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ORIGINAL PAPER

Src activation of Stat3 is an independent requirement from NF-κB activation for constitutive IL-8 expression in human pancreatic adenocarcinoma cells

Jose G. Trevino · Michael J. Gray · Steffan T. Nawrocki · Justin M. Summy · Donald P. Lesslie · Douglas B. Evans · Tomi K. Sawyer · William C. Shakespeare · Stephanie S. Watowich · Paul J. Chiao · David J. McConkey · Gary E. Gallick

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Abstract Human pancreatic tumors often overexpress the angiogenesis-promoting factor Interleukin 8 (IL-8), in part due to overexpression of NF-κB, a frequent occurrence in pancreatic adenocarcinoma. In this study, we demonstrate that reducing c-Src kinase activity, through either pharmacologic inhibition or small interfering RNA-targeted reduction of Src expression, significantly decreased IL-8 expression ($P < 0.05$) without affecting NF-κB-mediated transcription, but by decreasing phosphorylation of STAT3. To ascertain whether Src-mediated expression of IL-8 was dependent on STAT3, we used stable clones expressing a dominant-negative isoform of STAT3 that inhibits endogenous STAT3 phosphorylation and subsequent DNA binding and STAT3-mediated gene expression or a constitutively activated isoform of STAT3. IL-8 expression was significantly lower in clones expressing the dominant-negative isoform and significantly increased in clones expressing the activated isoform ($P < 0.05$ for both). Pharmacologic inhibition of NF-κB activity significantly reduced basal IL-8 expression and tumor necrosis factor-induced IL-8 expression ($P < 0.05$ for both), yet NF-κB activity was not dependent on Src. We therefore suggest that Src activation, through phosphorylation of STAT3, and NF-κB are all required for expression of IL-8 a critical angiogenic-promoting factor in pancreatic adenocarcinomas.

Keywords Angiogenesis · IL-8 · Src · NF-κB · STAT3 · NF-kappaB · Pancreatic adenocarcinoma

Jose G. Trevino and Michael J. Gray contributed equally to the work in this manuscript.

J. G. Trevino · M. J. Gray · S. T. Nawrocki · J. M. Summy · D. P. Lesslie · D. J. McConkey · G. E. Gallick (✉)
Department of Cancer Biology, Unit 179, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA
e-mail: ggallick@mdanderson.org

D. B. Evans · P. J. Chiao · J. G. Trevino · D. P. Lesslie
Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

S. S. Watowich
Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030, USA

S. T. Nawrocki · D. J. McConkey · G. E. Gallick
Program in Cancer Biology, The University of Texas Graduate School of Biomedical Sciences, Houston, TX, USA

T. K. Sawyer · W. C. Shakespeare
Ariad Pharmaceuticals, Inc., Cambridge, MA, USA

Introduction

Pancreatic adenocarcinoma is the fourth most common cause of cancer death in developed countries, and in the United States alone, approximately 30,000 deaths are attributed to this disease each year [1]. The disease is usually diagnosed at an advanced stage, at which point surgical resection is not a viable option. Treatment is often complicated by the predisposition of pancreatic adenocarcinomas to metastasize and its resistance to conventional therapeutic intervention [2, 3].

Pancreatic tumors are highly vascular and produce multiple cytokines that promote angiogenesis. One factor produced in abundance by most pancreatic tumors is Interleukin-8 (IL-8) [4, 5]. The highly

aggressive nature of these cancers is partly attributed to this factor. IL-8 is often overexpressed in surgical specimens of pancreatic cancer tissues [6], and its expression typically correlates with metastatic potential and tumor growth [7–9]. In pancreatic orthotopic models, reduction of IL-8 expression by protein tyrosine kinase inhibition results in decreased growth and metastasis of human pancreatic cancer cells [10]. The exact mechanisms that regulate IL-8 expression in pancreatic cancer cells have not been fully elucidated, although one study conducted in normal tissues suggests that phosphatidylinositol 3-kinase and its downstream mediator Akt control IL-8 expression through activation of transcription mediated by nuclear factor κB (NF-κB) [11]. In human aortic endothelial cells, Src family kinases (SFKs) were shown to regulate IL-8 expression independently of NF-κB through the activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) [12]. Whether activation of one or both of these pathways is required for the high levels of constitutive IL-8 expression in pancreatic tumor cells is unknown; therefore, in this work we examined the contributions of both Src and NF-κB in IL-8 expression in pancreatic tumor cell lines in vitro.

c-Src is a 60-kDa member of the structurally related SFK non-receptor protein tyrosine kinases. The SFKs are implicated in such critical cellular processes as proliferation, adhesion, migration, and angiogenesis [13]. Src overexpression occurs in 70% of pancreatic tumors [14]. In pancreatic cancers, inhibition of Src retards primary tumor growth in orthotopic models [15]. Furthermore, c-Src expression and activation directly correlated with resistance to chemotherapeutic agents in xenografts of human pancreatic cancer in mouse models [16]. Recently, we have demonstrated that Src inhibition by molecular and pharmacologic approaches decreases the ability of pancreatic tumor cells to metastasize in orthotopic mouse models [17]. Src is also a critical regulator of pro-angiogenic molecules [18, 19]. More specifically, Src activation leads to increased IL-8 expression in pancreatic cancer cells and Src inhibition significantly decreases this expression [19]. As we have also recently demonstrated that Src regulation of STAT3 phosphorylation is critical to VEGF expression in these cells [20], in this study, we examined the role of Src and STAT3 in IL-8 expression.

STAT3 belongs to a seven-member family of closely related transcription factors. Latent STAT3 is cytoplasmic, but its activation results in dimerization and nuclear translocation and is followed by STAT-specific gene transcription [21]. The receptor-associated specific Janus kinases (JAKs) were initially identified as STAT activators, but activation of STAT (including STAT3) can occur independently of JAKs by other kinases, including Src [20–23]. STAT3 is involved in numerous cellular processes, including proliferation, angiogenesis, and anti-apoptosis [21]. Constitutive activation and overexpression of STAT3 contribute to malignant transformation and directly correlate with tumor growth [24–26], by transcriptionally activating diverse genes associated with protease activation, angiogenesis, and survival.

In this study, we demonstrate that Src activity directly correlates with IL-8 expression levels and that STAT3 is a downstream target that controls Src-mediated induction of IL-8. We also show that NF-κB regulates IL-8 expression in a Src-independent manner. Thus, IL-8 transcription and expression appear to be mediated through multiple pathways, and therefore, specifically targeting upstream mediators of IL-8 for anti-angiogenesis therapy may require the downregulation of both NF-κB and Src/STAT3.

Materials and methods

Cell lines

PANC-1 human pancreatic cells of ductal origin were obtained from American Type Culture Collection (Rockville, MD) and grown in modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine, and 0.6% penicillin and streptomycin. L3.6pl human pancreatic adenocarcinoma cells were derived as previously described [27] and maintained in similar conditions. All cells were incubated under standard conditions (5% CO_2 and 95% air at 37°C).

Cell lysis and protein extraction

A total of 1×10^6 cells were plated in 10-cm dishes and maintained in modified Eagle's medium with 10% fetal bovine serum. At 70–80% confluence, the cells were washed with Dulbecco's phosphate-buffered saline (D-PBS) at 37°C and maintained in 5 ml of serum-free medium for 1 h with the desired concentration of inhibitor or an equal volume of dimethyl sulfoxide (DMSO). The cells were then washed and replaced with fresh serum-free medium and inhibitor (or an equal volume DMSO) for an additional 23 h. The cells and supernatants were harvested at 24 h. The cells were washed with ice-cold 1 × D-PBS, scraped from the plates, lysed, and harvested on ice in RIPA B buffer (20 mM sodium phosphate buffer, 150 mM

sodium chloride, 5 mM EDTA, 1% Triton X-100, and 0.5% sodium deoxycholate) supplemented with one tablet of complete mini-EDTA protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) and sodium orthovanadate (1 mM, pH 7.4). The homogenates were clarified by centrifugation at 15,000*g* for 15 min at 4°C and prepared for Western blot analysis and immunoprecipitation.

Reagents and primary antibodies

The selective small molecule Src inhibitor, AP23846 (ARIAD Pharmaceuticals, Cambridge, MA), the IκK inhibitor PS-1145 (Millennium Pharmaceuticals, Cambridge, MA), and the IκBα proteosome inhibitor PS-341 (Bortezomib; Millennium Pharmaceuticals) were used in these studies. All inhibitors were solubilized in DMSO and stored at – 80°C until use. Primary antibodies used were against c-Src (mAb 327; Oncogene Sciences, Cambridge, MA), phospho-Src (Y416) (Cell Signaling Technology, Boston, MA), STAT3 and phospho-STAT3 (Ser705; Upstate Biotechnology, Lake Placid, NJ), vinculin (Sigma-Aldrich, St. Louis, MO), and NF-κB p65 (Santa Cruz Biotechnology, Santa Cruz, CA).

Creation of Src-targeted siRNA expression plasmids

siRNA expression plasmids were created using the Ambion pSilencer 1.0-U6 (Austin, TX) according to the manufacturer's directions. c-Src-specific target sequences were designed using the Ambion siRNA web design tool (http://www.ambion.org). The target sequences used were (52–71 bp) 5′-AACAAGAG CAAGCCCAAGGAT-3′ and (226–244 bp) 5′-AA GCTGTTCGGAGGCTTCAAC-3′. Oligonucleotides corresponding to these sequences with flanking *Apa*1 (5′) and *Eco*R1 (3′) ends were purchased from Invitrogen/Life Technology (Carlsbad, CA) and ligated into the expression plasmid at compatible sites. Constructs were confirmed by DNA sequencing.

Creation of stable cell lines

For siRNA-targeted Src clones, L3.6pl cells at 80% confluency were transfected with 5.0 ng of each siRNA plasmid and 10 ng of pcDNA G418 resistance promoter-less plasmid for selection. Cells were grown in selective medium containing 600 μg/ml G418, and single clones were isolated and expanded. Controls were transfected with empty vector target sequences and pcDNA plasmids at identical concentrations. Total c-Src expression levels in siRNA clones were determined by Western blot analysis.

For activated and dominant-negative STAT3 clones, subconfluent PANC-1 cells were transfected with 1 μg of an expression plasmid encoding a dominant-negative isoform of STAT3 (STAT3-Y705F) or an activated isoform of STAT3 (STAT3C) [28, 29] using Fugene 6 (Roche Diagnostics) transfection reagent according to the manufacturer's directions. Twenty-four hours after transfection, the medium was removed and the cells were washed with 37°C PBS and then supplemented with complete medium containing 600 μg/ml G418 for selection. Single colonies were isolated, expanded, and screened by Western blot analysis. Clones expressing the highest levels of the STAT3 isoforms were used for subsequent experimental analysis.

Transient transfections and luciferase reporter gene assays

The STAT3 response plasmid, wild-type NF-κB (wild-type κB) and control mutant-κB (mutant NF-κB binding site) firefly luciferase promoter [30] and pTK-Renilla luciferase reporter gene plasmids were transfected into PANC-1 and L3.6pl cells using the materials and methods described above. The activities of firefly and Renilla luciferases were determined at 48 h after transfection using a dual-luciferase reporter assay system (Promega, Madison, WI). All experiments were performed in triplicate, and values are presented as means ± SD. Firefly luciferase activity was normalized to the activity of the Renilla luciferase and expressed in Renilla luciferase units (RLU).

Immunohistochemical staining and confocal microscopy

Pancreatic cancer cells were plated on chamber slides and treated with 100 nM PS-341, 1000 nM AP23846, or DMSO (control) for 12 h before stimulation with 100 ng/ml TNF. After treatment, the cells were fixed with 4% paraformaldehyde and then permeabilized using 0.2% Triton X-100. Cells were incubated overnight with antibody against NF-κB p65. Goat anti-rabbit Alexa Fluor 488 (Molecular Probes, Eugene, OR) fluorescent secondary antibody was used to visualize NF-κB p65 localization. To-Pro-3 (Molecular Probes) was used to counterstain the nucleus. Images were obtained using a Zeiss LSM510 confocal microscope (Oberkochem, Germany).

Immunoblot analysis

Total protein concentration was determined via the Bio-Rad D_c protein assay protocol (Bio-Rad Laboratories, Hercules, CA), which was followed by spectrophotometric analysis using a TECAN Genios plate reader and Magellan version 4.0 software. Equal amounts of protein (50 μg) were loaded into each well, separated via 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and electroblotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). The membranes were blocked with 0.1% Tris-buffered saline/Tween and 5% dried milk for 30 min and then probed with the desired primary antibody (anti-c-Src, anti-STAT3, or anti-phospho-STAT3) diluted 1:1000 in blocking buffer overnight at 4°C. Primary antibody incubation was followed by incubation with a horseradish peroxidase-conjugated secondary antibody (goat anti-mouse or sheep anti-rabbit) diluted 1:2,000 in blocking buffer for 1 h at room temperature with gentle rocking. Western blot analysis of vinculin expression was performed as a loading control by using anti-vinculin antibody. Proteins were visualized by incubation with epichemiluminescent detection reagents (Perkin-Elmer, Boston, MS) and exposed to film (Kodak Biomax MR, Rochester, NY). The membranes were then stripped and reprobed with antibody against specific proteins of interest. Western blot data were quantitated via densitometric analysis (Scion Image software, Scion Corporation, Frederick MD).

Immunoprecipitation

For detection of Src phosphorylation on tyrosine 416 (which is indicative of an activated form of Src), 500 μg of the samples in 650 μl of RIPA buffer was rotated with 6 μl of antibody against c-Src overnight at 4°C. A volume of 50 μl of a 1:1 slurry of protein G agarose in RIPA buffer was added and incubated with rotation for an additional 1 h at 4°C. Bound proteins were pelleted by centrifugation, washed three times with RIPA buffer, eluted by boiling in 1× Laemmli's sample buffer with subsequent immunoblotting with antibody against phospho-Src^{Y416} (1:1,000) (Cell Signaling Technology), and stripped and reprobed with the antibody against c-Src (1:1,000).

IL-8 ELISA

IL-8 production in cultured supernatants was examined using a human IL-8-specific ELISA kit (Quantikine; R&D Systems, Minneapolis, MN). IL-8 concentration (in picograms per milliliter) was determined by spectrophotometric analysis at 450 nm using a TECAN Genios plate reader and Magellan Version 4.0 software and normalized against the total protein concentration as determined by Bradford assay of each clone or the cells grown to 85% confluence. The results are presented as the means ± SD of experiments performed in triplicate.

Statistical analysis

Statistical analysis was performed using InStat 2.01 statistical software (GraphPad Software, San Diego, CA) using Student's *t*-test. Significance was determined with 95% confidence. Differences were considered statistically significant when $P < 0.05$.

Results

c-Src activity is necessary for maximum constitutive IL-8 expression in human pancreatic adenocarcinoma cells

To determine whether Src regulates IL-8 expression in pancreatic cancer cells, we decreased Src activity in L3.6pl and PANC-1 cells pharmacologically or by siRNA targeting. L3.6pl cells incubated for 24 h with increasing doses of the SFK inhibitor AP23846 showed decreased Src phosphorylation in a dose-dependent manner: treatment with 500 nM AP23846 reduced Src phosphorylation by 70% compared with untreated cells, and treatment with 1000 nM AP23846 reduced it by 80% (Fig. 1A). Maximum inhibition (1,000 nM AP23846) resulted in no cytotoxicity and did not affect cell growth as determined by MTT [19]. To complement pharmacologic inhibition, we developed stable subclones with reduced endogenous Src levels (Fig. 1B) in L3.6pl cells by Src-specific small interfering RNA (siRNA), as described previously [31]. In these clones, expression of Src, but not Yes or Lyn was reduced [17]. To determine the effects of both molecular and pharmacologic inhibition of Src on IL-8, IL-8 concentrations in the culture supernatants of these cells were determined by enzyme-linked immunoabsorbent assay (ELISA). Cells treated with AP23846 displayed a similar dose-dependent decrease in Src and IL-8, and treatment with 1,000 nM AP23846 decreased IL-8 expression by more than 85% compared with parental controls ($P < 0.005$) (Fig. 1C). Similar results were observed in PANC-1 cells (data not shown). Corresponding with decreased Src expression/activity, the IL-8 concentration (as measured by ELISA from

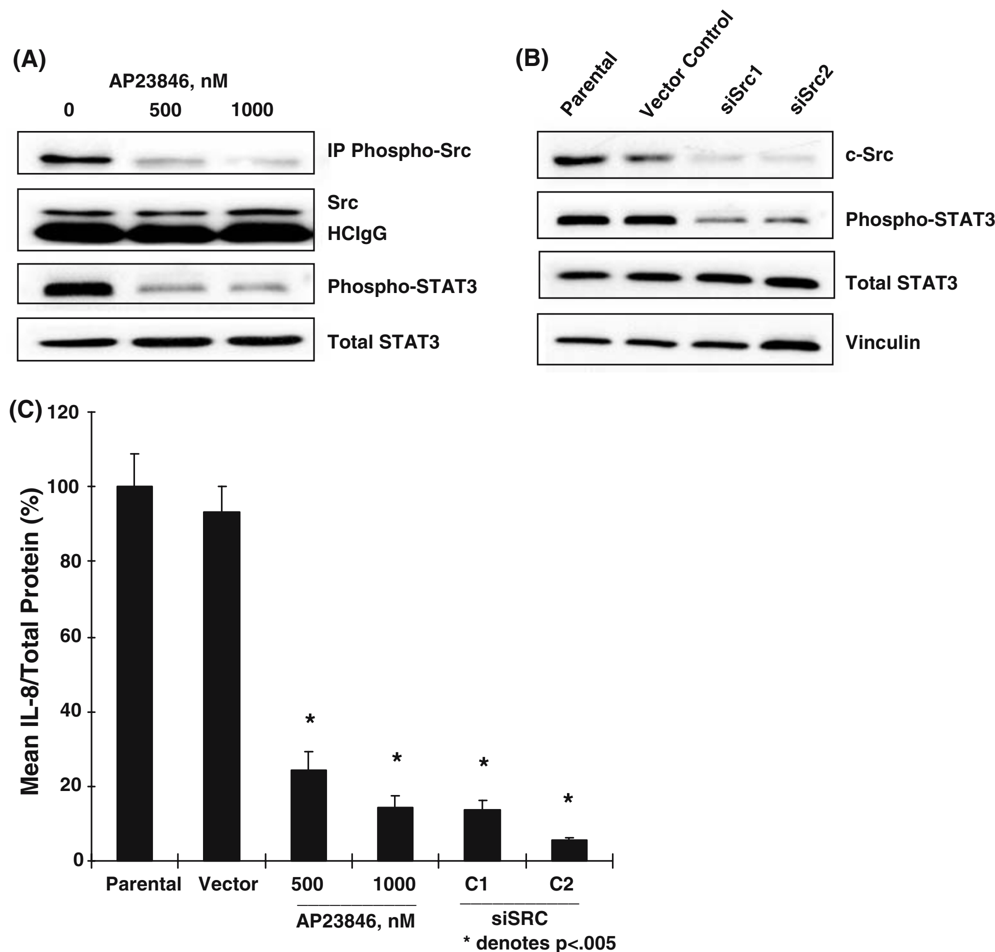

Fig. 1 Inhibition of Src activity and expression decreases IL-8 protein expression and STAT3 phosphorylation in L3.6pl pancreatic adenocarcinoma cells. Cells were grown to 70–80% confluency and then serum starved. After 24 h, cell lysates and supernatants were collected as described in Materials and methods. (**A**) Activated Src was determined by immunoprecipitation (IP) of total c-Src and subsequent Western blot analysis with anti-phospho-Src^{Y416} antibody. The blots were stripped and reprobed for total c-Src. Phosho- and total STAT3 were determined by immunoblotting as described in Materials and methods. (**B**) Stable G418-resistant clones expressing either c-*src*-targeted siRNA (siSrc C1 and C2) or vector controls were generated from parental L3.6pl cells as described in Materials and methods. Cells were plated and serum starved, and cell lysates and cell culture supernatants were harvested. Membranes were probed for total c-Src, phospho-STAT3, and total STAT3 and reprobed for vinculin as a loading control. (**C**) Culture supernatants were assayed for IL-8 using ELISA as described in Materials and methods. IL-8 concentration is expressed as the percentage of IL-8 per total cellular protein and is presented as the means ± SD of experiments performed in triplicate. *$P < 0.005$ versus controls by two-tailed Student's *t*-test

culture supernatants) was 85% lower in the siRNA-targeted clones than in parental cells and vector controls ($P < 0.005$ for both comparisons). These results suggest that activation of c-Src specifically contributes to constitutive IL-8 expression in pancreatic cancers.

Decreasing c-Src activity decreases STAT3 phosphorylation in human pancreatic adenocarcinoma cells

To determine the effect of Src activation and expression on STAT3 phosphorylation in pancreatic cancer cells, we incubated L3.6pl cells for 24 h with and without increasing doses of the Src-specific inhibitor, AP23846. Treatment with 500 nM or 1000 nM AP23846 reduced STAT3 phosphorylation by 60% compared with untreated cells (Fig. 1A). In addition, Src-specific siRNA-targeted clones were reduced in STAT3 phosphorylation by 70% compared with parental and vector controls (Fig. 1B). These data demonstrate that Src activity mediates STAT3 phosphorylation in these cells.

STAT3 regulates IL-8 protein expression in human pancreatic adenocarcinoma cells

Promoter analysis of the region of the human IL-8 promoter (– 2,000 to +1 bp) identified two potential

STAT3 elements (TTCN$_{2-4}$GAA). To determine whether Src-regulated expression of IL-8 is mediated through STAT3 activation in pancreatic adenocarcinoma cells, we generated stable clones expressing a dominant-negative isoform of STAT3 (STAT3-Y705F) (dominant-negative clones STAT3-DN-C1a and STAT3-DN-C1b) or an activated isoform of STAT3 (STAT3C) (activated clones STAT3-Act-C2a and STAT3-Act-C2b) as described in Materials and methods (Fig. 2A, B). Luciferase activity from the IL-8 promoter was significantly repressed in dominant-negative clones compared with control ($P < 0.005$ for both clones) and was significantly induced in cells expressing activated clones ($P < 0.005$ for both clones) (Fig. 2C). In cultured supernatants, IL-8 expression was decreased by 50% in dominant-negative clones compared with parental and vector controls ($P < 0.005$) and increased > 100% in activated clones ($P < 0.005$) as measured by ELISA of culture supernatants (Fig. 2D). Taken together, these results suggest that STAT3 regulates IL-8 in pancreatic cancer cells, and that some of the regulation is at the level of transcription.

NF-κB activity is Src independent but is required for IL-8 expression in human pancreatic adenocarcinoma cells

To confirm in this system that NF-κB also contributes to constitutive IL-8 expression in pancreatic carcinoma cells, we induced IL-8 with 100 ng/ml TNF in L3.6pl cells and PANC-1 cells with the pharmacologic NF-κB inhibitors PS-1145 (an IκK inhibitor) and PS-341 (bortezomib; an IκBα proteosome inhibitor). Treatment of L3.6pl cells with 5 μM PS-1145 or 100 nM PS-341 reduced NF-κB promoter luciferase activity by 50–60% vs. DMSO controls ($P < 0.05$ for both inhibitors) (Fig. 3A). Pharmacologic or molecular inhibition of Src had no effect on TNF-induced luciferase activity from the IL-8 promoter. PS-1145 and PS341 reduced IL-8 expression in culture supernatants by 80% vs. control ($P < 0.005$ for both inhibitors) (Fig. 3B). Similar results were observed in PANC-1 cells (data not shown). These results demonstrate that NF-κB contributes to constitutive IL-8 production in pancreatic cancer cells, and are consistent with many published observations.

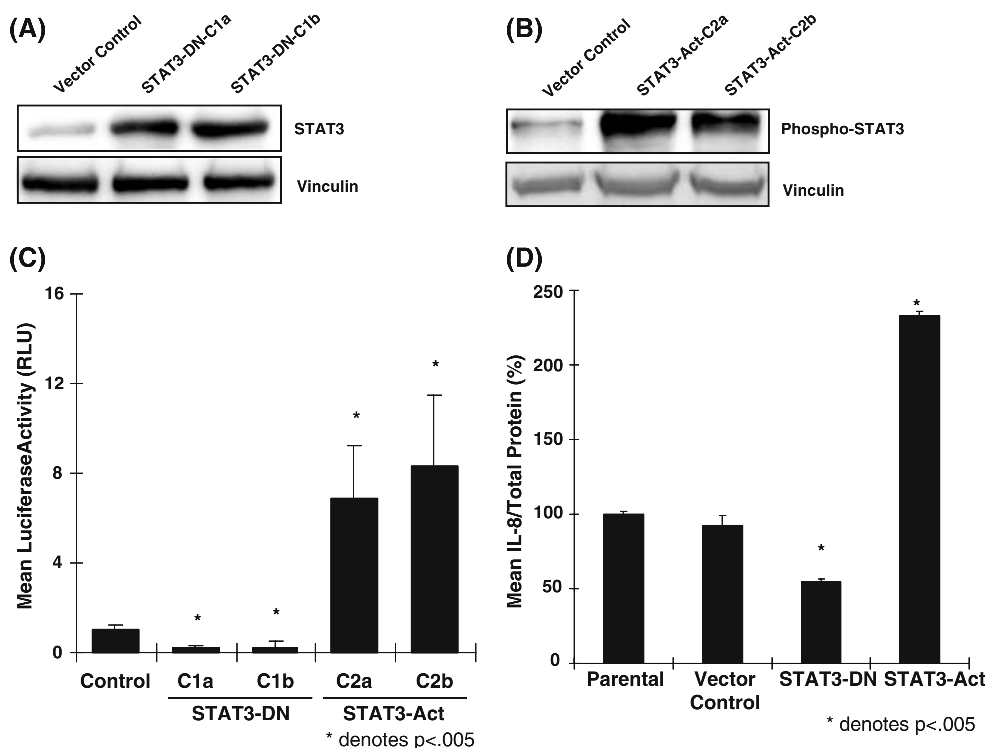

Fig. 2 Effect of STAT3 on IL-8 expression. Stable G418-resistant clones expressing (**A**) STAT3 dominant-negative (DN) or (**B**) activated (Act) STAT3 were generated from parental L3.6pl cells as described in Materials and methods and expression of the mutant protein forms examined by immunoblotting. (**C**) Luciferase activity from the IL-8 promoter from control, STAT3-DN and STAT3-Act clones is presented as the means ± SD of experiments performed in triplicate. *$P < 0.005$ versus control by two-tailed Student's *t*-test. (**D**) Culture supernatants were assayed using ELISA for IL-8 as described in Materials and methods. IL-8 concentration is expressed as the percentage of IL-8 per total cellular protein and is presented as the means ± SD of experiments performed in triplicate. *$P < 0.005$ versus vector control by two-tailed Student's *t*-test

To determine whether Src activity affects NF-κB nuclear translocation in pancreatic adenocarcinoma cells, we inhibited PANC-1 cells with the IκBα proteosome inhibitor PS-341 (100 nM) or the Src-specific inhibitor AP23846 (1000 nM) with or without stimulation with tumor necrosis factor (TNF; 100 ng/ml) or DMSO as a solvent control. NF-κB p65 nuclear translocation was unaffected by DMSO in the presence or absence of TNF and was drastically inhibited in the presence of PS-341, regardless of TNF stimulation (Fig. 4B, E). Src inhibition did not affect NF-κB p65 nuclear translocation into the nucleus with or without TNF stimulation (Fig. 4C, F).

The effect of Src inhibition on NF-κB transcriptional activity was also assessed. L3.6pl cells transfected with the NF-κB element-driven luciferase reporter plasmid (wild-type κB) and treated with 5 μM PS-1145 or 100 nM PS-341 showed a significant decrease in luciferase activity versus DMSO control ($P < 0.05$ for both inhibitors) (Fig. 3A). In contrast, no significant changes in luciferase activity were observed when L3.6pl cells transfected with this plasmid were treated with the Src inhibitor AP23846 (1,000 nM) or when the plasmid was transfected into c-Src siRNA clones (Fig. 3A). These data suggest that NF-κB nuclear translocation and, consequently, NF-κB binding to the promoter are not affected by Src inhibition.

Discussion

Pancreatic tumors produce multiple pro-angiogenic factors including bFGF, VEGF, and IL-8 [6, 32]. Multiple studies on VEGF [13] and IL-8 [7, 9] in pancreatic adenocarcinomas have demonstrated that expression of these factors correlates with poor survival. In this report, we examine regulation of IL-8 in pancreatic tumor cell lines. While IL-8 is a multifunctional cytokine acting as a chemokine that can induce chemotaxis of inflammatory cells and is an important regulator of the inflammatory response, ample evidence supports its pro-angiogenic capabilities [6, 9, 33] via its paracrine effects on endothelial cells [34]. Shi and colleagues demonstrated a correlation with IL-8 expression in pancreatic tumors' and the tumors ability to support angiogenesis. Specifically, the authors concluded that up-regulation of IL-8 expression in pancreatic cancer cells resulted in increased angiogenesis and subsequent tumor growth and metastasis in nude mice, whereas IL-8 antisense expression resulted in reduced tumor angiogenic potential and tumor growth

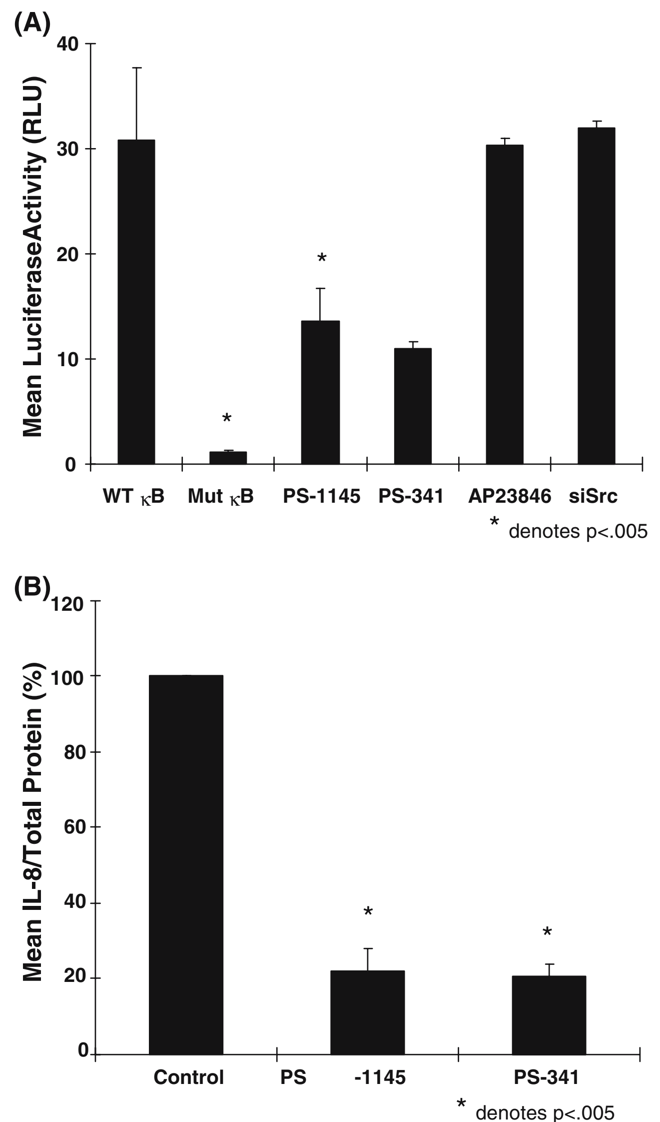

Fig. 3 Inhibition of NF-κB transcriptional activity inhibits IL-8 protein expression. (**A**) L3.6pl cells were plated and maintained as described in Materials and methods. Twenty-four hours after plating, the cells were transfected with wild-type κB or mutant κB (mutant NF-κB binding site) firefly promoter and pTK-Renilla luciferase reporter gene plasmids as described in Materials and methods. Firefly and Renilla luciferase activities were determined at 48 h after transfection using the dual-luciferase reporter assay system. Firefly luciferase activity was normalized to the activity of the Renilla luciferase, serving as an internal control. Luciferase activity is presented as the means ±SD of experiments performed in triplicate. *$P < 0.005$ versus control by two-tailed Student's *t*-test. (**B**) L3.6pl cells were cultured for 24 h in serum-free medium containing PS-1145, PS-341, or DMSO (control). IL-8 levels were assayed by ELISA as described in Materials and methods. IL-8 concentration is expressed as the percentage of IL-8 per total cellular protein and is presented as the means ±SD of experiments performed in triplicate. *$P < 0.005$ versus DMSO control by two-tailed Student's *t*-test

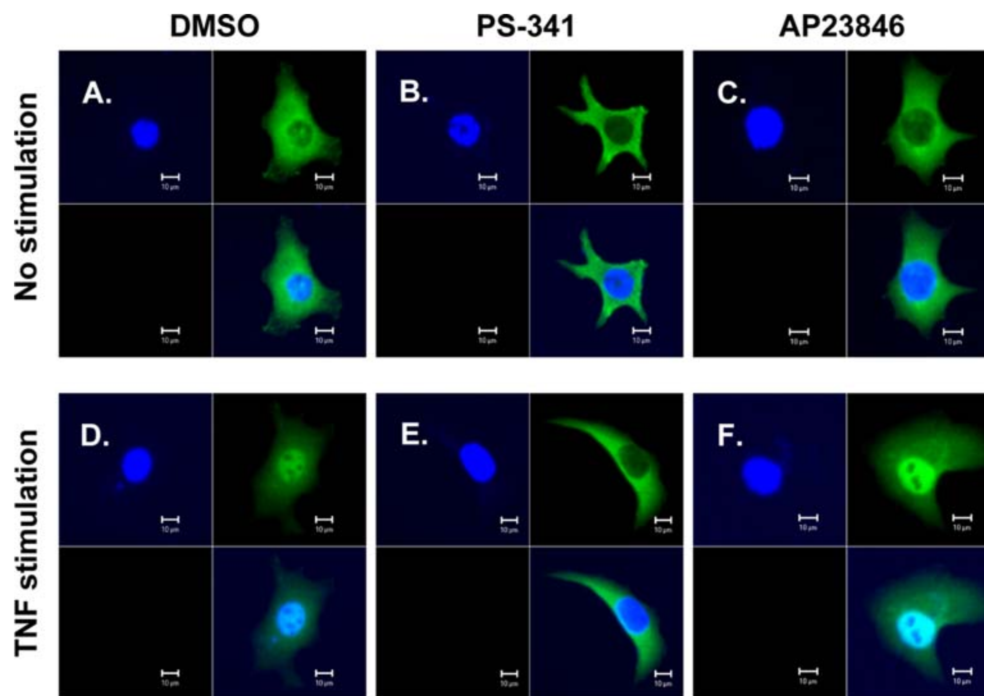

Fig. 4 Inhibition of Src activity does not affect NF-κB p65 nuclear translocation in PANC-1 cells. Pancreatic cancer cells were plated on chamber slides and treated with DMSO as a control (**A, D**); 100 nM IκBα proteosome inhibitor PS-341 (**B, E**), 1,000 nM; or the Src-selective inhibitor AP23846. Cells in the upper panels (**A, B, C**) were unstimulated, whereas cells in the lower panels (**D, E, F**) were stimulated with 100 ng/ml TNF to further activate NF-κB. The cells were then fixed with paraformaldehyde, permeabilized, and incubated overnight with antibody to visualize NF-kappaB p65 localization. A subsequent nuclear counterstaining was used as described in Materials and methods. Images were obtained utilizing a confocal microscope. Scale bar, 10 µm

and metastasis [8]. However, the signaling pathways mediating IL-8 expression in pancreatic tumors remain poorly defined.

In this report, we studied pancreatic tumor cell lines that constitutively express IL-8. Specifically, we studied the relationship of Src activation, which occurs in more than 70% of pancreatic tumors [14] and which we have demonstrated deregulates IL-8 expression, and NF-κB, also activated in pancreatic adenocarcinomas and long known to be a principal transcription factor in IL-8 expression.

Our results confirm that inhibition of NF-κB leads to decreases in constitutive IL-8 expression. However, we also demonstrate that decreased Src activity also leads to decreased IL-8 expression, through an NF-κB-independent pathway. Using multiple strategies we demonstrate that STAT3, a Src target, is critical in regulating IL-8 expression in the cells we studied. Interestingly, the suppression of either NF-κB or STAT3 leads to substantial decreases in IL-8 expression, suggesting that pathways that activate both transcription factors are important in maximal IL-8 expression in pancreatic tumor cells. While we cannot discount that these transcription factors act independently, our results suggest cross-talk between these two factors, which has been demonstrated in IL-6 and IL-1 studies [32]. NF-κB and STAT3 have also been shown to control expression of the anti-apoptotic factor Bcl-X_L in pancreatic cancers, further signifying some functional interaction between these two transcription factors [35]. While constitutive IL-8 expression required both NF-κB and STAT3, over-expression of activated STAT3 resulted in a large induction of IL-8. STAT3 is often overexpressed in human pancreatic cancers and contributes to cell proliferation and metastasis [24]. These data suggest that IL-8 can be induced by cytokines that activate STAT3 and illustrate the complexity of IL-8 regulation in pancreatic adenocarcinomas. The precise mechanism by which STAT3 regulates IL-8 expression remains unclear, but likely involves direct binding to consensus sites on the IL-8 promoter.

Regardless of the mechanism, evidence is mounting that Src activation contributes to tumor progression [36, 37] and therapeutic resistance, notably in cancers of pancreatic origin [16]. Src may represent an important target for therapy in pancreatic cancer because it regulates numerous diverse biologic properties,

including the regulation of angiogenesis-promoting factors, such as vascular endothelial growth factor (VEGF) and IL-8, through STAT3 [20, 25, 26]. We have demonstrated that IL-8 is capable of promoting angiogenesis in vivo assays and that inhibition of Src, which regulates IL-8 protein expression, abrogates this process [19]. Similarly, Src-targeted siRNA orthotopically implanted tumors also resulted in a significant decrease in their angiogenic potential when compared to controls [17].

Recently, some success has been achieved with Src/Abl inhibitors in treating chronic myelogenous leukemia [38], and such inhibitors may be promising in treating pancreatic cancers as well, as we have demonstrated they are effective in inhibiting metastasis of pancreatic tumor cells in orthotopic nude mouse models [17]. However, our study illustrates that gene regulation is infrequently under the control of a single factor. Blocking one factor, for example Src, may reduce basal levels of IL-8 expression, but if, for example, NF-κB is hyper-activated, then some IL-8 expression is still likely to occur. Likewise, NF-κB inhibition may still allow some IL-8 expression though Src activation of STAT3. Further, overexpression of growth factor receptors such as EGF-R and c-Met in pancreatic cancer may lead to STAT3 activation by a JAK-dependent, Src-independent mechanism. Thus, further study of the signaling pathways that contribute to tumor progression are likely to lead to new insights into which pathways are critical in regulating pro-angiogenic molecules such as IL-8, and may lead to logical combinations of therapeutic agents for treatment of tumors that constitutively express this cytokine, including pancreatic adenocarcinoma.

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