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RNA Interference Demonstrates a Novel Role for Integrin-Linked Kinase as a Determinant of Pancreatic Adenocarcinoma Cell Gemcitabine Chemoresistance

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Abstract

Integrin-linked kinase (ILK) facilitates signal transduction between extracellular events and important intracellular survival pathways involving protein kinase B/Akt. We examined the role of ILK in determining pancreatic adenocarcinoma cellular chemoresistance to the nucleoside analogue gemcitabine. Cellular ILK expression was quantified by Western blot analysis. We examined the effects of overexpression of active ILK and of ILK knockdown induced by RNA interference on gemcitabine chemoresistance. We also examined the effects of modulating ILK expression on gemcitabine-induced caspase 3– mediated apoptosis, phosphorylation status of Akt (Ser^{473}) and glycogen synthase kinase. Overexpression of ILK increased cellular gemcitabine chemoresistance, whereas ILK knockdown induced chemosensitization via increased caspase 3– mediated apoptosis. ILK knockdown attenuated Akt Ser^{473} and glycogen synthase kinase phosphorylation, whereas overexpression of constitutively active myristoylated Akt was sufficient to induce significant recovery in gemcitabine chemoresistance in the presence of ILK knockdown. Levels of ILK expression affect gemcitabine chemoresistance in pancreatic adenocarcinoma cells. This novel finding suggests that therapies directed against ILK and its downstream signaling targets may have the potential to enhance the efficacy of gemcitabine-based chemotherapy.

Integrin-linked kinase (ILK) is an intracellular serine/threonine kinase that interacts with the cytoplasmic domains of integrin β1 and β3 subunits (1). This 59-kDa protein contains a phosphoinositide phospholipid–binding domain, which is flanked by NH$_2$-terminal ankyrin repeat and COOH-terminal kinase domains. ILK kinase activity is stimulated by cellular attachment to extracellular matrix components and by growth factors. These stimuli result in suppression of apoptosis and promotion of cell survival via protein kinase B/Akt signaling events. ILK seems to couple integrin and growth factor receptors to this important cell survival pathway. ILK is also able to induce inhibitory phosphorylation of glycogen synthase kinase 3 (GSK-3), which modulates cyclin D1–mediated cell cycle regulation as well as activities of transcription factors, including activator protein and β-catenin-Lef-1/Tcf (2,3). Cellular adhesion plays an important role in determining cellular resistance to chemotherapeutic agents in a range of malignancies (4–8), and evidence strongly implicates integrin signaling in adhesion-dependent chemoresistance (9–11). Inhibition of ILK using small-molecule inhibitors and dominant-negative ILK expression constructs has been shown to attenuate the invasiveness of human tumor cells (12), suggesting that this molecule may be therapeutically

relevant. However, despite substantial evidence linking downstream targets of ILK to tumor cell chemoresistance, the role of ILK in this aspect of malignant cellular behavior has not been studied.

Pancreatic adenocarcinoma is among the most chemoresistant of malignancies. Even treatment with the current first line chemotherapeutic agent, the nucleoside analogue gemcitabine (2′,2′-difluorodeoxycytidine), offers minimal benefit in terms of survival and quality of life (13). Akt plays a central role in the resistance of pancreatic adenocarcinoma cells to apoptotic stimuli, including exposure to gemcitabine (14–16). Increased Akt activity protects cells from apoptosis in a variety of ways, including direct suppression of caspase activation (17,18). Given the importance of ILK in protein kinase B/Akt signaling, we hypothesized that targeting ILK might represent a novel approach to modulate the chemoresistance of pancreatic adenocarcinoma cells to gemcitabine. In this study, we present the first evidence indicating a novel role for ILK in determining pancreatic adenocarcinoma cellular chemoresistance to gemcitabine.

Materials and Methods

Cells and cell culture

PANC1, MIAPaCa2, and Capan2 pancreatic ductal adenocarcinoma cells were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM containing 10% fetal bovine serum (Life Technologies, Gaithersburg, MD). Cells were incubated in a humidified (37°C, 5% CO_2) incubator and passaged upon reaching 80% confluence.

Expression vectors and transfection

Constitutively active ILK [ILK(S343D)], constitutively active myristoylated Akt, and dominant-negative Akt (K179M) expression constructs were obtained from Upstate (Waltham, MA). Cells were transfected with the appropriate expression construct, or control empty vector (pUSE), using Lipofect-AMINE 2000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's protocol. Stable clones were selected by continuous treatment with G418 (Life Technologies, 0.8 mg/mL) over 4 weeks.

Small interfering RNA

Small interfering RNA (siRNA) targeting the following sequences were synthesized and purified by Qiagen-Xeragon (Germantown, MD): ILK sense 5′-CCUGACGAAGCUCAACGAGAAd(TT)-3′, ILK antisense 5′-UUCUCGUUGAGCUUCGUCAGGd(TT)-3′; control sense 5′-CCUGACGAAGCUCAACGAAAAd(TT)-3′, control antisense 5′-UUUUCGUUGAGCUUCGUCAGGd(TT)-3′. The target specificity of these sequences was confirmed by BLAST search (http://www.ncbi.nih.gov/BLAST). Homologous siRNAs were dissolved in buffer [100 mmol/L potassium acetate, 30 nmol/L HEPES-KOH, 2 nmol/L magnesium acetate (pH 7.4)] to a final concentration of 20 µmol/L, heated to 90°C for 60 seconds and incubated at 37°C for 60 minutes before use to disrupt any higher order aggregates formed during synthesis. Cells were plated into 35-mm 6-well trays and allowed to adhere for 24 hours. In all, 8 µL siPORT *Amine* transfection reagent (Ambion, Inc., Austin, TX) per well were added to serum-free medium for a final complexing volume of 200 µL, vortexed, and incubated at room temperature for 15 minutes. The transfection reagent/siRNA complexes were added to the wells containing 800 µL medium with 10% fetal bovine serum and incubated in normal cell culture conditions for 6 hours, after which 1 mL DMEM containing 10% fetal bovine serum was added. Assays were done 48 hours post-transfection.

Cytotoxicity assay

Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT, Trevigen, Inc., Gaithersburg, MD) in accordance with the manufacturer's instructions. Results of the MTT assay have been shown to correlate well with [^{3}H]-thymidine incorporation in pancreatic cancer cell lines (19). Logarithmically growing cells were plated at 5×10^3 cells per well in 96-well plates, allowed to adhere overnight, and were cultured in the presence or absence of 0 to 10 µmol/L gemcitabine. Gemcitabine-induced cytotoxicity was determined after 24 hours of exposure. Plates were read using a V_{max} microplate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm corrected to 650 nm and normalized to controls. Each independent experiment was done thrice, with 10 determinations for each condition tested. The concentration of gemcitabine required to inhibit proliferation by 50% (IC_{50}) was calculated from these results. At identical time points, cells were trypsinized to form a single cell suspension. Intact cells, determined by trypan blue exclusion, were counted using a Neubauer hemocytometer (Hausser Scientific, Horsham, PA). Cell counts were used to confirm MTT results.

Apoptosis staining

Following gemcitabine treatment, cells were washed, resuspended in 0.5 mL of PBS, and 1 µL/mL YO-PRO-1, and propidium iodide were added (Vybrant Apoptosis Assay Kit #4, Molecular Probes, Eugene, OR). Cells were incubated for 30 minutes on ice and analyzed by flow cytometry (FACScan, Becton Dickinson, Franklin Lakes, NJ), measuring fluorescence emission at 530 and 575 nm. Cells stained with the green fluorescent dye YO-PRO-1 were counted as apoptotic; necrotic cells stained with propidium iodide. The number of apoptotic cells was divided by the total number of cells (minimum of 10^4 cells), giving the apoptotic fraction. Data were analyzed using CellQuest software (Becton Dickinson). All observations were reproduced at least thrice in independent experiments.

Fluorometric caspase 3 activity assay

Lysates were assayed for caspase 3 activity using the BD ApoAlert fluorometric Caspase Assay Plate (BD Biosciences Clontech, Palo Alto, CA) in accordance with the manufacturer's instructions. Plates were read (excitation, 360 nm; emission, 480 nm) using a CytoFluor 4000 multiwell fluorescence plate reader (Applied Biosystems, Foster City, CA). A minimum of three determinations was done for each sample.

Western blot analysis

Cells were washed with ice-cold PBS and whole cell extracts were prepared using cell lysis buffer [20 mmol/L Tris (pH 7.5), 0.1% Triton X, 0.5% deoxycholate, 1 mmol/L phenyl-methylsulfonyl fluoride, 10 µg/mL aprotinin, and 10 µg/mL leupeptin] and cleared by centrifugation at $12,000 \times g$, 4°C. Total protein concentration was measured using the bicinchoninic acid assay kit (Sigma, St Louis, MO) with bovine serum albumin as a standard. Cell lysates containing 30 µg total protein were analyzed by immunoblotting. Anti-ILK, anti-Akt, and anti-p-Akt (Ser[473]) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-GSK-3α/β (Ser[21/9]) antibody was obtained from Cell Signaling Technology (Beverly, MA). Anti-actin antibodies were obtained from LabVision (Freemont, CA). Chemoluminescent detection (Upstate, Lake Placid, NY) was done in accordance with the manufacturer's instructions. The ILK signal was quantified using ImagePro Plus software version 4.0 and normalized to that of actin.

Statistical analysis

Differences between groups were analyzed using Student's t test, multifactorial ANOVA of initial measurements and Mann Whitney U test, for nonparametric data, as appropriate, using

Statistica 5.5 software (StatSoft, Inc., Tulsa, OK). In cases in which averages were normalized to controls, the SDs of each nominator and denominator were taken into account in calculating the final SD. $P < 0.05$ was considered statistically significant.

Results

Overexpression of active integrin-linked kinase increases pancreatic adenocarcinoma cell gemcitabine chemoresistance

The gemcitabine IC_{50} values (concentration of gemcitabine required to inhibit cellular proliferation by 50%) of the pancreatic adenocarcinoma cell lines PANC1, MIAPaCa2, and Capan2 have been determined previously and we confirmed these results (20). The IC_{50} of gemcitabine for PANC1, MIAPaCa2, and Capan2 are 50, 40, and 12 nmol/L, respectively. Levels of ILK expression in PANC1, MIAPaCa2, and Capan2 cells were compared by Western blotting (Fig. 1). PANC1 and MIAPaCa2, the most chemoresistant cell lines, exhibited greater levels of ILK expression than Capan2, which has lower chemoresistance to gemcitabine. To define the role of ILK in gemcitabine chemosensitivity, we established clones of Capan2 that were stably transfected with a constitutively active ILK (S343D) expression construct. Overexpression of ILK by two transfectant clones (Capan2-ILK1 and Capan2-ILK2) was confirmed by Western blot analysis (Fig. 2A). The gemcitabine IC_{50} values for the parental cell line (Capan2), empty vector transfectants (Capan2-pUSE), Capan2-ILK1, and Capan2-ILK were quantified by MTT cytotoxicity assay. Whereas empty vector transfection had no effect on gemcitabine IC_{50}, overexpression of active ILK resulted in a significant increase in chemoresistance to gemcitabine (Fig. 2B).

Integrin-linked kinase overexpression suppresses gemcitabine-induced caspase-mediated apoptosis

Apoptosis was quantified by flow cytometric analysis. Following exposure of ILK transfectant cells to 1 µmol/L gemcitabine for 24 hours, the apoptotic fraction of Capan2-ILK1 and Capan2-ILK2 were, respectively, 40% and 46% less than that of Capan2-pUSE and Capan2 cells, which showed no significant difference in their apoptotic response to gemcitabine (Fig. 3A).

Caspase 3 activities of cell lysates, obtained following exposure to 1 µmol/L gemcitabine for 24 hours, were quantified by fluorometric assay. Capan2-ILK1 and Capan2-ILK2 transfectants exhibited a 45% and 55% lower level of caspase 3 activation than Capan2-pUSE transfectants, respectively (Fig. 3B).

Integrin-linked kinase knockdown potentiates gemcitabine-induced cytotoxicity

PANC1 cells were chosen to study the effects of ILK knockdown as these cells inherently express a relatively high level of ILK. ILK expression was suppressed by transfection of ILK-specific siRNA. Forty-eight hours following transfection of siRNA, cells were subjected to gemcitabine cytotoxicity assay as described previously. Continued suppression of ILK expression of up to 75% was confirmed by Western blotting, 72 hours following siRNA transfection (Fig. 4A). ILK knockdown resulted in a 51% decrease in the gemcitabine IC_{50}, as determined by MTT assay, relative to control siRNA transfectants (Fig. 4B). Both the apoptotic fraction and caspase 3 activation induced by exposure to gemcitabine were significantly increased following transfection of ILK-specific siRNA but not control siRNA (Fig. 4C and D). Furthermore, when the apoptotic fraction was determined following exposure to gemcitabine in the presence of the caspase inhibitor z-VAD-fmk, the increase in apoptotic fraction induced by ILK knockdown was significantly reduced, signifying that increased gemcitabine-induced apoptosis in cells exposed to ILK siRNA is caspase dependent.

Integrin-linked kinase knockdown modulates Akt Ser[473] and glycogen synthase kinase phosphorylation

ILK facilitates the phosphorylation of Akt at Ser[473], which is required for Akt activation (21–23). In addition to modulating activity of Akt, ILK also induces phosphorylation of downstream targets such as glycogen synthase kinase and myosin light chain (22). In view of the important role of Akt in mediating pancreatic adenocarcinoma cellular resistance to gemcitabine (14–16,24), we examined the effect of ILK knockdown on Akt Ser[473] phosphorylation, as well as phosphorylation of its downstream target, GSK. We observed a decrease in both Akt Ser[473] phosphorylation (Fig. 5A) and phosphorylation of GSK (Fig. 5B) in cells transfected with ILK siRNA, which is consistent with a model in which ILK facilitates phosphorylation of Akt and GSK.

Activated Akt rescues cells from chemosensitization to gemcitabine induced by integrin-linked kinase knockdown

Given the effects of ILK knockdown on Akt phosphorylation status, we sought to determine the effects of concomitant overexpression of constitutively active Akt, and ILK knockdown, on gemcitabine-induced cytotoxicity. Cotransfection of a constitutively active myristoylated Akt expression construct at the time of ILK siRNA transfection was sufficient to maintain the gemcitabine IC_{50} of PANC1 cells at levels significantly higher than those of cells in which ILK knockdown was done in isolation (Fig. 6).

Discussion

Cellular adhesion to substrate is protective against a range of chemotherapeutic agents (7–11). ILK plays a critical role in coupling extracellular signaling events to intracellular cell survival pathways. In this study, we have shown for the first time that overexpression of active ILK increases gemcitabine chemoresistance in pancreatic adenocarcinoma cells. This chemoprotective effect occurs in association with suppression of caspase 3 activity. Furthermore, posttranscriptional knockdown of ILK expression by RNAi enhances gemcitabine-induced caspase 3–mediated apoptosis, decreases Akt Ser[473] phosphorylation, and suppresses levels of GSK phosphorylation. In addition to its recognized roles in transducing signals resulting from growth factor receptor ligation and extracellular matrix interaction, ILK can act as a determinant of pancreatic adenocarcinoma cellular resistance to gemcitabine.

Increased expression and activity of ILK protects epithelial cells from apoptotic events such as anoikis, permitting anchorage-independent cell cycle progression and conferring greater tumorigenic ability and cellular invasiveness (3,12,21,25). ILK interacts with β1 and β3 integrin subunits and is activated by contact with extracellular matrix components (1,21). Overexpression of the ILK has been shown to modulate β-catenin subcellular localization and function (3). ILK also facilitates phosphorylation of Akt at Ser[473], which is a requirement for Akt activation (21–23). Serine phosphorylation of Akt by ILK results in inhibitory phosphorylation of the downstream target GSK (22), which is associated with greater resistance to cellular insults such as exposure to ionizing irradiation (26,27). Akt has been recognized to play an important role in pancreatic adenocarcinoma cellular chemoresistance (14–16) and the ability of ILK knockdown to enhance gemcitabine-induced cytotoxicity is consistent with the role of ILK as a regulator of Akt activity. We confirmed that Akt plays a significant role in the changes in gemcitabine chemoresistance induced by ILK knockdown, as cotransfection of a constitutively active myristoylated Akt expression construct was sufficient to preserve gemcitabine chemoresistance significantly above that of cells in which ILK knockdown was done in isolation. Whereas ILK-deficient chondrocytes have been shown to exhibit unaltered Akt and GSK phosphorylation (28,29), others have shown Akt phosphorylation at Ser[473] to be

ILK dependent (30). Our results also suggest with an important role for ILK as a regulator of Akt activity.

ILK is implicated in the genesis and progression of a variety of malignancies. However, ILK expression has not been previously studied in pancreatic adenocarcinoma nor has its role in cellular chemoresistance. ILK protein hyperexpression is an early event in colonic polyposis (31) and carcinogenesis (32). ILK biochemical activity and protein expression is increased in polyps from familial adenomatous polyposis patients. Sulindac and aspirin, two agents that have been shown to have therapeutic and chemopreventive effects in colorectal carcinogenesis, target ILK and ILK-mediated signaling events (31). Inhibition of ILK has been reported to result in the transcriptional stimulation of E-cadherin expression and is correlated with inhibition of transcription of snail, a repressor of E-cadherin gene expression (33). ILK also plays a role in vascular endothelial growth factor–mediated tumor angiogenesis via protein kinase B/Akt–dependent signaling (34).

Considerable evidence derived from work done in a variety of malignancies, including pancreatic adenocarcinoma, indicates that Akt is a key regulator of cellular apoptosis (14–16, 18,35). Activated Akt protects cells from a variety of apoptotic stimuli, including exposure to gemcitabine (14,15,18,35). The caspase cascade of proteolytic enzymes comprises initiator and executioner elements. Following activation, these proteases degrade intracellular targets, resulting in apoptotic cell death. Active Akt has been shown to phosphorylate initiator caspase 9 directly, preventing its activation (17). Inhibition of this initiator caspase may interfere with apoptosome function and impair activation of effector caspase 3. Akt has also been reported to inhibit caspases by post-translational modification of an as yet unidentified cytosolic factor located downstream of cytochrome *c* release and upstream of caspase 9 activation (18). The results of this study indicate that modulation of Akt activity through alteration of ILK expression can influence pancreatic adenocarcinoma cellular gemcitabine chemoresistance.

In summary, our study is the first to characterize the role of ILK in mediating pancreatic adenocarcinoma cellular chemoresistance. We have shown that whereas overexpression of ILK protects cells against gemcitabine-induced apoptosis, posttranscriptional silencing of ILK expression induces a significant increase in caspase-mediated apoptosis following exposure to gemcitabine. Our findings suggest that strategies directed against ILK and its downstream signaling targets may represent a novel approach to enhance the efficacy of gemcitabine in pancreatic cancer.

Acknowledgments

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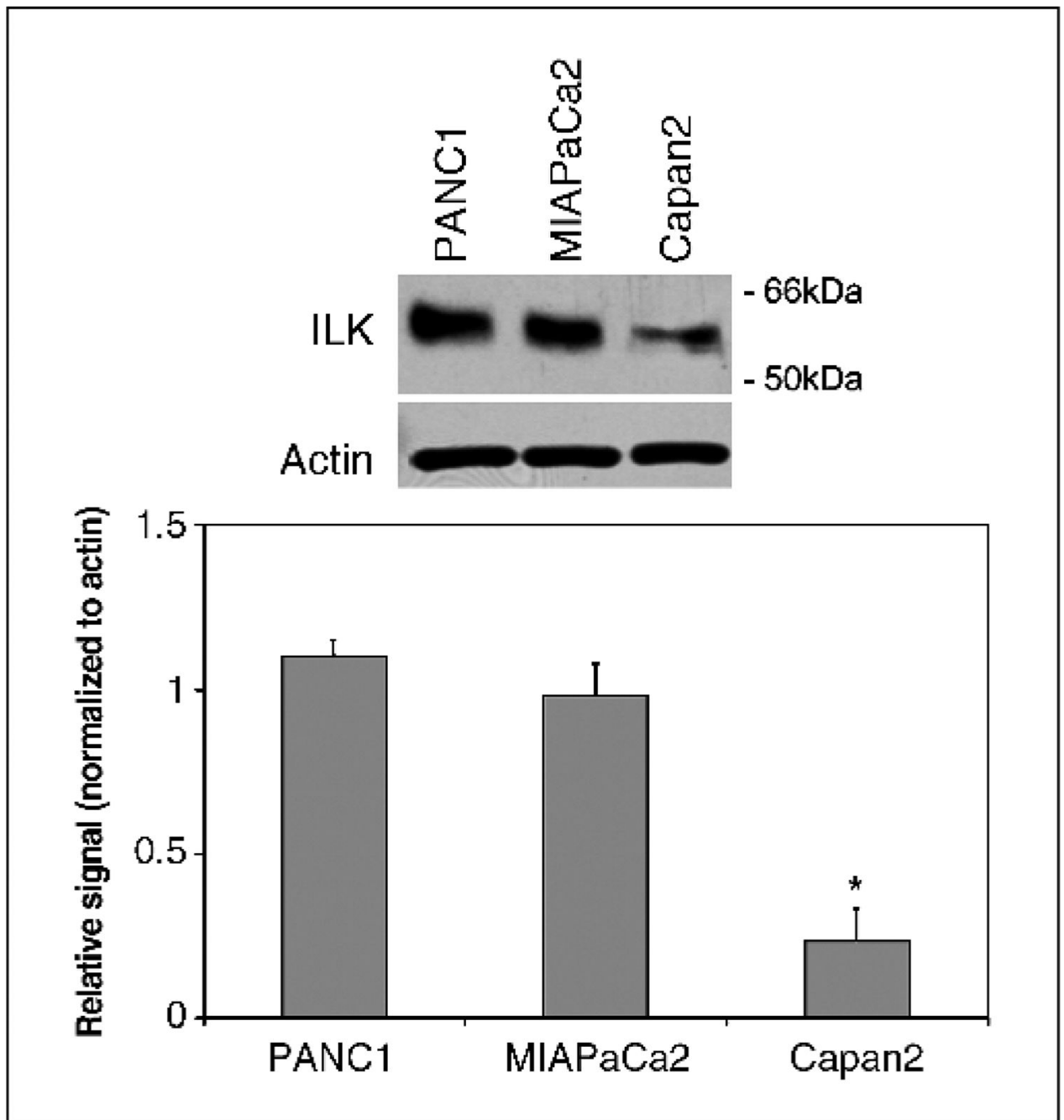
Fig. 1.
ILK expression among the pancreatic adenocarcinoma cell lines PANC1, MIAPaCa2, and Capan2 was quantified by Western blot analysis. PANC1 and MIAPaCa2, which have the greatest degree of gemcitabine chemoresistance (20), exhibited higher levels of ILK expression than Capan2. Representative example with mean densitometric values from triplicate blots.*, *P* < 0.05 versus Capan2 cells.
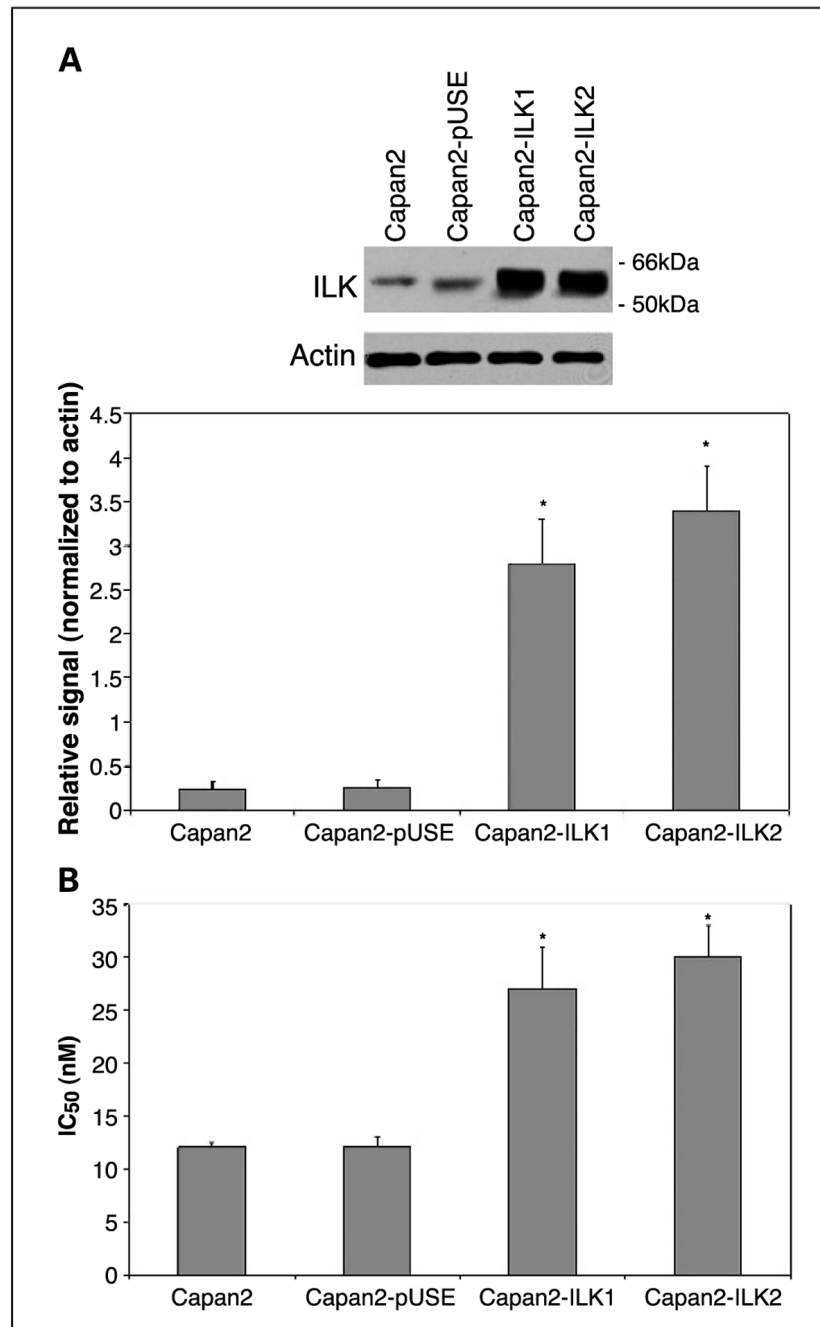
Fig. 2.
A, stable overexpression of active ILK was confirmed by western blotting in Capan2-ILK1and Capan2-ILK2 cells. Representative example with mean densitometric values from triplicate blots.*, $P < 0.05$ versus Capan2-pUSE transfectants. *B*, gemcitabine IC_{50} was determined by MTT cytotoxicity assay. Capan2-ILK1 and Capan2-ILK2 transfectants exhibited significantly increased gemcitabine chemoresistance, relative to both parental cells (*Capan2*) and empty vector transfectants (*Capan2-pUSE*).*, $P < 0.05$ versus Capan2-pUSE.
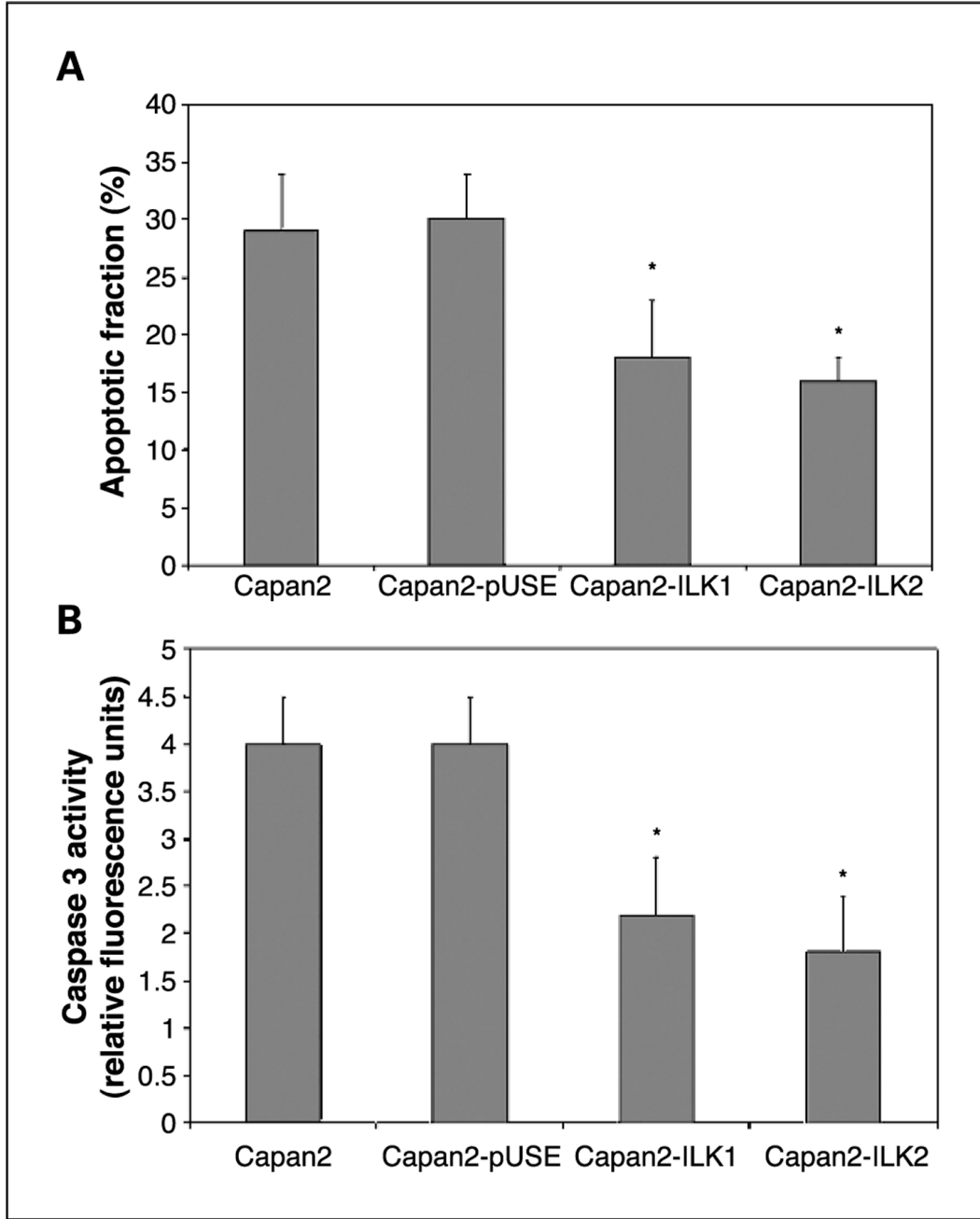
Fig. 3.
A, apoptotic fraction of cells was determined by flow cytometric analysis of at least 10,000 cells following exposure to1 µmol/L gemcitabine for 24 hours. The apoptotic fractions of both ILK transfectants were significantly lower than those of Capan2 and Capan2-pUSE. Columns, mean values from triplicate experiments; bars,± SD.*, *P* < 0.05 versus Capan2-pUSE. *B*, cellular caspase 3 activity following exposure to 1 µmol/L gemcitabine for 24 hours was significantly lower in Capan2-ILK1 and Capan2-ILK2 transfectants. *Columns*, mean values from triplicate experiments; *bars*, ±SD. *, *P* < 0.05 versus Capan2-pUSE.
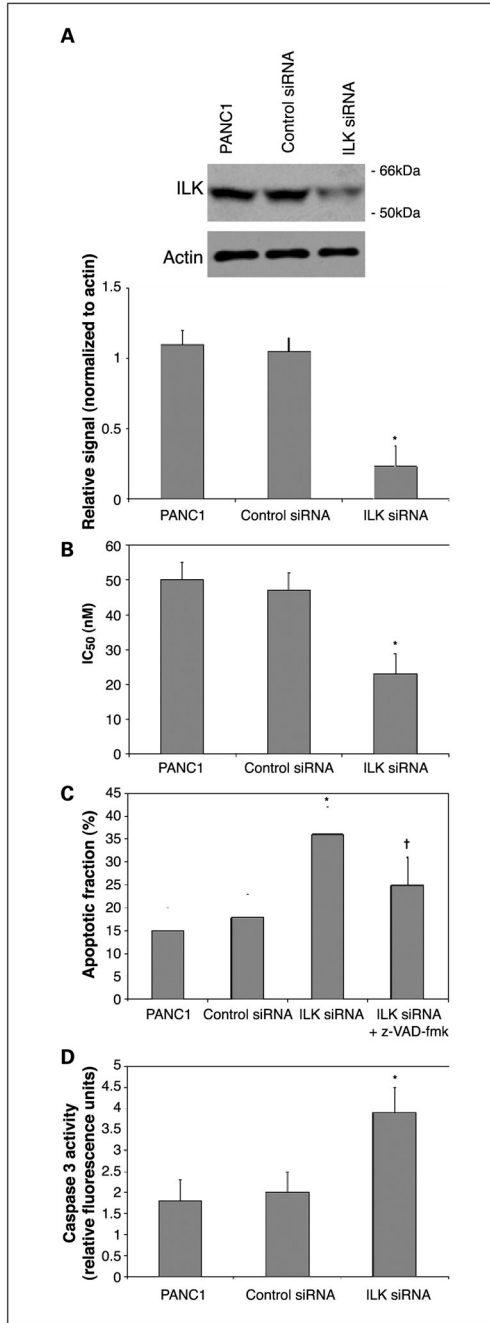
Fig. 4.
A, knockdown of ILK expression in PANC1 cells was confirmed by Western blot 48 hours following transfection of ILK-specific but not control siRNA. Representative example with mean densitometric values from triplicate blots. *, $P < 0.05$ versus control siRNA. *B*, gemcitabine-induced cytotoxicity was significantly enhanced following ILK knockdown. *, $P < 0.05$ versus control siRNA. Both the apoptotic fraction (*C*) and caspase 3 activation (*D*) in response to gemcitabine were significantly increase by exposure to ILK-specific siRNA. Apoptotic fraction of cells treated with ILK-specific siRNA was significantly reduced by exposure to the caspase inhibitor z-VAD-fmk. Columns, mean values from triplicate experiments; bars, ±SD. *, $P < 0.05$ versus control siRNA. †, $P < 0.05$ versus ILK siRNA.
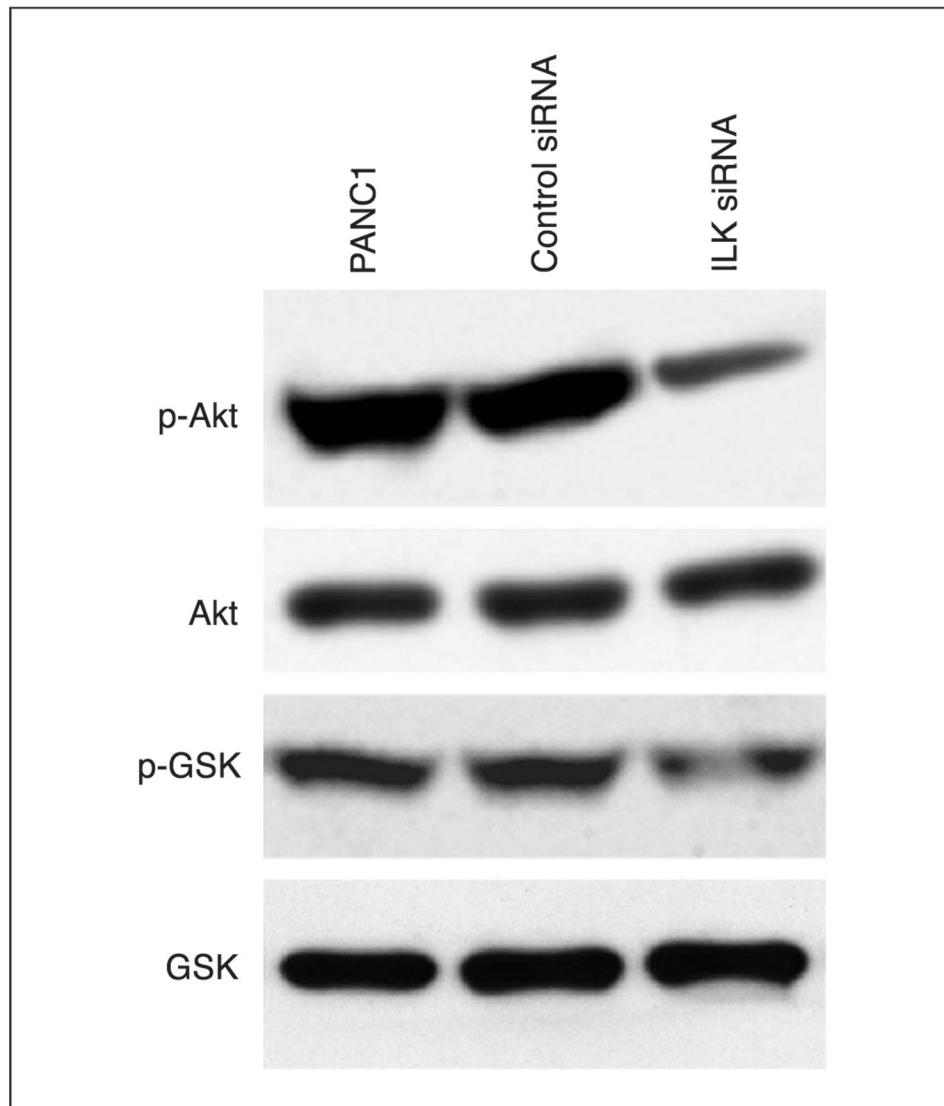
Fig. 5.
A, phosphospecific immunoblotting showed that knockdown of ILK expression suppressed Akt Ser^{473} phosphorylation. *B*, inhibitory phosphorylation of glycogen synthase kinase (*p-GSK*) was suppressed by ILK knockdown. Control siRNA had no effect on the phosphorylation status of either Akt or GSK. Representative example with mean densitometric values from triplicate blots. *, $P < 0.05$ versus control siRNA.
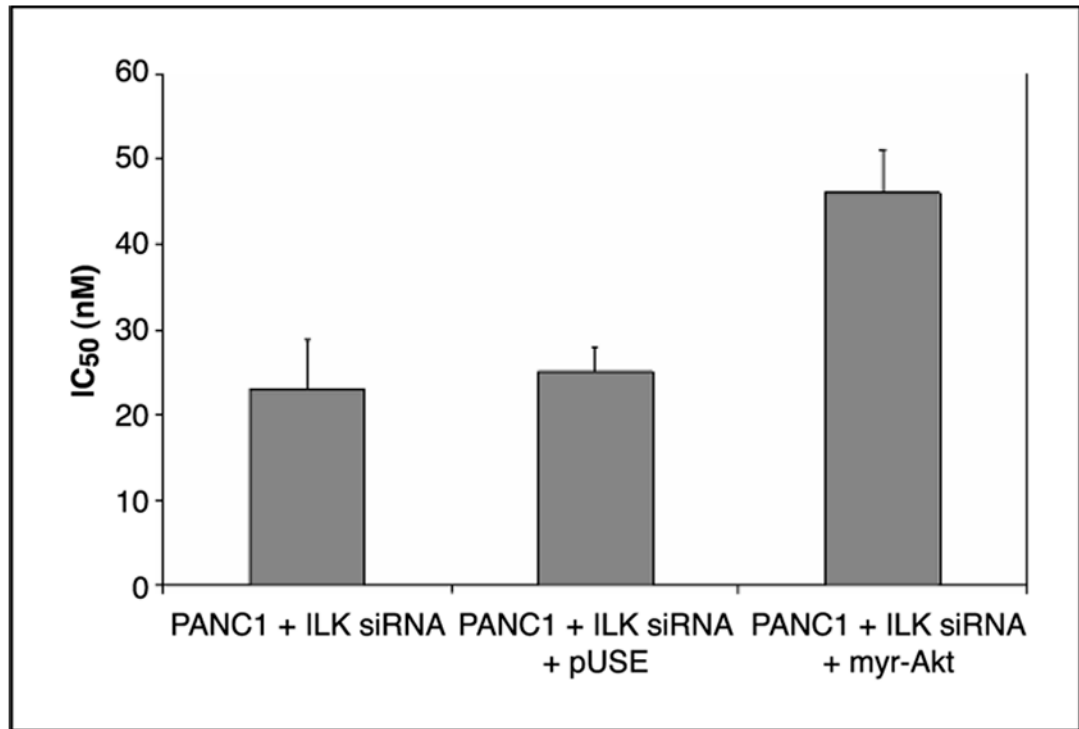
Fig. 6.
Transfection of a constitutively active myristoylated Akt expression construct (*myr-Akt*) induced significant recovery of the chemoresistant phenotype in PANC1 cells transfected with ILK-specific siRNA. Gemcitabine IC_{50} values were quantified by MTT cytotoxicity assay. *Columns*, mean values from triplicate experiments; *bars*, ±SD. *, $P < 0.05$ versus empty vector (*pUSE*).