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 communoprecipitation 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 (Y412), 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 invasive-ness (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 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 MgCl2, 150 mmol/L NaCl, 1 mmol/L EGTA, 20 mmol/L...
To further investigate the involvement of a tyrosine kinase in P13K activation, we examined the effects of various inhibitors on Akt phosphorylation in Panc-28 cells (Fig. 1B). 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 P13K 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 P13K in quiescent pancreatic cancer cells, we expressed and purified GST and the GST-p85NC-SH2 proteins for use in pull-down assays (Fig. 2A). GST-p85NC-SH2 lacks the NH2-terminal 329 amino acids but retains the receptor-interacting NH2- and COOH-terminal SH2 domains of the p85 subunit of P13K. 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 P13K. 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. 2B). The EGFR bound the column with high affinity only

![Diagram](image-url)
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. Immunocomplexes were washed and probed by Western blotting (WB) for the EGFR or the IGF-1R. Representative of three independent experiments.
IRS Activates PI3K in Pancreatic Cancer Cells

References


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IRS 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. 3B). 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^473 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. 3C). 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 family kinase 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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