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Requirement of different signaling pathways mediated by insulin-like growth factor-I receptor for proliferation, invasion, and VPF/VEGF expression in a pancreatic carcinoma cell line

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Abstract

Several oncogenes and growth factors are found to be mutated and overexpressed in adenocarcinoma of the pancreas, and may correlate with its highly aggressive nature. Insulin-like growth factor (IGF-I) and its receptor (IGF-IR) are highly expressed in this tumor type. We examined the IGF-IR-mediated signaling pathways in relation to cell proliferation, invasiveness, and expression pattern of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) in the pancreatic cancer line ASPC-1. Our findings show that IGF-IR is an important growth factor receptor for cell proliferation and invasion, and VPF/VEGF expression in ASPC-1. Further experiments indicate that IGF-IR mediates different signaling pathways to execute its functions. Activation of Ras by IGF-IR was found to be required for the cell invasion. On the other hand Src activation through IGF-IR is required for the cell proliferation, invasion, and also VPF/VEGF expression. Taken together, our data indicate the importance of IGF-IR in growth and invasiveness of the pancreatic cancer cell lines and also point out the multiple signaling pathways channeled through this receptor.

Adenocarcinoma of the pancreas is one of the leading causes of cancer death in the United States. This disease is usually diagnosed at a late, incurable stage; less than 5% of patients survive 5 years after contracting the disease [1,2]. Although the reason for the highly virulent nature of this cancer is not yet completely understood, previous studies have shown that the activation of oncogenes, specifically; K-ras [1,3], loss of tumor-suppressor p53 gene function [4], and overexpression of a number of growth factors (e.g., transforming growth factor, hepatocyte growth factor), and their receptors [5–8] are associated with this tumor. Insulin-like growth factor (IGF-I) is one such important cytokine and is overexpressed, along with its receptor, during the progression of this cancer [9]. Activation of the insulin-like growth factor receptor (IGF-IR) signaling pathway promotes both cell proliferation and differentiation, and causes the reversal of apoptosis in normal as well as in pathological conditions [10,11]. Furthermore, it has been demonstrated that embryonic fibroblasts established from IGR-IR$^{-/-}$ mice are resistant to transformation induced by different oncogenes, growth factor receptors, and viral proteins [11].

Like most other cancer types, pancreatic cancer is characteristically well vascularized [12]. This property has been attributed to consistent overexpression of the potent angiogenic molecules including basic fibroblast growth factor, acidic fibroblast growth factor, and vascular permeability factor/vascular endothelial growth factor (VPF/VEGF) [13,14] as well as the high affinity VPF/VEGF receptors, KDR and Flt-1 [15,16]. VPF/VEGF was originally described as a tumor-secreted protein that potentially increased the permeability of

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venues and small veins to circulating macromolecules. It also stimulates endothelial cell migration and division, reprograms endothelial cell gene expression, prevents endothelial cells from apoptosis and senescence, and induces angiogenesis in both in vitro and in vivo models [14,17–21]. Data suggest that high levels of VPF/VEGF expression correlate well with the incidence of liver metastasis and poor prognosis in ductal pancreatic adenocarcinoma [22]. Recently, in a transgenic mouse model of pancreatic tumor progression, it has been shown that both positive and negative regulators of angiogenesis play an important role in tumor progression [23,24]. However the molecular mechanisms underlined remain to be investigated.

In this study, we focused on the possible link between different oncogenes (e.g., Src and Ras) and growth factor receptor (e.g., IGF-IR) functions in VPF/VEGF upregulation and thus angiogenesis in pancreatic cancer. Our results demonstrate that in the pancreatic cancer cell line ASPC-1, cell proliferation, invasion, and VPF/VEGF expression are regulated by the signaling event downstream of IGF-IR, where Src and Ras act as key intermediary molecules.

Materials and methods

Reagents. [^{3}H]Thymidine (1 μCi/μl) was obtained from NEN, (Boston, MA) and Matrigel was obtained from Fisher Scientific (Pittsburgh, PA). The anti-phosphotyrosine antibody was obtained from Upstate Biotechnology (Lake Placid, NY). The anti-IGF-IR antibody is the product of Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-Src [pY^{418}] phosphospecific antibody is available from Biosource International (Camarillo, CA). Rabbit anti-c-Src antibody is available from Santa Cruz Biotechnology. Normal rabbit serum and rabbit IgG were obtained from Sigma (St. Louis, MO). PP-2 and PP-3 were obtained from Calbiochem (La Jolla, CA).

Cell culture. Human pancreatic carcinoma cell line, ASPC-1 (ATCC), was maintained in RPMI 1640 medium with 20% fetal bovine serum (HyClone Laboratories). ASPC-1 is a human pancreatic adenocarcinoma cell line established from ascites (ATCC # CRL-1682). BXPC-3 and SU86.86 cells were also obtained from ATCC and maintained in DMEM with 10% serum. Serum starvation was performed with 0.1% FBS in RPMI 1640 or DMEM, respectively.

Immunoprecipitation and Western blot analysis. Cells were washed twice with 10 ml of cold PBS, lysed with ice-cold lysis buffer (50 mM Tris [pH 7.5], 1% Nonidet p-40 [NP-40], 150 mM NaCl, 1 mM Na_3 VO_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin [10 μg/ml], 0.5% aprotinin, and 2 mM pepstatin A), incubated on ice for 10 min, and centrifuged at 4 °C for 10 min. Immunoprecipitation was performed in 0.5 mg cellular protein with a mouse monoclonal antibody (1 μg) directed against phosphorylated tyrosine. Immunocomplexes were captured with protein A–agarose beads (Pharmacia). After three washes with cell lysis buffer, bead-bound proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE). Size-separated proteins were transferred (Trans-Blot SD; Bio-Rad) to a nylon membrane (Immobilon-P; Millipore). For immunodetection, membranes were blocked in washing buffer (PBS and 0.1% Tween 20) with 4% milk or BSA and incubated in washing buffer with an antibody against IGF-IR (1:1000 dilution, stock conc. 0.2 mg/ml) or anti-phospho Src or anti-Src antibodies. The secondary antibodies were goat anti-mouse or anti-rabbit immunoglobulin (Ig) linked to horseradish peroxidase (Pierce) and were detected by chemiluminescence (Pierce).

Overexpression of Ras17N with retrovirus. Retrovirus preparation and infection were carried out as described [25]. Briefly, 293T cells (6×10^6/100 mm plate) were seeded 24 h before transfection. pMMP-LacZ (20 μg), or pMMP-Ras17N (20 μg), pMD.MLV gag.pol (15 μg), and pMD.G DNA (5 μg) (provided by Dr. Richard A. Mulligan) were diluted in 500 μl of H_2O with 62 μl of 2 M $CaCl_2$. To that diluted DNA mixture, 500 μl of 2× HBS (280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 12 mM dextrose, and 50 mM Hepes, pH 7.05) was added and incubated at room temperature for 20 min. The DNA mixture was added dropwise to 293T cells. Medium was changed after 16 h. Retrovirus was collected 48 h after transfection by passing through a 0.45 μm filter and used for infection directly.

Forty-eight hours before infection, ASPC-1 cells were seeded in a density of 1×10^6 cells/100 mm plate. Five milliliters of retrovirus solution and 5 ml fresh medium were added to cells with 10 μg/ml polybrene (Sigma). Medium was changed after 16 h and cells were used for the experiment 48 h after infection.

Proliferation assay. Cells (2×10^3) were transduced with a Ras dominant negative mutant and were seeded in a 24-well plate. Two days later, cells were serum starved with 0.1% FBS in RPMI 1640 for 20 h. One μCi [^{3}H]thymidine (PerkinElmer) was added to each well for 4 h. IGF-1R specific antibodies (1H7, 25 μg/ml), a control mouse IgG (25 μg/ml) or Src inhibitor (PP-2) (1 μM) and its analogue (PP3) (1 μM) were added at the time of serum starvation. Cells were washed with cold PBS three times, fixed with cold methanol at 4 °C for 15 min, precipitated with cold 10% TCA at 4 °C for 15 min, and then lysed with 0.1 N NaOH for 30 min. Cell lysis was subjected to scintillation counting. Data are expressed as means ± SD of triplicate values.

Isolation of RNA and Northern blot analysis. Total cell RNA was isolated from cultured cells essentially as described by Chomczynski and Sacchi (Chomczynski and Sachi, 1987). RNA was denatured at 65 °C for 15 min and then chilled on ice prior to loading. Electrophoresis of total RNA was carried out on a formaldehyde–agarose gel. RNA was transferred to a GeneScreen (PerkinElmer) membrane by using 10× SSC and probed with ^{32}P-labeled cDNA probes in a solution containing 0.5 M sodium phosphate, pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA, and sonicated herring sperm DNA (50 μg/ml) at 68 °C. Blots were washed three times with a solution containing 40 mM sodium phosphate (pH 7.2), 0.5% SDS, 0.5% BSA, and 1 mM EDTA at 68 °C and autoradiographed. ^{32}P-labeled DNA probes were prepared as described in the manufacturer's instruction (Boehringer Mannheim).

Transfection and luciferase assay. ASPC-1 cells (2×10^3) were seeded in a 96-well plate 2 days before transfection. Transfection was carried out with Effectene Transfection Kit (Qiagen, Valencia, CA). Briefly, 0.05 μg of the 2.6 kb VEGF promoter was resuspended in EC buffer (6.25 μl) and 0.4 μl Enhancer was added and incubated at room temperature for 5 min. Effectene (0.5 μl) was then added and the whole mixture was incubated for another 10 min. RPMI with 0.1% FBS (190 μl) was added to the DNA mixtures. This DNA mixture with or without addition of antibodies or inhibitor was added to cells that were washed with PBS twice. Twenty-four hours after transfection, cells were washed three times with PBS and lysed with 50 μl reporter lysis buffer at r.t. for 1 min. Cell lysis was subjected to luciferase assay. Data are expressed as means ± SD of quadruplicate values.

Invasion assay. A thin layer of matrigel solution (0.040 ml of 8 mg/ml stock solution; Becton–Dickinson Labware) was overlaid on the upper surface of the 6.5 mm Transwell chambers (8-μm pore size; CoStar, Corning, NY). The matrigel was allowed to solidify by incubating the plates for 4 h at room temperature. DMEM (0.6 ml) containing 0.5 μM IGF-I (Sigma, St. Louis, MO) was then added to the bottom chamber of the transwells. Cells with or without transduction with Ras17N-expressing viruses were resuspended in 0.2% BSA/DME at a concentration of 2×10^5 cells/ml, and 2×10^4 cells were added to the top well of the transwell chambers. In some experiments, IGF-1R

specific antibody (1H7, 25 μg/ml), control mouse IgG (25 μg/ml) or Src inhibitor (PP-2) or its analogue (PP-3) was added to the cells and incubated for 1 h before the cells were plated on the top of the transwells. Cells were then incubated for 5 h in the CO_2 incubator. The cells that had not invaded through the matrigel were removed from the upper surface using cotton swabs, and the cells that had invaded through the matrigel and entered into the lower surface of the filters were fixed in methanol and then stained with a 0.2% solution of crystal violet in 2% ethanol. Invasion was quantified by counting the cell number with bright-field optics in a Nikon Diaphot microscope equipped with a 16-square reticule. The surface area of this grid was determined to be 1 mm^2. Three separate fields were counted for each filters. Data are expressed as means ± SD of triplicate values.

Src kinase assay. Src kinase activity was assayed by measuring the incorporation of ^{32}P into the specific Src kinase substrate peptide, cdc2 (K-V-E-K-I-G-E-G-T-Y-G-V-V-Y-K) (Upstate Biotechnology, Lake Placid, NY). ASPC-1 cells were first serum starved (0.1% serum) overnight in the presence of either anti-IGF-IR antibody (25 μg/ml) or control mouse antibody (25 μg/ml). Cells were then lysed with RIPA buffer (50 mM Tris [pH 7.5], 1% Nonidet P-40 [NP-40], 150 mM NaCl, 1 mM Na_3 VO_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin [10 μg/ml], 0.5% aprotinin, and 2 mM pepstatin A) and followed by immunoprecipitation with the anti-Src antibody. Kinase assay was then performed with Src protein-containing protein A–Sepharose beads. Data are expressed as means ± SD of triplicate values.

Ras activation assay. Ras assay kit was obtained from Upstate Biotechnology (NY). The assays were carried out according to the protocol provided by the company. Briefly, confluent cells were serum-starved for 24 h with or without addition of different concentrations of antibodies against IGF-1R or control IgG. Cells were lysed with 0.8 ml magnesium-containing lysis buffer (MLB). The cell lysate was incubated with 10 μl Raf-1 RBD agarose at 4 °C for 30 min. After washing with MLB for three times, the proteins bound on the beads were subjected to Western blot analysis using an antibody against Ras.

RNA preparation and real time PCR. After washing twice with ice cold PBS, ASPC-1 cells were lysed using lysis buffer from the RNeasy Mini kit (Qiagen). Total RNA was extracted according to the RNeasy mini kit protocol. We used the Taqman real time PCR method. The sequences for forward, reverse, and Taqman middle primers for human VPF/VEGF and for human β-actin (houskeeping gene) were taken from the PubMed GenBank and synthesized (Integrated DNA Technology). VPF/VEGF forward: 5′-TAC CTC CAC CAT GCC AAG TG-3′. VPF/VEGF reverse: 5′-GAT GAT TCT GCC CTC CTC CTT-3′. VPF/VEGF middle primer: 5′-TCC CAG GCT GCA CCC ATG GC-3′. β-actin forward: 5′-TCA CCA TGG ATG ATG ATA TCG C-3′. β-actin reverse: 5′-AAG CCG GCC TTG CAC AT-3′. β-actin middle primer: 5′-CGC TCG TCG TCG ACA ACG GCT-3′. VPF/VEGF and β-actin middle primers both had a 5′-TET reporter and a 3′-Tamra quencher. Each real time PCR was done using 0.5 μg total RNA, 25 μl RT-PCR Master Mix (Applied Biosystems), 1.25 μl Rnase inhibitor (Applied Biosystems), 50 nM forward primer, 50 nM reverse primer, and 100 nM middle primer. For reverse transcription, a 30-min period at 48 °C was run before inactivating the reverse transcriptase at 95 °C for 10 min. Forty cycles at 95 °C for 15 s and 60 °C for 1 min were performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems). Light signal given by the TET-tagged middle primer was detected and the cycle number when the light signal crosses a defined threshold in the middle of the exponential phase of DNA amplification was measured (CT value). All experiments were carried out three times and from each of the three, triplicate readings were taken and the average was calculated. Relative RNA amount was calculated as follows: $\Delta = CT(VPF/VEGF\ sample) - CT(\beta\text{-actin sample})$. $\Delta\Delta = \Delta(transfected\ \ sample) - \Delta(empty\ vector\ sample)$. Relative RNA amount in comparison to the control $= 2^{-\Delta\Delta}$. Average and standard deviation from three experiments were calculated.

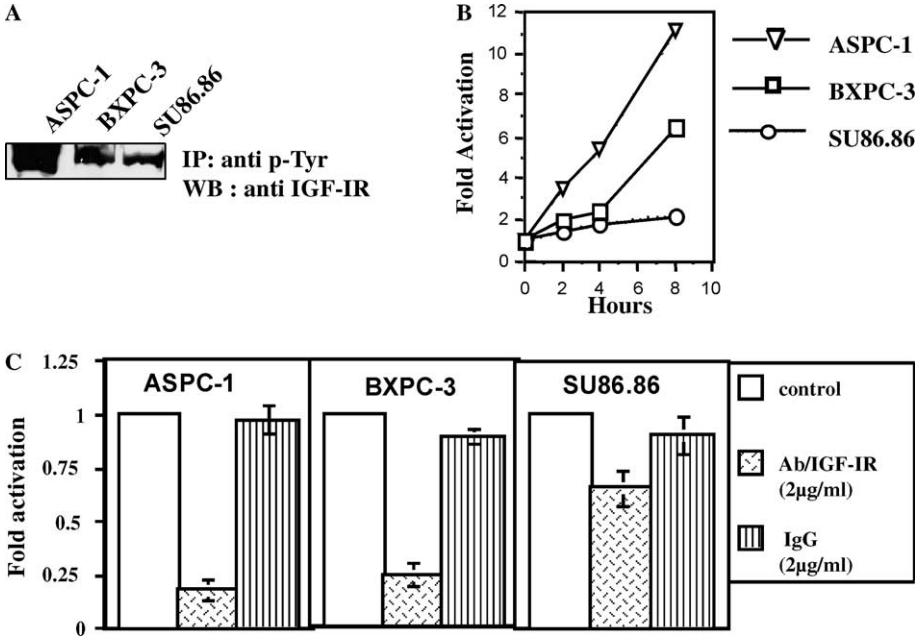

Fig. 1. IGF-IR mediated proliferation of PCA cells. (A) Immunoprecipitation of the cell lysates of three serum-starved-pancreatic cell lines; ASPC-1 (lane:1), BXPC-3 (lane:2), and SU86.86 (lane:3) with anti-phosphotyrosine antibody followed by Western blot with anti-IGF-IR antibody. (B) Cell proliferation assay. Three pancreatic cell lines were serum starved overnight and incubated with [^{3}H]thymidine (1 μCi/ml) for different time intervals. Fold activation was measured with respect to the thymidine incorporation at zero time point (considered as onefold) of each cell line. (C) Pancreatic cancer cell lines were serum starved and incubated overnight with IgG or anti IGF-IR antibody that specifically blocks IGF-IR signaling. [^{3}H]thymidine incorporation was measured after incubating the cells for 4 h. Fold activation was measured with respect to the thymidine incorporation of untreated cells of individual lines, which was considered as one fold. The data represent the average of three independent experiments (means ± SD).

Results

Requirement of IGF-IR for ASPC-1 cell proliferation

We first examined the phosphorylation of IGF-IR in different pancreatic cancer cells in the absence of serum. Three pancreatic cancer cell lines, ASPC-1, BXPC-3, or SU86.86, were serum starved for 24 h and then equal amounts of cellular extracts were immunoprecipitated with an antibody against phosphorylated tyrosine (p-Tyr), followed by immunoblot analysis using the anti-IGF-IR antibody. The data show that the levels of tyrosine phosphorylated IGF-IR are different among these three pancreatic cancer cells, highest in ASPC-1 and lowest in SU86.86 data (Fig. 1A). Next, we examined the rate of proliferation of these three cell lines in the absence of serum. The cells were incubated with serum-free media and proliferation was measured at 2, 4, 6, and 8 h by examining the [^{3}H]thymidine incorporation. It was found that ASPC-1 was highly proliferative, while the proliferation rates of BXPC-3 and SU86.86 are moderate and low, respectively (Fig. 1B). These results prompted us to determine the role of IGF-IR in the proliferation rate of these cells. Serum starved cells were treated with a neutralizing antibody against α-IGF-IR that specifically inhibits IGF-IR signaling and with a control IgG. Cell proliferation was measured. As shown in Fig. 1C, the proliferation of ASPC-1 and BXPC-3 was almost completely inhibited by the IGF-IR specific antibody while that of SU86.86 was not affected. The data suggest that the cell proliferation of ASPC-1 and BXPC-1 is induced by IGF-IR, probably acting in an autocrine manner. Since the levels of IGF-IR phopshorylation and cell proliferation in ASPC-1 are higher than those in BXPC-3, ASPC-1 was chosen for further study.

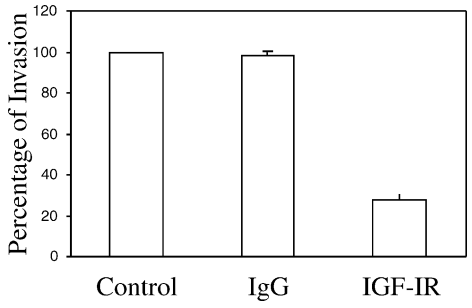

Fig. 2. IGF-IR mediated ASPC-1 cell invasion. (A) ASPC-1 cells were incubated for 24 h in serum-free medium with or without anti-IGF-IR antibody (25 μg/ml) or the nonspecific mouse IgG control (25 μg/ml) and then seeded onto matrigel which also contained respective antibodies in the same concentration. Bottom chamber contained the condition medium obtained from ASPC-1 cells with or without antibody treatment. Percent invasion was calculated based on the invasion of control samples as 100%. For each experiment, four transwells were used. The data represent the average of three independent experiments (means ± SD).

IGF-IR is involved in ASPC-1 invasion

Invasion of tumor cells through matrix is an early stage of metastasis. To determine whether IGF-IR plays a role in invasion, we performed the matrigel invasion assay of serum starved ASPC-1 cell in the presence or absence of anti-IGF-IR antibody. First, we incubated the cells with or without anti-IGF-IR-antibody for 24 h at 37 °C before putting it into the upper-well of the transwell. Conditional medium from 24-h serum starved ASPC-1 cultures with or without antibody treatment was placed in the bottom well of the transwell and cells were allowed to invade through the matrigel for 5 h at 37 °C. Fig. 2 shows that like proliferation, the invasion of ASPC-1 cells into the matrigel is induced by IGF-IR.

IGF-IR is required for VPF/VEGF upregulation in ASPC-1 cells

Many growth factors up-regulate VPF/VEGF [26–29]. Therefore, we tested whether IGF-IR, in addition to promoting cell proliferation and invasion, has any stimulatory effect on VPF/VEGF expression. We first examined the VPF/VEGF mRNA level in ASPC-1 cells in serum starved conditions and the effect of IGF-IR specific antibody on that expression pattern. Fig. 3A shows that the level of VPF/VEGF mRNA was maximum (>10-fold from the mRNA level at 0 h) at 24 h after serum starvation and this maximum level was sustained for at least 48 h (data not shown). When we

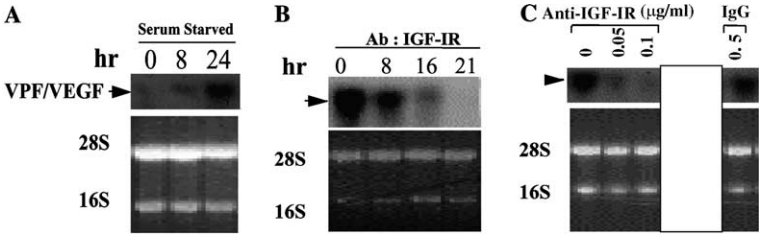

Fig. 3. VPF/VEGF expression. (A) ASPC-1 cells were serum starved (0.1% serum in DMEM) for different time intervals and (B) incubated with mouse monoclonal IGF-IR antibody (0.5 μg/ml) for different time intervals after serum starvation for 24 h. Total RNA was isolated and subjected to Northern blot analysis using human VPF/VEGF cDNA as a probe. (C) ASPC-1 cells were serum starved (0.1% serum in DMEM) for 24 h followed by incubation with mouse monoclonal IGF-IR antibody at two different concentrations as well as mouse IgG as control for 21 h. Total RNAs were isolated and subjected to Northern blot analysis using human VPF/VEGF cDNA as probe.

incubated 24 h-serum-starved ASPC-1 cells with 0.5 μg/ml mouse monoclonal antibody against IGF-IR for different time intervals (Fig. 3B), the total amount of VPF/VEGF mRNA was reduced by at least 50% and almost 100% with antibody treatment for 8 and 21 h, respectively. Moreover, we also found that anti-IGF-IR antibody levels as low as 0.1 μg/ml were sufficient for the inhibition of VFP/VEGF mRNA up-regulation, whereas IgG treatment had no effect (Fig. 3C). These results indicate that IGF-IR plays a positive role in VPF/VEGF expression in pancreatic cancer cells.

Role of Src and Ras in this IGF-IR induced proliferation, invasion, and VEGF upregulation

To further elucidate the signaling pathway(s) originated from IGF-IR that leads to cell proliferation, invasion, and VPF/VEGF upregulation in ASPC-1 cells, we tested the involvement of two proto-oncogenes, Ras and Src. We used a retrovirus system to express the dominant negative Ras mutant, Ras17N, to inhibit the function of endogenous Ras. This Ras17N blocks the guanine nucleotide exchange factors (GEFs) and thereby can inhibit all Ras family proteins. ASPC-1 cells were treated with Src inhibitor, PP-2, or transduced with virus containing Ras17N were serum-starved for 24 h and subjected to proliferation assay and invasion assay. Fig. 4A shows a clear inhibition of proliferation in PP-2 treated ASPC-1 cells as compared to that of control cells. This suggests the involvement of c-Src family member(s) in ASPC-1 proliferation in the absence of serum. In contrast, ASPC-1 cells transduced with Ras17N expressing virus had no difference in cell proliferation as compared to that of control cells (ASPC-1 cells transduced with same viral vector, pMMP, with *lac Z* cDNA) (Fig. 4A). These results indicate that the proliferation of ASPC-1 cells is Ras-independent, but Src family kinase-dependent. However, not only PP-2, but also overexpression of Ras17N inhibits ASPC-1 cell invasion in the absence of serum (Fig. 4B). These results indicate that both Src family kinases and Ras are involved in the signaling pathways of invasion of ASPC-1 and also emphasize the differences between proliferation vs. invasion signaling pathways.

Transcriptional regulation of VPF/VEGF mRNA

In order to determine the role of Src and Ras in VPF/VEGF mRNA expression, we performed real time PCR experiments in the presence of Src inhibitor (PP-2) or Ras dominant negative mutant. The results (Figs. 5A and B) suggest that VPF/VEGF expression is dependent on the Src activity as the total mRNA of VPF/VEGF is inhibited by 40% in the presence of the specific c-Src inhibitor, PP-2 but not its analogue molecule, PP-3. On the other hand the dominant negative mutant form of

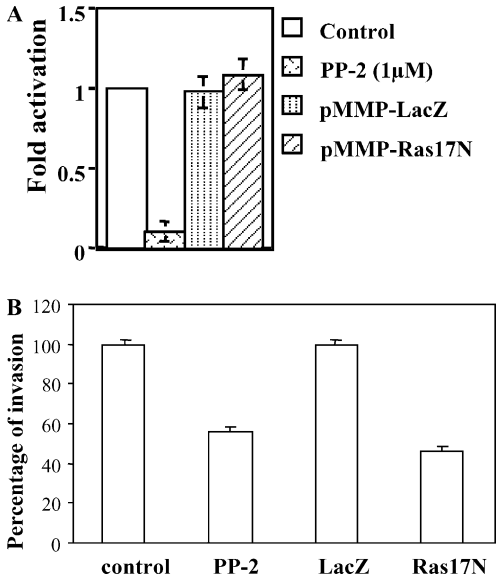

Fig. 4. Requirement of Src and Ras in the ASPC-1 cell proliferation and invasion (A) ASPC-1 cells were seeded for 2 days and serum starved for 20 h either pretreated with PP-2 or its analogue PP3 that acts as a control or is transduced with retroviruses. [^{3}H]thymidine incorporation was measured after incubating the cells for four hours. Fold activation was measured with respect to the thymidine incorporation in the untreated cell line, which was considered as one fold. The data represent the average of three independent experiments (means ± SD). (B) ASPC -1 cells treated with PP-2 and PP3 (1 μM) or transduced with retroviruses containing either pMMP-lacZ or pMMP-Ras17N were seeded on the matrigel. Bottom chamber contained the conditioned medium (CM) collecting from 24 h serum-starved ASPC-1 cells. Percent invasion was calculated based on the invasion of control sample without any treatment. For each experiment, three transwells were used. The data represent the average of three independent experiments (means ± SD).

Ras did not have any effect on total VPF/VEGF mRNA content in the ASPC-1 cell.

In order to determine whether the up-regulation of VPF/VEGF mRNA by IGF-IR is at the transcriptional level, we utilized a cDNA construct containing luciferase reporter gene whose expression is driven by the full length (2.6 kb) VPF/VEGF promoter. As expected, we found that after 24 h of serum starved condition, the VPF/VEGF promoter activity was high and the monoclonal antibody against IGF-IR inhibited this activity (Fig. 5C). These data suggest that the transcriptional activity of the VPF/VEGF promoter can be mediated by IGF-IR. We then also tested whether Ras and Src family kinases play any role in this transcriptional activation of VPF/VEGF. We transiently transfected ASPC-1 cells with the VPF/VEGF promoter (2.6 kb)-luciferase-reporter construct and measured the luciferase activity in serum starved conditions in the presence of either the specific inhibitor of c-Src family kinases, PP-2, or the dominant negative form of Ras protein, Ras 17N. Fig. 5C shows that PP-2 inhibits the VPF/VEGF transcription although Ras17N has no effect.

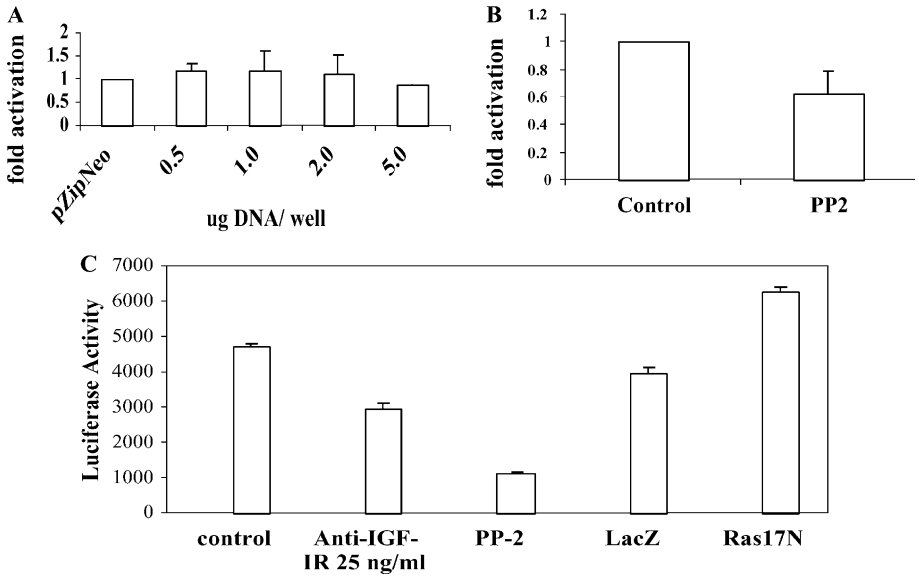

Fig. 5. Requirement of Src for VPF/VEGF expression in ASPC-1. (A) Real time PCR experiment to measure VPF/VEGF expression in serum starved ASPC-1 cells after overexpressing different concentrations of Ras17N. Relative total VPF/VEGF mRNA level was calculated as described in Materials and methods. (B) Real time PCR experiment to measure VPF/VEGF expression in serum starved ASPC-1 cells after incubating it with c-Src family kinase specific inhibitor, PP-2 (1 μM), and its analogue PP3 (1 μM) that acts as a control. (C) Luciferase activity assay to measure the transcriptional activity of the VPF/VEGF promoter: ASPC-1 cells were transfected with 2.6 kb VPF/VEGF promoter-luciferase construct and serum starved for 48 h either in the presence of anti-IGF-IR antibody (25 ng/ml), or c-Ssrc family kinase specific inhibitor, PP-2 (1 μM), and PP3 or overexpressed Ras dominant negative mutant, Ras17N and LacZ as control. The data represents the average of three independent experiments (means ± SD).

These data indicate that Src is required for the transcriptional activity of the VPF/VEGF promoter.

IGF-IR mediates the Ras and Src activity in ASPC-1 cells

Since IGF-IR, Src, and Ras are involved in the ASPC-1 cell proliferation, invasion, and VPF/VEGF expression, we further tested whether IGF-IR functions through activation of Src and Ras. We measured the Src and Ras activity in 24-h serum-starved ASPC-1 cells treated with or without an antibody against IGF-IR. Activation of Ras was measured by its ability to interact with the Ras-binding domain of Raf protein (RDB). Fig. 6A shows that in the absence of antibody, Ras binds to the agarose beads conjugated with RDB, indicating that Ras is activated in the serum starved condition. When ASPC-1 was treated with different concentrations of the IGF-IR antibody, Ras activation was inhibited partially and completely at 15 and 25 μg/ml, respectively (Fig. 6A). However, the same concentrations of IgG had no effect on the Ras activity (Fig. 6B). These data indicated that Ras is a downstream molecule of IGF-IR for a specific signaling event.

Next we measured the active Src level in ASPC-1 cells by Western blot experiments using anti-phospho Src [pY^{418}] antibody. ASPC-1 cells were first serum starved overnight in the presence of either anti IGF-IR antibody (25 μg/ml) or control antibody and after lysis of the cells with RIPA buffer, Western blot was performed. We

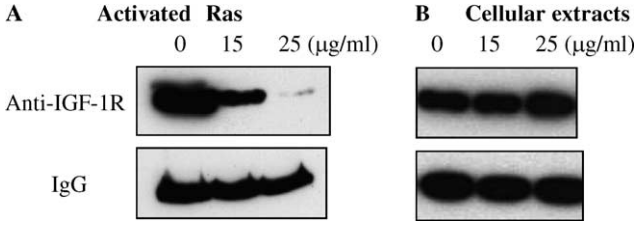

Fig. 6. IGF-IR mediated Ras activation. Cellular extracts from 24 h serum-starved ASPC-1 cells treated with or without different concentrations of the IGF-IR antibody or IgG were incubated with agrose conjugated RBD. Proteins bound on RBD beads (panel A) and cellular extracts (panel B) were analyzed with an antibody against Ras.

observed a significant decrease in the phospho-Src level in the presence of the IGF-IR antibody (Fig. 7A), suggesting the involvement of c-Src in certain IGF-IR mediated signaling events in the ASPC-1 cell. Fig. 7B shows the densitometric scan result of Fig. 7A where we compared the phospho-Src level in IgG treated cells and anti-IGF-IR treated cell after normalizing the value of the total c-Src level. Almost 50% decrease in phospho-Src level was detected by densitometric scanning. A parallel approach was also undertaken where we measured the c-Src activity that was determined by its ability to phosphorylate its substrate peptide as described in Materials and methods. As shown in Fig. 7C, Src is activated in the serum-starved condition. However, when cells were treated with 25 μg/ml IGF-IR antibody,

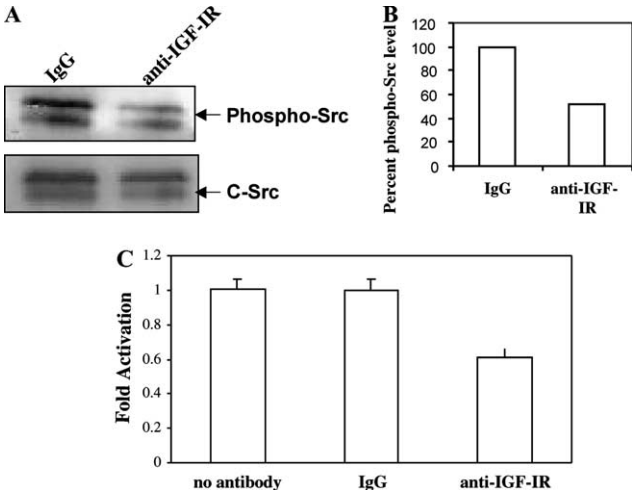

Fig. 7. IGF-IR mediated Src activation. (A) Cellular extracts from 16 h serum-starved ASPC-1 cells treated with or without IGF-IR antibody or IgG were Western blotted with an antibody against phospho-Src. (B) Densitometric scan result of (A) where the phospho-Src level was compared in IgG treated cells and anti-IGF-IR treated cells after normalizing the value of the total c-Src level. (C) Src assay. Cellular extracts from 16 h serum-starved ASPC-1 cells treated with or without IGF-IR antibody or IgG were immunoprecipitated with an antibody against Src. The immunoprecipitated complexes were used to detect the kinase activity as described in Materials and methods.

Src activity was inhibited. No effect of IgG could be detected. All these results clearly pointed out that in ASPC-1 cells, c-Src is one of the downstream molecules of IGF-IR.

Discussion

Growth factors can regulate tumor cell proliferation and invasion and promote angiogenesis either by autocrine or paracrine mechanism [30–33]. IGF-I is one of such growth factors that links to growth, invasion, and metastasis of different tumor cells [34–36] and is known to be secreted in pancreatic cancer as an autocrine loop [9]. In this study, we show that the IGF-IR is required for the ASPC-1 cell proliferation and invasion and VPF/VEGF mRNA expression (Figs. 1C, 2, and 3). We also determine the involvement of two important proto-oncogenes, Ras and Src, in pancreatic cancer cell proliferation, invasion, and VPF/VEGF expression.

Pancreatic cells are usually in a low proliferative state. In a normal cell cycle, there are several so-called checkpoints that monitor its progression. Loss of these checkpoints due to genetic alteration is considered to be the initial event of tumorigenesis. Overexpression of the growth factor receptor, IGF-IR is one such important genetic alteration that leads pancreatic cells to enter into the cell cycle and proliferate rapidly. We here showed that the expression level of the activated form of IGF-IR is directly correlated with the proliferation rate of different pancreatic cancer cells (Figs. 1A and B). Again, blocking of IGF-IR function can significantly inhibit the proliferation rate (Fig. 1C). We also found that the proto-oncogene Src, but not Ras, is involved in this IGF-IR induced proliferation (Fig. 4A). Very recently, Lopez and Hanahan showed that elevated levels of IGF-1 receptor convey invasive and metastatic capability in a mouse model of pancreatic islet tumorigenesis [58].

Another measure of tumor aggressiveness is metastasis in which invasion is an early step. Pancreatic cancer cells are generally found to be highly invasive [43]. In melanoma, hepatocarcinoma, breast cancer, renal cancer as well as others, IGF-I plays an important role in cell invasion [34,44–46]. Recent work has suggested that IGF-IR also plays a role in the invasiveness of pancreatic cancer [47]. We found that pancreatic cancer cell invasion was mostly modulated by IGF-IR. Furthermore, both Src family kinases and Ras were involved in this pancreatic cancer cell invasion, because both PP-2 and the dominant negative mutant of Ras completely inhibit ASPC-1 cell invasion in the absence of serum (Fig. 4B). Importantly, the dominant negative Ras17N blocks the guanine nucleotide exchange factors (GEFs) and thereby can inhibit all Ras family proteins including K-Ras. Our finding that Ras is required for invasion establishes a link between the frequently seen Ras mutations with highly virulent nature of pancreatic cancer.

A number of factors such as hypoxia, cytokines, hormones, and growth factors regulate VPF/VEGF expression in tumors [26,27,29,35]. In fact, both mRNA and protein levels of VPF/VEGF are greatly increased in a high percentage of malignant animal and human tumors and in many immortalized and transformed cell lines [48–50]. In PCA cells, VPF/VEGF expression is strikingly elevated as compared to that of normal pancreatic cells [51]. Our results indicate that elevated expression of VPF/VEGF in ASPC-1 cells is IGF-I-dependent. Several signaling intermediates, such as PKC, Src, and Ras, have already been identified in the signaling pathway that regulates VPF/VEGF expression [52–54]. For example, c-Src activation was found to be required for hypoxia-induced VPF/VEGF expression in U87 (human glioblastoma cells), 293 cells (adenovirus transformed human fetal kidney cell line) [53,55], and colon carcinoma cell lines [56]. Moreover, transfection of cells with activated forms of Src, i.e., v-Src, leads to a constitutive high level of VPF/VEGF expression even under normoxic conditions [57]. In this study, we have found that Src family of protein kinases, not Ras, is required for the VPF/VEGF upregulation in ASPC-1 cells and the involvement of Src is mainly at the transcriptional level (Fig. 5).

Several studies on breast cancer, glioblastoma, adenocarcinoma of lung, and many other cancer types have shown that Ras, an important proto-oncogene, plays an

important role in growth factor mediated cell proliferation [37–39]. It is also known that k-Ras mutation is found in most pancreatic cancers [40], though there are also reports that this mutation may not always be linked to pancreatic cancer growth [41,42]. In fact, k-Ras mutation is not found in ASPC-1 cells [9]. Our studies indicate that in ASPC-1, IGF-IR can induce Ras activity (Fig. 6A). However, this IGF-IR-mediated Ras activation is not required for the pancreatic cancer cell (ASPC-1) proliferation, but is important for cell invasion (Figs. 4A and B). We are thus able to detect the oncogene Ras as a downstream molecule in one of the important growth factor receptor (i.e., IGF-IR) mediated signaling events that helps invasion of pancreatic cancer cells.

Taken together, our findings indicate that IGF-IR is a major factor that mediates the cell proliferation, invasion, and VPF/VEGF upregulation in ASPC-1 cells. Src family kinases are the common molecules in these signaling pathways. The other pro-oncogene Ras cooperates with Src family kinases for the signaling event of invasion but not for proliferation and VPF/VEGF expression in pancreatic cancer cells. Discovery of other intermediary molecules will help us to visualize the more detailed and clearer picture of these complex interconnecting pathways leading to pancreatic cancer cell proliferation, the onset of angiogenesis and invasion. Further studies may thus allow targeting of these pathways to develop novel agents aimed at controlling this devastating disease.

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