THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 6, Issue of February 6, pp. 3941–3948, 2004

*Printed in U.S.A.*

# Role of Insulin Receptor Substrates and Protein Kinase C-ζ in Vascular Permeability Factor/Vascular Endothelial Growth Factor Expression in Pancreatic Cancer Cells*

Received for publication, April 15, 2003, and in revised form, October 10, 2003
Published, JBC Papers in Press, November 6, 2003, DOI 10.1074/jbc.M303975200

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**Vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), the critical molecule in tumor angiogenesis, is regulated by different stimuli, such as hypoxia and oncogenes, and also by growth factors. Previously we have shown that in AsPC-1 pancreatic adenocarcinoma cells, insulin-like growth factor receptor (IGF-IR) regulates VPF/VEGF expression. Insulin receptor substrate-1 and -2 (IRS-1 and IRS-2), two major downstream molecules of IGF-1R, are known to be important in the genesis of diabetes. In this study, we have defined a new role of IRS in angiogenesis. Both of the IRS proteins modulate VPF/VEGF expression in pancreatic cancer cells by different mechanistic pathways. The Sp1-dependent VPF/VEGF transcription is regulated mainly by IRS-2. Protein kinase C-ζ (PKC-ζ) plays a central role in VPF/VEGF expression and acts as a switching element. Furthermore, we have also demonstrated that the phosphatidylinositol 3-kinase pathway, but not the Ras pathway, is a downstream event of IRS proteins for VPF/VEGF expression in AsPC-1 cells. Interestingly, like renal cancer cells, in AsPC-1 cells PKC-ζ leads to direct Sp1-dependent VPF/VEGF transcription; in addition, it also promotes a negative feedback loop to IRS-2 that decreases the association of IRS-2/IGF-1R and IRS-2/p85. Taken together, our results show that in AsPC-1 pancreatic carcinoma cells, Sp1-dependent VPF/VEGF transcription is controlled by IGF-1R signaling through IRS-2 proteins and modulated by a negative feedback loop of PKC-ζ to IRS-2. Our data also suggest that IRS proteins, which are known to play crucial roles in IGF-1R signaling, are also important mediators for tumor angiogenesis.**

Tumor angiogenesis, the formation of neovascularization from the existing blood vessels, is well known to be a crucial event in the growth of solid tumors by ensuring its blood supply (1, 2). Several growth factors are able to induce tumor angiogenesis; of these, the vascular permeability factor/vascular endothelial growth factor (VPF/VEGF)[1] is the most potent and specific (3). VPF/VEGF increases the permeability of microvessels and modulates endothelial cell proliferation and migration through its receptors KDR and Flt-1, as well as the recently found receptor neuropilin (4, 5), and thus promotes the formation of new capillaries (6).

High blood vessel density in tumors is an independent predictor for metastasis (7). Ductal adenocarcinoma of the pancreas is the fifth leading cause of cancer death in the United States (8). In pancreatic adenocarcinoma, increased expression of VPF/VEGF is associated with liver metastasis and a poor prognosis for the patient (9). Recently we have shown that insulin-like growth factor-1 (IGF-1) promotes proliferation, invasion, and VPF/VEGF expression in AsPC1 human pancreatic adenocarcinoma cells through its receptor IGF-1R (10). Furthermore, Lopez and Hanahan (11) also showed in a mouse model that an elevated level of IGF-1 receptor is responsible for the invasive and metastatic capability of pancreatic islet tumor cells. Elevated expression of the IGF-1 receptor occurs in a wide range of tumors (12). Of importance, the physiological roles of IGF-1R are cell growth, proliferation, differentiation, and protection against apoptosis (13), but IGF-1R was also responsible for malignant transformation (14), and tumor progression (15). In addition, in PANC-1 pancreatic carcinoma cells, IGF-1R was found to cause invasion (16), and in AsPC-1 it has been shown to be responsible for tumor cell growth and invasion (17).

IGF-1 has also been shown to induce angiogenesis by increasing the VPF/VEGF production in colorectal carcinoma (18) and endometrial adenocarcinoma cells (19). However, there is no report that defines the signaling intermediates responsible for VPF/VEGF expression other than our recent observations that c-Src inhibitor PP-2 can inhibit VPF/VEGF transcription (10). Thus, in this study our focus was to understand the details of the signaling event mediated by IGF-IR for VPF/VEGF transcription. IGF-1R is a receptor tyrosine kinase with a 70% homology to the insulin receptor and shares parts of its functions (20). Ligand binding to IGF-1R results in receptor autophosphorylation of the β-subunit (21) and enables the association and tyrosine phosphorylation of two major down-

* This work was supported in part by the American Cancer Society and National Institutes of Health Grants HL70567 and CA7838 (to D. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: VPF/VEGF, vascular permeability factor/vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; IGF-1R, insulin like growth factor-1 receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; EMSA, electrophoretic mobility shift assay; PBS, phosphate-buffered saline; dN, dominant negative; PH, pleckstrin homology; PTB, phosphotyrosine binding; HA, hemagglutinin.

stream molecules: insulin receptor substrate-1 (IRS-1) and insulin receptor substrate-2 (IRS-2) (20). IRS proteins are already well known to play a crucial event in the development of diabetes (22), but they also play an important role in cancer. IRS-1 is constitutively activated in breast cancer, leiomyoma, Wilm's tumor, rhabdomyosarcoma, liposarcoma, leiomyosarcoma, and adrenal cortical carcinoma (23) as well as in mouse medulloblastoma (24). In estrogen receptor-positive breast cancer cells, IRS-1 is the predominant signaling molecule for IGF-1R (25), but progression of breast cancer was found to be associated with diminished IRS-1 levels (26). IRS-2 signaling causes enhanced adhesion and motility of metastatic breast cancer cells *in vivo* (27) as well as prostate cancer cells (28). Both IRS proteins are signaling intermediates for $\alpha_6\beta_4$ integrin-dependent cancer cell invasion (29). In pancreatic cancer, IRS-2 levels are shown to be elevated (30). In this study, we have investigated the function of IRS proteins in VPF/VEGF expression in pancreatic cancer cells and thus in angiogenesis.

IRS-1 and IRS-2 are scaffold molecules for both the insulin and the IGF-1 receptor (31). Although they are not completely functionally interchangeable, they both promote two already well known signaling pathways: the Ras/mitogen-activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (21, 31). PI3K was reported to induce VPF/VEGF expression in Ha-*ras*-transformed cells under hypoxic conditions (32) and in osteoblast-like cells (33). VPF/VEGF expression is increased under normoxic and hypoxic conditions by PI3K in astrocytoma cells, whereas the MAPK pathway only increased VPF/VEGF under normoxic conditions in these cells (34). Another group found that MAPK can upregulate VPF/VEGF in hamster fibroblasts under normoxic and hypoxic conditions through Sp1 and HIF-1, respectively (35). In renal cancer cells, we found Ras to be responsible for VPF/VEGF expression (36).

PDK-1 is a downstream molecule of PI3K, which activates PKC-$\zeta$ among many other kinases (37). This protein kinase is a member of the atypical PKCs. Unlike conventional or novel PKCs, it does not require diacylglycerol or phorbol esters for its activation, and its function is $Ca^{2+}$-independent (38). To achieve catalytic potential, PKC-$\zeta$ needs to be phosphorylated on Thr-410 through PDK-1 (37, 39).

In this study of pancreatic cancer cells, we provide evidence that similar to renal cancer cells, PKC-$\zeta$ is a central molecule for VPF/VEGF expression. On the contrary, unlike renal cancer cells, in pancreatic cancer cells PKC-$\zeta$ becomes activated by PDK-1 only but not by Ras. Moreover, PKC-$\zeta$ turned out to be a switching molecule for VPF/VEGF expression, because it promotes a feedback loop to IRS-2. We have described a novel and diverse function of IRS proteins in the regulation of VPF/VEGF transcription. We observed that it is IRS-2 but not IRS-1 that controls Sp1-dependent VPF/VEGF transcription in pancreatic cancer cells. Taken together, our studies define a new role of IRS proteins in promoting VPF/VEGF expression and thus angiogenesis.

## EXPERIMENTAL PROCEDURES

*Cell Culture*—Human AsPC-1 pancreatic adenocarcinoma cells from ATCC were cultured in RPMI 1640 medium with 20% fetal bovine serum (Hyclone Laboratories).

*Plasmids and Reagents*—2.6- and 0.35-kb VPF/VEGF promoter firefly luciferase constructs for transient transfection assays contain sequences derived from the human VPF/VEGF promoter described earlier (40). PDK-1- phosphorylation site-mutated dominant negative T410A PKC-$\zeta$ (threonine 410 to alanine), the active myristoylated PKC-$\zeta$ (PKC-$\zeta$-myr), and the kinase inactive myc-PDK-1.K/N (mutation of lysine 110 to asparagine) were generous gifts from Dr. Alex Toker (37). PH-PTB IRS-1 and PH-PTB IRS-2, both in pcDNA, were described earlier (41). 17N Ras, a dominant negative mutant of human Ras, a gift from Dr. Roya Khosravi-Far, was published earlier (42).

p85, IGF-1 receptor-$\alpha$ antibody (1H7) (for blocking receptor), and IGF-1 receptor-$\beta$ antibody (H60) (immunoprecipitation, Western blot) as well as nPKC-$\zeta$ antibody, anti-mouse-horseradish peroxidase, anti-rabbit-horseradish peroxidase secondary antibodies, and normal mouse and rabbit IgG were purchased from Santa Cruz Biotechnology. IRS-2 antibody was from Upstate Biotechnology. HA antibody was from Roche Applied Science, M2 FLAG antibody came from Sigma. Rabbit serum was purchased from Sigma-Aldrich. The phosphoserine antibody came from Zymed Laboratories Inc.

*Transient Transfection*—$6-8 \times 10^4$ cells/well in a 24-well plate and $3-4 \times 10^5$ cells/well in a 6-well plate (luciferase assay), $2-3 \times 10^5$ cells/60-mm plate (real-time PCR), and $6-8 \times 10^6$ cells/100-mm plate (immunoprecipitation) were plated 1 day prior to transfection. Cell confluency was 85–95% for all experiments. Plasmids were transiently transfected using the Effectene method (Qiagen) according to the manufacturer's instructions (a 1:10 DNA to Effectene ratio was used). For all experiments, a sample containing the empty vector was run.

*Luciferase Assay*—Cells were harvested 30 h after transient transfection. Luciferase activity was measured with a luminometer using a luciferase assay kit (Promega). An empty vector was used as a control in all assays. Results were normalized by total protein amount. All luciferase assays were performed at least four times, and the average and standard deviations were calculated.

*Immunoprecipitation*—AsPC-1 cells were washed twice with ice-cold PBS and lysed with radioimmune precipitation assay buffer (RIPA, Boston Bioproducts) containing 10 $\mu$g/ml leupeptin, 0.5% aprotinin, 2 mM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride, incubated for 10 min on ice, and scraped. Lysates were centrifuged for 10 min at 14,000 rpm at 4 °C. 1 $\mu$g of antibody was added to an equal amount of protein from the lysate for each tube, in total 500 $\mu$l, and incubated for 2 h (overnight for IRS-2 antibody) on a rocking table at 4 °C. 50 $\mu$l of protein A-agarose beads (Amersham Biosciences) were added and again incubated for 2 h under shaking conditions at 4 °C. Samples were washed three times with radioimmune precipitation assay buffer, and proteins were separated by Western blot.

*Western Blot*—Protein samples were mixed with 2× loading dye (125 mM Tris-HCL, pH 6.8, 20% glycerol, 10% $\beta$-mercaptoethanol, 4% SDS, and 0.0025% bromphenol blue), boiled, and run on a 4–15% linear gradient gel (Bio-Rad). Agarose beads with bound proteins were treated in the same way. Electrophoresis was run at 150 V. Separated proteins were transferred to a polyvinylidene difluoride membrane (PerkinElmer Life Sciences) at 300 mA for 1 h. For immunodetection, the membrane was blocked with 4% milk powder in PBS-T (phosphate-buffered saline and 0.05% Tween 20) and incubated with a primary antibody. After washing with PBS-T, the samples were coated with a peroxidase-linked secondary antibody. After a second washing, the reactive bands were detected by chemiluminescent substrate (Bio-Rad).

*RNA Preparation and Real-time PCR*—After AsPC-1 cells were washed twice with ice-cold PBS, total RNA was extracted according to the RNeasy kit protocol (Qiagen). We used the *Taq*man real-time PCR method. The sequence for forward, reverse and *Taq*man middle primers for human VPF/VEGF and for human $\beta$-actin (housekeeping gene) were taken from the PubMed GenBank™ (accession no. M10277) and synthesized by 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′; $\beta$-actin forward, 5′-TCA CCA TGG ATG ATG ATA TCG C-3′; $\beta$-actin reverse, 5′-AAG CCG GCC TTG CAC AT-3′; $\beta$-actin middle primer, 5′-CGC TCG TCG TCG ACA ACG GCT-3′; IRS-1 forward, 5′-CAG GCA CAT CCC CTA CCA TTA-3′; IRS-1 reverse, 5′-ACT CCT CAA TGG AAG CCA CTG A-3′; IRS-1 middle primer, 5′-CCA CCA GAA GAC CCC GTC CCA-3′; IRS-2 forward, 5′-CCC ATG CGC AGA GAG A-3′; IRS-2 reverse, 5′-GCT CCT CCC TCA CGT CGA T-3′; IRS-2 middle primer, 5′-CTG CCG GTT TCC AGA ATG GTC TCA AGT AC-3′. All middle primers had a 5′-TET reporter and a 3′-Tamra quencher. Each real-time PCR reaction was done using 0.5 $\mu$g of total RNA, 25 $\mu$l of reverse transcriptase-PCR Master Mix (Applied Biosystems), 1.25 $\mu$l of RNase inhibitor (Applied Biosystems), 50 nM forward primer, 50 nM reverse primer, and 100 nM middle primer. In all VPF/VEGF real-time PCR experiments, the $\beta$-actin amount was detected in parallel as a housekeeping gene for normalization. For reverse transcription, a 30-min period at 48 °C was run before inactivating the reverse transcriptase at 95 °C for 10 min. 40 cycles at 95 °C for 15 s and 60 °C for 1 min were performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems). CT (cycle threshold) values were measured, and the relative RNA amount was calculated as follows: $\Delta$ = CT(VPF/VEGF sample) − CT($\beta$-actin sample). $\Delta\Delta = \Delta$(transfected sample) − $\Delta$(empty vector sample). The relative RNA amount in comparison

with the empty vector = $2^{-\Delta\Delta}$. All experiments were carried out three times, and from each time the reading was taken in triplicate and the average and standard deviations were calculated.

*Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSAs)*—Nuclear extracts were prepared from AsPC-1 cells following a standard protocol (59), with modifications. Cells were washed in cold PBS, suspended in buffer A (10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 $\mu$g/ml aprotinin, 3 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride), and incubated for 15 min on ice. Cells were then lysed with 0.5% Nonidet P-40, and the pellets were resuspended in buffer C (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, 3 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). Following incubation on a rotating rack for 25 min, samples were centrifuged at 14,000 rpm for 10 min, and the clear supernatants containing the nuclear proteins were collected and stored at −70 °C.

EMSAs were performed as described previously (59). Briefly, EMSA binding reaction mixtures (25 $\mu$l) contained 20 mM HEPES (pH 8.4), 100 mM KCl, 20% glycerol, 0.1 mM EDTA, 0.2 mM $ZnSO_4$, 0.05% Nonidet P-40, and 1 $\mu$g of bovine serum albumin. The protein extracts and 200 ng of poly(dA-dT) were added at room temperature 10 min prior to the addition of ~0.1 ng of radiolabeled oligonucleotide probe. After a 20-min incubation at 4 °C, samples were run on 7% acrylamide gel in 1× TAE buffer (40 mM Tris acetate, 1 mM EDTA). The radiolabeled oligonucleotide used in EMSA studies was a double-stranded 22 mer that contains the consensus DNA-binding site for Sp1 (Promega, WI). The sequence of this Sp1 consensus double-stranded oligonucleotide is as follows: 5′-d(ATT CGA TCG GGG CGG GGC GAG C)-3′ and 3′-d(TAA GCT AGC CCC GCC CCG CTC G)-5′. The oligonucleotide was radiolabeled with a 3′ end-labeling kit (PerkinElmer Life Sciences).

## RESULTS

*Transcription Factor Sp1 Is Important for IGF-IR-induced VPF/VEGF Transcription*—Our previous study revealed the role of the IGF-1 receptor in VPF/VEGF expression and thus angiogenesis (10). Here we investigated the impact of the IGF-1 receptor downstream signaling molecules on VPF/VEGF expression in the pancreatic carcinoma cell line AsPC-1. VPF/VEGF transcription was measured by luciferase assays with two different VPF/VEGF promoter luciferase constructs (40): the 2.6-kb VPF/VEGF promoter, consisting of the full-length human VPF/VEGF promoter region; and the 0.35-kb VPF/VEGF promoter, which mainly contains the four putative Sp1 binding sites (59). With these two constructs, we were able to measure Sp1-dependent and -independent VPF/VEGF transcription in AsPC-1.

IGF-1 receptor signaling was blocked with anti-IGF-1R$\alpha$ neutralizing antibody, and at the same time, either the 2.6-kb or the 0.35-kb VPF/VEGF luciferase construct was transfected into AsPC-1 cells. Luciferase activity of the 2.6-kb VPF/VEGF promoter showed a 50% decrease, and the 0.3–5kb VPF/VEGF promoter (mainly Sp1 binding sites) showed a 40% decrease compared with that of the IgG control (Fig. 1). These results suggest that in AsPC-1 cells, the IGF-1 receptor signaling promotes VPF/VEGF expression mainly through transcriptional regulation, where the transcription factor Sp1 plays an important role. In fact, the importance of Sp1 in VPF/VEGF transcription in pancreatic cancer cells has been reported previously by Shi *et al.* (60). They showed that constitutive Sp1 activity is essential for differential constitutive expression of VPF/VEGF in human pancreatic adenocarcinoma. They also showed that mutation of individual or all of the putative Sp1 binding sites reduced or eliminated the constitutive high level of VPF/VEGF promoter activity in pancreatic adenocarcinoma (60).

*IRS-2, but Not IRS-1, Mediates Sp1-dependent VPF/VEGF Transcription*—Both IRS-1 and IRS-2 act as downstream molecules of IGF-IR and mediate most of its functions (20, 21). We have utilized the PH-PTB IRS-1 and PH-PTB IRS-2 (41) in our system as dominant negative mutants to block the downstream signaling events (44). These mutants contain only the $NH_2$-terminal pleckstrin homology (PH) and phosphotyrosine binding (PTB) domains of the IRS proteins but do not contain the COOH-terminal portion of the IRS proteins and thus are not able to transduce signals. The dominant negative effect of PH-PTB IRS-1 has been reported previously (45).

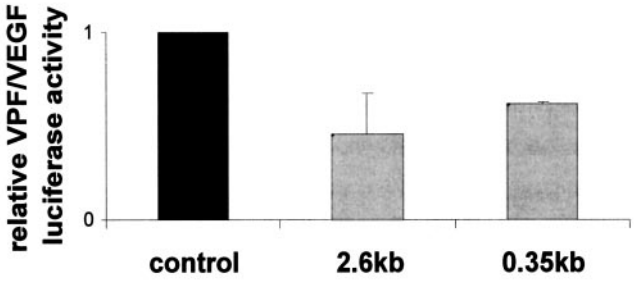


FIG. 1. **IGF-1R-mediated VPF/VEGF transcription.** AsPC-1 cells were transfected with a 2.6- or 0.35-kb VPF/VEGF promoter luciferase construct (0.3 $\mu$g), and mouse anti-IGF-1R$\alpha$ antibody (20 $\mu$g/ml) was added at the same time to block IGF-1R signaling (24-well plate). Mouse IgG instead of anti-IGF-1R antibody served as a control. After 30 h of incubation, cells were lysed and subjected to luciferase assays, and the enzyme activities were measured relative to the activity of the mouse IgG control. The results are presented as the average of four separate experiments.

Initially, we examined IRS-dependent VPF/VEGF mRNA expression by using mutants similar to those described above. Real-time PCR experiments, carried out after transfection of PH-PTP IRS-1 or IRS-2, showed a 50% decrease in the VPF/VEGF mRNA level (Fig. 2*a*). These experiments suggest that IRS-1 and IRS-2 are important upstream molecules for VPF/VEGF expression in AsPC-1 cells. To investigate the influence of IRS-1 and IRS-2 on Sp1-dependent or -independent VPF/VEGF transcription, we performed co-transfection assays using the 2.6-kb (full-length) or 0.35-kb (contains only Sp1 binding sites) VPF/VEGF promoter luciferase construct with increasing doses of PH-PTP IRS-1 or IRS-2 mutants (Fig. 2, *b–e*). For PH-PTB IRS-2, the luciferase activity of both VPF/VEGF promoter constructs (2.6b and 0.35 kb) decreased the dose dependence (Fig. 2, *d* and *e*). However, PH-PTB IRS-1 diminished only the 2.6-kb VPF/VEGF promoter luciferase activity; no significant change in the 0.35-kb promoter activity was detected (Fig. 2, *b* and *c*). These experiments provide evidence that IRS-1 and IRS-2 both are involved in the regulation of VPF/VEGF transcription in AsPC1 cells, although they do not induce VPF/VEGF transcription through the same pathway. Sp1-dependent VPF/VEGF transcription might in part involve IRS-2-mediated transactivation, as the 0.35-kb VPF/VEGF promoter construct predominantly has Sp1 binding sites. On the other hand, IRS-1-mediated VPF/VEGF transactivation is primarily dependent on other transactivator(s), as the 0.35-kb promoter activity of VPF/VEGF transcription was not influenced after IRS-1 was blocked.

It is noteworthy that in confluent AsPC-1 cells, the mRNA level of IRS-2 is significantly higher than that of IRS-1, suggesting the predominant role of IRS-2 in conducting the IGF-IR function in pancreatic cancer cells (Fig. 3). Our previous work has already shown the importance of Sp1 for VPF/VEGF transcription (36, 43) in renal cancer cells. It was also reported in pancreatic cancer cells that high constitutive expression of VPF/VEGF is due to the higher activity of Sp1 (60). From the above described experiments we concluded that in AsPC-1 cells, Sp1-dependent VPF/VEGF transcription is regulated mainly by IRS-2 and thus provides a mechanism of Sp1 activation for VPF/VEGF transcription in pancreatic cancer cells. Although we cannot rule out any influence of IRS-1 on VPF/VEGF expression, it is definitely not the predominant factor. Thus, in further experiments, we focused on IRS-2.

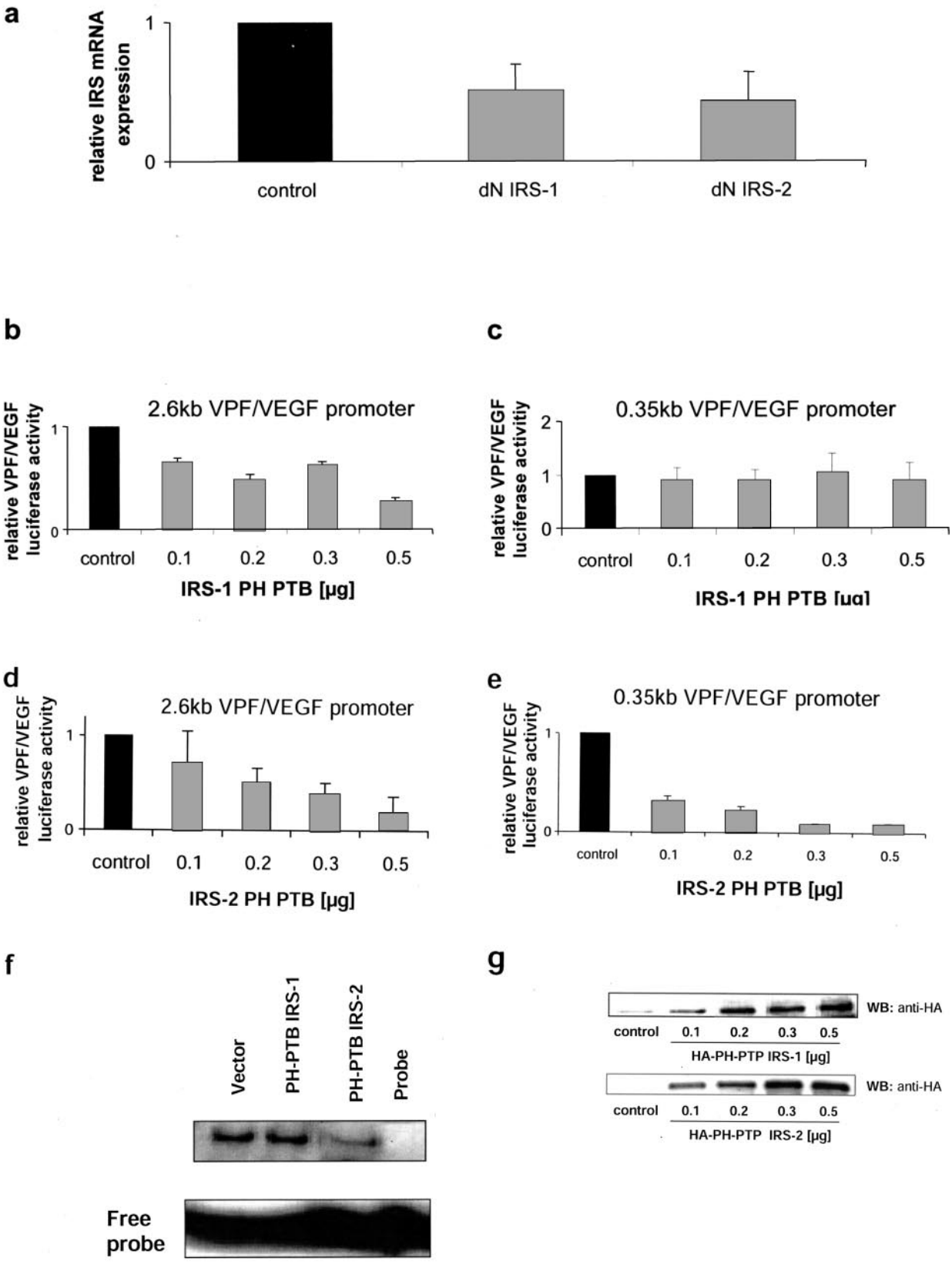


FIG. 2. **Effect of PH-PTP IRS mutants on VPF/VEGF expression.** *a*, transfection of AsPC-1 cells was carried out using expression vectors of PH-PTP IRS mutants (dN IRS-1 and dN IRS-2) or the empty pcDNA vector as a control (1 $\mu$g, 60-mm plate). After 24 h of transfection, total RNA was extracted, and VPF/VEGF mRNA expression was measured by real-time PCR. The average and standard deviation were calculated from three independent experiments. The -fold activation was calculated relative to the VPF/VEGF mRNA expression in cells transfected with IRS mutants and the control vector. *b–e*, co-transfection of a 2.6- or 0.35-kb VPF/VEGF promoter luciferase construct (0.3 $\mu$g) to ASPC-1 was carried out with increasing doses (0.1–0.5 $\mu$g) of PH-PTB IRS-1 or PH-PTB IRS-2 expression vector, respectively. Luciferase assays and relative enzyme activities were calculated as described above. The -fold activity is relative to the luciferase activity of the control vector. *f*, nuclear extracts (1 $\mu$g) from AsPC-1 cells transfected with PH-PTP IRS mutants were utilized to perform EMSA as described under "Experimental Procedures." *g*, protein expressions were verified with anti-HA Western blot (*WB*).

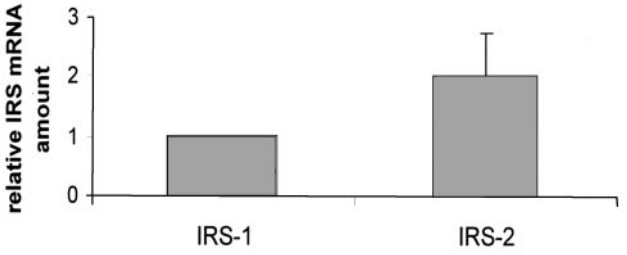


FIG. 3. **Expression of IRS proteins in AsPC-1 cells.** AsPC-1 cells were grown to ~80–90% confluence, mRNAs were isolated, and real-time PCR was carried out to detect IRS-1 and -2 expression as described under "Experimental Procedures." The relative mRNA expressions were calculated with respect to β-actin as a housekeeping gene expression. The figure shows the average of three independent experiments.

To examine whether IRS-2 mediated VPF/VEGF expression occurs mainly in a Sp1-dependent manner, we performed EMSAs. Fig. 2*f* shows that the interaction of Sp1 with its DNA binding region was reduced significantly by utilizing the nuclear extracts from AsPC-1 cells previously transfected with PH-PTB IRS-2, as compared with that of cells transfected with PH-PTB IRS-1. To our knowledge, this is a novel finding that suggests a specific role of IRS-2 for Sp1-dependent VPF/VEGF transcription in pancreatic cancer cells. Overall, our data indicate that in AsPC-1 cells, the signaling event originating from IGF-IR that leads to VPF/VEGF mRNA transcription mostly goes through the IRS-2 and Sp1 pathway.

*VPF/VEGF Expression in AsPC-1 Is Modulated by PKC-ζ in a Biphasic Manner*—Recently, we demonstrated the importance of Sp1 for VPF/VEGF transcription and its dependence on PKC-ζ in renal cancer cells (36, 43). Consequently, we examined whether PKC-ζ also plays any role in Sp1-mediated VPF/VEGF transcription in AsPC-1 cells.

PKC-ζ needs to be phosphorylated by PDK-1 on Thr-410 to function as a kinase (37). A mutated PKC-ζ, where threonine 410 is changed to alanine (T410A PKC-ζ), acts as a dominant negative of PKC-ζ (dN PKC-ζ) (37). Luciferase assays with co-transfection of dN PKC-ζ in increasing doses, and the two VPF/VEGF promoter constructs (2.6 or 0.35 kb) were performed. Both the 2.6- and 0.35-kb VPF/VEGF promoter luciferase activity revealed a biphasic kinetic; blocking PKC-ζ led to an increase in VPF/VEGF transcription in low doses and showed inhibition only in higher doses (Fig. 4*a*, data shown only for the 0.35-kb VPF/VEGF promoter construct). We also examined whether the promoter activity data of dN PKC-ζ expression reflects on the VPF/VEGF mRNA level. Real-time PCR results showed that the VPF/VEGF mRNA expression pattern is the same as its promoter activity after dose-dependent transfection of dN PKC-ζ (Fig. 4*b*). These results led us to hypothesize that blocking PKC-ζ in AsPC-1 cells, unlike in renal cancer cells, does not simply diminish VPF/VEGF expression, but rather, it influences a more complex event and might reflect the interference of different pathways.

It has been shown by different groups that PKC-ζ interacts with and phosphorylates the serine residues of IRS-1 and impairs the binding of IRS-1 to the receptor (46–50). As a consequence, PKC-ζ promotes a negative feedback loop because receptor binding of IRS proteins is required for activation of PI3K, which is upstream of PKC-ζ. Therefore, we also tested whether a regulatory loop similar to that of PKC-ζ with IRS proteins exists that might modulate VPF/VEGF transcription in AsPC-1 cells. Because our previous experiments revealed that IRS-2 is more abundantly expressed than IRS-1 in AsPC-1 cells and that IRS-1 does not influence Sp1-dependent VPF/VEGF transcription, we focused mainly on IRS-2 as a potential target molecule for a feedback loop.

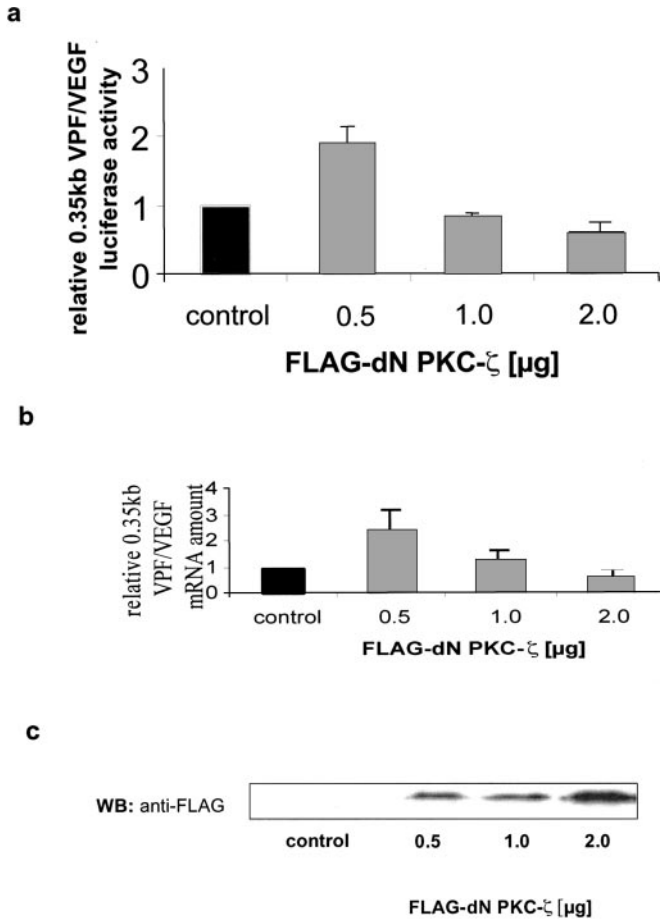


FIG. 4. **Dose-dependent effect of the dominant negative mutant of PKC-ζ on VPF/VEGF expression.** AsPC-1 cells were co-transfected (6-well plate) with an 0.35-kb VPF/VEGF promoter luciferase construct (0.3 μg) and increasing doses (0.5–5.0 μg) of dN PKC-ζ (T410A PKC-ζ). Empty cytomegalovirus vector served as a control. *a*, luciferase activity was measured from three independent experiments, and the average and standard deviation was calculated. The -fold activity is relative to the luciferase activity of the control experiment. *b*, dominant negative PKC-ζ (T410A PKC-ζ) was transfected into AsPC-1 cells (60-mm plate) in increasing doses (0.5–5.0 μg). The empty vector was transfected as a control. After 24 h of incubation, total RNA was extracted, and VPF/VEGF mRNA was measured using real-time PCR from three independent experiments. The average and standard deviation were calculated. *c*, dN PKC-ζ protein expression was verified with anti-FLAG Western blot (*WB*).

*Influence of PKC-ζ on IGF-1R and IRS-2 Interaction in AsPC-1 Cells*—Using immunoprecipitation and Western blot experiments from AsPC-1 cell lysates, we found that IRS-2 and PKC-ζ are in the same immunocomplex (Fig. 5*a*). To further examine whether PKC-ζ influences the association between IRS-2 and IGF-1R and also with the p85 subunit of PI3K, we utilized a constitutively active form of myristoylated PKC-ζ (PKC-ζ-myr). We postulated that if there were a feedback loop, PKC-ζ-myr should decrease the association between IRS-2 and the β-domain of the IGF-1 receptor and subsequently decrease the association of IRS-2 and p85. Indeed, Fig. 5*b* shows that overexpression of PKC-ζ-myr decreases the association of IRS-2 proteins with the IGF-1R. We have obtained similar data in the case of IRS-1, as shown previously (44–48) (data not shown). As expected, a dose-dependent inhibition of the p85 association with IRS-2 was also observed (Fig. 5*b*). Taken together, these results suggest that in AsPC-1 cells, PKC-ζ feeds back to the IRS proteins and controls their association with the IGF-1R as well as their downstream signaling.

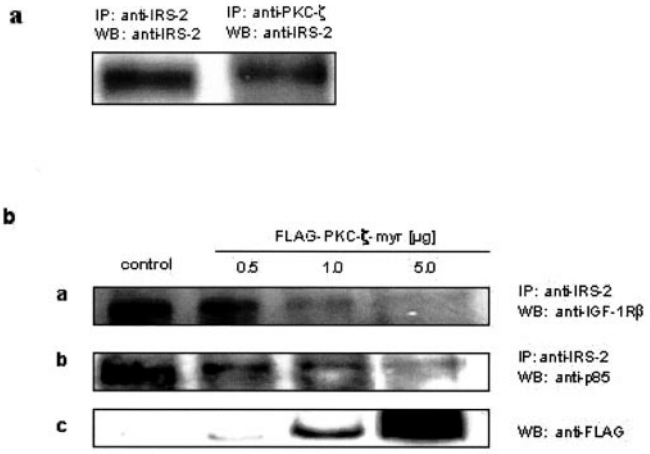


FIG. 5. **Role of PKC-ζ on the association between IRS-2/IGF-1R and IRS-2/p85.** *a*, AsPC-1 cell lysates were used for immunoprecipitations (*IP*) with anti-IRS-2 or anti-PKC-ζ antibodies. Western blots (*WB*) were carried out using anti-IRS-2. Rabbit serum was used for immunoprecipitation controls (data not shown). *b*, AsPC-1 cell lysates, after transfection with increasing doses of active PKC-ζ-myr (0.5–5.0 μg in 100-mm plate), was subjected to immunoprecipitation with anti-IRS-2 antibody and Western blot with either anti-IGF-1Rβ or anti-p85 antibodies. FLAG-tagged PKC-ζ-myr expression was verified by performing Western blot with anti-FLAG antibody.

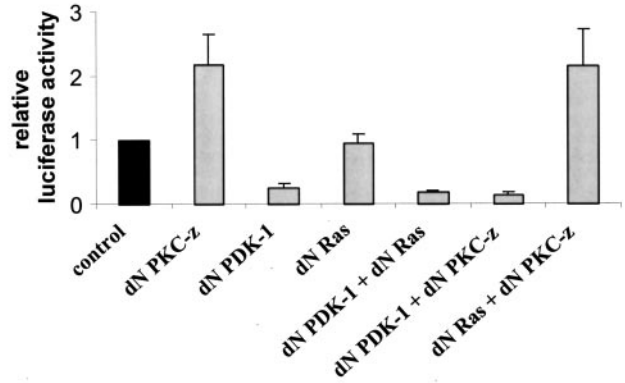


FIG. 6. **Effects of dominant negative mutants of PDK-1, Ras, and PKC-ζ on Sp1-dependent VPF/VEGF transcription.** Co-transfections of AsPC-1 cells with an 0.35-kb VPF/VEGF promoter luciferase construct (0.3 μg) and dominant negative constructs of PKC-ζ (T410A PKC-ζ), PDK-1 (PDK-1.K/N), or Ras (17N Ras) or a combination of dN PDK-1 and dN Ras, dN PDK-1, and dN PKC-ζ or dN Ras and dN PKC-ζ (24-well plate) were carried out. Transfections with the empty vector served as controls. After a 30-h incubation, cells were harvested, and luciferase activity was measured. The results shown are the average of four experiments. The -fold activity was calculated relative to the activity of the control experiments.

*PDK-1, but Not Ras, Is a Key Molecule Upstream of Sp1-mediated VPF/VEGF Transcription in AsPC-1*—We have previously shown the importance of Ras on Sp1-mediated VPF/VEGF transcription in renal cancer cells (36, 43). We further examined whether PI3K or Ras (which are the two major pathways downstream of the IRS proteins) or both are responsible for Sp1-dependent VPF/VEGF transcription in AsPC-1 pancreatic cancer cells. Luciferase assays with the 0.35-kb VPF/VEGF promoter construct were carried out, whereas key molecules of either the PI3K pathway or Ras were inhibited. We employed PDK-1.K/N as a dominant negative mutant (dN PDK-1) to block PI3K pathway and 17N Ras as a dominant negative mutant for Ras (dN Ras). dN PDK-1 diminished Sp1-dependent VPF/VEGF transcription significantly as compared with the control vector, whereas dN Ras was unable to do so (Fig. 6). Utilizing both of the dominant negative mutants, a similar effect was observed as when dN PDK-1 alone was transfected. These data corroborate our recent findings showing no significant change in VPF/VEGF mRNA level after dose-dependent transfection of dN *Ras* in AsPC-1 cells (10).

To verify our finding that in pancreatic cancer cells Ras has no influence on PKC-ζ-mediated VPF/VEGF expression, we co-transfected dN PKC-ζ together with dN Ras or dN PDK-1 (Fig. 6). As in the above mentioned experiments, dN PKC-ζ alone increased VPF/VEGF transcription significantly as it did in combination with dN Ras. However, dN PKC-ζ and dN PDK-1 together decreased VPF/VEGF luciferase activity to a similar level as when dN PDK-1 alone was utilized. These experiments show that PDK-1, but not Ras, is the key molecule that influences PKC-ζ-mediated Sp1-dependent VPF/VEGF transcription in AsPC-1 cells.

## DISCUSSION

The growth of solid tumors like the pancreatic adenocarcinoma depends on the production of VPF/VEGF to induce angiogenesis. Moreover, VPF/VEGF is a prognostic factor for tumor progression. VPF/VEGF secretion of tumor cells is mostly induced by hypoxia (51), but recently we have demonstrated that IGF-1 also is responsible for this activation pathway in AsPC1 cells (10). In this study, we investigated the mechanism of VPF/VEGF expression mediated by IGF-1R signaling in pancreatic adenocarcinoma cells and also defined a new role of the insulin receptor substrates in angiogenesis.

In AsPC-1 pancreatic carcinoma cells, we have shown that VPF/VEGF expression is dependent on IGF-1 receptor-mediated signaling. Our result is in accord with several other publications, which have reported that VPF/VEGF is controlled by IGF-1 in colon cancer cells, endometrial adenocarcinoma cells, and retinal epithelial cells (18, 19, 52–54). Earlier, it was shown that an auto- and paracrine loop of IGF-1 exists in human pancreatic cancer (17), indicating the importance of IGF-1 for the growth and progression of this cancer type. Furthermore, using a mouse model of pancreatic islet tumor, Lopez and Hanahan (11) showed that elevated levels of IGF-1 receptor convey invasiveness and metastatic capability of tumor cells. Therefore, the importance of IGF-1R in pancreatic cancer growth and invasiveness and also in angiogenesis is beyond doubt.

Signals from the IGF-1R are transduced through IRS-1 and IRS-2 (20). We found that both IRS-1 and IRS-2 are expressed in the pancreatic cancer cell line AsPC-1, although IRS-2 expression is more predominant. Overexpression of both IRS proteins in pancreatic cancer has already been reported (30, 55). In several tumors (breast cancer, leiomyoma, Wilm's tumor, rhabdomyosarcoma, liposarcoma, leiomyosarcoma, and adrenal cortical carcinoma), IRS-1 has been shown to be activated (23). Progression of breast cancer is associated with diminished IRS-1 levels and an increase in IRS-2 (26), which also enhances adhesion and motility of metastatic breast cancer cells (27). However, an influence of IRS proteins on VPF/VEGF, and thus angiogenesis, has not yet been described.

From our real-time PCR data and also from luciferase assays by utilizing PH-PTP IRS proteins as dominant negative mutants, we demonstrated IRS-1 and IRS-2 to be responsible for VPF/VEGF expression. However, their means of inducing VPF/VEGF differs. IRS-1 does not promote VPF/VEGF transcription only in the presence of Sp1 binding sites but requires other *cis* elements to induce it. On the other hand, IRS-2 induces VPF/VEGF transcription mainly through Sp1, because inhibiting IRS-2-mediated signaling can reduce the DNA binding ability of Sp1 to the VPF/VEGF promoter and Sp1-dependent VPF/

VEGF transcription. Moreover, highly confluent AsPC1 cells express mostly IRS-2. These results led us to conclude that IRS-2 plays a more important role in VPF/VEGF transcription than IRS-1 and that it does so mainly through the transcription factor Sp1. This finding is also important in the light of a previous report by Shi *et al.* (60) that constitutive Sp1 activity is essential for differential constitutive expression of VPF/VEGF in human pancreatic adenocarcinoma. Thus our findings have actually described the upstream mechanisms by which Sp1 is activated in the pancreatic cancer cells. We did not find any up-regulation of VEGF mRNA level in AsPC-1 cells during hypoxia (data not shown), which also suggests the presence of a separate mechanism other than hypoxia, such as the IGF-IR-IRS-2-Sp1 pathway, that is responsible for the high, constitutive level of VPF/VEGF expression in pancreatic cancer cells. Our data also suggest that other pathways may exist that will work in concert with VPF/VEGF up-regulation in pancreatic cancer cells, although their contribution will be less significant. One such pathway begins from IGF-IR and follows IRS-1 instead of IRS-2. Additionally, one has to consider that IRS proteins can act as mediators of other signaling events triggered by the insulin receptor (20), epidermal growth factor receptor (56), interleukin-4 receptor (57), and integrins (29), all of which can promote VPF/VEGF expression and thus angiogenesis. Therefore, IRS proteins can be potential targets to block VPF/VEGF expression in different tumor cells including pancreatic adenocarcinoma.

In our recent publications (36, 43), we showed that in renal cancer cells, Sp1-mediated VPF/VEGF transcription and its activation occur mainly through PKC-$\zeta$. In these cells, we did not find VPF/VEGF expression that depends on IGF-1R as it does in pancreatic cancer cells. The role of PKC-$\zeta$ in VPF/VEGF expression in the pancreatic cancer cell line AsPC-1 is different; blocking PKC-$\zeta$ with a dominant negative mutant increased VPF/VEGF transcription in low doses and showed an inhibitory effect only in higher doses. Moreover, we found similar results when we measured VPF/VEGF mRNA expression. In contrast to renal cancer cells, we demonstrated that in AsPC-1 cells PKC-$\zeta$ has a dual effect involving different pathways. By utilizing the activated form of PKC-$\zeta$ (PKC-$\zeta$-myr), we showed that the association between IRS-2 (which is more abundantly expressed in AsPC-1 cells than IRS-1) and IGF-1R decreased. This inhibition of association results in inhibition of downstream signaling and therefore in less activation of PKC-$\zeta$. These data provide evidence of a feedback loop of PKC-$\zeta$ to the IRS-2 protein. The biphasic kinetic of VPF/VEGF expression can thus be explained as the blocking of PKC-$\zeta$ in low doses inhibits the feedback loop, which results in an activation of VPF/VEGF expression, whereas in higher doses, direct inhibition of Sp1-mediated VPF/VEGF transcription is more predominant. Our results are in accord with the literature in which the existence of a feedback of PKC-$\zeta$ to IRS-1 has been suggested in relation to the insulin receptor and not in the context of angiogenesis (46, 47). It has also been reported by others that increased association of IRS-2/p85 occurs after inhibiting PI3K or the expression of PTEN (phosphatase and tensin homologue deleted from chromosome 10). p85, the regulatory subunit of PI3K, is a downstream molecule of IRS proteins and upstream of PKC-$\zeta$. The authors asserted a negative regulation without defining it (58). We suggest that PKC-$\zeta$ is the negative regulator, because it is downstream of PI3K and feeds back to IRS proteins where it has an impact on their activation and the association of IRS/p85.

PKC-$\zeta$ was activated by Ras in renal cancer cells, as described in our recent publications (36, 43). In AsPC-1 cells, we could not find any impact of Ras on VPF/VEGF transcription. PDK-1 was the key molecule responsible for activation of PKC-$\zeta$ and the expression of VPF/VEGF, suggesting that the PI3K pathway plays a major role as described by others (32, 33). On the contrary, the Ras/MAPK pathway is also important for VPF/VEGF expression (34, 35). Taken together, these findings imply the existence of different regulatory mechanisms of VPF/VEGF in different cancer cells.

In summary, our results here describe that in AsPC-1 pancreatic adenocarcinoma cells VPF/VEGF expression depends on IGF-1R signaling. It is mediated mainly through IRS-2. Furthermore, PKC-$\zeta$ promotes a feedback loop to IRS-2 and modulates VPF/VEGF expression in a biphasic manner. Because IRS-2 is a common mediator of different signaling pathways, it would be interesting to target IRS-2 to inhibit VPF/VEGF expression in different tumors including pancreatic adenocarcinoma, for which there is presently no active therapy.

*Acknowledgments*—We thank Dr. Alex Toker for the dominant negative T410A PKC-$\zeta$, the active PKC-$\zeta$-myr, and the dominant negative myc-PDK-1.K/N. We are grateful to Dr. Roya Khosravi-Far for the dominant negative 17N Ras.

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