Pim-3, a Proto-Oncogene with Serine/Threonine Kinase Activity, Is Aberrantly Expressed in Human Pancreatic Cancer and Phosphorylates Bad to Block Bad-Mediated Apoptosis in Human Pancreatic Cancer Cell Lines

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Abstract

Pancreatic cancer still remains a serious health problem with <5% 5-year survival rate for all stages. To develop an effective treatment, it is necessary to identify a target molecule that is crucially involved in pancreatic tumor growth. We previously observed that Pim-3, a member of the proto-oncogene Pim family that expresses serine/threonine kinase activity, was aberrantly expressed in human and mouse hepatomas but not in normal liver. Here, we show that Pim-3 is also expressed in malignant lesions of the pancreas but not in normal pancreatic tissue. Moreover, Pim-3 mRNA and protein were constitutively expressed in all human pancreatic cancer cell lines that we examined and colocalized with the proapoptotic protein Bad. The ablation of endogenous Pim-3 by small hairpin RNA transfection promoted apoptosis, as evidenced by increases in a proportion of cells in the sub-G_1 fraction of the cell cycle and in phosphatidyl serine externalization. A pro-apoptotic molecule, Bad, was phosphorylated constitutively at Ser^{112} but not Ser^{136} in human pancreatic cancer cell lines and this phosphorylation is presumed to represent its inactive form. Phosphorylation of Bad and the expression of an antiapoptotic molecule, Bcl-X_L, were reduced by the ablation of endogenous Pim-3. Thus, we provide the first evidence that Pim-3 can inactivate Bad and maintain the expression of Bcl-X_L and thus prevent apoptosis of human pancreatic cancer cells. This may contribute to the net increase in tumor volume or tumor growth in pancreatic cancer. (Cancer Res 2006; 66(13): 6741-7)

Introduction

Pancreatic cancer is one of the commonest causes of death from cancer, and 5-year survival for all stages for pancreatic cancer remains at <5% (1). Moreover, most patients are diagnosed at an advanced stage and are not candidates for local treatment such as surgical resection and irradiation. Although several chemotherapy regimens have been recently developed for advanced pancreatic cancer, the results are not satisfactory with a median survival <1 year. Thus, to develop a more effective treatment, it is necessary to identify additional target molecules that are crucially involved in the growth of pancreatic cancer cells.

Pim-3, a member of the proto-oncogene Pim family that expresses serine/threonine kinase activity, was originally identified as a depolarization-induced gene, *KID-1*, in PC12 cells (a rat pheochromocytoma cell line; ref. 2). Subsequently, several independent groups reported its potential role in neuronal cell functions including consolidation of long-term potentiation (3, 4). Meanwhile, Deneen et al. (5) showed that *Pim-3* gene transcription was enhanced in EWS/ETS-induced malignant transformation of NIH 3T3 cells, suggesting the involvement of Pim-3 in tumorigenesis. In line with these observations, we showed that Pim-3 was selectively expressed in human and mouse hepatocellular carcinomas but not in normal liver tissue (6). Moreover, the ablation of endogenous Pim-3 by short interfering RNA reduced the cell growth of human hepatoma cell lines, partly by promoting apoptosis (6). These observations would indicate a potential role for Pim-3 in tumorigenesis, similar to that of other members of the Pim kinase family (7–10).

Reverse transcription PCR (RT-PCR) analysis of mouse normal tissues further showed that *Pim-3* mRNA was undetectable in the normal pancreas.[5] As the pancreas is derived from endoderm, similar to that of the liver, we hypothesized that Pim-3 might be aberrantly expressed in malignant lesions of the pancreas and, if confirmed, Pim-3 might be a candidate target molecule for the treatment of pancreatic cancer. To test this hypothesis, we examined Pim-3 expression in human pancreatic cancer tissues as well as normal pancreatic tissues, by immunohistologic analysis. Pim-3 protein was detected in human pancreatic cancer but not in normal pancreatic tissue. Moreover, *Pim-3* mRNA and protein were constitutively expressed in all human pancreatic cancer cell lines that we examined. Furthermore, the ablation of endogenous Pim-3 promoted the apoptosis of a human pancreatic cancer cell line. This antiapoptotic function by aberrantly expressed Pim-3 may result in the net increase in tumor volume or growth. Thus, Pim-3 may be an important molecular target for pancreatic cancer as conventional treatments have little effect on the prognosis of this incurable disease (1).

The proapoptotic activity of Bad is regulated by its phosphorylation at Ser^{112} or Ser^{136}. Unphosphorylated Bad binds and eventually inactivates antiapoptotic family members, primarily Bcl-X_L but also Bcl-2 (11–13). Phosphorylation of Bad at Ser^{112} or

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Ser^{136} resulted in the liberation of Bcl-X_L and Bcl-2, which can prevent apoptosis (14, 15). Thus, phosphorylated Bad represents its inactive form. Other Pim family members, Pim-1 and Pim-2, can inactivate Bad by phosphorylating Bad Ser^{112} (16, 17). Moreover, another serine/threonine kinase, Akt, shows similar substrate specificity as Pim kinases, and its inhibitor can selectively decrease Bcl-X_L protein expression (18). These observations prompted us to evaluate the effects of *Pim-3* gene ablation on Bad phosphorylation and Bcl-X_L protein expression. Indeed, the ablation of *Pim-3* gene reduced phosphorylation of Bad at Ser^{112} and Bcl-X_L protein expression. Thus, Pim-3 may prevent apoptosis in human pancreatic cancer cell lines by modulating these molecules.

Materials and Methods

Cell culture and reagents. Human pancreatic cancer cell lines, PANC-1 (19), MiaPaca-2 (20), PCI35, PCI55, and PCI66 (21), were maintained in RPMI 1640 (Sigma Chemical Co., St. Louis, MO). Human embryonic kidney HEK293 and HeLa cells were maintained in DMEM (Sigma). All media were supplemented with 10% fetal bovine serum (Sigma) and the cells were cultured in 5% CO_2 at 37°C. The following antibodies were used: mouse anti-Bad and rabbit anti-β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); Alexa Fluor 488 donkey anti-rabbit immunoglobulin G (IgG), Alexa Fluor 546 donkey anti-goat IgG, and Alexa Fluor 594 donkey anti-mouse IgG (Invitrogen Japan, Tokyo, Japan); rabbit anti–phospho-Ser^{112} Bad, anti–phospho-Ser^{136} Bad, and anti–phospho-Ser^{155} Bad antibodies (Cell Signaling Technology, Beverly, MA); and rabbit anti-Bcl-X_L antibodies (Medical and Biological Laboratories, Co. Ltd., Nagoya, Japan). Rabbit anti-human Pim-3 IgG was prepared by immunizing a rabbit with a Pim-3 peptide (6).

Immunohistochemical analysis of Pim-3 in human pancreatic cancer tissues. Human pancreatic tissues were obtained from patients on surgery for pancreatic cancer with their informed consent. Pancreatic tissue, which was at least 2 cm apart from the edge of tumor foci and was judged histologically to be free from adenocarcinoma cells, was used as a normal tissue control. Ten pancreatic adenocarcinoma tissues were obtained and were pathologically graded as well-differentiated (one case), moderately differentiated (eight cases), and poorly differentiated adenocarcinoma (one case) according to the WHO classification (22). Paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol (70-100%). After incubation with 0.3% hydrogen peroxide, sections were incubated sequentially with 3% normal goat serum (DakoCytomation, Glostrup, Denmark) and 2% bovine serum albumin (BSA) in PBS(−), and with an Avidin-Biotin Blocking Kit (Vector Laboratories). Subsequently, the slides were treated with rabbit anti-Pim-3 IgG (3 μg/mL), followed by incubation with goat anti-rabbit IgG at room temperature for 1 hour. Pim-3 immunoreactivity was visualized by using the Vectastain Elite ABC kit and the Vectastain DAB substrate kit (Vector Laboratories, Burlingame, CA). The slides were counterstained with ChemMate Hematoxylin (DakoCytomation), mounted, and observed under a microscope (BX-50, Olympus, Tokyo, Japan). The proportion of Pim-3-positive cells was classified into five levels (0%, 1-4%, 5-30%, 31-60%, and >60%) by an examiner who was without any clinical information.

Immunofluorescence analysis. Cells were cultured in Lab-Tec chamber slides (Nalge Nunc, Roskide, Denmark) and were fixed with 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS. Then, slides were incubated with 3% donkey serum in 1% BSA/PBS at room temperature for 30 minutes. The slides were then incubated with the combination of rabbit polyclonal anti-Pim-3 antibodies (3 μg/mL) and mouse monoclonal anti-Bad antibody, diluted at 1:30, or with the combination of anti-Pim-3 and goat polyclonal anti–phospho-Ser^{112} Bad antibodies, diluted at 1:20, overnight at 4°C. Thereafter, the slides were incubated with the combination of Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 594 donkey anti-mouse IgG or with the combination of Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 546 donkey anti-goat IgG. In some experiments, immunofluorescence analysis was done using the combination of anti-Pim-3 and Alexa Fluor 488 donkey anti-rabbit IgG. The signals were visualized using a laser-scanning microscope (Axiovert 100M in LSM 510 system, Carl Zeiss Japan, Tokyo, Japan) and fluorescent images were digitally merged in both horizontal (*xy* axis) and orthogonal planes (*xz* and *yz* axes).

Immunoprecipitation using anti-Pim-3 antibodies. Human pancreatic cancer cell lines, PCI-35, PCI-55, PCI-66, PANC-1, MiaPaca-2, and HeLa cells, were collected and solubilized with 1 mL of CelLytic-M mammalian Cell Lysis/Extraction Reagent (Sigma) and with complete protein inhibitor cocktail (Roche Diagnostics, Tokyo, Japan). Our preliminary experiments revealed that HeLa cells contained Bad but not Pim-3 (data not shown). Thus, cell lysates from HeLa cells were subjected to immunoprecipitation with anti-Bad antibodies, followed by immunoblotting with anti-Bad antibodies as a positive control for Bad but not Pim-3. Aliquots (50 μg) of the supernatants were incubated with rabbit anti-Pim-3 IgG (5 μg) overnight at 4°C. The resultant immune complexes were collected with protein G-Sepharose 4 Fast flow (GE Healthcare Biosciences, Tokyo, Japan) and the beads were washed thrice with cell lysis buffer. Materials bound to the beads were eluted with SDS-PAGE loading buffer containing 1% 2-mercaptoethanol. The resultant proteins were separated in a 12% SDS-polyacrylamide gel and transferred onto an Immobilon-P Transfer membrane (Millipore, Bedford, MA). After being soaked with 3% BSA, the membrane was incubated with rabbit anti-Pim-3, followed by incubation with ImmunoPure peroxidase–conjugated goat anti-rabbit IgG, diluted at 1:500. The blotted membrane was treated with the SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, Inc., Milwaukee, WI) and signals were detected by LAS-3000 mini CCD camera (Fuji Film, Tokyo, Japan). Aliquots of cell lysates (10 μg) were directly subjected to immunoblotting with anti-β-actin antibodies to ensure that an equal amount of protein was used for immunoprecipitation. The intensities of the bands were determined using NIH Image Analysis Software version 1.62 (NIH, Bethesda, MD) and the ratios to β-actin were determined. In some experiments, rabbit anti-Bad antibodies were used instead of anti-Pim-3 antibodies.

Construction of small hairpin RNA plasmids and transfection. The selected short interfering RNA target sequence in Pim-3 (5′-GCACGUGGUGAAGGAGCGG-3′ corresponding to 642-661 residues) and nonspecific control short interfering RNA duplexes (5′-GCGCGCUUUGUAGGAUUCG-3′) were designed as previously described (6) and small hairpin RNA (shRNA)–encoding oligonucleotides were prepared by Ambion (Austin, TX). The annealed shRNA were inserted into the *Bam*HI and *Hin*dIII sites of the pSilencer neo H1 vector (Ambion). PCI35 or PCI55 cells in a 6-cm dish were transfected with the resultant shRNA vector using Lipofectamine 2000 (Invitrogen Corporation, Carlsbad, CA) according to the instructions of the manufacturer.

Cell viability assay. Two days after the transfection, the cells were trypsinized and 3×10^3 cells were plated in a 96-well plate. This time point was designated as day 0. Aliquots of cells were processed for immuoblotting with anti-Pim-3 antibodies to estimate the amount of Pim-3 protein. The cell viability was determined every day by adding 10 μL of WST-1 reagent (an MTT analogue from Boehringer Mannheim Corporation, Indianapolis, IN) to each well. After incubation at 37°C for 2 hours, the absorbance at 450 nm was measured and ratios of cell numbers were determined by comparison of the number of cells at day 0.

Cell cycle and cell apoptosis analysis. At the indicated time intervals following transfection with either Pim-3 or scramble shRNA, the cells were harvested and fixed with 70% ethanol at −20°C. The fixed cells were incubated with 50 μg/mL propidium iodide (Molecular Probes) and 1 μg/mL RNase A for 30 minutes at room temperature. DNA content was then analyzed on a FACS Caliber system (Becton Dickinson, Bedford, MA). The distribution of cells in each cell-cycle phase was determined by using cell ModFitLT Software (Becton Dickinson). In some experiments, phosphatidyl serine exposure level was determined by staining the cells with human Annexin V-FITC Kit (Bender MedSystem Inc., Burlingame, CA) according to the instructions of the manufacturer. At least 50,000 stained cells were analyzed on a FACS Caliber system for each determination.

Determination of Bad and Bcl-X_L proteins after Pim-3 shRNA transfection. At the indicated time intervals following shRNA transfection,

whole-cell lysates were prepared by using CelLytic-M mammalian Cell Lysis/Extraction Reagent containing complete protein inhibitor cocktail. The supernatants were obtained after centrifugation for 15 minutes at 14,000 rpm. Aliquots (50 μg) of the obtained supernatants were separated on a 15% SDS-polyacrylamide gel and transferred onto an Immobilon-P Transfer membrane (Millipore). After being soaked with 3% BSA, the membrane was incubated with mouse anti-Bad, rabbit anti–phospho-Ser^{112} Bad, anti–phospho-Ser^{136} Bad, anti–phospho-Ser^{155} Bad, or anti-Bcl-X_L antibodies, followed by incubation with ImmunoPure peroxidase–conjugated anti-rabbit IgG or anti-mouse IgG. The blotted membrane was then treated with the SuperSignal West Dura Extended Duration Substrate and signals were detected using LAS-3000 mini CCD camera.

Construction of human Pim-3 expression vector and transfection. Full-length human Pim-3 cDNA was subcloned into pcDNA4 (Invitrogen). PANC-1 or MiaPaca-2 cells in a 6-cm dish were transfected with the resultant expression vector using Lipofectamine Plus (Invitrogen). The used amounts of the vectors were adjusted to 2 μg each transfection, with pcDNA4 empty vector. The cell lysates were obtained 30 hours after the transfection and aliquots (10 μg) of the obtained supernatants were separated on a 15% SDS-polyacrylamide gel and transferred onto an Immobilon-P Transfer membrane (Millipore). After being soaked with 3% BSA, the membrane was incubated with mouse anti-Bad, rabbit anti–phospho-Ser^{112} Bad, anti–phospho-Ser^{136} Bad, or anti–phospho-Ser^{155} Bad antibodies, followed by incubation with ImmunoPure peroxidase–conjugated anti-rabbit IgG or anti-mouse IgG. The blotted membrane was then treated with the SuperSignal West Dura Extended Duration Substrate and signals were detected using LAS-3000 mini CCD camera.

Results

Aberrant expression of Pim-3 protein in human pancreatic cancer tissue. We previously observed that Pim-3 protein was aberrantly expressed in both human and mouse hepatocarcinomas but not in normal liver tissues. Moreover, Pim-3 mRNA was not detected in normal murine pancreas, another endoderm-derived organ.[5] Hence, we postulated that Pim-3 protein might be aberrantly expressed in malignant lesions in pancreas, similar to that observed in liver. In support of this hypothesis, Pim-3-positive cells were not detected in islet and non-islet portion of four normal human pancreatic tissues that we examined (Fig. 1*A*). In contrast, Pim-3 protein was abundantly detected in malignant glandular epithelium in 10 of 10 human pancreatic cancer tissues examined (Fig. 1*B, C, E*, and *F*; Pim-3-positive cell proportion 1-4%, 3 cases; 5-30%, 4 cases; 31-60%, 3 cases). The immunoreactivity was not detected by the use of rabbit control IgG (Fig. 1*D*) or the antibodies preadsorbed with the peptides used for the immunization,[5] indicating the specificity of the reaction. Moreover, an observation under a higher magnification showed that the staining pattern is cytoplasmic in most positive cells (Fig. 1*C* and *F*). These observations suggest that Pim-3 protein was aberrantly expressed in malignant lesions of the pancreas, another endoderm-derived organ.

Constitutive Pim-3 expression in human pancreatic cancer cell lines. The aberrant expression of Pim-3 in human pancreatic cancer tissues prompted us to evaluate its expression in human pancreatic cancer cell lines. In all five human pancreatic cancer cell lines that were examined, RT-PCR analysis detected Pim-3 mRNA (data not shown) and immunoblot analysis showed the presence of Pim-3 protein (Fig. 2*A*). An immunofluorescence analysis further showed that Pim-3 protein was present in cytoplasm of human pancreatic cancer cell lines, PCI55 and PCI35 (Fig. 2*C*). Similar staining patterns were also observed on another three cell lines (data not shown).

Enhanced apoptosis of human pancreatic cancer cell lines resulting from the ablation of endogenous Pim-3 protein. We have previously shown that the ablation of endogenous Pim-3 promoted apoptosis in human hepatoma cell line (6). Therefore, to clarify the role of endogenous Pim-3 in human pancreatic cancer cell survival, we ablated Pim-3 protein by the transfection with Pim-3 shRNA into the human pancreatic cancer cell lines, PCI55 and PCI35 cells. Transfection with Pim-3 shRNA, but not scramble shRNA, markedly diminished Pim-3 protein in both PCI55 and PCI35 by 48 hours after the transfection (Fig. 3*A* and *B*). Under the same transfection conditions, cell numbers were significantly reduced in the cells transfected with Pim-3 shRNA compared with those transfected with scramble shRNA or no shRNA (Fig. 3*C* and *D*). Several lines of evidence have indicated that apoptosis can be negatively modulated by other Pim kinases (15). Hence, we examined the effects of the ablation of Pim-3 protein on the cell cycle distribution of the pancreatic cancer cell lines. Transfection with Pim-3 shRNA resulted in a higher ratio of sub-G_1 cell populations with reduced G_1 population compared with the cells transfected with scramble shRNA (Fig. 4*A-C*). Moreover, Pim-3 shRNA transfectants contained a markedly higher ratio of apoptotic cells as evidenced by enhanced phosphatidyl serine externalization (Fig. 4*D-F*). In contrast, the treatment with Pim-3 shRNA reduced marginally the ratio of S-phase cell population. Similar results were obtained using PCI35 cells (data not shown). These observations indicate that the ablation of Pim-3 may promote apoptosis but have little effects on cell cycle progression, thereby leading to reduced cell numbers in the cell line cultures.

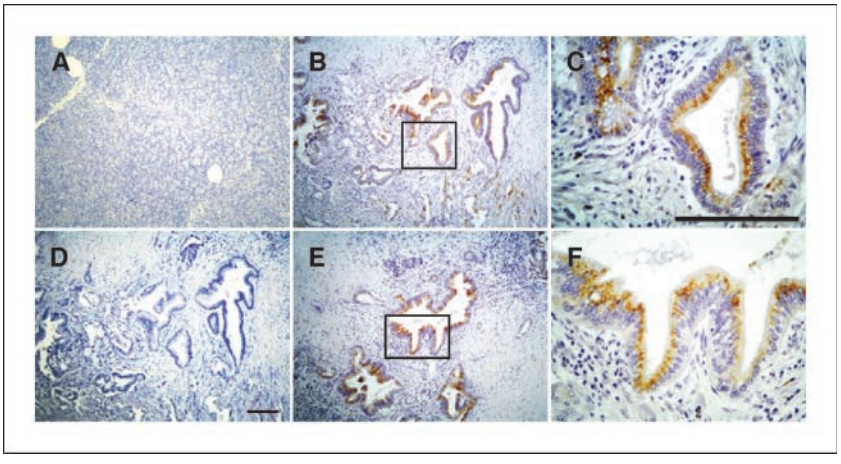

Figure 1. Aberrant expression of Pim-3 protein in human pancreatic cancer tissues. Human normal pancreatic tissue (*A*) and moderately-differentiated pancreatic cancer tissues (*B-F*) were immunostained with anti-Pim-3 antibodies (*A, B, C, E*, and *F*) or control rabbit IgG (*D*) as described in Materials and Methods. Serial sections from the same patient were used in (*B, C*, and *D*). The section from the other patient was used in (*E* and *F*). *C* and *F,* boxes areas in *B* and *E*, respectively, observed under a higher magnification. Similar staining patterns were observed on well-differentiated and poorly differentiated as well as moderately differentiated pancreatic adenocarcinoma tissues. Representative results from 4 normal pancreas tissues and 10 pancreatic cancer tissues. Original magnification, ×100 (*A, B, D*, and *E*); ×400 (*C* and *F*).

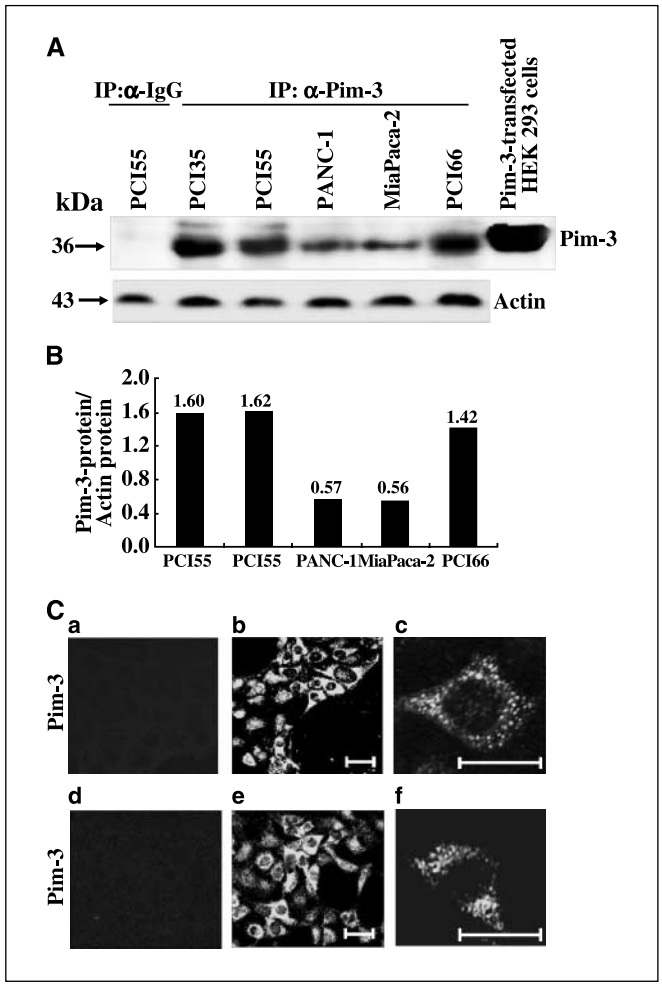

Figure 2. Constitutive Pim-3 expression in human pancreatic cancer cell lines. *A* and *B,* immunoblotting analysis of Pim-3 protein in human pancreatic cancer cell lines. Cell lysates were obtained from human pancreatic cancer cells and were subjected to immunoprecipitation with anti-Pim-3 antibodies followed by immunoblotting with anti-Pim-3 antibodies as described in Materials and Methods. HEK293 cells transfected with human Pim-3 cDNA were used as a positive control. Cell lysates from PCI55 cells were subjected to immunoprecipitation with rabbit anti-IgG antibodies followed by immunoblotting with anti-Pim-3 antibodies as negative control for Pim-3. The intensities of the bands were determined as described in Materials and Methods. *A,* representative results from three independent experiments; *B,* ratios to β-actin. *C,* immunofluorescence analysis of Pim-3 protein expression in human pancreatic cancer cell lines. Immunofluorescence analysis was carried out on human pancreatic cancer cell lines, PCI55 (*a-c*) and PCI35 cells (*d-f*), with (*b, c, e*, and *f*) or without anti-Pim-3 antibodies (*a* and *d*), as described in Materials and Methods. Representative results from three independent experiments. Original magnification, ×200 (*a, b, d*, and *e*); ×800 (*c* and *f*). Bar, 20 μm.

Phosphorylation of Bad by Pim-3. Accumulating evidence indicates that other members of the Pim family, Pim-1 and Pim-2, can phosphorylate Bad, the proapoptotic BH3-only protein at Ser^{112}, and thereby prevent apoptosis (16, 17). Hence, we explored whether Pim-3 could phosphorylate Bad. A double-color immunofluorescence analysis showed that Pim-3 protein was colocalized with Bad protein in the human pancreatic cancer cell lines in horizontal plane (*xy* axis; Fig. 5*A*). The analysis on orthogonal planes (*xz* and *yz* axes) further showed the colocalization of Bad with Pim-3 protein [Fig. 5*C*(*i*)]. Moreover, immunoprecipitation with anti-Pim-3 antibodies, but not control IgG, coprecipitated Bad [Fig. 5*D*(*i*)]. Furthermore, immunoprecipitation with anti-Bad antibodies, but not control IgG, coprecipitated Pim-3 protein in cell extracts [Fig. 5*D*(*ii*)]. These observations indicate an association between Pim-3 and Bad in these cell lines. Pim-3 protein colocalized with phospho-Ser^{112} Bad protein in these cell lines in both horizontal and orthogonal planes [Fig. 5*B* and *C*(*ii*)]. As Bad contains three representative serine phosphorylation sites, Ser^{136} and Ser^{155}, as well as Ser^{112}, we next examined the effects of the ablation of endogenous Pim-3 on the expression levels of Bad and its different phosphorylated forms. In PCI55 cells, Bad was phosphorylated at Ser^{112} and marginally at Ser^{155}, but not at Ser^{136} (Fig. 6*A*). The ablation of endogenous Pim-3 protein failed to decrease the total amount of Bad protein. In contrast, the amount of phospho-Ser^{112} Bad was markedly reduced by Pim-3 shRNA transfectants but not by scramble shRNA transfectants (Fig. 6*A*). To substantiate our finding that Pim-3 phosphorylates Bad at Ser^{112}, we next examined the effect of the transfection of Pim-3 cDNA into PANC-1 and MiaPaca-2, two pancreatic cancer cell lines that contain about 35% to 40% of Pim-3 protein compared with other three cell lines (Fig. 2*A* and *B*). *Pim-3* gene transfection increased in a dose-dependent manner the amount of phospho-Ser^{112} Bad in PANC-1 cells (Fig. 6*B*) and MiaPaca-2 cells (data not shown). In contrast, the same treatment did not change significantly the total amount of Bad and other phosphorylated form of Bad (Fig. 6*B*). These observations indicate that Pim-3 could phosphorylate Bad at Ser^{112}. Akt shows similar substrate specificity as Pim kinases, including Pim-3 and its inhibitor, decreased Bcl-X_L expression in HTLV-1-transformed cell lines (18). Thus, we assumed that the ablation of Pim-3 may decrease Bcl-X_L expression in human pancreatic cancer cell lines, similarly as an Akt inhibitor did. To address this possibility, we explored the effects of *Pim-3* gene ablation on the expression of antiapoptotic Bcl family proteins. RT-PCR analysis showed that *Pim-3* gene ablation decreased the gene expression of Bcl-X_L without any effects on those of Bcl-2, Bcl-w, BAK, and BAX (data not shown). Moreover, Bcl-X_L protein level was decreased by the transfection with Pim-3 shRNA (Fig. 6*A*) similarly as an Akt inhibitor did (18).

Discussion

Pancreatic cancer still remains a serious health problem with the 5-year survival rate for all stages of disease at <5% (1). Most patients are diagnosed at an advanced stage and are treated mainly with chemotherapy with a median survival of <1 year. Thus, there is an urgent need to develop a more effective therapy based on the molecular pathologic understanding of pancreatic cancer biology. Here, we showed that Pim-3 was selectively expressed in pancreatic cancer tissues, but not in normal pancreatic tissue, and that the ablation of endogenous Pim-3 could induce apoptosis of human pancreatic cancer cell lines. Thus, Pim-3 might be a novel target molecule for the development of the drugs effective for pancreatic cancer.

The function of Bad, the proapoptotic BH3-only protein, is regulated by its phosphorylation at serine residues. Unphosphorylated Bad binds and eventually inactivates antiapoptotic family members, primarily Bcl-X_L but also Bcl-2 (11–13). Phosphorylation of Bad at Ser^{112}, Ser^{136}, and Ser^{155} impairs its binding to Bcl-X_L and promotes the sequestration of Bad from the surface of the mitochondria to the cytosol by the protein 14-3-3 (14, 15). Pim-1 and Pim-2 can phosphorylate Bad Ser^{112} (16, 17). In this report, we observed that Pim-3 colocalized with Bad and Bad phospho-Ser^{112} in human pancreatic cancer cell lines. The common Pim kinase

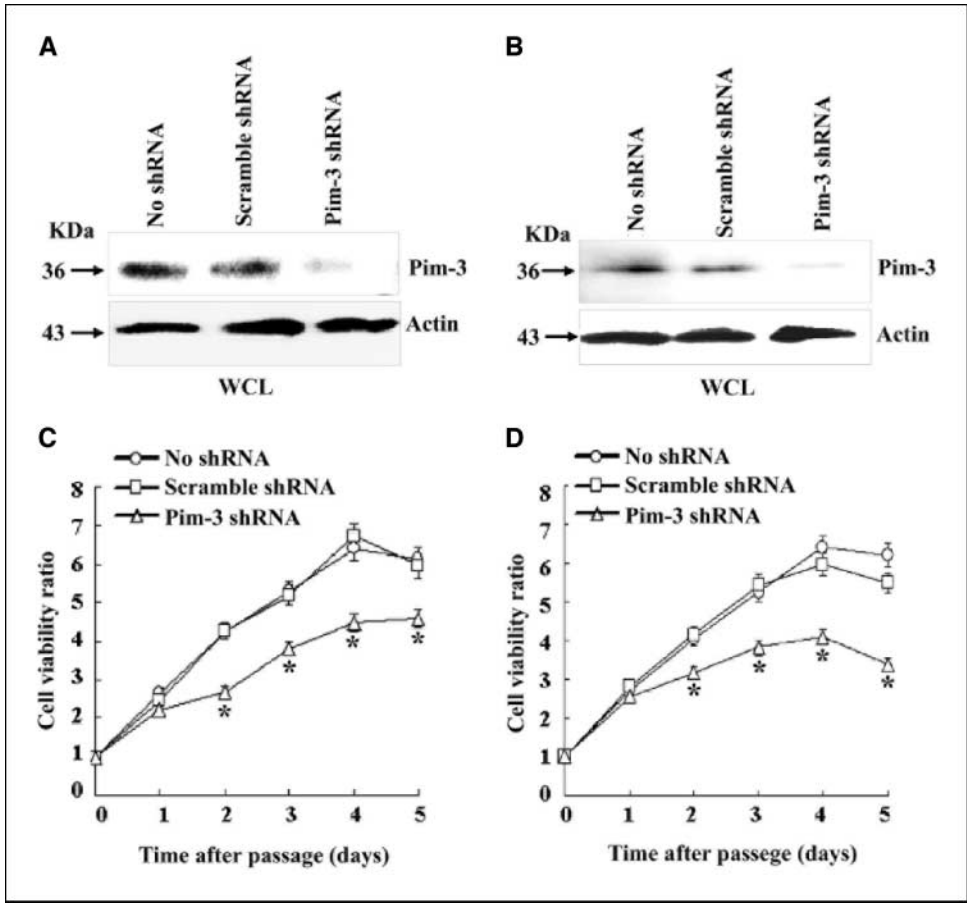

Figure 3. The effects of endogenous Pim-3 ablation on cell viability of human pancreatic cancer cell lines. *A* and *B,* immunoblotting analysis for Pim-3 protein in shRNA-treated PCI55 (*A*) or PCI35 (*B*) cells. Cell lysates were obtained from cells transfected with Pim-3 shRNA, scramble shRNA, or no shRNA 48 hours after transfection and the resultant lysates were subjected to immunoblotting with anti-Pim-3 antibodies as described in Materials and Methods. Representative results from three independent experiments. *C* and *D,* cell numbers were determined on PCI55 (*C*) and PCI35 (*D*) cells transfected with Pim-3 shRNA (△), scramble shRNA (□), or no shRNA (○). The cells were trypsinized and 3×10^3 cells were plated in a 96-well microplate 2 days after transfection. This time point was designated as day 0. The cell numbers were determined daily for 5 days with the use of WST-1 assay and the ratios were compared to day 0. *Points,* mean ($n = 5$); *bars,* SD. Representative results from three independent experiments. *, $P < 0.05$, compared with no shRNA samples at the same time point (ANOVA).

motif was characterized by selectivity for arginine at the −5 and −3, histidine at the −2, proline at the −1, and glycine at the +1 positions in relation to the phosphorylation site (23, 24). Among the three Bad serine residues, Ser^{112} conforms to this motif better than Ser^{136} and Ser^{155}. Indeed, Bad Ser^{112}, but not Ser^{136} and Ser^{155}, was abundantly phosphorylated in human pancreatic cancer cell lines. Moreover, the ablation of endogenous Pim-3 reduced the amount of phosphorylated Bad, but not total Bad, along with an enhancement of apoptosis. Thus, Pim-3 may phosphorylate mainly Bad Ser^{112} and thus prevent apoptosis.

Bad can also be phosphorylated by additional serine/threonine kinases, including Akt and mitogen-activated protein kinase

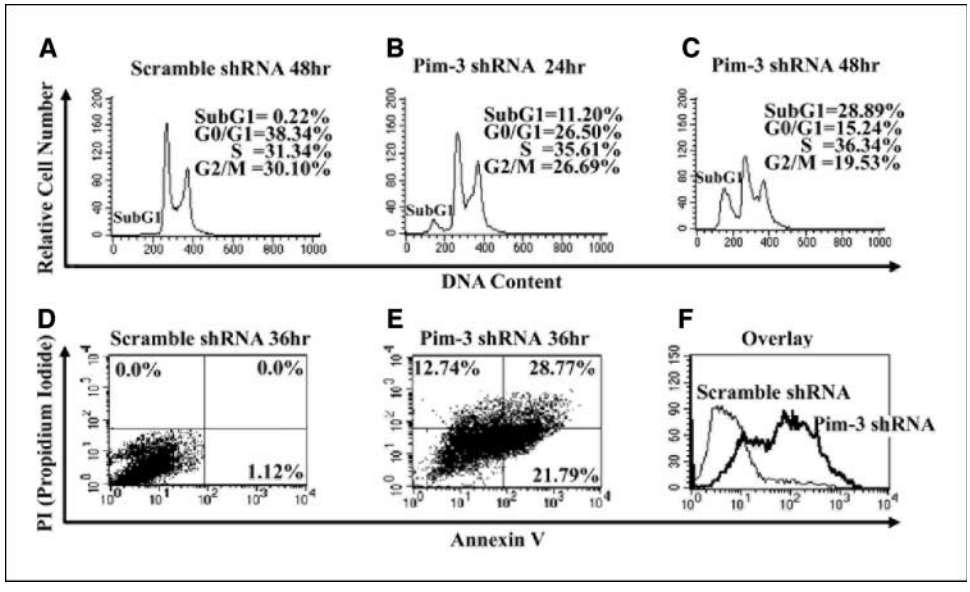

Figure 4. Induction of apoptosis in human pancreatic cancer cell lines by ablation of endogenous Pim-3 protein. PCI55 cells were transfected with either scramble shRNA (*A* and *D*) or Pim-3 shRNA (*B, C*, and *E*). Cells were harvested 24 hours (*B*), 48 hours (*A* and *C*), or 36 hours (*D* and *E*) after transfection and subjected to staining with propidium iodide (*A-C*) or combined staining with propidium iodide and Annexin V (*D* and *E*) as described in Materials and Methods. The proportion of cells in each cell cycle phase was determined (*A-C*) as described in Materials and Methods. The number in each quadrant (*D* and *E*) indicates the proportion of the cells present in the quadrant. The intensities of Annexin V staining are shown in (*F*) by overlaying the data in (*D* and *E*). Representative results from three independent experiments.

(MAPK)/extracellular signal–regulated kinase kinase (MEK)/MAPK (15, 25). Although the Akt consensus phosphorylation site contains arginine at the −5 and −3 positions in relation to the phosphorylation site similar to the Pim kinases (26), Akt preferentially phosphorylates Bad Ser[136], but not Ser[112] (15), whereas MEK/MAPK phosphorylates mainly Bad Ser[112] (25). However, the phosphorylation of either Ser[112] or Ser[136] is sufficient to sequester Bad to 14-3-3 (27). A pancreas-specific knockout of *Pten*, a negative regulator of Akt, resulted in the development of pancreatic ductal malignancy in a small portion of *Pten*-null mice (28). On the contrary, pancreatic cancer exhibits a mutation in *Pten* in <20% of cases (1). Moreover, Ser[136], a preferential phosphorylation site for

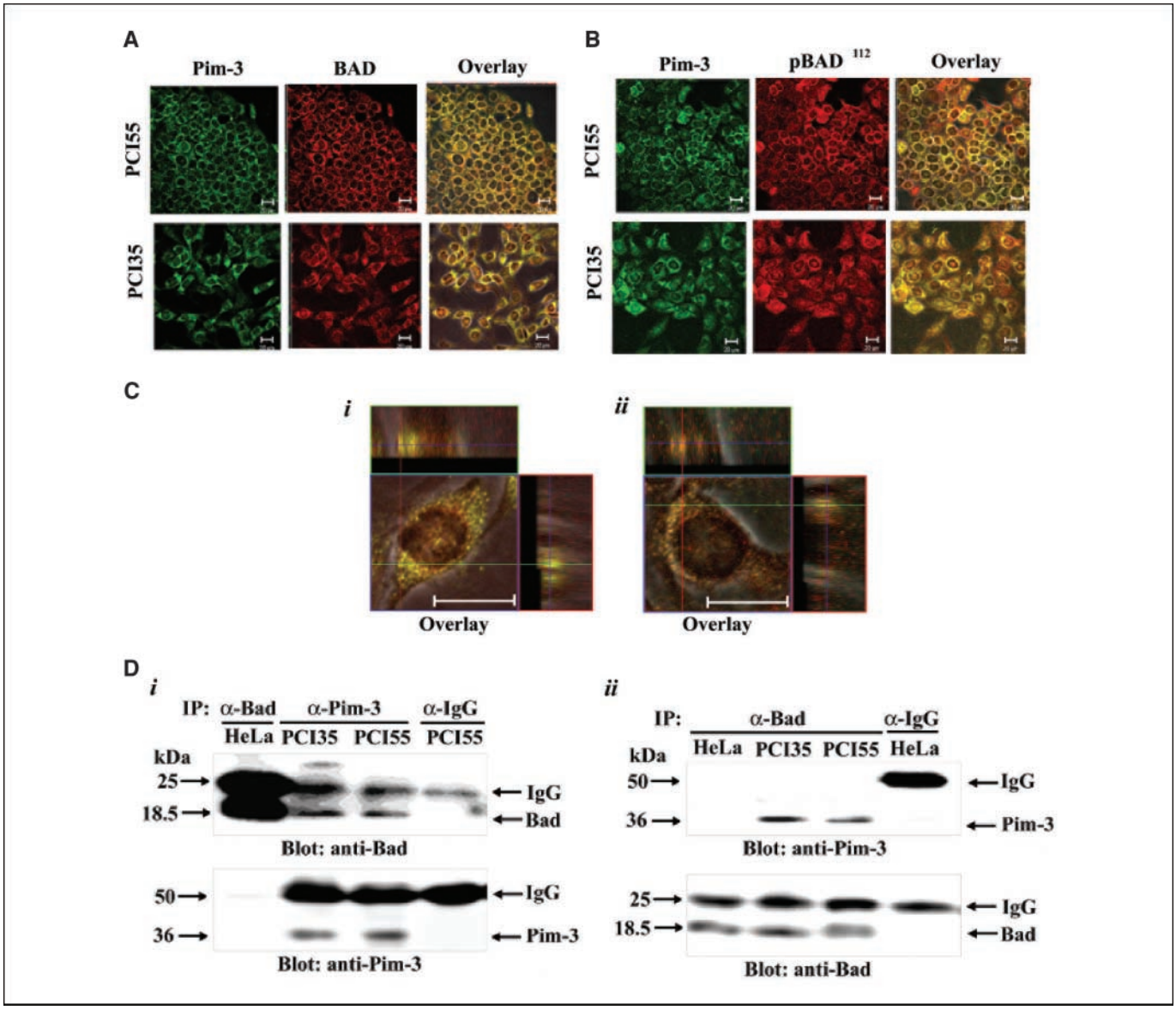

Figure 5. Association of Pim-3 with Bad and phosphorylated Bad in human pancreatic cancer cell lines. *A* and *B,* PCI55 and PCI35 cells were immunostained with either the combination of anti-Pim-3 and anti-Bad antibodies (*A*) or the combination of anti-Pim-3 and anti–phospho-Ser[112]Bad antibodies (*B*) as described in Materials and Methods. The fluorescent images were digitally merged and the resultant images are shown in the third panels. Yellow coloration in overlay panels indicates the colocalization of Pim-3 with Bad (*A*) or phospho-Ser[112]Bad (*B*). Representative results from three independent experiments. Bar, 20 μm. *C,* PCI35 cells were immunostained with either the combination of anti-Pim-3 and anti-Bad antibodies (*i*) or the combination of anti-Pim-3 and anti–phospho-Ser[112]Bad antibodies (*ii*) as described in Materials and methods. The merged fluorescent images in horizontal and orthogonal planes are shown. Yellow coloration indicates the colocalization of Pim-3 with Bad (*A*) or phospho-Ser[112]Bad (*B*). Representative results from three independent experiments. Bar, 20 μm. *D,* coimmunoprecipitation of Pim-3 and Bad in PCI55 and PCI35 cells. *i,* cell lysates were obtained from PCI55, PCI35, or HeLa cells. The resultant cell lysates were subjected to immunoprecipitation with anti-Pim-3 antibodies, followed by immunoblotting with either anti-Bad (*top*) or anti-Pim-3 (*bottom*) antibodies as described in Materials and Methods. Our preliminary experiments revealed that HeLa cells contained Bad but not Pim-3 (data not shown). Thus, cell lysates from HeLa cells were subjected to immunoprecipitation with anti-Bad antibodies followed by immunoblotting with anti-Bad antibodies as a positive control for Bad but not Pim-3. Cell lysates from PCI55 cells were subjected to immunoprecipitation with rabbit anti-IgG antibodies followed by immunoblotting with anti-Bad or anti-Pim-3 antibodies as a negative control. *ii,* cell lysates from PCI55 or PCI35 cells were subjected to immunoprecipitation with anti-Bad antibodies followed by immunblotting with either anti-Pim-3 (*top*) or anti-Bad (*bottom*) antibodies. Our preliminary experiments revealed that HeLa cells contained Bad but not Pim-3 (data not shown). Thus, cell lysates from HeLa cells were subjected to immunoprecipitation with anti-Bad antibodies followed by immunoblotting with anti-Bad antibodies as a positive control for Bad. Cell lysates from HeLa cells were subjected to immunoprecipitation with control rabbit IgG as a negative control. Representative results from three independent experiments.

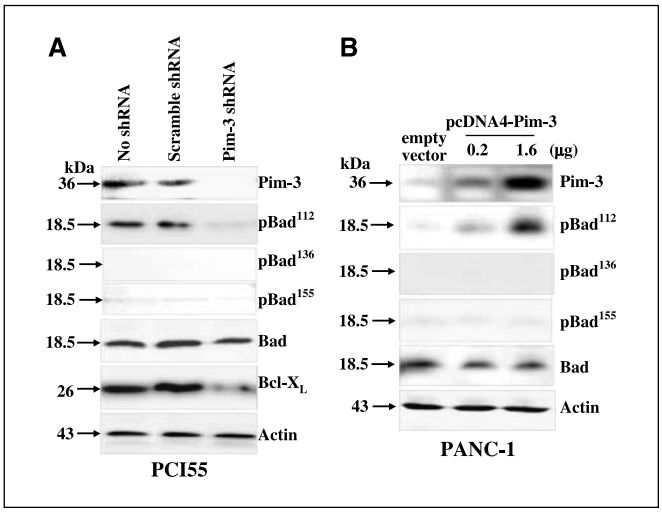

Figure 6. The effects of Pim-3 shRNA or Pim-3 expression vector transfection on Bad and Bcl-X_L proteins. *A,* cell lysates were obtained from PCI55 cells that were transfected with Pim-3 shRNA, scramble shRNA, or no shRNA, and the resultant lysates were subjected to immunoblotting as described in Materials and Methods. Representative results from three independent experiments. Similar results were also obtained using PCI35 cells (data not shown). *B,* cell lysates were obtained from PANC-1 cells transfected with the indicated doses of Pim-3 expression vectors. The resultant lysates were subjected to immunoblotting as described in Materials and Methods. Representative results from three independent experiments. Similar results were also obtained using MiaPaca-2 cells (data not shown).

Akt, was not phosphorylated in human pancreatic cancer cell lines that we examined. Thus, it is likely that the *Pten* may not be mutated and that Akt may not be responsible for the phosphorylation of Bad in the pancreatic cancer cell lines that we examined.

Accumulating evidence implicates Akt as an essential kinase that phosphorylates substrates that regulate both apoptosis and other cellular mechanisms (29). Therefore, pharmacologic manipulation of Akt has been proposed to be potentially effective for a wide variety of diseases, including cancer, but its wide tissue distribution may preclude its clinical use. Pim kinases exhibit similar substrate specificity as Akt but Pim-3 was expressed selectively in malignant lesions of the pancreas and liver. Moreover, only mild growth retardation was observed in mice deficient in all three Pim family proteins, including Pim-3 (30), although Pim-3 mRNA was abundantly expressed in several essential organs such as heart and kidney (6). Thus, blocking Pim-3 kinase may be more suitable than inhibiting Akt for the treatment of these malignancies.

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