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AKT Activation Up-Regulates Insulin-like Growth Factor I Receptor Expression and Promotes Invasiveness of Human Pancreatic Cancer Cells[1]

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ABSTRACT

Insulin-like growth factor I receptor (IGF-IR) is frequently overexpressed in several types of human malignancy and is associated with invasion and metastasis of tumor cells. Recently, IGF-IR expression was reported to be up-regulated in the human pancreatic cancer cell line PANC-1 when cells were stably transfected with active *Src*. The downstream targets of Src that lead to the up-regulation of IGF-IR expression were previously unknown. We demonstrate here that AKT regulates IGF-IR expression in PANC-1 and AsPC-1 cells. Cells transfected with active *Src* exhibited significantly more IGF-IR protein compared with vector-transfected cells. Overexpression of wild-type or constitutively active AKT (*i.e.*, AKT1 or AKT2) also resulted in elevated IGF-IR expression. IGF-IR protein levels were higher in cells transfected with constitutively active *AKT* than in cells transfected with active *Src*. *In vitro* kinase assays showed that AKT kinases are activated by active Src and inhibited by dominant negative Src or the tumor suppressor PTEN. Furthermore, AKT-induced IGF-IR expression was down-regulated by dominant-negative Src or PTEN. In addition, cells transfected with activated AKT in the presence of IGF-I were shown to have enhanced invasiveness compared with control cells. These data provide evidence for a link between AKT signaling and the regulation of IGF-IR expression and demonstrate that active AKT promotes the invasiveness of pancreatic cancer cells through the up-regulation of IGF-IR expression.

INTRODUCTION

More than 80% of pancreatic cancers are diagnosed at an advanced pathological stage, with locally advanced or metastatic disease, and are associated with a poor prognosis, regardless of therapy (1). Thus, new therapeutic modalities need to be investigated for the treatment of pancreatic cancer. Enhanced understanding of the signaling mechanisms that regulate pancreatic cancer cell growth may provide important insights for more effective therapeutic strategies.

IGF-IR,[3] a member of the tyrosine kinase family, is a heterotetramer consisting of α- and β-subunits (2). The α-subunits function in ligand binding, whereas the β-subunits span the plasma membrane and transmit cellular signals. Numerous studies have demonstrated that overexpression and excessive activation of IGF-IR are associated with malignant transformation, increased tumor aggressiveness, and protection from apoptosis (2–6). It also has been reported that IGF-IR is often overexpressed in human pancreatic tumors (7). In experiments using pancreatic cancer cell lines overexpressing IGF-IR, cell growth was significantly inhibited by an anti-IGF-IR antibody or IGF-IR antisense oligodeoxynucleotides (7). Thus, IGF-IR may play a critical role in the growth of pancreatic cancer cells.

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[3] The abbreviations used are: IGF-IR, IGF-I receptor; IGF-I, insulin-like growth factor I; PI3K, phosphatidylinositol 3-kinase; PtdIns-3,4-P_2, phosphatidylinositol-3,4-bisphosphate; PtdIns-3,4,5-P_3, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homologue deleted on chromosome 10; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; ECM, extracellular matrix.

AKT (also known as protein kinase B) consists of a family of highly conserved serine/threonine kinases including AKT1 and AKT2. These kinases are activated in response to a wide variety of growth factors through PI3K (8–10). The pleckstrin homology domain of AKT has an affinity for PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 produced by PI3K (11, 12). PtdIns-3,4-P_2 and/or PtdIns-3,4,5-P_3 trigger the translocation of AKT to the plasma membrane, where the AKT kinases can be activated by phosphorylation of Thr-308/309 and Ser-473/474 (13). Activated AKT has been shown to mediate cell survival by phosphorylating several downstream targets, such as BAD (14) and caspase-9 (15). In contrast, the tumor suppressor PTEN inhibits PI3K-dependent activation of AKT by dephosphorylating PtdIns-3,4-P_2 and PtdIns-3,4,5-P_3 (16).

The nonreceptor tyrosine kinase Src has been reported to be overexpressed and activated in most pancreatic tumors and pancreatic cancer cell lines (17). Furthermore, active Src has been shown to increase IGF-I-dependent growth of PANC-1 cells through the up-regulation of IGF-IR expression (18). However, downstream targets of active Src that regulate IGF-IR expression have not been identified to date. In other cell signaling pathways, Src has been shown to activate AKT through PI3K (19). In particular, AKT2 is a potentially intriguing target because we and others have reported previously amplification and overexpression of the *AKT2* oncogene in 10–20% of pancreatic tumors and cell lines (20–22).

We report here that AKT, specifically AKT1 or AKT2, up-regulates the expression of IGF-IR, and inactivation of AKT signaling inhibits expression of IGF-IR. We also show that increased IGF-IR expression induced by active AKT markedly enhances the invasiveness of human pancreatic cancer cells.

MATERIALS AND METHODS

Cell Culture. PANC-1 cells were obtained from the American Type Culture Collection. AsPC-1 cells were provided by Dr. A. Klein-Szanto (Fox Chase Cancer Center). The cells were maintained under standard culture conditions in DMEM or RPMI 1640 supplemented with 10% fetal bovine serum.

Antibodies. Antibodies used for immunoprecipitation and Western blotting were as follows. Anti-AKT1, anti-AKT2, and anti-Src antibodies were obtained from Upstate Biotechnology (Lake Placid, NY); anti-α-subunit of IGF-IR, anti-β-subunit of IGF-IR, and anti-PTEN antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-HA antibody was from Babco (Richmond, CA); anti-FLAG-M2 antibody was from Eastman Kodak; and anti-pan Ras antibody was from Oncogene Science (Cambridge, MA).

Plasmid Constructs and Transient Transfection Assays. HA epitope-tagged *AKT1* (HA-*AKT1*) and *AKT2* (HA-*AKT2*) cDNAs were constructed by PCR using pcDNA3 expression vector (Invitrogen, Carlsbad, CA). Flag epitope-tagged *AKT1* (Flag-*AKT1*) and *AKT2* (Flag-*AKT2*) were prepared using the same vector. Constitutively active HA-*AKT1* (myr-HA-*AKT1*) and HA-*AKT2* (myr-HA-*AKT2*) were created by adding a double-stranded DNA fragment corresponding to a myristylation signal at the 5′ end of each cDNA. Myr-HA-EGFP-*AKT1* and myr-HA-EGFP-*AKT2* were also generated. Wild-type *Src* (c-*Src*), active *Src* (*Src*Y527F), dominant-negative *Src* (N17*Src*), and active *Ras* (v-Ha-*ras*) were a kind gift from Dr. C. Patriotis (Fox Chase Cancer Center).

Cells were plated at a density of 2×10^5 cells/well in six-well plates 1 day before transfection. Transient transfection of the cells was carried out with 2

μg of DNA/well using GenePORTER (Gene Therapy Systems, San Diego, CA), according to the protocol suggested by the manufacturer. Transfection efficiencies were determined by immunocytochemistry, using anti-HA antibody, and nuclear counterstaining with diamidino-2-phenylindole. Transfection efficiencies in all experiments were consistently >40%.

***In Vitro* AKT Kinase Assay.** Cells transiently transfected with the expression construct of Flag-*AKT* were washed once with ice-cold PBS and lysed with lysis buffer [50 mM Tris-HCl (pH 7.5), 137 mM sodium chloride, 1 mM EDTA, 1% NP40, 10% glycerol, 0.1 mM sodium orthovanadate, 10 mM sodium PP_i, 20 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, and 2 μg/ml aprotinin]. Insoluble material was removed by centrifugation at 4°C for 10 min at 18,400 $\times$ *g*. The supernatants were incubated with monoclonal anti-FLAG M2 antibody at 4°C for 1 h. The immunocomplex was precipitated with protein A:protein G (1:1) agarose beads (Life Technologies, Inc., Grand Island, NY) at 4°C for 1 h and washed twice with lysis buffer. The immunoprecipitates were incubated with 5 μCi of [γ-^{32}P]ATP in kinase buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, and 10 mM $MnCl_2$] at 30°C for 25 min using histone H2B as a substrate. The reactions were terminated by addition of 2$\times$ Laemmli sample loading buffer and then subjected to 15% SDS-PAGE. Phosphorylation of histone H2B was visualized by autoradiography.

Immunoprecipitation and Western Blot Analysis. At 48 h after transfection, cells were washed with ice-cold PBS and lysed with lysis buffer as described above. Protein concentration was determined with a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). For immunoprecipitation, cell lysates (200 μg of protein) were incubated with 0.5 μg of anti-α-subunit of IGF-IR for 1 h at 4°C. After incubation with protein A:protein G (1:1) agarose beads for 1 h at 4°C, immunoprecipitates were washed three times with lysis buffer. Proteins were subjected to 6% SDS-PAGE and Western blotting. Membranes were blocked and incubated with anti-β-subunit of IGF-IR antibody (1:400) in Tris-buffered saline containing 1% nonfat dry milk/0.1% Tween 20. Detection of antigen-bound antibody was carried out with Renaissance Chemiluminescence Reagent Plus (NEN Life Science, Boston, MA).

***In Vitro* Cell Invasion Assay Using Matrigel.** Cell invasion assays were performed using Transwell membrane filter inserts with 8-μm pore size (Corning Costar, Cambridge, MA). The upper surface of the Transwell membrane was coated with 250 μg/ml of growth factor-reduced Matrigel matrix (Becton Dickinson, Bedford, MA) overnight at 4°C, rehydrated once with 0.1% BSA in DMEM for 1 h at room temperature, and then placed in the upper compartment of six-well tissue culture plates. Twenty-four h after transfection with myr-HA-EGFP-*AKT*, PANC-1 cells were removed from tissue culture flasks by a short exposure to 5 mM EDTA and washed once in PBS. Then 2×10^5 cells in serum-free medium containing 0.1% BSA were added to each Transwell chamber and allowed to migrate toward the underside of the membrane for 18 h with and without 20 ng/ml of IGF-I (Life Technologies) in the lower chamber as a chemoattractant. After cells were fixed in 3.5% paraformaldehyde, cells on the upper surface of the membrane were removed by wiping with a cotton swab, and membranes were mounted onto glass slides. The relative number of invasion was determined by counting the number of invading EGFP-positive cells. The number of invading cells transfected with empty vector was assigned a value of 1.0 in each experiment. Twenty random fields/membrane were counted for each assay. Each determination represents the average of three separate experiments.

RESULTS

IGF-IR Expression Is Up-regulated by AKT. PANC-1 cells have been shown previously to exhibit increased IGF-IR expression in response to stable transfection with active *Src* (18). To confirm this finding under our experimental conditions, we examined IGF-IR expression in PANC-1 cells transiently transfected with active *Src*. As shown in Fig. 1*A*, cells transfected with active *Src* had significantly more IGF-IR protein than cells transfected with vector alone.

To examine the effects of AKT on the expression of IGF-IR, we transfected PANC-1 cells with constructs expressing wild-type AKT (HA-AKT1 or HA-AKT2) or constitutively active AKT (myr-HA-AKT1 or myr-HA-AKT2). IGF-IR expression was elevated in both wild-type *AKT*-transfected and constitutively active *AKT*-transfected cells compared with vector-transfected controls (Fig. 1*A*). The amount of IGF-IR protein in wild-type *AKT*-transfected cells was comparable with that of active Src-transfected cells, whereas IGF-IR expression was markedly higher in cells transfected with constitutively active *AKT*. These data suggest that activation of AKT is associated with the up-regulation of IGF-IR expression. AKT1 and AKT2 appear to be equally capable of inducing increased amounts of IGF-IR protein, because no significant difference in IGF-IR expression was observed in cells transfected with *AKT1* or *AKT2* constructs.

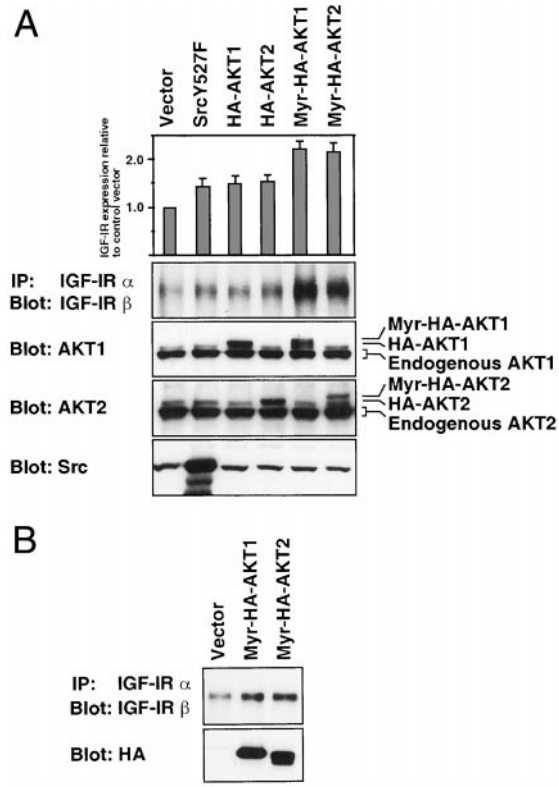

Fig. 1. *A*, IGF-IR expression in PANC-1 cells after transfection with active *Src* (*Src*Y527F), wild-type *AKT* (HA-*AKT1* or HA-*AKT2*), or constitutively active *AKT* (myr-HA-*AKT1* or myr-HA-*AKT2*). Cells were harvested 48 h after transfection. After immunoprecipitation with anti-IGF-IR α-subunit antibody, Western blotting was carried out with anti-IGF-IR β-subunit antibody. Western blots were performed to assess the expression of Src, AKT1, and AKT2 proteins. *Columns*, densitometric analysis of signals normalized to those of the vector-transfectant (assigned a value of 1.0); *bars*, SD. *B*, IGF-IR expression in AsPC-1 cells transfected with constitutively active *AKT1* and *AKT2*. *IP*, immunoprecipitation.

We also examined the effects of active Src and AKT on the expression of IGF-IR in AsPC-1 cells. IGF-IR expression was elevated in AsPC-1 cells transfected with constitutively active *AKT1* and *AKT2* (Fig. 1*B*).

AKT Is Activated by Active Src. AKT is activated by various extracellular stimuli through the PI3K pathway (8–10). To determine whether AKT functions as a downstream effector of active Src to regulate IGF-IR expression, we examined whether AKT is activated by active Src in PANC-1 cells. We assayed the *in vitro* kinase activity of AKT1 and AKT2 in cells cotransfected with Flag-*AKT* constructs and active, wild-type, or dominant-negative *Src*. Cells cotransfected with Flag-*AKT* and v-Ha-*ras* served as a positive control. As shown in Fig. 2, both AKT1 and AKT2 were activated by active Src or active Ras in PANC-1 cells. This suggests that AKT functions as a downstream effector of Src and Ras signaling in these cells. Furthermore, both AKT1 and AKT2 kinase activities were inhibited by dominant-negative Src (N17Src) or PTEN, a known inhibitor of PI3K/AKT signaling (16).

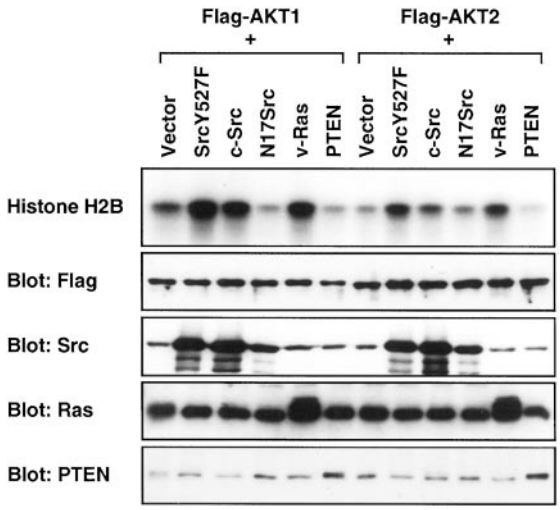

Fig. 2. *In vitro* kinase assay of AKT immunoprecipitates from PANC-1 cells cotransfected with Flag-*AKT* constructs and *Src*Y527F, c-*Src*, N17*Src*, v-*Ras*, and/or *PTEN*. Histone H2B was used as substrate, and phosphorylation of histone H2B was visualized by autoradiography. Western blots were performed to assess expression of transfected cDNAs.

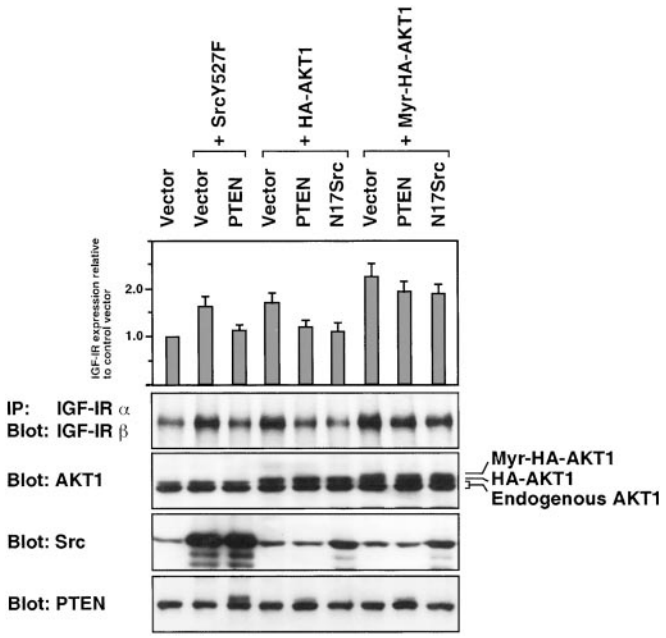

Fig. 3. PTEN or dominant-negative Src inhibits up-regulation of IGF-IR expression induced by active Src or AKT. Cell lysates were immunoprecipitated with anti-IGF-IR α-subunit antibody and immunoblotted with anti-IGF-IR β-subunit antibody. *Columns*, densitometry results; *bars*, SD. Values were normalized to those of the vector-transfectant. Western blots of cell lysates were probed with anti-AKT1, Src, and PTEN antibodies.

AKT-induced IGF-IR Expression Is Down-Regulated by Dominant-Negative Src or PTEN. As shown in Fig. 2, dominant-negative Src and PTEN inhibit the activation of AKT. We examined PANC-1 cells cotransfected with *AKT* and dominant-negative *Src* to determine whether dominant-negative Src inhibits the up-regulation of IGF-IR expression. Indeed, dominant-negative Src blocked induction of IGF-IR expression in cells cotransfected with wild-type *AKT* (Fig. 3). However, IGF-IR expression was only slightly down-regulated by dominant-negative Src in cells transfected with constitutively active *AKT*. These results suggest that Src activates AKT, which results in the up-regulation of IGF-IR expression in these cells.

We also examined cells cotransfected with *AKT* and *PTEN* to determine whether the PTEN tumor suppressor is capable of inhibiting AKT-induced expression of IGF-IR. We demonstrated that IGF-IR expression induced by wild-type and, to a lesser degree, constitutively active AKT is inhibited by PTEN (Fig. 3). In addition, IGF-IR expression induced by active Src was shown to be inhibited by PTEN. These data provide further evidence that Src-induced up-regulation of IGF-IR expression is mediated through the PI3K/AKT signal transduction pathway.

PANC-1 Cells Transfected with Constitutively Active AKT Have Increased Invasiveness. It has been reported that increased expression of IGF-IR in tumorigenic cells can enhance their invasiveness (23, 24). We performed an *in vitro* cell invasion assay using Matrigel matrix to examine whether the invasiveness of PANC-1 cells transfected with constitutively active *AKT* increases via up-regulation of IGF-IR expression. PANC-1 cells were transiently transfected with EGFP-containing myr-HA-*AKT* constructs, *i.e.*, myr-HA-EGFP-*AKT1* and myr-HA-EGFP-*AKT2* (Fig. 4*A*). As a control, cells were transfected with a myristylated HA-tagged EGFP construct. Transfected cells were easily distinguished from untransfected cells based on the expression of EGFP. *In vitro* kinase assays were used to assess the activity of myristylated AKT1 and AKT2. Extremely high kinase activity was observed in both myr-HA-EGFP-*AKT1* and myr-HA-EGFP-*AKT2*-transfected cells without stimulation, whereas control vector (myr-HA-EGFP)-transfected cells exhibited low kinase activity (Fig. 4*A*). IGF-IR expression also was elevated in myr-HA-EGFP-*AKT*-transfected PANC-1 cells as detected by Western blotting (data not shown). Migratory cells were identified by counting the number of EGFP-positive cells on the underside of the Transwell membrane. Repeated experiments revealed that constitutively active *AKT*-trans-

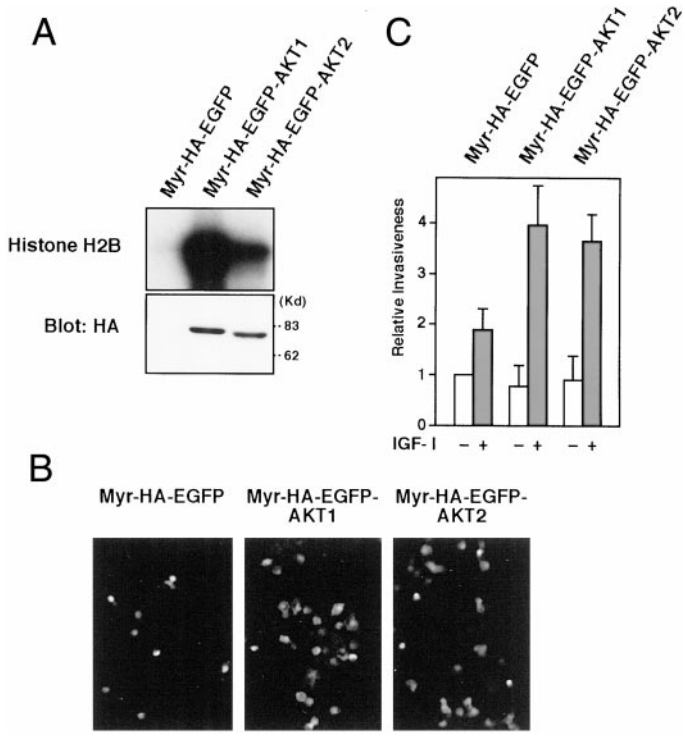

Fig. 4. Increased invasiveness potential of cells transfected with active *AKT1* and *AKT2* in a Matrigel invasion assay. *A*, PANC-1 cells were transiently transfected with EGFP-containing constitutively active *AKT1* and *AKT2* constructs (myr-HA-EGFP-*AKT1* and myr-HA-EGFP-*AKT2*). EGFP with myristylation signal (*myr-HA-EGFP*) was used as a control. *Upper panel*, activity of myr-HA-EGFP-AKT1 and myr-HA-EGFP-AKT2 was examined by *in vitro* kinase assay. *Lower panel*, Western blot probed with anti-HA antibody. *B*, invading PANC-1 cells transfected with control vector, constitutively active *AKT1*, and constitutively active *AKT2*. Invasive cells were assayed by counting EGFP-positive cells using a fluorescence microscope. *C*, invasiveness depicted as the average number of invading cells relative to controls in three individual experiments. □, invasiveness without addition of IGF-I; ▨, cells treated with IGF-I. *Bars*, SD.

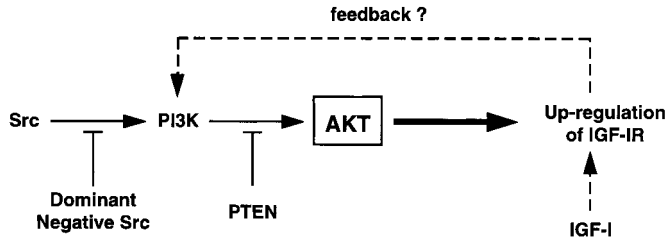

Fig. 5. Model depicting role of AKT signaling in the regulation of IGF-IR expression in pancreatic cancer cells. Up-regulation of IGF-IR expression induced by active Src can be down-regulated by PTEN, indicating that active Src-induced IGF-IR expression is mediated by the PI3K/AKT signaling pathway. *Thick arrow*, strong induction of IGF-IR expression, specifically upon activation of AKT.

fected cells showed significantly higher invasiveness potential than cells transfected with vector alone (Fig. 4, *B* and *C*). Without IGF-I in the lower chamber, there were no significant differences in invasiveness potential between constitutively active *AKT*- and vector-transfected cells. These results suggest that constitutively active AKT enhances the invasiveness of tumorigenic cells through the up-regulation of IGF-IR expression.

DISCUSSION

We have determined in our investigations that active AKT, like active Src, up-regulates IGF-IR expression in pancreatic cancer cell lines. Significantly more IGF-IR protein was detected in cells transfected with constitutively active *AKT* than in cells transfected with wild-type *AKT*. Furthermore, constitutively active *AKT*-transfected cells showed a higher invasiveness potential than control cells. Interestingly, we also found that inhibition of active Src-induced IGF-IR expression by PTEN and inactivation of AKT by PTEN or dominant-negative Src suppress AKT-induced up-regulation of IGF-IR expression. These results suggest that AKT mediates the Src signaling pathway leading to the up-regulation of IGF-IR protein, and that activation of AKT plays a significant role in the regulation of the IGF-IR expression in pancreatic cancer cells. A schematic representation of these events is depicted in Fig. 5, in which we propose that AKT participates in a feed-back loop whereby activation of AKT up-regulates IGF-IR expression. The increased IGF-IR is available to interact with the ligand IGF-I, and IGF-I binding to IGF-IR further activates the PI3K/AKT signaling pathway.

The downstream effectors of AKT signaling that lead to the up-regulation of IGF-IR have not been identified. Several consensus GC boxes (GGGCGG) for the binding of Sp1 transcription factor are contained within the proximal 5′-flanking region of the IGF-IR promoter, and Sp1 has been shown to be a strong activator of IGF-IR expression (25). Thus, Sp1 transcription factor may be involved in the up-regulation of IGF-IR. Moreover, mutant p53 interacts with the TATA box binding protein to up-regulate IGF-IR expression at the level of transcription, whereas wild-type p53 represses IGF-IR expression (26). Both PANC-1 and AsPC-1 cells have mutant p53 (27). In addition to these IGF-IR transcriptional regulators, PI3K/AKT signaling has been shown to regulate several transcription factors, such as E2F, cyclic AMP-responsive element binding protein, and Forkhead family member Daf-16 (28–30). PI3K/AKT also has been reported to be involved in the critical process of protein synthesis, the phosphorylation of 4E-BP1 and its dissociation from the mRNA cap binding protein elF4E, leading to the activation of mRNA translation (31). Therefore, PI3K/AKT may transcriptionally and posttranscriptionally regulate IGF-IR expression.

In human pancreatic cancer, it has been reported that IGF-I is not expressed in cancer cells and that IGF-I is abundantly expressed in the stromal tissue surrounding the tumor cells (7). This suggests that IGF-I exerts a paracrine effect on pancreatic cancer cell growth. IGF-I has been shown to be expressed in hepatocytes (32). Thus, paracrine growth stimulation by hepatocyte-derived IGF-I may account, at least in part, for the fact that pancreatic cancer cells readily metastasize to the liver at early stages of the disease. In addition, it has been shown that IGF-I stimulates the invasion and metastasis of cancer cells (33–35), and that increased expression of IGF-IR in tumorigenic cells could enhance their invasiveness (23). We demonstrated that PANC-1 cells transfected with constitutively active *AKT1* and *AKT2* show increased invasiveness in the presence of IGF-I. This observation further supports our contention that activation of AKT up-regulates IGF-IR expression and demonstrates that increased IGF-IR expression induced by active AKT is sufficient to enhance the invasiveness of pancreatic cancer cells in the presence of IGF-I. In this study, we used Matrigel matrix to assay for invasiveness potential. Matrigel contains ECM components such as laminin and collagen IV (36, 37), which closely resemble the tumor environment. It has been suggested that IGF-IR and IGF-I regulate the expression of ECM proteinases, such as matrix metalloproteinases and/or urokinase-type plasminogen activator, to enhance invasiveness potential (24, 38). These ECM proteinases are directly or indirectly involved in degrading the ECM. Indeed, urokinase-type plasminogen activator (39, 40) or activated forms of matrix metalloproteinases (41, 42) are detectable in human pancreatic cancer and/or its metastatic outgrowths. We reported previously that expression of AKT2 in PANC-1 and AsPC-1 cells is greatly decreased by antisense *AKT2* RNA, and that tumorigenicity in nude mice and tumor cell invasiveness are diminished in cells expressing antisense *AKT2* RNA (20). Taken together, our data suggest that overexpression and activation of AKT plays a significant role in the invasiveness of pancreatic cancer cells. Likewise, other investigators have reported that activation of PI3K signaling is implicated in hepatocyte growth factor-dependent invasiveness, and inactivation of PI3K results in reduced invasiveness of human intestinal cells (43).

The fact that inactivation of AKT can down-regulate IGF-IR expression suggests that AKT could represent an important therapeutic target in human pancreatic cancer. Because amplification and overexpression of *AKT2* have been reported in pancreatic cancers, perturbations of the AKT2 kinase may play a significant role in the pathogenesis of such tumors (20–22). Thus, selective inhibitors that specifically target downstream effectors of AKT to regulate IGF-IR expression may have important therapeutic implications in pancreatic cancer.

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