Insulin Receptor Substrate Is a Mediator of Phosphoinositide 3-Kinase Activation in Quiescent Pancreatic Cancer Cells

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Abstract

Phosphoinositide 3-kinase (PI3K) is activated in pancreatic cancer cells and plays a central role in their proliferation, survival, and drug resistance. Although the mechanism is unclear, PI3K activation in these cells could be due to physical interaction between its regulatory subunit (p85) and specific tyrosine kinases or their mediators. Consistent with this possibility, PI3K was precipitated with anti-phosphotyrosine antibodies and Akt phosphorylation was blocked by the tyrosine kinase inhibitors SU6656 and PD158780 in quiescent pancreatic cancer cells. Pull-down assays with a fusion protein (GST-p85NC-SH2), and coimmunoprecipitation studies, indicated that the insulin receptor substrate (IRS), and not the epidermal growth factor and insulin-like growth factor receptors or the Src tyrosine kinase, was physically associated with PI3K in these cells. Our data also indicated that SU6656 and PD158780 inhibited Akt activation in pancreatic cancer cells by interfering with the ability of IRS-1 to recruit PI3K. Furthermore, IRS-1 was phosphorylated on a p85-binding site (Y^{612}), and IRS-specific small interfering RNA potently inhibited activation of PI3K and Akt in transfected cells. Taken together, these observations indicate that IRS is a mediator of PI3K activation in quiescent pancreatic cancer cells. (Cancer Res 2005; 65(20): 9164-8)

Introduction

As an important regulator of cellular proliferation and survival, the phosphoinositide 3-kinase (PI3K) signaling pathway plays a central role in the development and dissemination of various human cancers (1, 2). That PI3K and its mediator Akt have an important function in pancreatic tumorigenesis is well supported by various studies. For example, Testa et al. showed that the *AKT2* gene was amplified and overexpressed in about 10% of pancreatic ductal adenocarcinomas and that reducing its expression in pancreatic cancer cells inhibited their tumorigenicity and invasiveness (3). There is evidence that Akt promotes the invasiveness of pancreatic cancer cells by up-regulating the expression of insulin-like growth factor-1R (IGF-IR; ref. 4). Other studies have shown that the PI3K pathway is constitutively activated in pancreatic cancer cells (5–8) and that its inhibition increases gemcitabine-induced antitumor activity in many (5), if not all, cases. In addition, a pivotal role has been established for PI3K in the growth and survival of pancreatic cancer cells using both *in vitro* and *in vivo* systems (5–8).

Whereas significant information is now available on PI3K function in pancreatic cancer, the molecular mechanisms that facilitate its activation remain poorly understood. Recent evidence suggests that Akt activation in this cancer is facilitated, in part, by aberrant expression of the PTEN tumor suppressor gene, a natural antagonist of PI3K activity. Using transgenic mice and patient tumor specimens, Ebert et al. suggested that transforming growth factor-β1 overexpression reduced PTEN expression in pancreatic cancer (9). We recently showed that PTEN expression was reduced or lost in over 60% of pancreatic tumor tissues and cell lines examined and that the reduction might be due to promoter methylation (8). Because PTEN functions downstream, the mechanism of activation of PI3K itself remained to be investigated.

In this study, we examined the possibility that PI3K was activated in quiescent pancreatic cancer cells through interaction with specific cellular receptors. Indeed, PI3K is activated when the SH2 domains of its regulatory subunit (p85) interact with phosphorylated YXXM motifs on tyrosine kinases like the epidermal growth factor receptors (EGFR) and Src, or receptor-associated molecules such as the insulin receptor substrate (IRS; ref. 10). PI3K is also recruited and activated by heterotrimeric G proteins and small G proteins of the Ras family (10). Although various tyrosine kinases are overexpressed and Ki-Ras constitutively activated in pancreatic cancer (11, 12), there is little evidence linking them to PI3K activation. Our results suggest that PI3K is activated in quiescent pancreatic cancer cells through physical interaction with the IRS adaptor molecule.

Materials and Methods

Materials. The pancreatic cancer cell lines AsPC-1, BxPC-3, and Panc-1 were obtained from the American Type Culture Collection (Manassas, VA) and cultured as recommended by them. Panc-28 was kindly provided by Dr. Paul Chiao (M.D. Anderson Cancer Center) and maintained under standard culture conditions in RPMI 1640 supplemented with 10% fetal bovine serum. Antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA (p85 PI3K, Akt1/2, IRS-1, EGFR, IGF-1R, and phospho-ERK); Cell Signaling Technology, Beverly, MA (phospho-Akt, IRS-1); BD Transduction Laboratories, San Diego, CA (anti-phosphotyrosine PY20); and Biosource International, Camarillo, CA (anti-phospho-IRS-1-pY612). Insulin was purchased from Sigma (St. Louis, MO), and EGF from Upstate Biotechnology (Charlottesville, VA). The tyrosine kinase inhibitors SU6656 and PD158780 were obtained from Calbiochem (San Diego, CA); Tyrphostin51 and Erbstatin were from Biomol (Plymouth Meeting, PA). Small interfering RNA (siRNA) against IRS-1 was obtained from Santa Cruz Biotechnology, and control siRNA was purchased from Ambion (Austin, TX).

Immunoprecipitation and Western blotting. Pancreatic cancer cells were seeded in 100-mm dishes and serum starved for 16 hours. Whole cell extracts were then prepared and used for immunoprecipitation (3 μg of PY20 or 2 μg of other antibodies) or Western blotting as described earlier (13). Briefly, cells were lysed in buffer containing 50 mmol/L HEPES (pH 7.5), 1.5 mmol/L $MgCl_2$, 150 mmol/L NaCl, 1 mmol/L EGTA, 20 mmol/L

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NaF, 10 mmol/L $Na_4P_2O_7$, 10% glycerol, 1% Triton X-100, 3 mmol/L benzamidine, 1 mmol/L Na_3VO_4, 1 μmol/L pepstatin, 10 μg/mL aprotinin, 5 mmol/L iodoacetic acid, and 2 μg/mL leupeptin. Lysates were clarified by centrifugation at 14,000 × *g* for 5 minutes. Whole cell extracts were then incubated for 3 hours at 4°C with specific antibodies for the immunoprecipitation experiments or resolved by SDS-PAGE and probed directly by Western blotting. After incubation with antibodies, immune complexes were gathered with 25 μL of Protein A-Sepharose bead (50%) slurry, washed, and eluted by boiling in SDS sample buffer. Eluted proteins were then resolved by SDS-PAGE and probed by Western blotting with specific antibodies using the enhanced chemiluminescence detection reagent (Amersham Biosciences, Piscataway, NJ). Densitometric analysis of scanned Western blots was done with the NIH ImageJ program.[3]

Preparation of GST-p85 NC-SH2 and pull-down assays. DH5α bacteria were transformed with pGEX control vector or pGEX-p85NC-SH2 and cultured in an incubator at 37°C with continuous shaking; 0.3 mmol/L isopropyl-β-thiogalactoside was added to the culture to induce (4 hours at 37°C) expression of glutathione *S*-transferase (GST) or GST-p85NC-SH2. Bacteria were pelleted and lysed in buffer [40 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 10% glycerol, 1 mmol/L DTT, 0.4 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin] along with lysozyme (5 mg/L) and a bacterial protease inhibitor cocktail (Sigma). Suspensions were vortexed, incubated on ice for 30 minutes, and sonicated briefly (3 × 20 seconds). The insoluble fraction was removed by centrifugation, and the supernatants incubated with glutathione-Sepharose beads (Amersham Biosciences) for 30 minutes at room temperature. Following centrifugation, the GST-p85NC-SH2 beads were extensively washed, resuspended (50% slurry), and stored at 4°C. The GST proteins were subjected to SDS-PAGE and visualized after staining (Coomassie blue).

For the pull-down assays, 30 μL of the 50% GST or GST-p85NC-SH2 bead slurry were incubated with whole cell extracts of serum-starved pancreatic cancer cells (60-mm culture dishes) at 4°C for 3 hours. To detect proteins that bound specifically, the beads were washed several times with lysis buffer, boiled in sample buffer, and subjected to SDS-PAGE and Western blotting.

Small interfering RNA transfection. Panc-28 cells were seeded overnight in six-well plates and transfected the next day with 50 nmol/L control or IRS-specific siRNA using the RNAifect reagent (Qiagen, Valencia, CA). One day later, cells were serum-starved for 16 hours, and whole-cell extracts were prepared as described above for the analysis of specific proteins (Akt, phospho-Akt-Ser^{473}, and actin) by immunoprecipitation and/or Western blotting.

Results and Discussion

Phosphoinositide 3-kinase activation in quiescent pancreatic cancer cells involves tyrosine kinase activity. Our previous study suggested that Akt activation in quiescent pancreatic cancer cells was due to a stimulation of PI3K activity and a reduction in PTEN expression levels (8). That PI3K is activated in pancreatic cancer cells is also supported by this current study, because we found that its p85 subunit could indeed be immunoprecipitated by anti-phosphotyrosine (PY20) antibodies (Fig. 1*A*). The precipitation of PI3K was specific, because other proteins such as extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) were absent from the same complexes (data not shown) and it seemed to involve phosphorylated tyrosine, because a nonspecific antibody was unable to immunoprecipitate p85 (Fig. 1*A*). Because PY20 antibodies are routinely employed to assess PI3K stimulation by tyrosine kinases, these data suggest that tyrosine kinase activity was responsible for PI3K activation in quiescent pancreatic cancer cells.

[3] http://rsb.info.nih.gov/ij.

To further investigate the involvement of a tyrosine kinase in PI3K activation, we examined the effects of various inhibitors on Akt phosphorylation in Panc-28 cells (Fig. 1*B*). Selective inhibitors (14, 15) of the Src kinase (SU6656) and the EGFR or EGFR tyrosine kinase (PD158780) strongly inhibited Akt phosphorylation (87 ± 11% and 92 ± 6%, respectively) and implicated tyrosine kinase activity in PI3K activation. Together with the fact that neither inhibitor blocked the phosphorylation of the ERK/MAPKs, this result suggested that the effects of SU6656 and PD158780 involved Src and the EGFR tyrosine kinases. Interestingly, however, two other tyrosine kinase inhibitors (Tyrphostin51 and Erbstatin) had no effect on Akt phosphorylation. Because Tyrphostin51 is also a potent inhibitor of the EGFR (16), it is conceivable that the effect of PD158780 might have involved a distinct tyrosine kinase.

IRS-1 physically interacts with phosphoinositide 3-kinase. To identify the tyrosine kinase that might interact with PI3K in quiescent pancreatic cancer cells, we expressed and purified GST and the GST-p85NC-SH2 proteins for use in pull-down assays (Fig. 2*A*). GST-p85NC-SH2 lacks the NH_2-terminal 329 amino acids but retains the receptor-interacting NH_2- and COOH-terminal SH2 domains of the p85 subunit of PI3K. Whole cell extracts from control and EGF-stimulated Panc-28 cells were incubated with GST-p85NC-SH2 to determine whether EGFR and Src, which are overexpressed in pancreatic cancer cells (12), were capable of interacting with PI3K. Although EGFR and Src from the serum-starved control (EGF−) seemed to interact with GST (data not shown) and the GST-p85NC-SH2 affinity column, they could be washed off easily (Fig. 2*B*). The EGFR bound the column with high affinity only

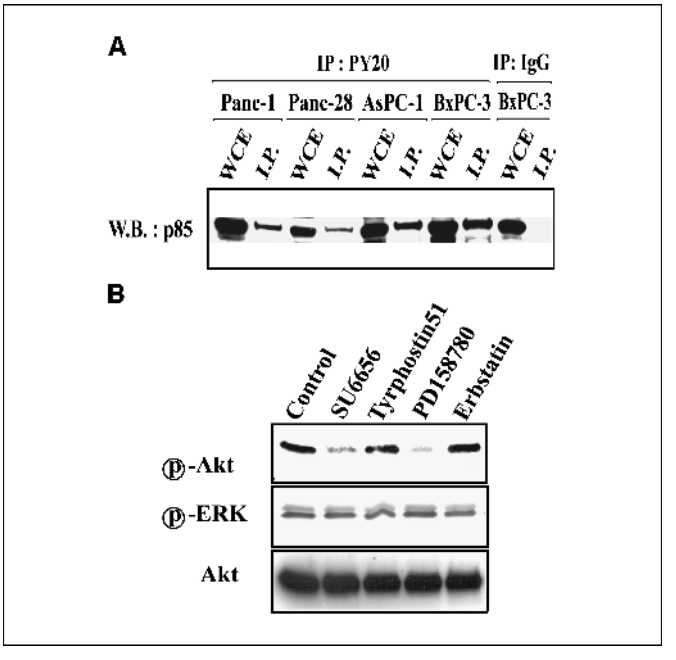

Figure 1. Tyrosine kinase activity in PI 3-kinase activation. *A,* for immunoprecipitation (*IP*) assays, whole cell extracts (*WCE*) prepared from various serum-starved pancreatic cancer cell lines containing equal amounts of protein were incubated with anti-phosphotyrosine (PY20) antibodies. One set of BxPC-3 cell extracts was also incubated with a nonspecific antibody (IgG). Immune complexes were collected by Protein A-Sepharose beads and probed by Western blotting (*W.B.*) for the p85 subunit of PI3K. *B,* Panc-28 cells were serum starved for 16 hours and treated for 2 hours with the following tyrosine kinase inhibitors: SU6656 (1 μmol/L), Tyrphostin51 (5 μg/mL), PD158780 (10 μmol/L), and Erbstatin (2 mg/mL). Whole cell extracts were analyzed by immunoblotting for phosphorylated Akt (Ser^{473}), phosphorylated ERK (Tyr^{204}), and total Akt. Representative of three independent experiments.

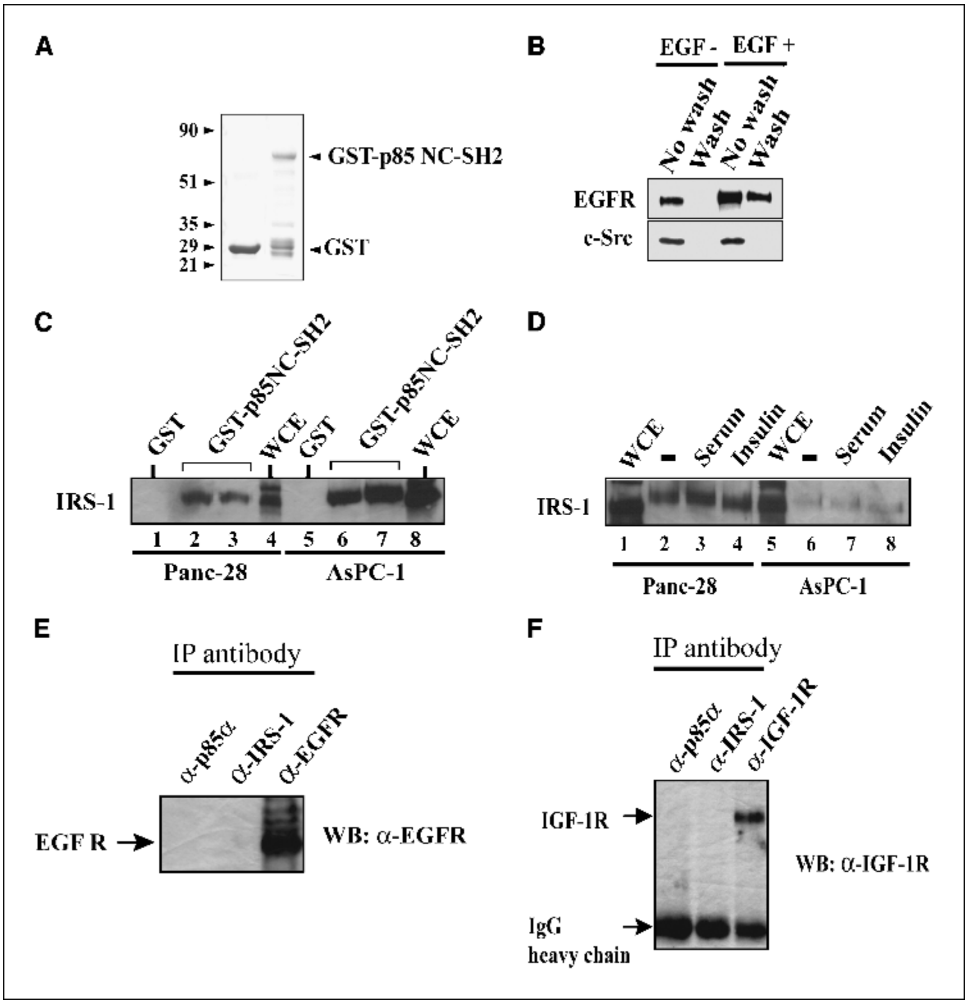

Figure 2. IRS interacts with PI3K in quiescent pancreatic cancer cells. *A,* GST (*lane 1*) and GST-p85 NC-SH2 (*lane 2*) were expressed, purified, and visualized by Coomassie blue staining. *B,* whole cell extracts (*WCE*) from control and EGF-treated (5 minutes) Panc-28 cells were incubated with GST-p85 NC-SH2 beads. The beads were then either left unwashed (*No wash*) or washed thrice with lysis buffer (*Wash*). Bound protein was probed by immunoblotting for EGFR and Src. *C,* whole cell extracts from serum-starved Panc-28 (*lane 4*) and AspC-1 (*lane 8*) cells were incubated with GST (*lanes 1* and *5*) or GST-p85 NC-SH2 (in duplicate, *lanes 2* and *3* and *lanes 6* and *7*). After being washed (thrice), protein that remained bound was probed by Western blotting for IRS-1. *D,* whole cell extracts (*lanes 1* and *5*) from serum-starved Panc-28 and AsPC-1 cells left either unstimulated (*lanes 2* and *6*) or stimulated by serum (*lanes 3* and *7*) or insulin (*lanes 4* and *8*) for 5 minutes were used for GST-p85 NC-SH2 pull-down assays. Bound protein was probed for IRS-1. *E-F,* antibodies that recognized p85/PI3K, IRS-1, EGFR, and IGF-1R were employed (immunoprecipitation, *IP*, antibody) for coimmunoprecipitation experiments as indicated. Immune complexes were washed and probed by Western blotting (*WB*) for the EGFR or the IGF-1R. Representative of three independent experiments.

when Panc-28 cells were stimulated with EGF, which confirmed that GST-p85NC-SH2 was fully capable of specific interactions (Fig. 2*B*). Because EGFRs and Src are both capable of physically interacting with PI3K (17), these data suggest that they are not directly associated with PI3K in quiescent pancreatic cancer cells.

The IGF-1R strongly induces PI3K activity and has an important role in the development of human cancer (18). Along with Src and receptors of the EGFR family, IGF-1R is also overexpressed in pancreatic tumors (19) and has been implicated in the invasiveness of pancreatic cancer cells (4). Following stimulation, IGF-1R recruits IRS, whose phosphorylated YXXM motifs then dock and induce PI3K (18). Like IGF-1R, IRS is also overexpressed in pancreatic tumors and might contribute to the uncontrolled growth of pancreatic cancer cells (20, 21). We, therefore, investigated the possibility that the IGF-1R/IRS signaling complex might be responsible for PI3K activation in pancreatic cancer. Pull-down assays with GST-p85NC-SH2 showed that, unlike EGFR and Src, IRS-1 might indeed interact with PI3K in quiescent pancreatic cancer cells (Fig. 2*C*). Because IRS-1 did not bind the GST control, its interaction seemed to be specifically with p85. The addition of serum or insulin induced ERK phosphorylation (data not shown) but did not significantly increase the amount of IRS-1 that bound to the affinity column (Fig. 2*D*). Thus, most of the IRS-1 in Panc-28 and AsPC-1 cells may already have been phosphorylated on YXXM motifs and fully activated. Interestingly, however, Kornmann et al. have shown that the addition of insulin, IGF-1, or IGF-2 induced PI3K activity in AsPC-1 and COLO-357 cells (21). The reason for the inconsistency between the two observations is unclear, but it is possible that ligand-induced PI3K activation in their study was due to the recruitment and phosphorylation of IRS-2. The role of IRS-2, if any, in the activation of PI3K in quiescent pancreatic cancer cells is unclear and remains to be investigated.

In support of our conclusions from the pull-down assays, anti-p85α antibodies did not precipitate EGFR (Fig. 2*E*) or ErbB2 (data not shown). Although neither receptor, by itself, is capable of binding PI3K, both can dimerize with ErbB3 to recruit the enzyme. The identification of IRS-1 as a potential PI3K recruiter in pancreatic cancer cells would, in fact, implicate the IGF-1R and be consistent with an important study by Nair et al. (22). To our surprise, however, immunoprecipitation-competent anti-p85α and anti-IRS-1 antibodies did not precipitate IGF-1R (Fig. 2*F*). Furthermore, anti-EGFR, anti-ErbB2, and anti-IGF-1R antibodies did not precipitate p85 or IRS-1 (data not shown) either, suggesting that a tyrosine kinase distinct from EGFRs and IGF-1R is likely to be involved in recruiting PI3K in quiescent pancreatic cancer cells. We cannot however eliminate the possibility that IGF-1R/IRS/p85 interactions were disrupted during immunoprecipitation or that the antibodies employed failed to recognize IGF-1R/IRS/p85 complexes. Whether or not IGF-1R is involved, our results implicate IRS-1 as a mediator of PI3K activation in pancreatic cancer cells.

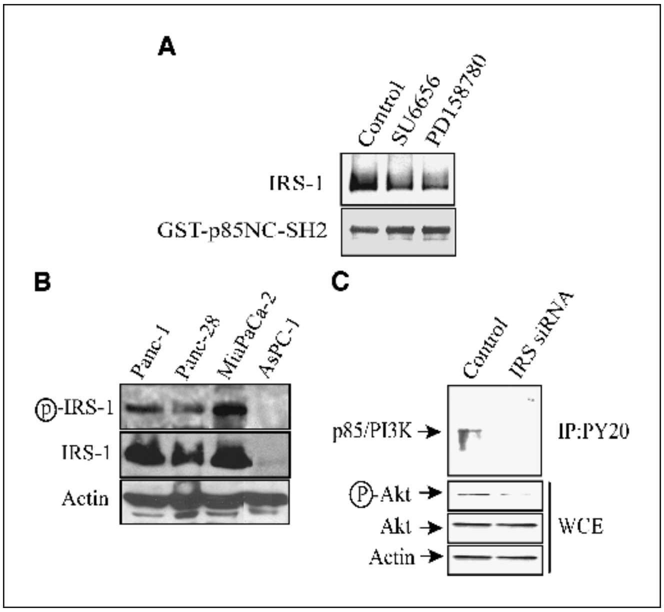

Figure 3. IRS is a mediator of PI3K activation in quiescent pancreatic cancer cells. *A,* serum-starved Panc-28 cells were left untreated (*control*) or treated with SU6656 or PD158780 for 2 hours. GST-p85 NC-SH2 pull-down assays were performed with whole cell extracts (*WCE*) to determine the effect of these inhibitors on the ability of IRS-1 to bind PI3K. *Bottom,* western blot of equal amounts of GST-p85 NC-SH2 used in each lane (anti-p85 antibodies). Representative of three independent experiments. *B,* whole cell extracts were prepared from pancreatic cancer cells that had been serum starved for 16 hours and probed by Western blotting for phospho-IRS (Y^{612}), IRS-1, and actin. *C,* control or IRS-specific siRNA was transfected into Panc-28 cells. After 24 hours, cells were serum starved for 16 hours and whole cell extracts were employed either for immunoprecipitation (*IP*) with PY20 antibodies (followed by immunoblotting with anti-p85/PI3K antibodies, *top*) or directly analyzed by Western blotting for phospho-Akt, Akt, and actin (*bottom three*). Representative of two independent experiments.

Whereas proteins of the Ras family also induce PI3K, oncogenic Ras activates PI3K by interacting with its p110 catalytic subunit (10). Ki-Ras, which has activating mutations in pancreatic cancer (11), was not detectable, however, in any of the immune complexes described above. Moreover, farnesyl transferase inhibitors did not suppress phospho-Akt levels (data not shown). Thus, Ki-Ras does not seem involved in PI3K activation in pancreatic cancer cells.

IRS-1 is essential for the activation of phosphoinositide 3-kinase and Akt in quiescent pancreatic cancer cells. The fact that SU6656 and PD158780 potently blocked Akt phosphorylation in quiescent pancreatic cancer cells (Fig. 1*B*) suggests that they might have nonspecifically inhibited a tyrosine kinase upstream of IRS. Indeed, the amount of IRS-1 that interacted with PI3K in the pull-down assays was significantly reduced by both SU6656 (75 ± 7%) and PD158780 (83 ± 9%) in Panc-28 cells (Fig. 3*A*). Specifically, therefore, our data suggests that the two inhibitors blocked a tyrosine kinase responsible for IRS phosphorylation on PI3K-recruiting YXXM motifs. The effects of SU6656 and PD158780 on phospho-Akt, and IRS-1 binding in the pull-down assays, were observed within 2 hours of treatment and did not seem to involve changes in the expression or phosphorylation of other proteins (data not shown).

Because IRS interacts with PI3K when its YXXM motifs are phosphorylated, we next investigated the possibility that IRS-1 was covalently modified in quiescent pancreatic cancer cells. Western blotting analysis indicated that IRS-1 was phosphorylated on Tyr^{612} (Y^{612}) in different pancreatic cancer cell lines (Fig. 3*B*). Y^{612} of human IRS-1 resides in a canonical YXXM motif and its phosphorylation is essential for insulin-induced PI3K recruitment/activation and GLUT4 translocation (23, 24). In three different pancreatic cancer cell lines (Panc-1, Panc-28, and MiaPaCa-2), IRS-1 was highly expressed and phosphorylated. In sharp contrast, IRS-1 was poorly expressed in AsPC-1 cells and its phosphorylation barely detectable. That IRS-1 was phosphorylated even in AsPC-1 cells was indicated earlier by pull-down assays that first enriched it from cell extracts before the Western blotting analysis. Whereas Y^{612} and other tyrosine residues trigger specific signaling pathways when phosphorylated, the modification of other IRS sites such as Ser^{616} is pertinent to signal desensitization. The phosphorylation status of IRS-1 in pancreatic cancer cells would, therefore, be critical for its ability to activate PI3K at any given time.

To investigate further the involvement of IRS-1 in PI3K activation, Panc-28 cells were transfected with control and IRS-specific siRNA. Relative to the control, Akt phosphorylation was significantly inhibited (70 ± 10%) and the ability of PY20 antibodies to immunoprecipitate PI3K was abolished in IRS-1 siRNA-transfected cells (Fig. 3*C*). Thus, our data support an important role for IRS in PI3K/Akt activation and function in quiescent pancreatic cancer cells.

In addition to the IGF-1R, a wide variety of hormone and cytokine receptors, such as those of insulin, interleukin-4 (IL-4), IL-9, IL-13, IL-15, and IFN, also use IRS to recruit PI3K (25). Unlike insulin and IGF-1R receptors that possess intrinsic kinase activity, cytokine receptors induce IRS phosphorylation through the recruitment of the Janus kinase family of tyrosine kinases, further increasing the complexity of IRS regulation. Interestingly, EGF has also been shown to induce IRS phosphorylation on tyrosine residues (26). Studies are under way to identify the signaling molecules that recruit IRS and elevate PI3K activity in pancreatic cancer.

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