Cell migration and Matrigel invasion assay

Migration and invasion assay were performed using modified Transwell chambers with a polycarbonate nucleopore membrane (BD Falcon) as described previously (9). Cells treated with each siRNA were detached with trypsin-EDTA. Then, the cells in 100 μL of RPMI 1640 with 10% FBS were seeded onto the upper part of each chamber. After incubation for 6 hours for migration, and 18 hours (BxPC3) or 30 hours (Capan1) for invasion, the cells on the membrane were fixed. The totals of migrated or invaded cells were determined by counting the cells on the lower side of the membranes from two wells (two fields per membrane) at a magnification of ×100, and the extent of migration or invasion was expressed as the average ratio (number of cells transfected with siRNA per field/average number of cells transfected with control siRNA per field). The results were from three independent experiments.

### ECM degradation assays

The 12-mm-round cover glasses were coated with fluorescein-conjugated type I collagen from bovine skin (Invitrogen) diluted at 1 μg/μL in PBS for 5 minutes at room temperature, and the cover glasses were dried out for 10 minutes at room temperature. The collagen-coated glasses were then fixed with 0.5% glutaraldehyde solution on ice for 10 minutes and at room temperature for 30 minutes. After washing six times with PBS, the glasses facing upward were transferred to each well of a 24-well plate containing 70% ethanol and incubated for 15 minutes. After two washes with PBS, $2 \times 10^4$ cells were plated onto the coated glass in RPMI 1640 containing 10% FBS. After 18 hours (BxPC3) or 30 hours (Capan1) of culture, cells were fixed and stained with Alexa phalloidin (1:100; Invitrogen) in PBS. The staining was visualized using a Radiance 2100 confocal microscopic system (Bio-Rad). The cells degrading collagen were determined to

overlap the degradation area. Data in 20 fields at a magnification of ×800 were used to calculate the cells degrading collagen per total cells. The results were from three independent experiments.

### Gelatin zymography

Gelatin zymography was conducted with a polyacrylamide gel containing gelatin (0.8 mg/mL) as described previously (10). The SDS-polyacrylamide gel was incubated for 24 hours in the incubation buffer with or without 2.7 nmol/L MMP inhibitor II (MMP-1, MMP-3, MMP-7, and MMP-9 inhibitor; Calbiochem) or DMSO at 37°C. Enzyme activity was visualized as negative staining with Coomassie brilliant blue.

### Immunocytochemical staining

Immunocytochemical staining was performed as previously described (11). The cover glasses were coated by FBS for 1 hour before seeding the cells. For transfection, $5.0 \times 10^4$ cells were seeded on a glass and then fixed and stained. The staining was visualized using a confocal microscopic system (Bio-Rad).

### Patients and tissue samples

Pancreatic tumor specimens were obtained from 158 patients who underwent surgery at the National Cancer Center Hospital (1990–2005; clinicopathologic findings from these 158 patients are summarized in Supplementary Table S1). The follow-up period for survivors ranged from 0.067 to 172.833 months (median, 14.433 mo). Tumors were classified according to the International Union Against Cancer tumor-node-metastasis classification (12), the classification of pancreatic carcinoma of the Japan Pancreas Society (13), and the WHO classification (14). The study was approved by the ethical review board of the National Cancer Center. Informed consent was obtained from each patient.

### Immunohistochemistry

Immunohistochemical staining was performed on the formalin-fixed, paraffin-embedded slides using the avidin-biotin complex method, as described previously (15). A specific antibody against CDCP1 (rabbit polyclonal antibody, 1:500) was used as the primary antibody. Staining in the absence of the primary antibody provided the negative control. The staining on each slide was evaluated by one researcher with two independent observations. Samples were blinded to clinicopathologic data and patient outcomes during observation. Immunoreactivity was scored semiquantitatively according to the estimated percentage of positive tumor cells (1, <50% reacting cells; 2, 50–80% reacting cells; 3, >80%) and intensity (1, weaker than the intensity of surface staining in the islet of Langerhans; 2, equal to the intensity of the islet of Langerhans; 3, stronger than the intensity of the islet of Langerhans). The slides, whose islet of Langerhans was not significantly stained, were considered to be in bad condition and were not evaluated. A total immunohistochemical score was calculated by summing the percentage score and the intensity score. The quantity of CDCP1 expression was classified into two groups by the total score (low group, 2–4; high group, 5 and 6).

### Statistical analysis

We used the StatView software and SAS version 9.1.3 (SAS Institute, Inc.) for statistical analyses. Cochran-Armitage trend test and $\chi^2$ test were used to assess the association between CDCP1 expression levels and clinicopathologic parameters (Supplementary Table S2). Kaplan-Meier methods were used to calculate overall survival, and differences in survival curves were evaluated with the log-rank test. The hazard ratios (HR) with 95% confidence intervals (CI) of the CDCP1 high-expression effect were estimated using univariate and multivariate Cox's proportional hazards model (Table 1). $P$ values of <0.05 were considered to be statistically significant.

## Results

### CDCP1 expression is correlated with prognosis of patients with pancreatic cancer

During the screening for the protein expression of CDCP1 in various cancer cell lines, we noticed that CDCP1 is highly expressed and phosphorylated at tyrosines in most pancreatic cell lines (Fig. 1A). CDCP1 is also expressed in other cancer cells such as lung cancers and gastric cancers as we previously reported, whereas it is not expressed in some cancers such as neuroblastomas (data not shown). We further examined CDCP1 expression in human pancreatic cancer tissues by immunohistochemical analysis. Both well-differentiated and poorly differentiated types of pancreatic cancers were significantly stained with the CDCP1 antibody whereas normal pancreatic ducts were not obviously stained (Fig. 1B, a and b). CDCP1 staining was also detected in the perineural invasion site and at sites of lymph node metastasis (Fig. 1B, c–e). Expression levels of CDCP1 in poorly differentiated cancers are generally higher than in well-differentiated types, especially at invasion sites and metastatic loci. CDCP1 protein expression was examined in surgical specimens from 158 patients with pancreatic cancer. Expression levels of CDCP1 were evaluable in 145 cases, and they were classified into the low-expressing (63.4%, $n$ = 92) and high-expressing (36.6%, $n$ = 53) groups, as described in Materials and Methods. There were no significant associations in the clinicopathologic parameters between CDCP1 expression groups (Supplementary Table S2). The Kaplan-Meier plots also showed that there was a significant difference in overall survival rates ($P$ = 0.0391) between groups with high and low CDCP1 expression (Fig. 1C). The effect of CDCP1 expression on overall survival is similar between univariate and multivariate analyses (Table 1).

### Tyrosine-phosphorylated CDCP1 regulates cell migration and invasion

As pancreatic cancer cells with high metastatic potential, such as BxPC3 and CFPAC1, seemed to show relatively high phosphorylation levels of CDCP1 at tyrosines (Fig. 1A), we analyzed the role of phosphorylated CDCP1 in the invasion and metastasis of pancreatic cancers. Localization of CDCP1 was mainly detected at cell-cell contact in human tissue samples (Fig. 1B). CDCP1 was also expressed at cell-cell contact

**Table 1.** Univariate and multivariate analyses of prognostic factors for overall survival

| | Overall survival | | | |
|---|---|---|---|---|
| | Univariate | | Multivariate | |
| | HR (95% CI) | *P* | HR (95% CI) | *P* |
| Stage* | 1.549 (1.275–1.883) | <0.0001 | 1.458 (1.185–1.795) | 0.0004 |
| Primary tumor* | 2.833 (1.403–5.718) | 0.0037 | — | — |
| Regional lymph nodes* | 2.963 (1.592–5.515) | 0.0006 | — | — |
| Distant metastasis* | 1.994 (1.166–3.408) | 0.0117 | — | — |
| Histology†‡ | 1.340 (0.997–1.801) | 0.0525 | | |
| Lymphatic invasion (ly0 + ly1 or ly2 + ly3)† | 1.954 (1.381–2.765) | 0.0002 | 1.507 (0.977–2.325) | 0.0639 |
| Venous invasion (v0 + v1 or v2 + v3)† | 1.536 (1.093–2.160) | 0.0136 | 1.308 (0.861–1.987) | 0.2084 |
| Intrapancreatic nerve invasion (n0 + n1 or n2 + n3)† | 1.340 (0.953–1.884) | 0.0923 | | |
| Spread within the main pancreatic duct [mpd (–) or mpd (b) + (+)]† | 1.065 (0.679–1.668) | 0.7848 | | |
| CDCP1 | 1.470 (1.017–2.125) | 0.0404 | 1.482 (1.022–2.150) | 0.0381 |

Abbreviations: mpd, main pancreatic duct; b, borderline.
*Classified according to the classification of International Union Against Cancer.
†Classified according to the classification of pancreatic carcinoma of Japan Pancreas Society.
‡Classified according to the classification of WHO.

in pancreatic cancer cell lines, whereas much lower expression was detected at the free edges of cells (Supplementary Fig. S1).

Suppression of CDCP1 expression by siRNA strongly inhibited cell migration and invasion of pancreatic cancer cells (Fig. 2A; Supplementary Fig. S2). Similar to the previous report in lung cancer cells (5), downregulation of CDCP1 in pancreatic cancer cells had no significant effect on the phosphorylation of AKT and extracellular signal-regulated kinase 1/2, which are essential components of the growth factor signaling pathway mediating cell survival, proliferation, and motility (data not shown). For rescue experiments, vectors expressing wild-type CDCP1 and Y734F mutant CDCP1, which lacks the SFKs-binding site, were designed to contain silent mutations to be resistant to siRNA for CDCP1 as shown in Fig. 2B (CDCP1 res-F and Y734F res-F, respectively). Tyrosine phosphorylation of CDCP1 is shown to be attenuated in Y734F mutant through dissociation from SFKs (Supplementary Fig. S3). After suppression by CDCP1 siRNA, CDCP1 expression was restored by transfection of either CDCP1 res-F or Y734F res-F constructs in BxPC3 as expected (Fig. 2C). Both migration and invasion were recovered by the CDCP1 res-F construct to the level of original BxPC3 cells, but not by the Y734F res-F mutant (Fig. 2D), suggesting that CDCP1 regulates migration and invasion in a tyrosine phosphorylation–dependent manner.

### Tyrosine-phosphorylated CDCP1 promotes ECM degradation

Because the biological role of CDCP1 in invasion is totally unknown, we first examined whether CDCP1 influences ECM degradation of cancer cells. Loss of CDCP1 decreased the ability to degrade fluorescence-conjugated collagen on cover glasses (Fig. 3A), whereas it caused no significant effect on the degree of cell-ECM attachment (data not shown). To quantify the ability of ECM degradation, cells that are >50% covered by dark area with degraded collagen were counted, and the ratio to the total number of cells was calculated. This ratio of degradation showed 40% to 90% decrease in BxPC3 and Capan1 by suppression of CDCP1 (Fig. 3B). Protease secretion was then analyzed by zymography to identify factor (s) that regulates ECM degradation. Suppression of CDCP1 attenuated gelatin degradation bands at ~90 kDa detected in culture medium of BxPC3 and CFPAC1 (Fig. 3C, top; Supplementary Fig. S4). The bands at ~90 kDa in the zymogram correspond to the molecular size of MMP-9, a major MMP expressed in invasive cancer, and were actually detected by Western blotting using the anti–MMP-9 antibody (Fig. 3C, bottom left). Treatment of MMP inhibitor II, which inhibits a series of MMPs including MMP-9, suppressed the bands at ~90 kDa in both BxPC3 and HT1080, which is already known to secrete MMP-9 (Fig. 3C, bottom right), whereas the bands at ~60 kDa, presumably MMP-2, were not inhibited in HT1080 cells. The quantity of MMP-9 mRNA was not affected by CDCP1 siRNA (data not shown). These results indicate that CDCP1 controls ECM degradation through secretion of proteases, including MMP-9, in pancreatic cancer cells.

### CDCP1 induces activation of PKCδ through tyrosine phosphorylation of PKCδ

It was shown in pancreatic cancer cell lines that tyrosine phosphorylation of CDCP1 at $Tyr^{734}$ triggers its association with a downstream target PKCδ, recruitment of PKCδ to the CDCP1-SFKs complex, and tyrosine phosphorylation of PKCδ (Fig. 2C; Supplementary Fig. S3), which is required for anoikis resistance of cancer cells (5). Suppression of PKCδ inhibited cell migration and invasion (Supplementary Fig. S5)

and also blocked ECM degradation and protease secretion in BxPC3 and CFPAC1 (Fig. 4A; data not shown). The overexpression of the HA-tagged C2 domain of PKCδ, which was designed to block the CDCP1-PKCδ interaction (4, 5), resulted in decrease of migration and invasion of Capan1 cells (Fig. 4B), suggesting the CDCP1-PKCδ association is essential for these characteristics. Moreover, the overexpression of Y734F-F mutant in BxPC3 decreased ECM degradation and the secretion of proteases, including MMP-9 (Fig. 4C). This Y734F-F mutant suppressed tyrosine phosphorylation of PKCδ without significantly affecting the phosphorylation states of wild-type CDCP1 (Fig. 4C), possibly by interfering the extracellular signal, which might also modulate CDCP1-PKCδ signal in a dominant-negative manner. Phosphorylation of PKCδ at Thr$^{505}$, which was reported to indicate kinase activity of PKCδ (16), was examined by using a phosphospecific antibody. Phosphorylation of PKCδ at Thr$^{505}$ was actually induced by treatment of phorbol 12-myristate 13-acetate (PMA), an activator of PKCs, whereas it was reduced by suppression of CDCP1 expression (Fig. 4D). It was indicated that tyrosine phosphorylation of PKCδ by CDCP1 affects the kinase activity of PKCδ.

### Cortactin is a candidate protein downstream of the CDCP1-PKCδ pathway in cell migration and invasion

Treatment of CDCP1 siRNA did not cause significant effects on the phosphorylation status of several signaling proteins such as paxillin, focal adhesion kinase, and adducin, which were reported as downstream molecules of PKCδ (data not shown). On the other hand, we discovered that cortactin, which has been detected as one of the major substrates for SFKs and has been reported to play an essential

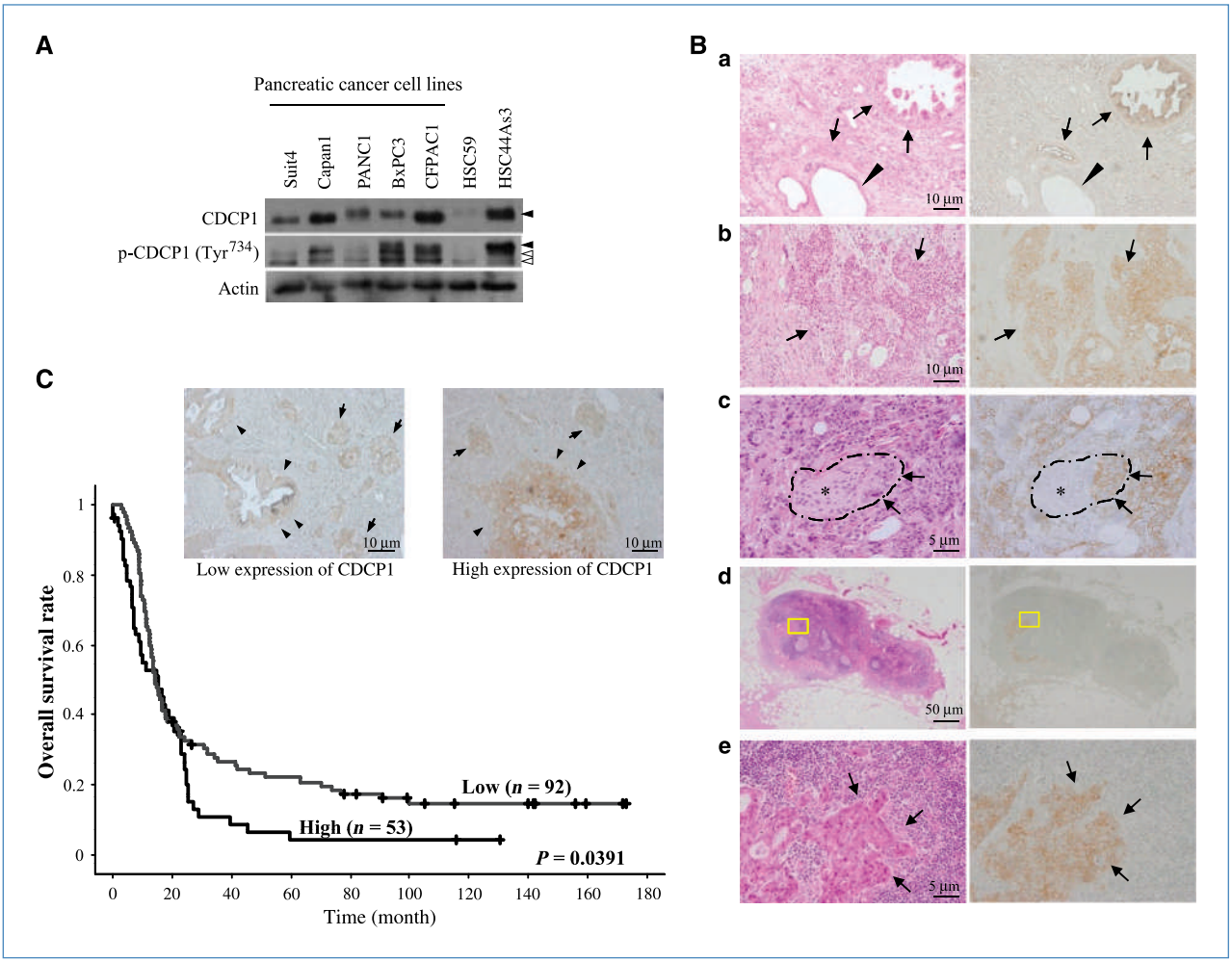


Figure 1. CDCP1 expression is correlated with prognosis of patients with pancreatic cancer. A, expression and tyrosine phosphorylation of CDCP1 in pancreatic cancer and noninvasive (HSC59) or invasive (HSC44As3) gastric cancer cell lines. Black arrowheads, CDCP1; white arrowheads, cross-reactive bands. B, CDCP1 expression in human pancreatic cancer tissues. Left, H&E staining; right, immunohistochemistry with anti-CDCP1 antibody. a, black arrowhead, normal pancreatic cells; black arrow, well-differentiated types of pancreatic cancers. b, poorly differentiated types of pancreatic cancers; c, perineural invasion (dotted line, epineurium; *, nerve); d and e, lymph node metastasis. e, enlargements of yellow squares in d. Magnifications, ×20 (d), ×100 (a and b), and ×200 (c and e). C, black arrowheads, pancreatic cancer; black arrows, islet of Langerhans for control staining. Kaplan-Meier plots of overall survival of patients with pancreatic cancer. High, CDCP1 high-expression group; Low, CDCP1 low-expression group.

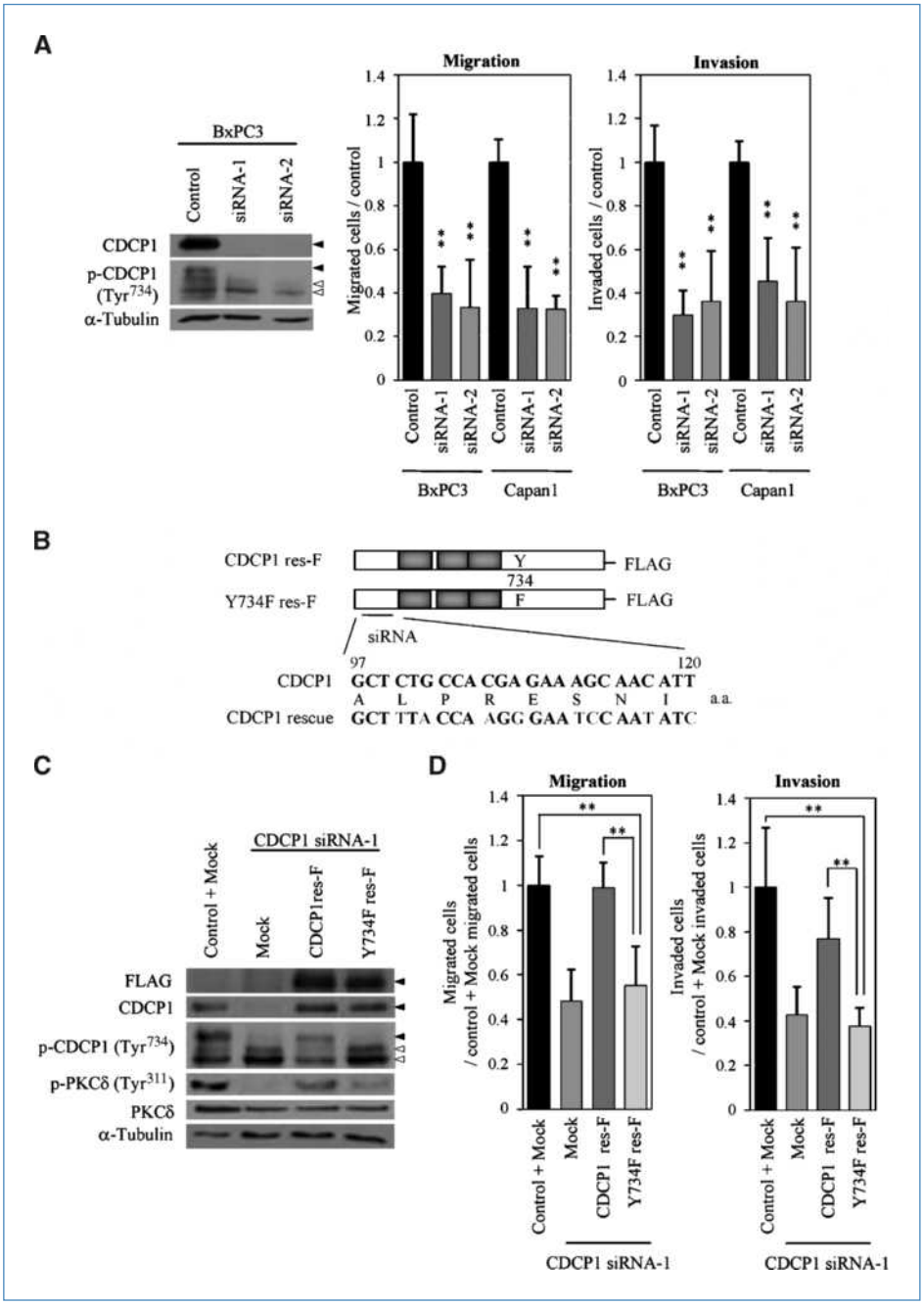


**Figure 2.** CDCP1 regulates migration and invasion in a tyrosine phosphorylation–dependent manner. A, left, BxPC3 cells treated with CDCP1 siRNAs were used for immunoblotting with the indicated antibodies; right, migration and invasion assay of BxPC3 and Capan1 ($4.0 \times 10^4$ cells) treated with CDCP1 siRNAs. Black arrowheads, CDCP1; white arrowheads, cross-reactive bands. B, schematic structure of CDCP1 and CDCP1 rescue mutant tagged with FLAG (CDCP1 res-F and Y734F res-F). The DNA base sequence that is not suppressed by CDCP1 siRNA is described. C, BxPC3 cells were treated with each siRNA and, 48 h later, transfected with the indicated plasmid. Cancer cells were used for immunoblotting with the indicated antibodies. Black arrowheads, CDCP1; white arrowheads, cross-reactive bands. D, migration and invasion assay using CDCP1 rescue mutant. BxPC3 cells treated with either control siRNA + pcDNA3.1 + pEGFP (bars, control + mock) or CDCP1 siRNA + each plasmid DNA + pEGFP (bars, mock, CDCP1 res-F, Y734F res-F) were used for each assay. Only migrated and invaded cells expressing green fluorescent protein (GFP) were counted using a fluorescence microscope. Columns, mean; bars, SD. The asterisks indicate statistically significant differences from the cells compared with control siRNA or control siRNA + Mock. *, $P < 0.05$; **, $P < 0.005$.

role in cell migration and invasion (17–19), was localized with CDCP1 and PKCδ at cell-cell contact in BxPC3 (Fig. 5A), and showed physical association with PKCδ through immunoprecipitation (Fig. 5B). Interestingly, suppression of CDCP1 disrupted the physical association between PKCδ and cortactin. On the other hand, tyrosine phosphorylation of cortactin was not affected by suppression of CDCP1 (Supplementary Fig. S6). Suppression of cortactin decreased migration and invasion in BxPC3 (Fig. 5C), as also reported in other cell types (20, 21), whereas it did not suppress ECM degradation (Fig. 5D) and gelatin degradation by proteases in the zymogram (data not shown) in BxPC3 and CFPAC1 cells. These results suggest that cortactin might be one of the mediators of the CDCP1-PKCδ signaling complex in cancer cell migration but not in the ECM degradation.

## Discussion

CDCP1 was originally identified as a membrane protein selectively expressed in the surface of metastatic cancers such as colon and lung cancers. It was later shown that CDCP1 is a potent substrate of SFKs *in vitro*, and our previous analysis

revealed that phosphorylation of CDCP1 by SFKs is essential for the anoikis resistance, which supports distant metastasis of solid cancers. In this study, we showed that CDCP1 is a significant prognostic factor that predicts the overall survival of patients with pancreatic cancer. We further showed for the first time that CDCP1-PKCδ signaling plays a crucial role in cell migration and ECM degradation in pancreatic cancer cells.

CDCP1 mRNA and protein expressions were found in various human solid cancers, including colon, lung, and breast cancers (1, 7, 22). CDCP1 was recently shown to be a prognostic factor of lung adenocarcinoma and renal cell carcinoma by using a large-scale analysis of CDCP1 expression in tumor samples (7, 8). By histologic analysis using human pancreatic cancer tissues, CDCP1 expression was detected not only in the original lesion but also in lymph node metastasis and perineural invasion (Fig. 1B). Pancreatic cancer is one of the most frequent causes of cancer-related deaths worldwide. The poor prognosis is attributed to the high incidence of distant metastasis at the point of diagnosis and the highly invasive nature of this cancer. In that sense, it is important that the patients with high CDCP1 expression showed significantly worse overall survival on both univariate and multivariate analyses, suggesting that it might be a novel and independent prognostic factor of pancreatic cancer (Table 1). The individual clinicopathologic factors were not statistically significantly correlated with CDCP1 expression, which may be due to remarkably short overall survival in patients with pancreatic cancers compared with those with renal cell carcinomas or lung cancers. Although general prognostic markers such as p16, MMP-7, and vascular endothelial growth factor are also applicable to patients with pancreatic cancer (23), CDCP1 is an essential marker of poor

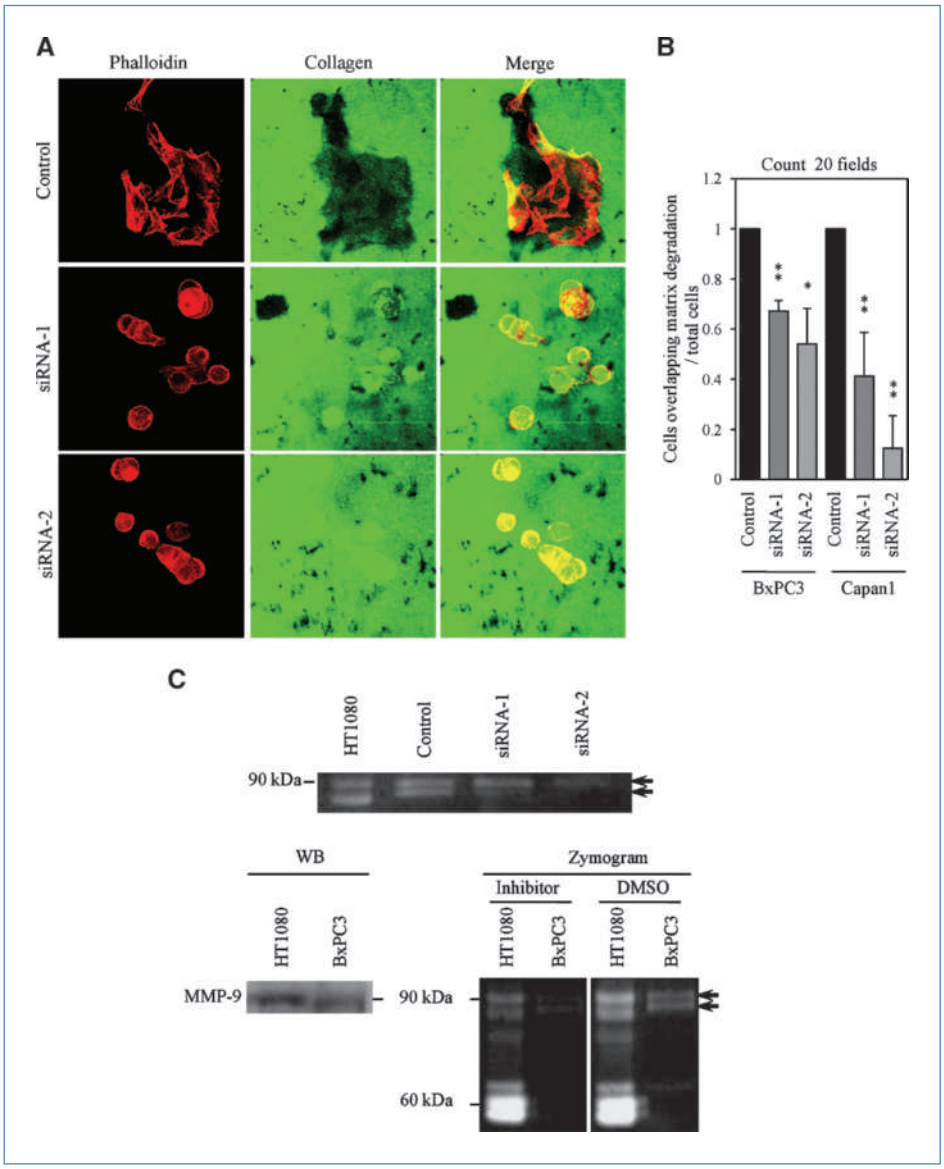


**Figure 3.** Tyrosine-phosphorylated CDCP1 promotes ECM degradation. A, BxPC3 cells transfected with CDCP1 siRNAs were incubated on covered glasses with fluorescein-conjugated collagen and stained with phalloidin to identify actin filaments. ECM degradation is identified as the dark area. B, quantification of ECM degradation. Cells overlapping the dark area, occupying more than half of the cell area, were counted. Columns, mean; bars, SD. The asterisks indicate statistically significant differences from the cells compared with control siRNA. *, $P < 0.05$; **, $P < 0.005$. C, top, gelatin zymography in BxPC3 transfected with CDCP1 siRNAs. Left, culture medium using BxPC3 and HT1080 cultures was concentrated and analyzed by Western blotting (WB) with the anti–MMP-9 antibody; right, zymogram of samples treated with MMP inhibitor II and DMSO. Black arrows, gelatin degradation.

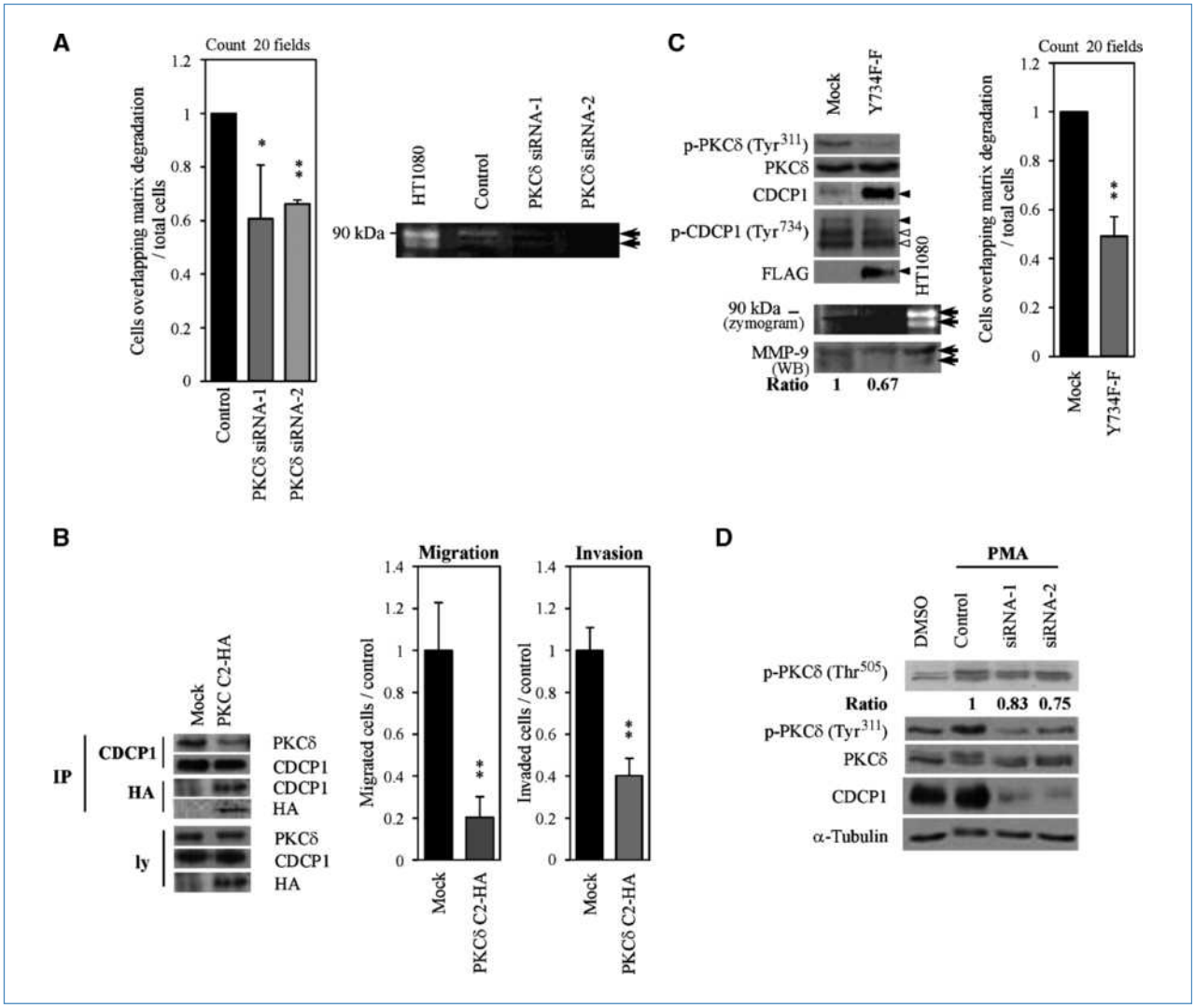


Figure 4. CDCP1 regulates migration and invasion via the PKCδ kinase activity. A, quantification of ECM degradation assay and zymogram of BxPC3 cells transfected with PKCδ siRNAs. Black arrows, MMP-9. B, left, Capan1 cells transfected with the C2 domain of PKCδ tagged with HA (PKCδ C2-HA) or pcDNA3.1 (Mock) were selected by G418. The lysate immunoprecipitated (IP) with anti-CDCP1 or anti-HA antibodies was used for immunoblotting with the indicated antibodies. Right, migration and invasion assay using Capan1 ($4.0 \times 10^4$ cells) transfected with the indicated plasmids. C, left, top, BxPC3 cells transfected with the CDCP1 mutant (Y734F res-F) or pcDNA3.1 (Mock) were selected by G418 and used for immunoblotting with the indicated antibodies; bottom, culture medium of the BxPC3 was analyzed by gelatin zymography and Western blotting with anti–MMP-9 antibody. Ratio, total MMP-9 of mock or Y734F/total MMP-9 of mock in Western blotting. Right, quantification of ECM degradation of BxPC3. Columns, mean; bars, SD. The asterisks indicate statistically significant differences from the cells transfected with control siRNA or mock. Black arrowheads, CDCP1; white arrowheads, cross-reactive bands; black arrows, MMP-9. *, $P < 0.05$; **, $P < 0.005$. D, BxPC3 cells transfected with CDCP1 siRNAs are cultured in 0.5% FBS for 24 h and treated with 100 nmol/L PMA or DMEM for 20 min at 37°C. The cells were used for immunoblotting with the indicated antibodies. Ratio, [phospho-PKCδ $Thr^{505}$/PKCδ (each siRNA)]/[phspho-PKCδ $Thr^{505}$/PKCδ (control siRNA)].

prognosis that is functionally related to particular malignant characteristics such as migration, ECM degradation, and anoikis resistance of cancer cells. This study also indicates that CDCP1 induces ECM degradation through secretion of proteases including MMP-9 (Figs. 3C and 4C). MMP-9 secretion has also been reported in human pancreatic tissue (24, 25) and was also considered to be a potential prognostic factor in pancreatic cancer (26).

A recent observation indicates that CDCP1 protein is expressed in some normal human tissues, such as the colon, breast, and lung, but not in the pancreatic duct (Fig. 1B), whereas the levels of CDCP1 phosphorylation in these normal tissues were much lower than those in cancer cells (27). We previously reported that phosphorylation of CDCP1 at $Tyr^{734}$ is increased during peritoneal dissemination of gastric cancer *in vivo* and that it could promote migration of gastric cancer cells *in vitro* (6). In this study, we showed that phosphorylation of CDCP1 at $Tyr^{734}$ plays a significant role in promotion of cell migration and ECM degradation, in addition to anoikis resistance of cancer cells. Therefore, it is

reasonable that not only the expression but also the tyrosine phosphorylation of CDCP1 is required for these characteristics of cancer cells associated with invasion and metastasis.

The complex formation between tyrosine-phosphorylated CDCP1 and PKCδ is required for the promotion of migration and ECM degradation. Our results also suggest that tyrosine phosphorylation of PKCδ, which was triggered by the association with phosphorylated CDCP1 coupled with SFKs, is essential for CDCP1-induced cell migration and ECM degradation. Judging from the levels of phosphorylation at $Thr^{505}$ of PKCδ (Fig. 4D), a putative autophosphorylation site in an activation loop (16), tyrosine phosphorylation of PKCδ by the SFKs-CDCP1 pathway might activate PKCδ. Previous studies on PKCδ have shown that phosphorylation of PKCδ at $Tyr^{311}$ results in enhanced phosphorylation at $Thr^{505}$ (28) and that activation of PKCδ enhances the MMP-9 activity (29). These findings suggest that the formation of the SFKs-CDCP1-PKCδ complex triggered by tyrosine phosphorylation of CDCP1 causes tyrosine phosphorylation of PKCδ and activation of kinase activity of PKCδ, which promotes cell migration and ECM degradation.

We identified cortactin as a binding partner of PKCδ. Cortactin is one of the key molecules that control cell migration and invasion. Some studies have reported that serine phosphorylation, but not tyrosine phosphorylation, of cortactin increases N-WASP binding and facilitates N-WASP–dependent actin polymerization (30, 31). Because the kinase activity of PKCδ (Fig. 4D) and the association of PKCδ and cortactin (Fig. 5B) can be affected by CDCP1, CDCP1 possibly regulates cell migration by altering serine/threonine phosphorylation of cortactin through PKCδ. Unfortunately, this could not be confirmed due to the lack of a good specific

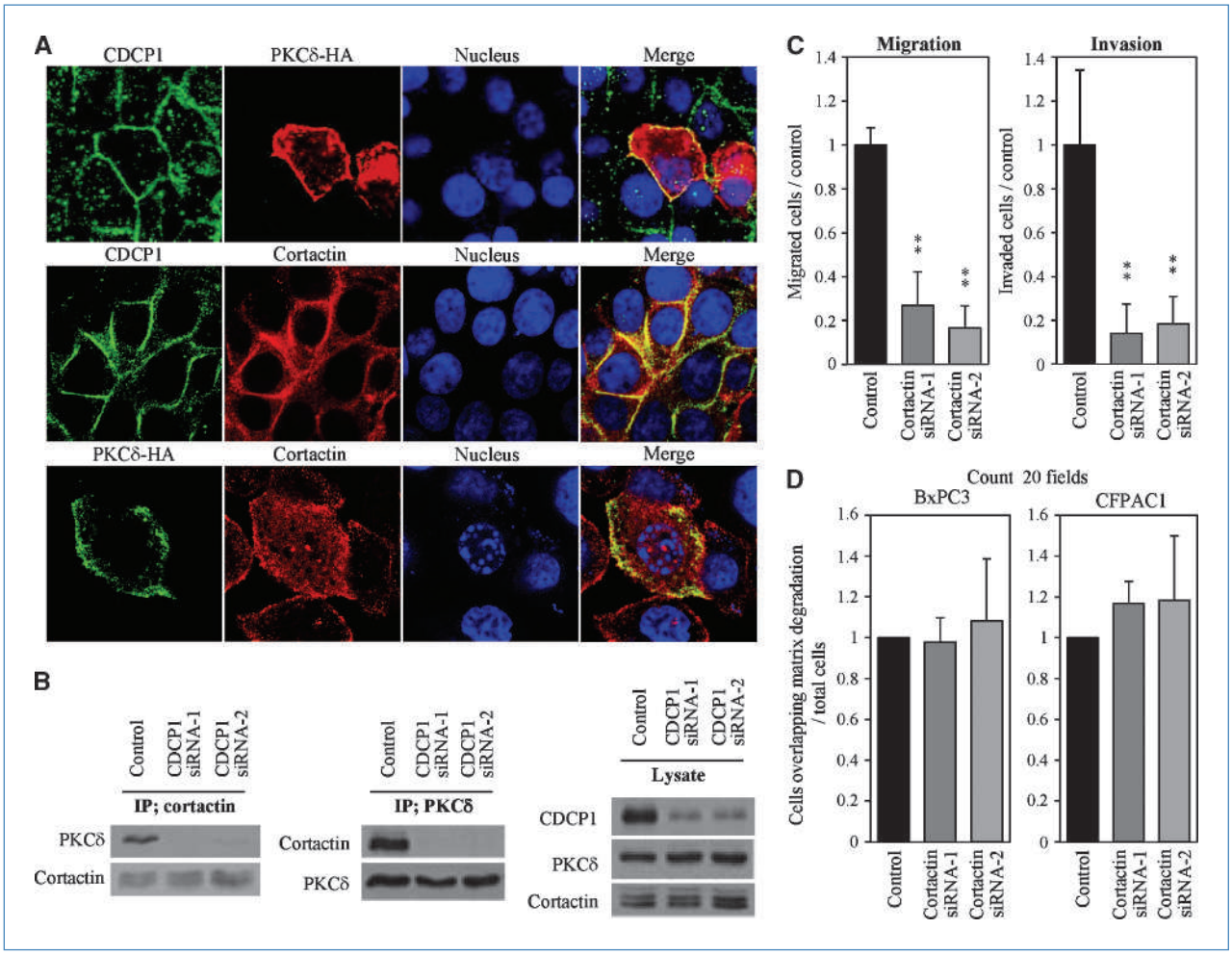


Figure 5. Cortactin, a PKCδ-associated protein, regulates cell migration and invasion. A, top, BxPC3 stained with anti-HA antibody (PKCδ-HA; red) and anti-CDCP1 antibody (green). The nucleus was stained with TOTO3. Middle, BxPC3 stained with anti-CDCP1 antibody (green) and anti-cortactin antibody (red). Bottom, BxPC3 stained with anti-HA antibody (PKCδ-HA; green) and anti-cortactin antibody (red). B, immunoprecipitation with anti-cortactin or anti-PKCδ antibodies in BxPC3 transfected with each siRNA. Samples were immunoblotted with the indicated antibodies. C, migration and invasion assay of BxPC3 ($1.0 \times 10^4$ cells) transfected with cortactin siRNAs. Columns, mean; bars, SD. The asterisks indicate differences from cells treated with control siRNA. *, $P < 0.05$; **, $P < 0.005$. D, quantification of ECM degradation of protease in BxPC3.

antibody against serine/threonine–phosphorylated cortactin. Cortactin has also been reported to play a role in the function of invadopodia in several cancers (32, 33), although a structure similar to invadopodia was not found in the pancreatic cancer cells used in this study. Loss of cortactin did not suppress ECM degradation and protease secretion in pancreatic cancer cells, suggesting that some molecules other than cortactin might be regulating ECM degradation under the control of CDCP1, whereas cortactin is involved in CDCP1-induced migration of cells possibly through regulation of actin dynamics.

As CDCP1 binds the regulatory domain of SFKs (5), there is a possibility that CDCP1 controls the activity of SFKs by unfolding these proteins. Positive correlation between CDCP1 expression and $Tyr^{418}$ phosphorylation, which indicates autophosphorylation activity of SFKs, was obtained in BxPC3 cells (data not shown), whereas no clear correlation was obtained in other pancreatic cell lines. Further study will be required to understand the functional association between SFKs and CDCP1, including identification of other modulators of association.

Collectively, CDCP1 is a novel prognostic factor of pancreatic cancer, and inhibition of a specific cellular signal originating from the expression of phosphorylated CDCP1 has been shown to regulate cell migration, invasion, and ECM degradation in pancreatic cancer cells. Because early clinical diagnosis of pancreatic cancer is difficult, invasion or metastasis to other organs frequently precedes diagnosis. In addition to its diagnostic usefulness as a membrane protein, CDCP1 might be an optimal therapeutic target of invasive and metastatic pancreatic cancers alone or combined with general chemotherapy drugs.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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