Insulin Receptor Substrate-2 Mediated Insulin-like Growth Factor-I Receptor Overexpression in Pancreatic Adenocarcinoma through Protein Kinase Cδ

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Abstract

Pancreatic adenocarcinoma (PCA) is an almost invariably fatal disease. Recently, it has been shown by several groups as well as ours that insulin-like growth factor-I receptor (IGF-IR) overexpression is related to higher proliferation, survival, angiogenesis, and highly invasive pancreatic tumors. Several studies have been carried out to understand the pathways that lead to growth factor–mediated signaling, but the molecular mechanism of receptor overexpression remains mostly unknown. Treatment with neutralizing antibodies or a specific kinase inhibitor against IGF-IR could block the receptor expression in PCA cells. Furthermore, we also showed that insulin receptor substrate (IRS)-2, but not IRS-1, is involved in regulation of IGF-IR expression, which is most likely not transcriptional control. By blocking mammalian target of rapamycin (mTOR) pathway with rapamycin as well as other biochemical analysis, we defined a unique regulation of IGF-IR expression mediated by protein kinase Cδ (PKCδ) and mTOR pathway. Moreover, we showed that the down-regulation of IGF-IR expression due to IRS-2 small interfering RNA can be compensated by overexpression of dominant-active mutant of PKCδ, suggesting that PKCδ is downstream of IGF-IR/IRS-2 axis. Overall, these findings suggest a novel regulatory role of IRS-2 on the expression of IGF-IR through PKCδ and mTOR in pancreatic cancer cells. [Cancer Res 2009;69(4):1350–7]

Introduction

Pancreatic cancer is associated with high morbidity. Due to late-stage diagnoses, rapid tumor progression, and resistance to conventional chemotherapeutic agents, the 5-year survival rate remains at <5% (1). A hallmark of tumor growth is the overexpression of growth factors and their receptors. Overexpression of insulin-like growth factor-I (IGF-I) and IGF-I receptor (IGF-IR) has been shown in human pancreatic cancer tissue (2–5). Although the structure and function of IGF-IR are the main focus of intensive investigations (6–8), less data are available on the mechanisms and causes for the observed dysregulation of IGF-IR protein expression (9–11). Defining the molecular mechanisms underlying the highly aggressive nature of pancreatic adenocarcinomas (PCA) may provide more selective methods for an effective treatment.

IGF-IR is composed of an extracellular ligand-binding domain that controls the activity of an intracellular tyrosine kinase (12–14). During ligand binding by either IGF-I, IGF-II, or insulin (at high concentrations), IGF-IR becomes tyrosine phosphorylated through an autophosphorylation reaction, an essential step in its activation cascade (15). Most intracellular signals are generated through cellular scaffold proteins. These scaffold proteins, including the insulin receptor substrates (IRS), bind to autophosphorylation sites and are themselves phosphorylated on multiple tyrosine residues by the activated receptor kinase (16, 17). IRS proteins do not contain intrinsic kinase activity but rather act at the interface between the cell surface receptor and intracellular signaling molecules. At least three IRS proteins occur in the human: IRS-1/Irs-1 and IRS-2/Irs-2, which are widely expressed, and IRS-4/Irs-4, which is limited to the thymus, brain, kidney, and possibly β cells of the pancreas (18). IRS-1 and IRS-2 are overexpressed in pancreatic cancer cells (3, 19).

Given that tumorigenesis is a complex and multifaceted process, tumor cells have a variety of cellular defects promoting uncontrolled growth. Germ-line inactivating mutations of either the tuberous sclerosis complex (TSC) 1 or TSC2 tumor suppressor protein are linked to TSC, a genetic disorder that is characterized by the development of benign tumors called hamartomas in several tissues and organs, including the central nervous system, skin, lungs, and kidneys (20–23). Although there are very few reports of mutation of *TSC1* and *TSC2* genes in PCA, a recent study shows that reduced expression of *TSC2* might be involved in the progression of pancreatic cancer (24). The main target of TSC described thus far is the mammalian target of rapamycin (mTOR; also known as rapamycin and FK506-binding protein target 1, RAFT1) pathways (25, 26). The atypical serine/threonine kinase mTOR regulates the translation of key mRNA transcripts for proteins required for cell cycle progression. Rapamycin, a bacterial macrolide with antifungal, immunosuppressant, and antitumor activities, is known to target mTOR. Rapamycin forms a complex with the cytosolic 12-kDa FK506-binding protein (FKBP12) and binds to mTOR, inducing a partial dephosphorylation and deactivation of p70S6 kinase, an enzyme critical for G_1 to S transition (27). Furthermore, rapamycin inhibits the mitogen-stimulated phosphorylation of eIF4E-binding protein 1 (4E-BP1; ref. 28). Dephosphorylated 4E-BP1 interacts with the translation initiation factor eIF4E and thereby inhibits cap structure-dependent protein synthesis and cell growth (28, 29). Interestingly, Akt/protein kinase B pathways are the main modulator of mTOR activation; however, recent experiments showed that protein kinase Cδ (PKCδ) associates with RAFT1 and thereby regulates

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the phosphorylation of 4E-BP1 and cap-dependent initiation of protein translation (30). PKCδ contains phospholipid-dependent serine/threonine kinase activity and plays a key role in cellular signal signaling (31).

In the present study, we showed that IRS-2, but not IRS-1, is involved in the regulation of IGF-IR protein expression and found that the overexpression of IGF-IR in PCA is an autocrine expression loop and is mainly regulated at the translational level by a signaling pathway via mTOR (32). To define this pathway, we provided evidence that PKCδ plays a crucial role by regulating IGF-IR overexpression in PCA cells.

Materials and Methods

Cell culture and reagents. AsPC-1 and Su86.86 cells, purchased from the American Type Culture Collection, were cultured in RPMI 1640 with 20% or 10% fetal bovine serum (FBS; Hyclone Laboratories), respectively, and 1% penicillin-streptomycin (Invitrogen Corp.). Serum starvation was performed with 0.1% FBS in RPMI 1640. Rapamycin was obtained from Sigma. TATFLAGVHL peptide (107-122) was described previously (33). IGF-IR kinase inhibitor, picropodophyllin (PPP; first inhibitor reported to discriminate between IGF-IR and insulin receptor), and proteasome inhibitor I (34) were purchased form Calbiochem, Inc.

Antibodies. The antibodies used were from the following sources: anti-IGF-IRα 1H7 (for blocking), anti-IGF-IRα 2C8 (for Western blot detection), anti-IGF-IRα H60 (for immunoprecipitation), and anti-nPKCδ (Santa Cruz Biotechnology); anti-mTOR, anti-phospho-mTOR, anti-phospho-FKHR, and anti-phospho-PKCδ (Cell Signaling Technology); anti-IRS-2 (Upstate Biotechnology); anti-flag-tag, anti-β-actin, normal rabbit serum, and rabbit IgG (Sigma); and anti-HA-tag (Boehringer Mannheim).

Plasmids. HA-PH-PTB IRS-1 and HA-PH-PTB IRS-2, both in pcDNA3, were described previously (35). In brief, IRS-1 and IRS-2 proteins were lacking the tail of tyrosine phosphorylation sites; however, these truncated IRS-1 and IRS-2 proteins retained the NH_2-terminal pleckstrin homology (PH) and phosphotyrosine-binding (PTB) domains. pZipNeo-17N Ras was as described (36). pGEFPC1-PKCδ (PKCδ KR; point mutation of Lys^{376} to arginine) was a kind gift from R. Dutta (Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA). Dominant-negative T410A PKCζ (Thr^{410} to alanine) was described previously (37). Dominant-negative mutant of Akt (T308A, S473A, K179A) expression vector was a kind gift from Dr. K. Walsh (Tufts University, Boston, MA).

Transient transfections. Cells (3×10^5 to 4×10^5 per well) in a six-well plate (Western blot analysis), 2×10^6 to 3×10^6 cells/60-mm plate (real-time PCR), and 6×10^6 to 8×10^6 cells/100-mm plate (immunoprecipitation) were plated 1 d before transfection. Cell confluency was 85% to 95% for all experiments. Plasmids were transiently transfected using Effectene (Qiagen) according to the manufacturer's instructions at a 1:10 DNA to Effectene ratio. For all experiments, a sample containing the empty vector was run. The IRS-1 small interfering RNA (siRNA) and IRS-2 siRNA were purchased from Santa Cruz Biotechnology.

Immunoprecipitation. AsPC-1 cells were washed twice with ice-cold PBS and lysed with radioimmunoprecipitation assay (RIPA) buffer (Boston Bioproducts) containing 10 μg/mL leupeptin, 0.5% aprotinin, 2 mmol/L pepstatin A, and 1 mmol/L phenylmethylsulfonyl fluoride; incubated for 10 min on ice; and scraped. Lysates were centrifuged for 10 min at 14,000 rpm at 4°C. In a total volume of 500 μL, 1 μg of antibody was added to equal amounts of protein from each sample protein lysate and incubated for 2 h or overnight for the IRS-2 antibody at 4°C while rocking. Protein A-agarose beads (50 μL; Amersham Biosciences) were then added to the samples and again incubated for 2 h under shaking conditions at 4°C. Samples were washed thrice with RIPA buffer and proteins were separated by electrophoresis and visualized with a Western blot.

Western blot. Protein samples were mixed with 2× loading dye [125 mmol/L Tris-HCl (pH 6.8), 20% glycerol, 10% β-mercaptoethanol, 4% SDS, and 0.0025% bromphenol blue], boiled, and separated by SDS-PAGE at 150 V. Agarose beads with bound proteins were treated in the same way. Size-separated proteins were transferred onto a polyvinylidene difluoride membrane (Perkin-Elmer Life Sciences) at 300 mA for 1 h. For immunodetection, the membranes were blocked with 4% milk or bovine serum albumin in PBS-T (PBS and 0.1% Tween 20) and incubated with a primary antibody. After washing with PBS-T, the membrane was incubated with a peroxidase-linked secondary antibody. After a second round of washing, the reactive bands were detected with a chemiluminescent substrate (Bio-Rad).

RNA preparation and real-time PCR. After washing AsPC-1 cells twice with ice-cold PBS, total RNA was extracted according to the RNeasy Mini kit protocol (Qiagen). For Taqman PCR, the sequences for forward, reverse, and Taqman middle primers for human IGF-IR and for human β-actin were taken from the PubMed Genbank and synthesized by Integrated DNA Technology: IGF-IR forward, 5′-CATCGACATCCGCAACGA-3′; IGF-IR reverse, 5′-CCCTCGATCACCGTGCA-3′; and Taqman middle primer, 5′-TTCTCCAGGCGCTTCAGCTGC-3′. The middle primer had a 5′-TET reporter and a 3′-Tamra quencher. Each real-time PCR was prepared with 0.5 μg of total RNA, 25 μL of reverse transcription-PCR (RT-PCR) Master Mix (Applied Biosystems), 1.25 μL of RNase inhibitor (Applied Biosystems), 50 nmol/L forward primer, 50 nmol/L reverse primer, and 100 nmol/L middle primer. In all IGF-IR real-time PCR experiments, the *β-actin* amount was detected in parallel as a housekeeping gene for normalization. For reverse transcription, a 30-min incubation 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). C_T (cycle threshold) values were measured, and the relative RNA amount was calculated as follows: $\Delta = C_T$(IGF-IR sample) − C_T(β-actin sample). $\Delta\Delta = \Delta$(transfected sample) − Δ(empty vector sample). The relative RNA amount in comparison with the empty vector = $2^{-\Delta\Delta}$. All experiments were repeated thrice, and from each experiment, each reading was taken in triplicate.

Results

Autocrine protein expression loop of IGF-IR in human pancreatic cancer. Herein, we would like to understand the molecular mechanisms of IGF-IR overexpression in PCA. Several reports indicate autocrine control of tumor cell growth by the IGF-I/IGF-IR system in pancreatic cancer (3, 38). To examine whether IGF-IR contributes to the dysregulation of its own expression, we blocked IGF-IR function under serum-starved conditions by using the highly specific antibody (anti-IGF-IRα 1H7) that binds to the ligand-binding α-subunit of IGF-IR. As a control, anti-β-actin was analyzed. After 24-hour treatment of AsPC-1 cells with 1.0, 5.0, and 10.0 μg of anti-IGF-IRα 1H7, we found a strong inhibition of IGF-IR protein expression by Western blot analysis. Control cells were treated with mouse IgG (Fig. 1*A*). To confirm the possibility that the receptor internalization is not responsible but rather IGF-IR kinase activity is, we used a potent IGF-IR tyrosine kinase inhibitor PPP to examine IGF-IR expression in PCA cells. Figure 1*B* showed the inhibition of IGF-IR expression with increasing concentration of PPP treatment, which is in favor of an autocrine loop hypothesis that is accountable for IGF-IR overexpression via its kinase activation. The decrease in protein expression of IGF-IR, after treating the cells with anti-IGF-IRα antibody, was also detected at 20 hours even in the presence of a proteasome inhibitor (Fig. 1*C*; ref. 34), suggesting that the inhibitory effect of IGF-IR expression is not due to activation of proteasome degradation pathways. Similarly, treatment of cells with propidium iodide could not overcome the effect of PPP as well (Fig. 1*D*). Hence, our results indicate that in PCA cells IGF-IR is overexpressed due to its autocrine loop. We then examined the role of downstream molecules such as mTOR pathway in this regulation.

Regulation of IGF-IR protein expression by mTOR. To further determine whether mTOR participates in the regulation of IGF-IR

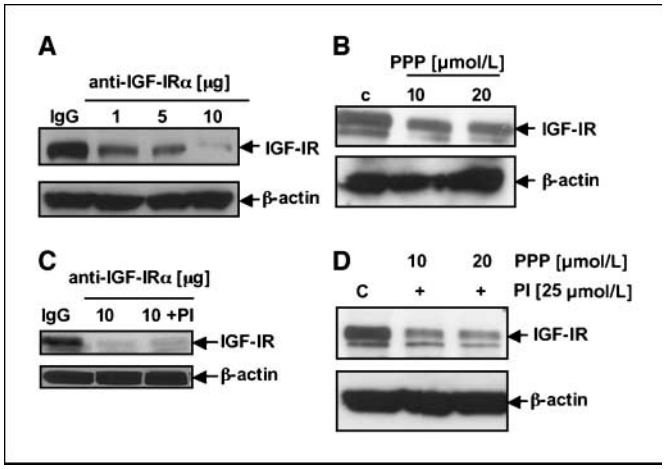

Figure 1. Autocrine loop of IGF-IR in PCA. Total cell lysates from AsPC-1 cells (*A*) treated with 1.0, 5.0, and 10.0 μg of anti-IGF-IRα 1H7 or mouse IgG (control); (*B*) treated with PPP (IGF-IR inhibitor) for different concentrations for 24 h; and (*C*) and (*D*) pretreated with proteasome inhibitor (34) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-IGF-IR 2C8 (*IGF-IR*). β-Actin served as control protein.

overexpression in PCA cells, we treated AsPC-1 cells with 20 and 40 nmol/L of rapamycin for 16 hours. To examine the effect of rapamycin on mTOR, we measured the phosphorylation level of mTOR by using anti-phospho-mTOR antibody, and as expected, the phospho-mTOR level was inhibited (Fig. 2*A*). Moreover, as shown in Fig. 2*A*, we also observed that IGF-IR protein expression is down-regulated, indicating the role of mTOR in IGF-IR–mediated overexpression. To further establish our hypothesis that mTOR participates in IGF-IR autoregulation, we blocked IGF-IR function with 1.0, 5.0, and 10.0 μg of anti-IGF-IRα 1H7 for 24 hours and observed a decrease in mTOR phosphorylation (Fig. 2*B*). To link up between the mTOR and Akt pathway, we used expression vector containing a triple mutant (T308A, S473A, K179A) of Akt-1 (a kind gift from Dr. K. Walsh) to block Akt pathways. Notably, AsPC-1 cells transfected with an expressing vector of dominant-negative Akt mutant did not change the phosphorylation levels of mTOR, whereas with its other downstream effector, FKHR, phosphorylation was inhibited (Fig. 2*C*). These results suggest that mTOR modulates IGF-IR expression by a pathway where Akt might not be involved. At this point, we thought other IGF-IR adapters and signaling molecules might be engaged in this pathway.

IRS-2, but not IRS-1, is involved in the translational regulation of IGF-IR expression. To determine whether IRS-1 or IRS-2 is involved in the downstream regulation of IGF-IR expression, we transiently transfected AsPC-1 cells with 0.2 and 1.0 μg of HA-IRS-1-PH-PTB or HA-IRS-2-PH-PTB constructs. The PH-PTB constructs are composed of both the NH_2-terminal PH (39) domain and the PTB domain. The PH domain binds membrane phospholipids or acidic motifs of different proteins, and the PTB domain interacts with the IGF-IR receptor α-subunit. These constructs lack the COOH-terminal portions of both IRS proteins that enable them to interact with specific downstream SH-2 domain-containing proteins. Therefore, HA-tagged IRS-1-PH-PTB and IRS-2-PH-PTB constructs block the IRS-1 and IRS-2 function, respectively. As shown in Fig. 3*A*, the expression of HA-IRS-1-PH-PTB did not affect the IGF-IR protein level, whereas the expression of HA-IRS-2-PH-PTB (Fig. 3*A*) led to a significant reduction of IGF-IR protein expression in AsPC-1 cells. Furthermore, we examined whether phosphorylation of mTOR is modulated by IRS proteins. The HA-IRS-1-PH-PTB–transfected AsPC-1 cells did not show any changes in the phosphorylation status of mTOR, whereas we found a strong reduction of phosphorylated sites in HA-IRS-2-PH-PTB–transfected cells (Fig. 3*A*). To confirm the role of IRS-2 in the regulation of IGF-IR expression, we used a different pancreatic cancer cell line. Su86.86 cells that were transiently transfected with 1.0 μg HA-IRS-2-PH-PTB were found to have a significant decrease in IGF-IR protein expression (data not shown). Similarly, we also found that in HA-IRS-2-PH-PTB–expressing cells, there was no apparent change in the mRNA level of IGF-IR compared with that of parental cells (Fig. 3*B*).

To confirm the role of IRS-2 in the regulation of IGF-IR, we have used the siRNA approach to block the expression of the IRS family of proteins. Figure 3*C* displays that by blocking IRS-2 expression, there are significant changes in protein expression levels of both families of proteins, whereas blocking IRS-1 has no apparent effect. Of importance, the level of IRS-1 is much less in both mRNA as well as protein level in most of the PCA cells. To confirm the importance of IRS-2 in PCA growth, we have performed [^{3}H]thymidine incorporation of AsPC-1 cells after treating with IRS-2 siRNA (50 and 100 nmol/L) in the presence of serum, which showed significant inhibition of cell proliferation (Fig. 3*D*). Overall,

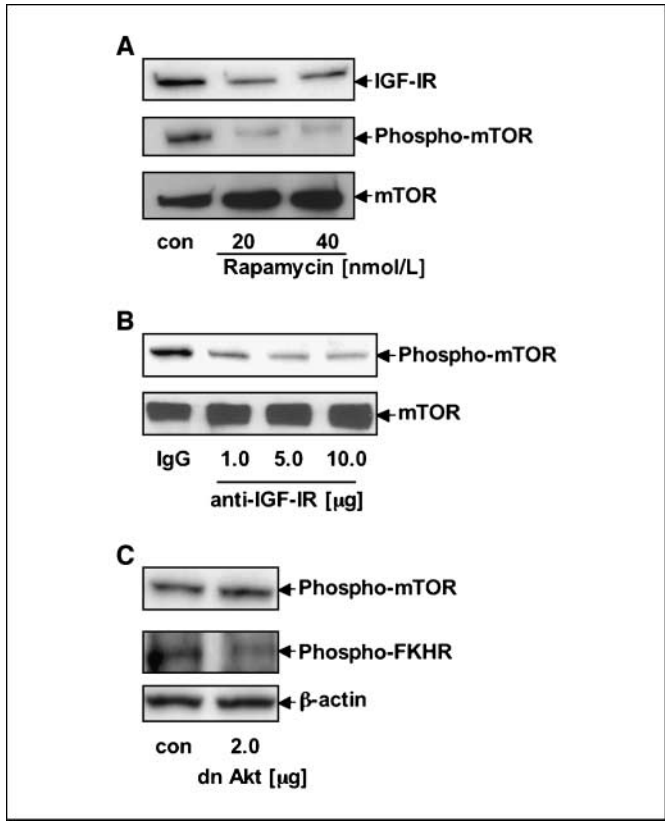

Figure 2. mTOR-meditated IGF-IR protein expression in PCA. *A,* total cell lysates from AsPC-1 cells untreated (*con*) or treated with 20 and 40 nmol/L of rapamycin were resolved by SDS-PAGE and analyzed by immunoblotting with anti-IGF-IR 2C8 (*IGF-IR*) and anti-phospho-mTOR. Total mTOR served as control protein. *B,* total cell lysates from AsPC-1 cells treated with anti-IGF-IRα 1H7 or mouse IgG (control) and analyzed by immunoblotting with anti-phospho-mTOR. *C,* total cell lysates from AsPC-1 cells transduced with vector expressing dominant-negative Akt (*dn Akt*) or constitutively active Akt (*con*) and analyzed by immunoblotting with anti-phospho-mTOR and anti-phospho-FKHR. β-Actin served as control protein.

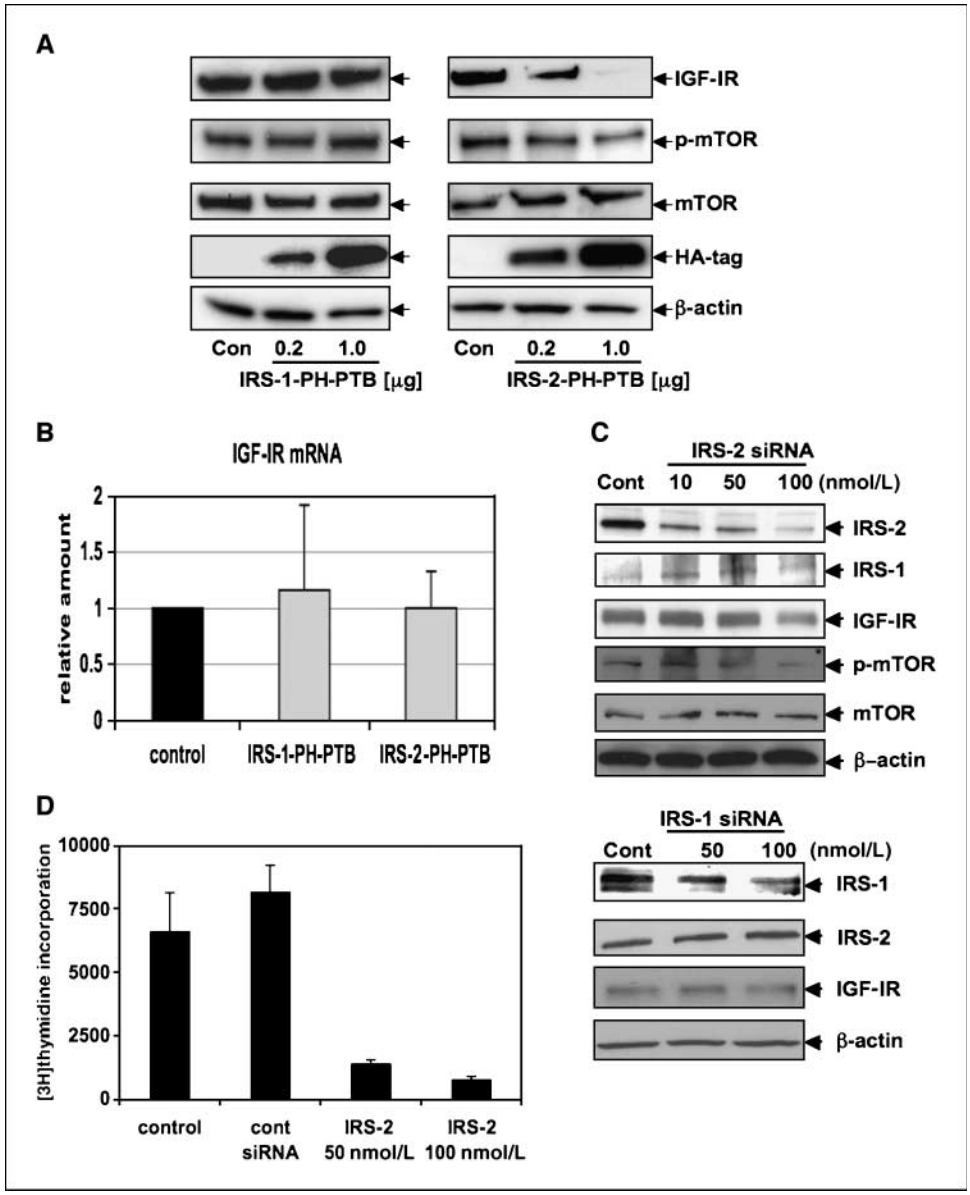

Figure 3. IRS-2, but not IRS-1, in IGF-IR overexpression in PCA. Total cell lysates from AsPC-1 cells transiently transfected with 0.2 and 1.0 μg of expression vector containing either (*A*) IRS-1-PH-PTB or IRS-2-PH-PTB or empty vector (*Con*) were analyzed by immunoblotting with anti-IGF-IR 2C8 (*IGF-IR*) and anti-phospho-mTOR (*p-mTOR*). Anti-HA-tag was measured to monitor transfection efficiency, and β-actin served as control protein. *B,* IGF-IR mRNA expression was analyzed from total RNA extracted from AsPC-1 cells transiently transfected with 1.0 μg of expression vectors containing either IRS-1-PH-PTB or IRS-2-PH-PTB and empty vector (*control*) and was analyzed by RT-PCR using β-actin as control. *C,* siRNA of IRS-2, but not IRS-1, inhibits IGF-IR protein expression. Total cell lysates from AsPC-1 cells transiently transfected with siRNA of IRS-2, siRNA of IRS-1, or control siRNA (*Cont*; all purchased from Santa Cruz Biotechnology) were analyzed by immunoblotting with anti-IGF-IR 2C8 (*IGF-IR*) epidermal growth factor receptor and other proteins. siRNA of IRS proteins did not influence each other. β-Actin served as control protein. *D,* [^{3}H]thymidine incorporation of AsPC-1 cells after treating with IRS-2 siRNA (50 and 100 nmol/L), which showed the significant inhibition of cell proliferation.

our data suggest that IRS-2 is involved in the regulation of IGF-IR expression through mTOR.

PKCδ, mTOR, and IGF-IR regulation. As IRS-2 only acts as an adapter molecule, we were now interested in downstream molecules that possess kinase activity. Others, as well as our group, showed that PKCδ is involved in the signaling pathways associated with IGF-IR activation (33, 34). Initially, to prove whether PKCs are involved in the regulation of IGF-IR expression, we treated AsPC-1 cells overnight with 7 and 10 nmol/L of the general PKC inhibitor Goe6983 and examined IGF-IR protein expression and the phosphorylation status of mTOR by Western blot analysis. Up to 7 nmol/L, Goe6983 specifically blocks calcium-dependent PKCs, such as PKCα and PKCβ. Up to 10 nmol/L, Goe6983 will block novel PKCs, including PKCδ, whereas with higher concentrations it blocks atypical PKCs, including PKCζ. Compared with untreated AsPC-1 cells, we detected a strong reduction of IGF-IR protein levels as well as a reduction of mTOR phosphorylation and its related molecules, such as S6 kinase (S6K) and 4E-BP, beginning with 10 nmol/L Goe6983, indicating that novel and/or atypical PKCs may be involved in the regulation of IGF-IR expression (Fig. 4*A*). Moreover, rottlerin (a more specific inhibitor of PKCδ) treatment revealed a similar effect as shown with Goe6983 (data not shown).

To further confirm the importance of PKCδ in this pathway, we transiently transfected PCA cells with 0.5, 1.0, and 1.5 μg of dominant-negative mutant of PKCδ and investigated IGF-IR protein expression as well as the phosphorylation levels of mTOR. As shown in Fig. 4*B*, we observed a significant inhibition of IGF-IR expression accompanied by a marked decrease of phosphorylated mTOR. We also confirmed a decrease of phosphorylation of 4E-BP after dominant-negative mutant transfected cells (Fig. 4*B*). In contrast, with overexpression of dominant-negative mutant of PKCζ, we were unable to show similar results indicating that PKCζ is not involved in this pathway (Fig. 4*B*). We also found that PKCδ regulates IGF-IR expression typically at the translational level, as there was no apparent change in the mRNA level of IGF-IR when

cells were expressed with 1.0 and 1.5 μg of dominant-negative mutant of PKCδ (Fig. 4*C*).

In a different approach to confirm these results, we treated AsPC-1 cells overnight with 20 and 50 nmol/L of TATFLAGVHL peptide (107-122), which directly binds to PKCδ and blocks its kinase activity (33). Untreated cells and cells treated with only TATFLAG peptide (33) served as controls. As expected, we detected a decrease of phosphorylated mTOR, confirming our hypothesis that mTOR phosphorylation is dependent on PKCδ activity (Fig. 4*D*).

PKCδ is downstream of IGF-IR/IRS-2 axis to promote IGF-IR expression. To prove whether the phosphorylation of PKCδ depends on IGF-IR activation, we treated AsPC-1 cells with 1.0, 5.0, and 10.0 μg of anti-IGF-IRα 1H7 for 24 hours and examined the phosphorylation status of PKCδ with anti-phospho-PKCδ by Western blot analysis. Figure 5*A* shows a marked decrease in the phosphorylation levels of PKCδ after blocking IGF-IR function, which clearly suggests that PKCδ is the downstream of IGF-IR. It also indicates that PKCδ may be a potential kinase of mTOR through the adapter IRS-2. We also observed a functional interaction between PKCδ and IGF-IR in AsPC-1 cells, as reported by other workers (34) in different cell lines (data not shown). To further establish the relationship of PKCδ within IGF-IR/IRS-2 axis, we observed that PKCδ phosphorylation is IRS-2 dependent

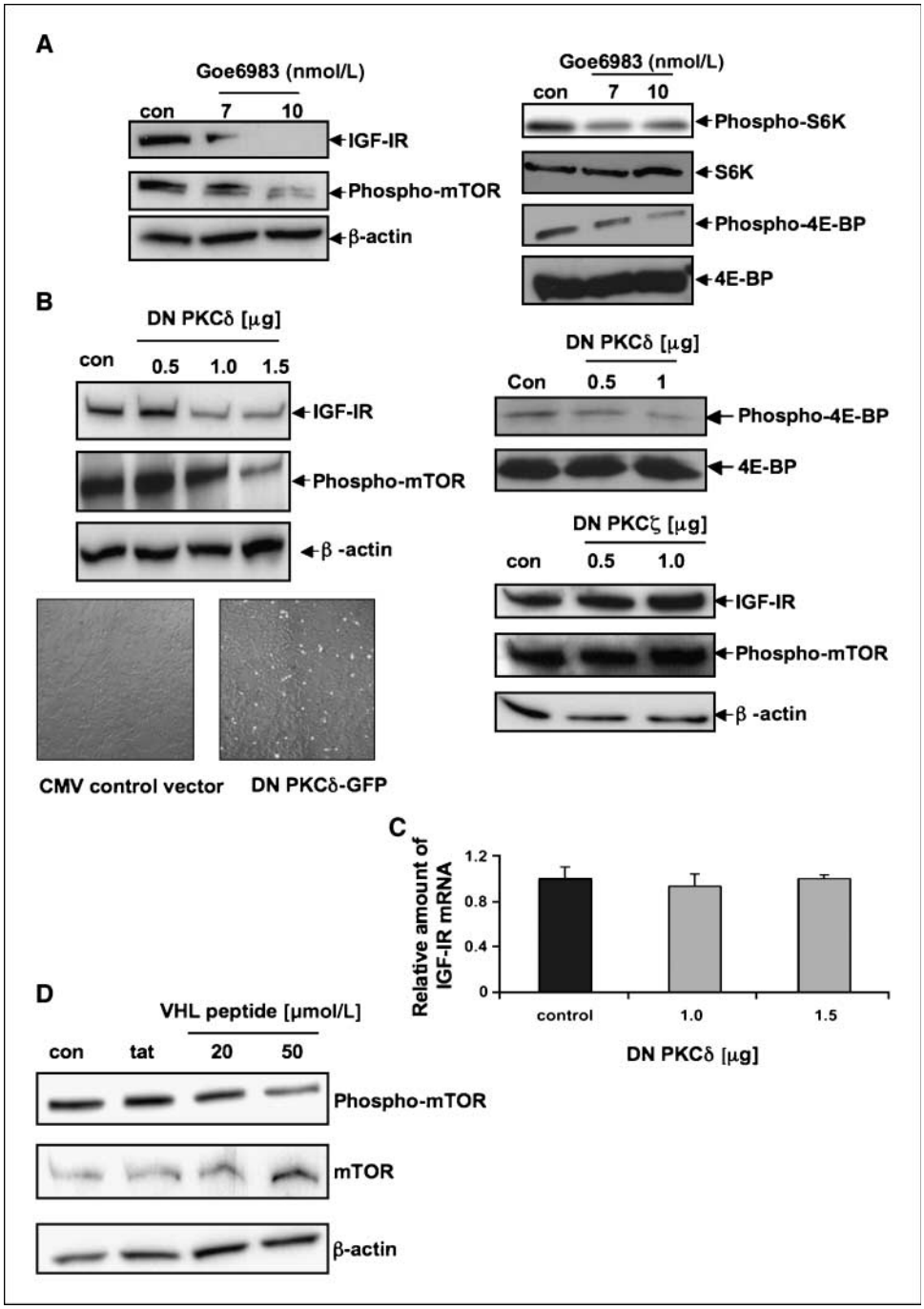

Figure 4. PKCδ controls IGF-IR overexpression in PCA. *A,* total cell lysates from AsPC-1 cells untreated (*con*) or treated with 7 or 10 nmol/L of the PKC inhibitor Goe6983 were resolved by SDS-PAGE and analyzed by immunoblotting with anti-IGF-IR 2C8 (*IGF-IR*) and anti-phospho mTOR. β-Actin served as control protein. Up to a concentration of 7 nmol/L, Goe6983 blocks specifically calcium-dependent PKCα and PKCβ. Up to 10 nmol/L, Goe6983 also inhibits novel PKCs, including PKCδ, and in higher concentrations than 10 nmol/L to atypical PKCs, including PKCζ. β-Actin served as control protein. Same extracts were immunoblotted with anti-phospho-S6K, anti-S6K, anti-phospho-4E-BP, and anti-4E-BP antibodies. *B,* total cell lysates from AsPC-1 cells transiently transfected with 0.5, 1.0, and 1.5 μg of dominant-negative PKCδ (*DN PKCδ*) or empty vector (*control*) were analyzed by immunoblotting with anti-IGF-IR 2C8 (*IGF-IR*) and anti-phospho-mTOR. β-Actin served as control protein. Same extracts were immunoblotted with anti-phospho-4E-BP and anti-4E-BP antibodies. *Bottom,* total cell lysates from AsPC-1 cells transiently transfected with 0.5 and 1.0 μg of dominant-negative PKCζ or empty vector (*control*) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-IGF-IR 2C8 (*IGF-IR*) and anti-phospho-mTOR. β-Actin served as loading control. *C,* IGF-IR mRNA extracted from AsPC-1 cells transiently transfected with 1.0 and 1.5 μg of dominant-negative PKCδ (*DN PKCδ*) or empty vector (*control*) was analyzed by RT-PCR using β-actin as control. *D,* total cell lysates from AsPC-1 cells untreated (*con*), 50 μmol/L TATFLAG peptide (control), and treated with 20 and 50 μmol/L of TATFLAGVHL peptide were resolved by SDS-PAGE and analyzed by immunoblotting with anti-phospho-mTOR and anti-mTOR. TATFLAGVHL peptide (107-122) binds directly to PKCδ and blocks its kinase activity.

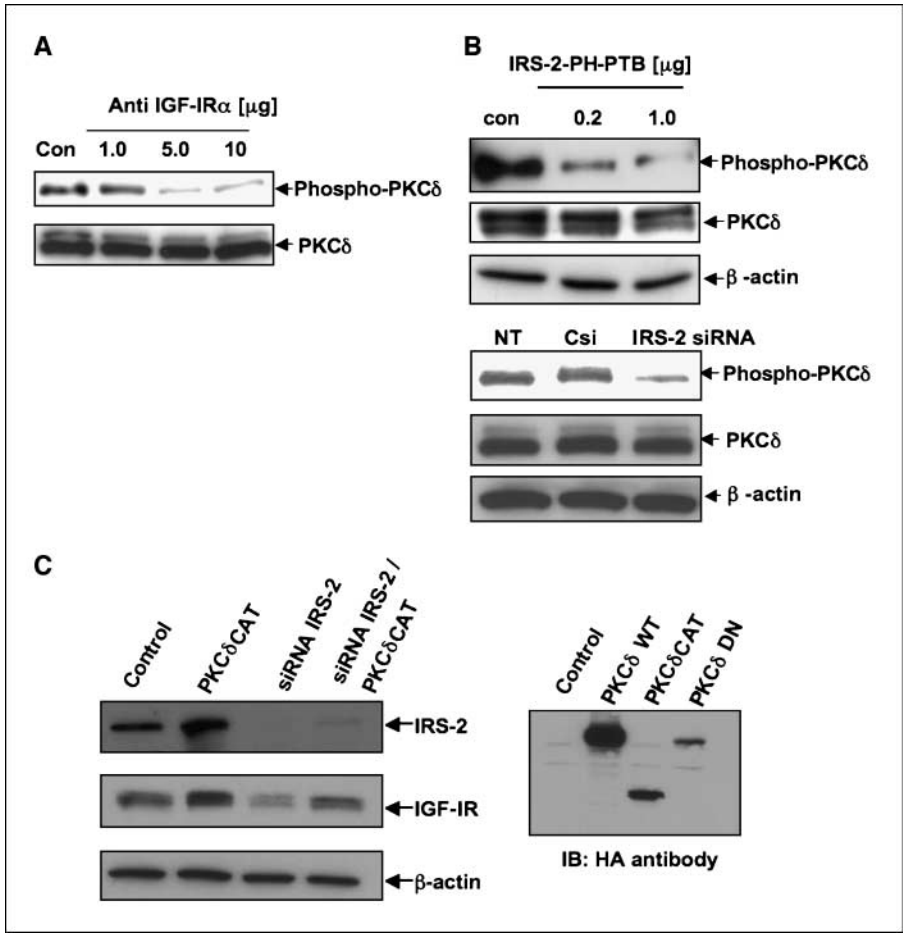

Figure 5. PKCδ is in the loop of IGF-IR expression in PCA. *A,* total cell lysates from AsPC-1 cells treated with 1.0, 5.0, and 10.0 μg of anti-IGF-IRα 1H7 or mouse IgG (*Con*) were analyzed by immunoblotting with anti-phospho-PKCδ and anti-PKCδ. *B,* total cell lysates from AsPC-1 cells transiently transfected with 0.2 and 1.0 μg of IRS-2-PH-PTB or empty vector (*con*) were immunoblotted with either anti-PKCδ or anti-phospho-PKCδ antibodies, respectively. Total cell lysates from AsPC-1 cells transfected with 0.1 μmol/L of IRS-2 siRNA or control siRNA were immunoblotted with either anti-PKCδ or anti-phospho-PKCδ antibodies, respectively. β-Actin served as loading control. *C,* total cell lysates from AsPC-1 cells transiently transfected with 0.1 μmol/L of IRS-2 siRNA or control siRNA (*Control*) with or without dominant-active mutant (amino acids 334–674) of PKCδ (*PKCδCAT*) were resolved by SDS-PAGE and analyzed by immunoblotting with IRS-2 and IGF-IR antibodies. β-Actin served as control protein.

because the IRS-2-PH-PTP mutant-overexpressing cells and IRS-2 knockdown cells showed less phosphorylation of PKCδ (Fig. 5*B*) as we observed in anti-IGF-IRα antibody treatment (Fig. 5*A*).

Next, we examined whether dominant-active mutant (amino acids 334–674, CAT) of PKCδ (PKCδCAT) can rescue the IRS-2 null effect with respect to IGF-IR expression and mTOR phosphorylation. We then transfected expression vector of PKCδCAT in IRS-2 siRNA-expressing AsPC-1 cells and performed similar experiments as described earlier. Indeed, overexpression of PKCδCAT can override the IRS-2 null effect by expressing more IGF-IR as shown in Fig. 5*C*. Taken together, our results suggest that IRS-2 is playing an important role as an adaptor of IGF-IR and PKCδ that might lead to mTOR activation and thus IGF-IR expression in PCA.

Discussion

Our results elucidate several important aspects of IGF-IR–mediated signaling and may eventually suggest a novel mechanism of IGF-IR protein expression in PCA. Although the reasons for the highly aggressive nature of pancreatic cancer are not fully understood, it is already known that tumorigenesis is a complex process also characterized by an activation of oncogenes, specifically K-ras (40), and the loss of tumor suppressor p53 gene function (41) and PTEN mutation (42). Additionally, the recent data showed that reduced expression of *TSC2* might be involved in the progression of pancreatic cancer (24). Previously, it has been shown by several groups that mTOR activation leads to the formation of a rapamycin-sensitive complex with Raptor (regulatory-associated protein of mTOR) followed by increases in mRNA translation via phosphorylation of two effector molecules: S6K and 4E-BP (32, 43–47). Interestingly, TSC1 or TSC2 removal constitutively activates rapamycin-sensitive functions of mTOR independently of Akt (48). A similar situation might exist in PCA cells as well as in the absence of TSC. Our data indicate that mTOR signaling in the context of IGF-IR expression is independent of Akt, whereas PKCδ might play an important role. There is increasing evidence that tumors with mutant p53 use a phosphatidylinositol 3-kinase (PI3K)–dependent but Akt-independent pathway, whereas tumors with loss of p16 use a PI3K/Akt/reactive oxygen species (ROS)–dependent pathway. Future studies will reveal whether p53 and p16 mutation in pancreatic cancer can be the detrimental factors for selection of PKCδ versus Akt/ROS inhibitors for therapeutics.

The overexpression and excessive activation of IGF-IR are associated with malignant transformation, increased tumor aggressiveness, and protection from apoptosis (49, 50). Previously, we have shown that overexpressed IGF-IR promotes the proliferation and invasion in AsPC-1 cells (51). Although the mechanisms inducing aberrant IGF-IR expression are not yet clear, several reports indicate an autocrine control of tumor cell growth by the IGF-I/IGF-IR system in pancreatic cancer (3, 38). We have determined in our investigations that an autocrine IGF-IR protein expression loop is mediated by at least two different signaling pathways. A recent report showed that mTOR inhibition activates PI3K/Akt by up-regulating IGF-IR signaling in acute myeloid

leukemia (AML) mostly due to up-regulation of IRS-2 (52). Although the authors did not describe the effect of IGF-IR expression in that regard, there might be a different regulation in AML versus pancreatic cancer cells as well.

Further investigations to determine downstream molecules that mediate IGF-IR expression at the translational level revealed a crucial role for PKCδ. AsPC-1 cells transfected with dominant-negative PKCδ expressed markedly less IGF-IR protein, whereas IGF-IR mRNA levels did not change. We found evidence for functional interactions between PKCδ and IGF-IR wherein IRS-2 plays a role like an adaptor in the loop. Li and colleagues (34) showed that activated IGF-IR was able to phosphorylate purified PKCδ *in vitro* and stimulate its kinase activity. Our data also indicate that phosphorylation of PKCδ depends on IGF-IR function. Furthermore, AsPC-1 cells transfected with dominant-negative PKCδ showed a decrease in phosphorylated mTOR, suggesting that PKCδ acts upstream of mTOR and mTOR phosphorylation is PKCδ dependent. Kumar and colleagues (30) showed that PKCδ constitutively associates with mTOR; however, in our experimental conditions, we were unable to detect mTOR and PKCδ in same immunocomplexes reproducibly. Nonetheless, other PKCs, such as PKCα, PKCβ, or PKCζ, are not involved in the regulation of IGF-IR protein expression.

Another important aspect of our findings is that IRS-2, but not IRS-1, is involved in this signaling pathway of IGF-IR regulation. An overexpression of IRS-2 and IRS-1 in pancreatic cancer has been shown by several groups (3, 19). Several reports indicate that IRS-1 and IRS-2 are not fully interchangeable and signaling intermediates for the biological effect of IGF-I and insulin (53). In this report, we have shown that inhibition of IRS-2, but not IRS-1, function leads to a decrease of IGF-IR protein expression. Additionally, we have shown that the presence of IRS-2 is required to activate PKCδ by IGF-IR. In conclusion, our results indicate that overexpression of IGF-IR in human pancreatic cancer cells is an autocrine mechanism and primarily regulated by a signaling pathway via mTOR. In this pathway, PKCδ plays a key role by functionally downstream of IGF-IR/IRS-2 axis. Further studies are in progress that use this unique pathway to develop a novel therapy of PCA where there is no therapy currently available for patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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