

Src Stimulates Insulin-like Growth Factor I (IGF-I)-dependent Cell Proliferation by Increasing IGF-I Receptor Number in Human Pancreatic Carcinoma Cells¹

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Abstract

We examined the potential function of Src in human pancreatic carcinoma. Overexpression of kinase-activated SrcY527F resulted in a significant increase of insulin-like growth factor I (IGF-I)-dependent cell proliferation in the cell line PANC-1. Western blotting and competition binding studies demonstrated 2.3 ± 0.2 -fold increase in IGF-I receptor expression and 2.8 ± 0.4 -fold increase in IGF-I-specific binding sites/cell. SrcY527F transfection alone did not change receptor affinity or basal receptor tyrosine phosphorylation, whereas IGF-I-stimulated receptor phosphorylation was increased by 2.1 ± 0.5 -fold. IGF-I mRNA expression and protein secretion did not change to exclude autocrine activation. We conclude that Src stimulates IGF-I-dependent proliferation of PANC-1 cells by increasing the number of IGF-I receptors/cell.

Introduction

Src is a cytoplasmic membrane-associated, nonreceptor protein tyrosine kinase that participates in the regulation of cell adhesion, cell growth, and differentiation (1). Src functions as a cotransducer of mitogenic signals from a number of tyrosine kinase growth factor receptors (2). Interaction with the receptors for platelet-derived growth factor, epidermal growth factor, or colony-stimulating factor 1 is bidirectional, *i.e.*, Src is activated by these receptors and may itself regulate receptor activation by phosphorylation or binding (2). Our previous results demonstrate that Src is overexpressed in human exocrine pancreatic carcinoma cells. Furthermore, Src is overexpressed in 76% of pancreatic carcinoma cell lines and activated in most of them (3). Src expression increases early during tumorigenesis (4), and a role for growth control seems likely because herbimycin A is able to suppress growth and kinase activity (3).

The ability of Src to increase cell growth of fibroblasts in soft agar or in serum-free medium is dependent on its activity and on coexpression of growth factor receptors. Wild-type Src, even when overexpressed, is not very effective in transforming cells (5). When tyrosine residue 527 is mutated to phenylalanine (SrcY527F) in cellular Src (c-src) to prevent phosphorylation and folding of the protein, Src activity is increased, and mutated Src becomes able to partially transform NIH 3T3 cells (5). However, this transformation by SrcY527F is dependent on the expression of functional IGF-I³ receptors. Full transformation of cells that lack IGF-I receptors is only observed after transfection with the permanently active viral src

(v-src; Ref. 6). The transforming ability of Src correlates with phosphorylation of the IGF-IR in chicken embryonic fibroblasts (7), and coexpression of IGF-IRs, together with temperature-sensitive mutants of Src in Rat-1 fibroblasts, indicates that Src may phosphorylate and activate the IGF-IR (8).

The IGF-IR is a tetrameric glycoprotein with an $\alpha_2\beta_2$ structure in which the α subunits (M_r 130,000) contain the ligand-binding domain and where the β subunits (M_r 95,000) possess intrinsic tyrosyl protein kinase activity (9). The IGF-IR binds its homologous ligand, IGF-I, with a K_d of ~ 1 nM, which is followed by rapid autophosphorylation on tyrosine residues of the β -subunit and activation of the kinase activity of the receptor. Many tumors express IGF-IR and secrete IGF-I (10), and growth regulation by autocrine stimulation has been suggested (11). Expression of IGF-IR mRNA as well as overexpression of IGF-I mRNA could be demonstrated in human pancreatic tumors. Because antibody neutralization of IGF-I or an antisense oligonucleotide-induced decrease in IGF-IR number inhibited cell growth, the IGF-I system seems to participate in growth regulation of pancreatic tumor cells (12).

The role of Src in pancreatic cancer cell growth is unknown. We speculated that Src may stimulate proliferation of pancreatic tumor cells through modification of the IGF-IR and examined whether Src was able to regulate IGF-I-dependent cell growth in the pancreatic cell line PANC-1, one of the rare cell lines with low intrinsic expression and activity of Src (3). Permanently active SrcY527F was overexpressed to observe IGF-IR expression, binding characteristics and activation, as well as potential autocrine activation of the IGF-IR by secretion of IGF-I.

Materials and Methods

Materials. IGF-I was from Bachem (Heidelberg, Germany), and [¹²⁵I]-labeled IGF-I (2 μ Ci/mmol) was from Amersham Buchler (Braunschweig, Germany). Monoclonal antibodies against phosphotyrosine (clone PT66) were from Sigma (Deisenhofen, Germany); against the IGF-IR β -subunit, from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA); and against the α -subunit of the IGF-IR (clone α IR3) and Src (clone 327), from Dianova (Hamburg, Germany). All tissue culture reagents were obtained from Life Technologies, Inc. (Eggenstein, Germany). Permanently active src plasmid (pSGT-SrcY527F) was a kind gift of S. Courtneidge (EMBO, Heidelberg, Germany).

Cell Culture. PANC-1 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom). Cells were maintained under standard culture conditions in DMEM with high glucose and 10% fetal bovine serum.

Plasmids and Cell Transfection. Deregulated Src (pSGT-SrcY527F) was subcloned into pcDNA3 (Invitrogen, Leek, the Netherlands) by *Eco*RI. Transfection with pcDNA3-SrcY527F or vector control was performed using the Ca_2PO_4 -precipitation method. Transfected clones were selected by incubation with 850 mg/L of G-418 sulfate. Isolated clones were screened for expression of Src protein or phosphotyrosine content by Western blotting, and the presence of vector or Src construct was confirmed using PCR. Of 82 clones, 2 clones with maximum Src expression and elevated phosphotyrosine content were selected for further analysis.

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³ The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor.

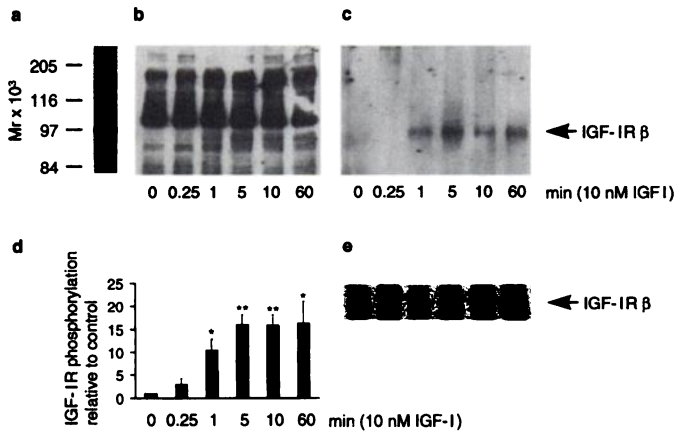


Fig. 1. IGF-IR expression and tyrosine phosphorylation in PANC-1 cells. Total cell lysates were examined for expression of the β -subunit of the IGF-IR by Western blotting (a) or for protein tyrosine phosphorylation (b) after stimulation with IGF-I. Tyrosine phosphorylation of the IGF-IR β is demonstrated in panel c after coimmunoprecipitation with the α subunit and phosphotyrosine Western blotting. Densitometrical analysis of receptor phosphorylation from three experiments is demonstrated in panel d. Values indicate means \pm SE. Significance levels for comparisons with controls are indicated by * ($P \leq 0.05$) or ** ($P \leq 0.005$). Control of immunoprecipitation was performed in Western blots with anti- β subunit antibodies (e).

Western Blotting and Immunoprecipitation. Cells were plated at 2×10^6 cells/10-cm cell culture dish, were allowed to grow for 24 h, and were serum starved overnight. Following stimulation with 10^{-8} M IGF-I or buffer control, cells were washed twice in ice-cold PBS, were lysed by scraping into lysis buffer [50 mM Tris-HCl (pH 7.2), 150 mM sodium chloride, 5 mM EDTA, 1% NP40, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml leupeptin, 1.4 μ g/ml aprotinin, 10 μ g/ml pepstatin A, and 0.5 mM benzamide], and were left on ice for 10 min. For immunoprecipitation, cell lysates containing 200 μ g of protein were incubated with 0.5 μ g of antibody to the α -subunit of IGF-IR for 1 h at 4°C. After addition of rabbit anti-mouse antibody-preincubated protein G plus agarose for 1 h, immunoprecipitates were washed three times with lysis buffer. Proteins were subjected to 7.5% SDS-PAGE and Western blotting (3). Membranes were blocked and incubated with antibodies in Tris-buffered saline [10 mM Tris-HCl (pH 7.2) and 150 mM sodium chloride] containing 2% BSA/0.2% Tween 20 for anti-phosphotyrosine antibodies and in Tris-buffered saline containing 2% nonfat dry milk for all other antibodies. Staining was visualized using the Super Signal enhanced chemiluminescence system (Pierce, Rockford, IL).

Growth Assays. Cells were plated in six-well plates at a density of 10^5 cells/well and were allowed to attach in medium with 10% serum for 12 h. After two washing steps, serum-free medium with or without 10^{-8} M IGF-I was added. After the indicated time periods, cells were trypsinized, and the number of vital cells/well was determined by counting. Cell viability was assessed by trypan blue exclusion.

Receptor Binding Studies. Ligand binding studies were performed as described (13). Briefly, 3×10^5 cells/well were plated in six-well plates in medium containing 10% FCS and were allowed to adhere for 24 h. They were then serum starved overnight, washed twice with binding buffer [50 mM HEPES (pH 7.8), 120 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium sulfate, 10 mM glucose, 15 mM sodium acetate, 10 mM calcium chloride, and 0.1% human serum albumin], and incubated with 10^{-11} M [125 I]-labeled IGF-I (2 μ Ci/mmol) together with increasing concentrations of unlabeled hormone at room temperature for 1 h. Bound and free hormones were determined by gamma counting. Degradation of [125 I]-labeled IGF-I was $<10\%$. Scatchard analysis was performed as described (13).

IGF-I Levels. Cell culture supernatants were concentrated 10-fold by centrifugation in 5K Ultrafree tubes (Millipore, Bedford, MA). IGF-I concentrations were determined by ELISA (Mediagnost, Tübingen, Germany) with cross-reactivity to IGF-II of $<0.05\%$.

RNA Isolation and Northern Blot Analysis. Total RNA was extracted using the acid guanidinium thiocyanate extraction method or the RNeasy-Kit (Qiagen, Hilden). RNA was fractionated in 1% agarose/5.7 M formaldehyde

gels, transferred onto nylon membranes (Hybond N⁺; Amersham Buchler) using the capillary blot technique, and cross-linked by UV irradiation. The blots were prehybridized and hybridized with a 32 P-labeled human IGF-I cDNA probe and washed under high stringency conditions. After washing, blots were exposed to Kodak X-OMAT AR films. Equivalent loading of RNA in each lane was confirmed by ethidium bromide staining of gels.

Statistics. Results are given as means \pm SE of at least three independent experiments. For comparisons, Student's *t* test for unpaired samples was used. $P < 0.5$ was judged significant.

Results

PANC-1 Cells Express Functional IGF-IRs. We first examined expression of IGF-IR in PANC-1 cells (Fig. 1a). Using antibodies against the IGF-IR β chain, a major band migrating at M_r 95,000 could be demonstrated by Western blotting. The band migrating at M_r 205,000 represents the precursor molecule. To demonstrate receptor function, cells were stimulated with IGF-I. As shown in Fig. 1b, 10 nM IGF-I stimulated rapid and reversible tyrosine phosphorylation of several proteins, one of them migrating at M_r 95,000. This M_r 95,000 protein comigrated with the IGF-IR β -subunit. Its identity was confirmed by immunoprecipitation with antibodies against the α -subunit that coprecipitated a M_r 95,000 phosphoprotein (Fig. 1c) and by staining of this band with antibodies directed against the β -subunit (Fig. 1e). Stimulation with IGF-I increased receptor β -subunit phosphorylation to 16.0 ± 2.1 -fold of control after 5 min, and phosphorylation remained elevated for up to 1 h.

Activation of Src Stimulates IGF-I-dependent Cell Growth. Under serum-free conditions, cell number in vector controls increased 1.6 ± 0.14 -fold at day 3. Stable transfection of PANC-1 cells with permanently active Src resulted in a slight nonsignificant increase in cell number at day three to 2.4 ± 0.37 -fold basal in transfected cells. A similar nonsignificant increase was observed after the addition of 10^{-8} M IGF-I to vector controls (2.3 ± 0.27 -fold). In contrast, the addition of IGF-I to Src-transfected cells was synergistic and increased cell number to 3.9 ± 0.47 -fold basal (Fig. 2). This increase was significant when compared with unstimulated Src-transfected cells ($P < 0.04$) or to IGF-I-stimulated vector controls ($P < 0.02$). The observed increase in cell number was clearly higher than a purely additive effect, which would have increased the expected cell count to 3.0-fold. Hence, activation of Src increases responsiveness of cell growth to stimulation with IGF-I.

Activation of Src Increases IGF-IR Number. In response to transfection with activated Src, protein levels of IGF-IR in whole-cell lysates increased significantly. As examined by Western blotting (Fig.

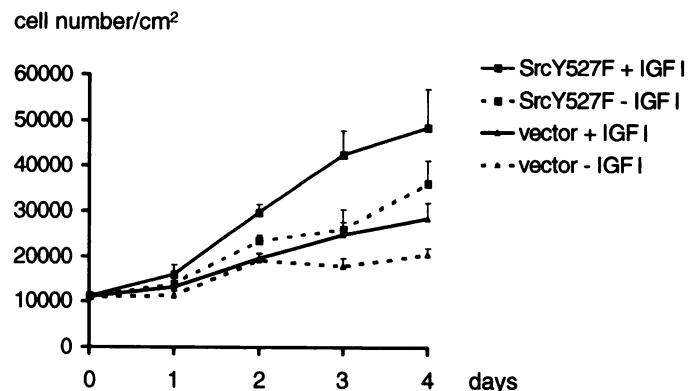


Fig. 2. Effects of IGF-I and SrcY527F on cell proliferation. Cell numbers were counted under serum-free conditions after the indicated time periods with or without addition of IGF-I (10 nM) in cells that had been transfected with the pcDNA3 vector alone or with the pcDNA3-SrcY527F construct. Shown are the mean values of five independent experiments; bars, SE.

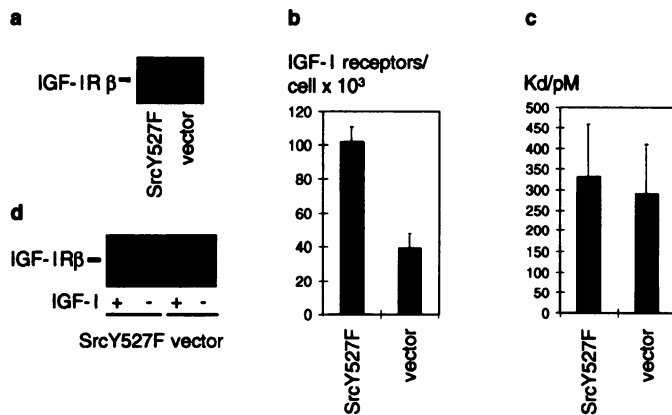


Fig. 3. IGF-IRs after transfection with SrcY527F. IGF-IR β -subunit expression was examined in whole-cell lysates by Western blotting (a). Receptor density (b) and affinity (c) were determined by competition binding studies in whole cells. Bars, SE. IGF-IR tyrosine phosphorylation was examined after stimulation with IGF-I (10 nM) for 5 min by phosphotyrosine Western blotting after immunoprecipitation of the IGF-IR (d).

3a), β -subunit expression increased 2.3 ± 0.24 -fold as compared with vector controls. This increase in receptor protein expression resulted in a significant increase in IGF-I-specific high affinity binding sites to 2.78 ± 0.42 -fold the control value (Fig. 3b). Under the same conditions, receptor affinity did not change (Fig. 3c). Therefore, posttranslational modification of the IGF-IR by Src may occur but does not seem to change receptor affinity. This is further supported by receptor phosphorylation studies, where basal IGF-IR tyrosine phosphorylation did not noticeably change in response to activation of Src, whereas IGF-I-stimulated phosphorylation increased after transfection in a magnitude similar to the increase in receptor protein (Fig. 3d). Although these data virtually exclude direct or indirect activation of the IGF-IR by Src, we did measure IGF-I expression to examine the possibility of autocrine stimulation. PANC-1 cells did not express detectable amounts of IGF-I mRNA, either under control conditions or after activation of Src (not shown). In addition, although IGF-I concentrations in the supernatant did slightly increase after transfection ($1.5 \pm 0.3 \times 10^{-12}$ M in vector controls versus $2.4 \pm 0.8 \times 10^{-12}$ M in transfected cells), concentrations were at least two orders of magnitude below the IC_{50} of the IGF-IR (10^{-9} M), and growth-stimulatory effects through activation of the IGF-IR by IGF-I, therefore, are rather unlikely.

Discussion

The tyrosine kinase Src is overexpressed and active in most pancreatic tumors and pancreatic tumor cell lines (3). Here, we examine the potential growth-regulatory function of this kinase. We report for the first time that activation of Src increases IGF-I-dependent cell growth of the pancreatic carcinoma cell line PANC-1 through up-regulation of IGF-IR number.

IGF-I is a major mitogenic factor *in vivo* that exerts its action largely through binding to the IGF-IR (9). IGF-I and its receptor can contribute to neoplastic transformation (14). Targeted overexpression of IGF-I induces hyperplasia, dermal abnormalities, and spontaneous tumor formation in transgenic mice (15). Constitutive activation of the IGF-IR or overexpression and ligand occupation results in growth and transformation in a variety of epithelial and mesenchymal cell types (9) and protects cells from apoptosis induced by cytostatic drugs or ischemia (16). In pancreatic cancer tissue, aberrant activation of IGF-IRs by paracrine or autocrine mechanisms has been suggested because IGF-I mRNA expression is increased in tumor tissue compared with normal pancreas (12). The functional importance of IGF-IR was demonstrated by the addition of IGF-IR antisense oligonucleotides, which decreased basal and IGF-I-stimulated cell growth

of the pancreatic carcinoma cell line ASPC-1 (12). The mechanisms that lead to increased IGF-I or IGF-IR expression in the pancreas are unknown.

Our experiments, where activation of Src increased expression of functionally active IGF-IR, demonstrate one possible mechanism of increasing sensitivity to stimulation with IGF-I. It is well known that receptor density can be the determining factor of response to growth-regulatory peptides. In fibroblasts, IGF-IR number needs to be $>20,000$ receptors/cell to enable mitogenesis after stimulation with IGF-I (17). Similarly, in the breast carcinoma cell line MCF-7, IGF-I becomes mitogenic only after the number of IGF-IRs has been increased by estrogen treatment (18). In our model, the observed increase in receptor number from 40,000 receptors/cell to 100,000 receptors/cell is in the range required in 3T3 cells to enable IGF-I-stimulated growth in soft agar and therefore may be of central importance for pancreatic tumor growth control. However, in contrast to the data by Bergmann *et al.* (12), our results do not support constitutive activation of the IGF-IR because basal phosphorylation of the receptor did not increase after activation of Src and because IGF-I was required to stimulate growth, even after up-regulation of receptor number.

In vivo, activation of pancreatic IGF-IR might occur through autocrine or paracrine mechanisms. Published evidence suggests that IGF-I in pancreatic adenocarcinoma is produced in the stromal tissue with some expression in single cancer cells (11, 12). Our data do not support regulation of pancreatic tumor growth by autocrine stimulation through IGF-I because: (a) addition of IGF-I was required to increase cell number significantly; and (b) activation of Src did not increase IGF-I mRNA or protein levels. Therefore, paracrine stimulation, *e.g.*, through release of IGF-I from stromal cells, likely accounts for stimulation of the IGF-IR in cancer cells. This requirement of pancreatic cancer cells for stimulation with IGF-I may be one of the reasons why pancreatic adenocarcinoma is composed largely of stromal tissue and why pancreatic tumor cells tend to invade perineural spaces, which are known to express high concentrations of IGF-I (19). Similarly, the high concentration of hepatocyte-derived IGF-I in liver tissue may facilitate growth of liver metastases (20). Together, our observations suggest that Src-induced up-regulation of IGF-IR contributes to the growth characteristics of pancreatic adenocarcinoma. Therapies directed toward Src may be of value in human pancreatic cancer.

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