Enhanced Expression of the Insulin Receptor Substrate-2 Docking Protein in Human Pancreatic Cancer

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Abstract

Insulin receptor substrate-2 (IRS-2) is a multisite docking protein implicated in mitogenic signaling after activation of the insulin and insulin-like growth factor (IGF-I) receptors. In the present study, we characterized IRS-2 expression and function in human pancreatic cancer. IRS-2 mRNA and protein were expressed in ASPC-1 and COLO-357 human pancreatic cancer cell lines. Insulin, IGF-I, and IGF-II enhanced the growth of both cell lines, stimulated tyrosine phosphorylation of IRS-2, and increased IRS-2-associated phosphatidylinositol (PI) 3-kinase activity. The mitogenic effects of insulin, IGF-I, and IGF-II were markedly attenuated by the PI 3-kinase inhibitor LY 294002. Northern blot analysis of total RNA extracted from normal and cancerous tissues revealed that IRS-2 mRNA levels were increased in the cancer tissues (P = 0.032). In the normal pancreas, IRS-2 immunoreactivity was present at low levels in some ductal and acinar cells and at moderate levels in a heterogeneous pattern in all of the endocrine islets. In the pancreatic cancers, IRS-2 was abundant in the ductal-like cancer cells. These findings indicate that IRS-2 is overexpressed in human pancreatic cancer and suggest that it may contribute to enhanced mitogenic signaling via the PI 3-kinase pathway, thereby leading to excessive growth stimulation in this malignancy.

Introduction

Insulin and IGFs are mitogenic polypeptides that bind to specific, high-affinity receptors composed of two disulfide-linked extracellular α-subunits that contain the ligand binding sites and two β-subunits consisting of a transmembrane region and an intracellular region exhibiting tyrosine kinase activity (1). Ligand binding to the insulin or IGF-IR results in activation of the intracellular receptor tyrosine kinase domains, subsequent auto-phosphorylation, and association and tyrosine phosphorylation of the IRS docking molecules (2–4). Pancreatic cancers overexpress IGF-I, IGF-IR, and IRS-2 (5, 6). Moreover, in vitro studies have demonstrated that insulin and IGFs are potent mitogens for cultured human pancreatic cancer cells (5–7). Despite the potential importance of IGF-1 in human pancreatic cancer, nothing is presently known about the expression of IRS-2 in this malignancy. Therefore, in this study, we compared IRS-2 expression in normal and cancerous human pancreatic tissues and characterized the actions of IGF-1 and related ligands on IRS-2 phosphorylation in ASPC-1 and COLO-357 human pancreatic cancer cell lines. We now report that IRS-2 is overexpressed in human pancreatic cancer, where it localizes in the cancer cells, and that insulin, IGF-I, and IGF-II enhance IRS-2 tyrosine phosphorylation and IRS-2-associated PI 3-kinase activity in cultured pancreatic cancer cells.

Materials and Methods

Materials. The following were purchased: ASPC-1 human pancreatic cancer cells from American Type Culture Collection (Manassas, VA); [32P]ATP and [α-32P]dCTP from Amersham Corp. (Arlington Heights, IL); PI substrate from Avanti Polar Lipids Inc. (Alabaster, AL); insulin from Becton Dickinson and Company (Franklin Lakes, NJ); LY 294002 from Biomol Research Laboratories Inc. (Plymouth Meeting, PA); BioMax ML and MS films from Kodak (Rochester, NY); silica gel 60 TLC plates from Merck (Darmstadt, Germany); immobilon-P nitrocellulose membranes from Millipore (Bedford, MA); enhanced chemiluminescence substrate from Pierce Chemical Co. (Rockford, IL); MTT, protein A-Sepharose, and secondary horse-radish-conjugated anti-rabbit antibody from Sigma Chemical Co. (St. Louis, MO); PY20 antibody from Transduction Laboratory (Lexington, KY); IGF-II from United States Biochemical Co. (Cleveland, OH); rabbit polyclonal anti-IRS-2 antibodies from Upstate Biotechnology Inc. (Lake Placid, NY). COLO-357 human pancreatic cancer cells were a gift from R. S. Metzgar at Duke University (Durham, NC), and IGF-I was a gift from Genentech Inc. (South Francisco, CA).

Cell Culture and Growth Assay. COLO-357 cells were grown in DMEM, and ASPC-1 cells were grown in RPMI medium. All media were supplemented with 8% FBS, penicillin G (100 units/ml), and streptomycin (100 µg/ml). Cells were maintained at 37°C in humidified air with 5% CO₂. Cell growth was determined by the MTT colorimetric assay, as described previously (5–7).

Immunoblotting and Immunoprecipitation. Immunoblot analysis of IRS-2 was carried out as described previously for IRS-1 (6, 7). Briefly, cells were washed twice with ice-cold PBS and lysed in lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 2 mM EDTA, 50 mM NaF, 1 mM Na V O₄, 50 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 10 µg/ml benzamidine, and 1 mM phenylmethylsulfonyl fluoride. Cell lysates (30 µg) were subjected to 7% SDS-PAGE and electro-transferred to immobilon-P membranes. Membranes were blotted with the indicated primary antibodies and corresponding secondary horseradish-conjugated antibodies as described (6, 7). Bound antibodies were visualized using enhanced chemiluminescence.

For immunoprecipitation with IRS-2 antibodies, cells were grown to 50% confluency in medium containing 8% FBS and then incubated for 24 h in serum-free medium containing antibiotics, 0.1% BSA, 5 mg/ml transferrin, and 5 µg/ml selenious acid, before stimulation with growth factors and cell lysis. Cell lysates (500 µg in 500 µl of lysis buffer) were incubated for 2 h at 23°C with IRS-2 antibodies (2 µg/sample), followed by a 1-h incubation with protein A-Sepharose (30 µl) at 23°C. Precipitates were washed with ice-cold PBS, resuspended in loading buffer and boiled for 5 min at 100°C. After centrifugation, the supernatants were subjected to Western blotting as described above. For reprobing, membranes were incubated for 30 min at 50°C in buffer containing 2% SDS, 62.5 mM Tris (pH 6.7), and 100 mM 2-mercaptoethanol.

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3 The abbreviations used are: IGF, insulin-like growth factor; IRS-1, insulin receptor I; IRS-2, insulin receptor substrate; MTT, 3-(4,5-methylthiazol-2-yl)-2,5-diaphenyltertrazolium bromide; PI, phosphorytidinolinositol; PY20, antiphosphotyrosine; FBS, fetal bovine serum.
Northern Blot Analysis. Total RNA was extracted by the acid guanidinium thiocyanate method, and poly(A)^+ RNA was prepared by affinity chromatography on oligo-dT cellulose (5). For Northern blotting, membranes were hybridized under high stringency conditions with a 1.0 kb SacI/Nhel fragment of the mouse IRS-2 cDNA (8). cDNAs were labeled with [α-^32P]dCTP (3000 Ci/mmol) by random priming before hybridization. A human β-actin cDNA and a mouse βS cDNA were used as loading controls (5). The membranes were exposed to BioMax MS films at −80°C using intensifying screens.

PI 3-kinase Activity Assay. PI 3-kinase activity was assayed in IRS-2 immunoprecipitates that were prepared as described above. Immunoprecipitates were sequentially washed with buffer A (PBS containing 1% NP-40 and 1 mM DTT), buffer B (100 mM Tris (pH 7.4), 0.5 M LiCl, and 1 mM DTT), and buffer C (10 mM Tris, 100 mM NaCl, and 1 mM DTT), and resuspended and incubated for 5 min at 4°C in 10 µl of buffer C (9). To assay for phosphorylation activity, 20 µl of PI substrate (0.5 mg/ml) were dispersed by sonication for 15 min in 50 mM HEPES (pH 7.6), 1 mM EGTA, and 1 mM NaH2PO4 before use and added to the buffer solution. After a 5-min incubation at 23°C, the reaction was started by the addition of 10 µl of 5 X ATP solution (50 mM MgCl2, 100 mM HEPES, and 250 µM ATP including 10 nCi/µl [γ-^32P]ATP). The reaction was stopped after 5 min by adding 4 N HCl (15 µl). To separate the phospholipids, the solution was vortexed after the addition of 130 µl of chloroform:methanol (1:1). The phospholipid containing chloroform phase (35 µl) was spotted onto TLC plates coated with 1% potassium oxalate and activated at 110°C for 90 min. Phosphorylated products were separated in chloroform:methanol:NH4OH:H2O (600:470:20:113) until the solvent reached the top of the plates. The dried plates were exposed to Kodak Biomax Light films at −80°C using intensifying screens.

Patients and Tissue Samples. Normal human pancreatic tissue samples were obtained through a donor program. There were six female and eight male organ donors with a median age of 26 years (range, 2–54). Pancreatic adenocarcinoma tissues were obtained from 10 female and 6 male patients having a median age of 64 years (range, 44–77). All cancer patients underwent surgery for pancreatic cancer as recently described (10). Samples were either immediately frozen upon surgical removal in liquid nitrogen and stored at −80°C until RNA extraction, or fixed in Bouin’s solution for 18–20 h and embedded in paraffin for histological analysis. Studies involving human tissues were approved by the Ethics Committee of the University of Ulm and the Human Subjects Committee of the University of California.

Immunohistochemistry. Paraffin-embedded 4-µm tissue sections were immunostained using the streptavidin-peroxidase technique, as described previously by Kornmann et al. (10). After deparaffinization and blocking endogenous peroxidase activity, the sections were incubated for 15 min at 23°C in 10% normal goat serum and for 16 h at 4°C with a rabbit polyclonal antibody against IRS-2 (0.5 µg/ml) that was also used for immunoblotting. Bound antibodies were detected with biotinylated goat antirabbit IgG secondary antibodies and streptavidin-peroxidase complex, using diaminobenzidine tetrahydrochloride as the substrate. Sections were counterstained with Mayer’s hematoxylin. Omission of primary antibodies or incubation in the presence of nonimmunized rabbit IgG instead of primary antibodies did not yield any immunoreactivity.

Statistics. Statistical analysis was performed with SigmaStat software (Jandel Scientific, San Rafael, CA). The Rank Sum Test was used and a P < 0.05 was taken as the level of significance.

Results
IRS-2 expression in ASPC-1 and COLO-357 cells was characterized by immunoblotting using highly specific rabbit polyclonal antibodies against IRS-2 (11) and by Northern blotting using a 1.0-kb mouse IRS-2 cDNA fragment (3, 8). The bands representing the M, 190,000 IRS-2 protein and the 7.2-kb IRS-2 mRNA transcript (3) were readily evident in both cell lines (Fig. 1, A and B). The effects of insulin, IGF-I, and IGF-II on tyrosine phosphorylation of IRS-2 and IRS-2-associated PI 3-kinase activity were investigated next. Analysis of the tyrosine phosphorylation status of the IRS-2 immunoprecipitates with PY20 antibodies revealed that insulin and IGF-I enhanced tyrosine phosphorylation of IRS-2 in ASPC-1 cells, whereas IGF-II was without effect (Fig. 1C). In contrast, in COLO-357 cells, all three growth factors enhanced IRS-2 tyrosine phosphorylation (Fig. 1D). In both cell lines, the magnitude of this stimulatory effect was greatest with insulin. A PI 3-kinase activity assay after IRS-2 immunoprecipitation revealed that all three growth factors enhanced PI 3-kinase activity in both cell lines (Fig. 1, E and F). However, the effect of IGF-II on PI 3-kinase activity in ASPC-1 cells was relatively modest.

To further assess the role of PI 3-kinase activation in insulin- and IGF-mediated mitogenesis in ASPC-1 and COLO-357 cells, we next compared the growth stimulatory effects of these ligands in the absence or presence of the selective PI 3-kinase inhibitor LY 294002. In both cell lines, the same dose of the stimulatory ligands, the growth rate was significantly reduced in the presence of LY 294002 (Fig. 2, A–D). The data clearly demonstrate that PI 3-kinase activation is necessary for the growth stimulatory effects of insulin and IGF-II in both cell lines.
Northern blot analysis revealed the presence of variable levels of the 7.2-kb IRS-2 mRNA transcript (3) in normal and cancerous pancreatic tissues (Fig. 3A). After prolonged exposure of the autoradiographs, the IRS-2 mRNA transcript was detectable in all normal and cancerous tissue samples. Densitometric analysis with normalization to 28S indicated that the median level of IRS-2 mRNA was increased by 1.4-fold in the cancer tissues in comparison with the median level in the normal pancreatic samples \((P = 0.032)\). Overall, 4 of the 16 cancer samples displayed IRS-2 mRNA levels that clearly exceeded the IRS-2 mRNA levels observed in the 12 normal samples tested (Fig. 3B).

Immunohistochemical analysis using the same highly specific anti-IRS-2 antibody that was used for immunoblotting was performed next. In the normal pancreas, faint IRS-2 immunoreactivity was present in some ductal and acinar cells (Fig. 4, A and B), and moderate IRS-2 immunoreactivity was present in at least some islet cells in all of the endocrine islets (Fig. 4A and B, arrowheads). In the pancreatic cancer samples, a similar pattern of IRS-2 immunoreactivity was seen in the endocrine islets adjacent to the pancreatic cancer cells (data not shown). In addition, moderate to strong IRS-2 immunoreactivity was present in many of the ductal-like cancer cells (Fig. 4, C and D).

Discussion

IRS-1 and -2 are homologous docking proteins that mediate the actions of IGF-I and insulin (4, 12). The presence of over 30 unique phosphorylation motifs on each of the two IRS proteins enables them to interact with a variety of down-stream signaling molecules (4). In addition to their importance for insulin/IGF-I signaling, IRS proteins contribute to the signal transduction of several cytokine receptors (4, 13), underscoring the potential importance of these docking molecules for mediating intracellular signaling of a variety of extracellular stimuli.

Previously, we reported that IRS-1 is overexpressed in human pancreatic cancer (6). In the present study, we determined that IRS-2 is also overexpressed in this malignancy. Although there was only a 40% increase in IRS-2 mRNA levels in the pancreatic cancers, this increase was statistically significant by comparison with the levels observed in the normal pancreas. By immunohistochemistry, IRS-2 was consistently present in the endocrine islets in the normal pancreas, raising the possibility that IRS-2 may participate in the regulation of endocrine functions in the human pancreas. In addition, faint IRS-2 immunoreactivity was present in some ductal and acinar cells. These observations suggest that a significant component of the IRS-2 signal observed by Northern blotting of normal pancreatic RNA derives from both the endocrine and exocrine cells. In contrast, only faint IRS-2 immunoreactivity was evident in the abundant stromal elements in the

![Fig. 3. IRS-2 mRNA expression in pancreatic tissues. A, Northern blot analysis of total RNA (20 μg/lane) from six normal and nine pancreatic cancer samples was carried out as described in the legend to Fig. 1B. The 28S cDNA was used as loading control. The migration site of the 28S rRNA is indicated on the left. B, comparison of the relative IRS-2 mRNA expression in normal and cancerous (n = 16) pancreatic tissues. Autoradiographs for IRS-2 and 28S were scanned and analyzed by densitometry. The ratio of IRS-2 and 28S (arbitrary units) was then plotted for normal \((n = 14)\) and cancerous \((n = 16)\) samples. Pancreatic cancers displayed a higher median IRS-2 expression \((dashed lines)\) than the normal samples \((P = 0.032)\).](image-url)
pancreatic cancer samples, whereas a strong IRS-2 signal was present in the pancreatic cancer cells. Taken together, these findings suggest that the pancreatic cancer cells within the tumor mass express higher levels of IRS-2 than indicated by Northern blotting.

Several lines of evidence from our in vitro studies suggest that IRS-2 contributes to enhanced mitogenic signaling in pancreatic cancer. First, IRS-2 was expressed in both tested pancreatic cancer cell lines. Second, insulin, IGF-I, and IGF-II enhanced tyrosine phosphorylation of IRS-2 and IRS-2-associated PI 3-kinase activity in COLO-357 cells, and IGF-I and insulin exerted similar effects in ASPC-1 cells. Inasmuch as IGF-II did not enhance tyrosine phosphorylation of IRS-2 in ASPC-1 cells and only minimally stimulated IRS-2-associated PI 3-kinase activation in these cells, it is likely that IGF-II enhances the growth of these cells via a different mechanism. Third, in both cell lines inhibiting PI 3-kinase activity with concentrations of LY 294002 that did not alter basal growth markedly attenuated the effect of all three growth factors on mitogenesis. Taken together, these observations suggest that IRS-2 is an important mediator of mitogenic signaling in pancreatic cancer cells, and that this effect is mediated, in part, via PI 3-kinase.

Overexpression of IRS-1 results in an increase in mitogenic response to insulin and IGF-I (14-16), whereas a decrease in IRS-1 levels diminishes this response (17, 18). Despite the 43% amino acid sequence homology between IRS-2 and IRS-1 (4), these molecules may mediate distinct signals. Thus, studies with knockout mice suggest that IRS-1 may best mediate the biological actions of IGF-I, whereas IRS-2 may preferentially mediate the activation of insulin on glucose transport (3, 19). In contrast, studies with cultured cell lines have suggested that IRS-2 may be better adapted to mediate mitogenic signaling than IRS-1 (8). However, when IRS-1 or IRS-2 were expressed in IRS-1-deficient cells, both proteins were required for a complete mitogenic response, indicating that they are not functionally interchangeable with respect to mitogenesis (20). Furthermore, in estrogen receptor positive breast cancer cells IRS-1 is the major signaling molecule activated by insulin and IGF-I (21), whereas in RINm5F insulin-secreting cells IRS-2 seems to mediate the actions of IGF-I on mitogenesis (22). We have previously shown that IGF-I enhances tyrosine phosphorylation of IRS-1 in A5PC-1 and COLO-357 cells (7). Thus, the concomitant overexpression of IRS-1 (6) and IRS-2 (present findings) in human pancreatic cancer underscores their potential importance in conferring a growth advantage onto pancreatic cancer cells.

Previous studies have demonstrated that pancreatic cancers overexpress IGF-I and IGF-IR (5). Although insulin and IGF-II are not overexpressed in this malignancy, both are present in endocrine islet cells, which are dispersed throughout the exocrine pancreas (23). Furthermore, as a consequence of the existence of an intrapancreatic portal circulation, portions of the exocrine pancreas are exposed to high levels of islet cell hormones (23). This anatomical arrangement may allow for islet cell hormones to act via a so-called proxocrine mechanism on pancreatic exocrine cells, including cancer cells (23). Inasmuch as insulin and IGF-II bind and activate the IGF-IR (12), our
findings suggest that there is a potential for IGF-I, insulin, and IGF-II to excessively activate mitogenic signaling in pancreatic cancer cells by acting via autocrine, paracrine, and proxicrine mechanisms on the overexpressed IGF-IR, IRS-1, and IRS-2. Therefore, pharmacological or genetic interference with this pathway may have an important therapeutic potential in pancreatic cancer.

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References

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