CHK negatively regulates Lyn kinase and suppresses pancreatic cancer cell invasion

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Abstract. Among the most important signaling pathways operating in pancreatic cancer cells are those resulting from mutations in the Ras oncogene or from overexpression of ErbB-2 and associated Src-family kinases. In this study, we aimed to characterize CHK expression and function in pancreatic cancer. Our data demonstrated CHK expression in human pancreatic cancer tissues, and also showed that CHK associated with ErbB-2 via its SH2 domain in human PANC-1 pancreatic cancer cells. PANC-1 cells were found to express both Src kinase and Lyn kinase, although the expression of Lyn kinase was more abundant. Furthermore, CHK downregulated Lyn kinase activity and significantly inhibited the *in vitro* growth and invasion of PANC-1 cells upon EGF stimulation. These results indicate that CHK is a negative regulator of ErbB-2 and Lyn kinase signaling in pancreatic cancer cells.

Introduction

Pancreatic cancer is a devastating disease that is currently the fourth leading cause of cancer deaths in North America (1,2). Late diagnosis and low resection rates cause the low survival rates of patients with pancreatic cancer. This poor outlook results from a combination of factors, including an anatomical location that makes early detection difficult, a poor profile of sensitivity to chemotherapeutics as well as the advanced medical conditions of patients at the time of diagnosis (3). Perineural invasion to the extrapancreatic nerve plexus, already present at the time of diagnosis, is the cause of painful symptoms that preclude curative resection (4) and of local recurrence after tumor resection (5). Conservative oncological strategies such as chemotherapy and radiotherapy have failed

to significantly improve the prognoses for patients with pancreatic cancers (6-9). In addition, neither anti-hormonal modalities using tamoxifen nor the systemic use of specific anti-pancreatic cancer cell monoclonal antibodies have changed the outcomes for pancreatic cancer patients thus far (10-15). Hence, new treatment strategies are urgently being sought.

One of the most frequently overexpressed proto-oncogenes in pancreatic cancer is the ErbB-2 (also named HER-2/neu) receptor tyrosine kinase (16). This receptor belongs to the ErbB (HER) family that includes four members: $p170^{ErbB-1}$ [epidermal growth factor receptor (EGF-R)], $p185^{ErbB-2}$, $p180^{ErbB-3}$ and $p180^{ErbB-4}$ (17). It has been found that excessive amounts of these receptors predominate in approximately 70% of pancreatic cancers (17). The activity of ErbB family members is stimulated by a group of growth factors classified as EGF-like ligands that are able to heterodimerize and cross-phosphorylate ErbB receptors (18-21). Although none of these growth factors is capable of direct binding to ErbB-2, several observations indicate that it is the pan-ErbB subunit that creates high-affinity binding receptors for heregulin or EGF (17,18,21). Upon its activation, ErbB-2 undergoes rapid phosphorylation at five distinct tyrosine residues located on its non-catalytic cytoplasmic terminus. These phosphorylated tyrosines create docking sites for numerous proteins containing SH2 domains such as: Shc, c-Src, Ras-GTPase activating protein (Ras-GAP), Rho-GAP, phosphatidylinositol 3'-kinase (PI3K), Grb7, PTP1D, and phospholipase Cγ (PLC-γ) (19,22,23). All of these proteins function as adapter proteins or tyrosine kinases that further amplify and diversify signaling mechanisms, and contribute to malignant growth. An essential step in the initiation of the mitogenic response to growth factor stimulation is caused by the activation of c-Src or other tyrosine kinases belonging to the Src-family. Interestingly, phosphorylated tyrosine residues of ErbB-2 can also associate with several proteins with negative regulatory activity, such as CHK.

Several EGF-related growth factors enhance the proliferation of pancreatic cancer cell lines (16). Some of these (EGF, HB-EGF, TGF-α) are able to stimulate their own production or induce the expression of other members of this family, thereby creating a series of positive feedback-loops that promote the survival of pancreatic cancer cells (24,25). This theory is supported by observations that the co-expression of EGF-R with either EGF or TGF-α is associated with the decreased survival of patients with pancreatic cancer (25).

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On the other hand, interference with these signaling pathways decreases the anchorage-independent growth and proliferation of pancreatic cancer cells (26). It has been shown that increased levels of ErbB-2 or other family members correlate with a poor or even lost responsiveness of various cancers to chemotherapy, radiotherapy and hormone therapy (24-27). Moreover, accumulating evidence indicates that the signaling pathways from these receptors might interfere with the initiation and execution of apoptosis (26).

These observations indicate that therapeutic approaches aimed to inhibit signaling by EGF and related growth factors might be a novel and promising avenue in the treatment of pancreatic cancer (28,29).

The Src-family protein kinases are overexpressed in a great majority of pancreatic cancer specimens and in most pancreatic cancer cell lines (30,31). The Src-specific tyrosine kinase inhibitor herbimycin A decreases cell growth in a dose-dependent manner, suggesting that Src-family kinases participate in the regulation of pancreatic cancer cell growth. The cytoplasmic tyrosine kinase CHK (Csk homologous kinase), as well as Csk (C-terminal src kinase), are able to phosphorylate the inhibitory C-terminal tyrosine of Src (32-42) and of several Src-related enzymes, including Lck (42), Fyn and Lyn (43). Our previous immunohistochemical studies revealed that the CHK protein is expressed in breast carcinoma specimens, but not in normal or benign (fibroadenoma) breast tissues (32,33,44,45). Additionally, CHK was found to co-localize with ErbB-2 in the investigated specimens. We have also shown that the CHK-SH2 domain can precipitate ErbB-2 in T47D breast cancer cell lines after heregulin stimulation (44), and that the SH2 domain of CHK binds to Tyr^{1253} of ErbB-2, the most critical site for the oncogenicity of this receptor. CHK is also able to inhibit ErbB-2 activated Src-kinases, which are rapidly activated upon growth factor stimulation (33). This effect is mediated by phosphorylation of the C-terminal regulatory tyrosines in Src-kinases.

Although protein tyrosine kinases such as Src and ErbB-2 have been linked to the development and progression of pancreatic cancer, little is known about the downstream participants in their signaling cascades. In this study, we determined the CHK expression as well as the CHK-mediated effects on Src and ErbB-2 in PANC-1 cells.

Materials and methods

Cell lines and primary tumor samples. Cells were purchased from ATCC and were cultured in RPMI-1640 medium supplemented with 10% FBS, 2 mM L-glutamine and anti-biotics (all reagents from Cellgro, Herndon, VA). Primary tumor samples and normal adult pancreatic samples were obtained from the Cooperative Human Tissue Network (CHTN, Philadelphia, PA).

Antibodies. Polyclonal anti-Csk, anti-CHK (Lsk), and anti-Src antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-PY20 antibodies were obtained from BD Transduction Laboratories (San Diego, CA). Goat polyclonal anti-GST antibody was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Anti-Src antibody was a product of Biosource (Camarillo, CA).

Immunoprecipitation and Western blotting. For the immuno-precipitation (IP) and Western blot analyses (WB), cells were scraped off the plates and lysed in cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 10% glycerol) containing protease inhibitors (Complete Tablets, Roche, Basel, Switzerland), and 1 mM sodium orthovanadate inhibitor for 45 min at 4˚C. Protein concentration was determined using a protein assay (Bio-Rad, Hercules, CA). For the IP, lysates (500 µg of total protein in 500 µl of lysis buffer) were incubated with the desired antibody (diluted 1:100) overnight at 4˚C, followed by incubation with protein G beads for 30 min at 4˚C, and then the beads were washed three times with the lysis buffer, suspended in Laemmli sample buffer (Bio-Rad), and subjected to SDS-PAGE followed by Western blot analysis.

For the Western blotting, total cell lysates (70 µg of total protein extracts) were electrophoretically separated on 10% polyacrylamide-SDS gels, transferred to nitrocellulose membrane, and probed with appropriate antibodies. Immuno-detection was performed using the enhanced chemilumin-escence system (ECL, Amersham Pharmacia Biotech).

In vitro tyrosine kinase assay. The kinase assay was performed as described previously (45). One milligram of protein was immunoprecipitated from PANC-1 cells and PANC-1 cells overexpressing CHK by using polyclonal antibodies against Lyn. The immunoprecipitates were washed three times with lysis buffer and then resuspended in 30 µl of kinase buffer [50 mM Tris-HCl (pH 7.4), 0.5 mM MnCl$_2$, 10 mM MgCl$_2$, 0.1% Triton X-100, and 1 mM dithiothreitol] containing phos-phatase and protease inhibitors, 0.25 mg/ml poly(Glu/Tyr) (4:1; Sigma) as an exogenous kinase substrate, 10 µM unlabeled ATP, and 10 µCi of [γ-^{32}P]ATP (6000 Ci/mmol; Perkin-Elmer Life Sciences, Wellesley, MA). After 15 min at 30˚C, the reaction was stopped by adding SDS sample buffer and boiling the samples for 10 min. Subsequently, the samples were resolved on 12% polyacrylamide-SDS gels, and the gels were stained with Coomassie Blue. The labeled poly(Glu/Tyr) was excised from the gel, and radioactivity was counted.

Construction of adenoviral constructs. cDNA encoding CHK was generated from template DNA by PCR. The PCR products were digested with *Sal*I and *Eco*RV and inserted into an Ad Track vector (Quantum Biotech, Carlsbad, CA). Briefly, the cassette containing the CHK expression unit was co-transfected into human kidney 293 cells together with an adenovirus genome DNA-terminal protein complex. The targeted recombinant adenovirus carrying the CHK gene was generated by overlapping recombination. After maintaining the 293 cells for 10-15 days, the virus clone was isolated. Recombinant GFP-tagged CHK adenovirus (Ad5ΔE1/ΔE3-CHK) and control adenovirus (Ad5ΔE1/ΔE3) were titrated to achieve a transfection efficiency of >50%, without affecting cell viability. Transfection efficiency was monitored by analyzing the GFP fluorescent signal using a fluorescence microscope. The expression of CHK was detected by Western blotting. Prior to the signal-transduction studies, the multiplicity of infection (m.o.i.) level for the adenoviruses was adjusted for each cell line used throughout our studies to achieve ~95%
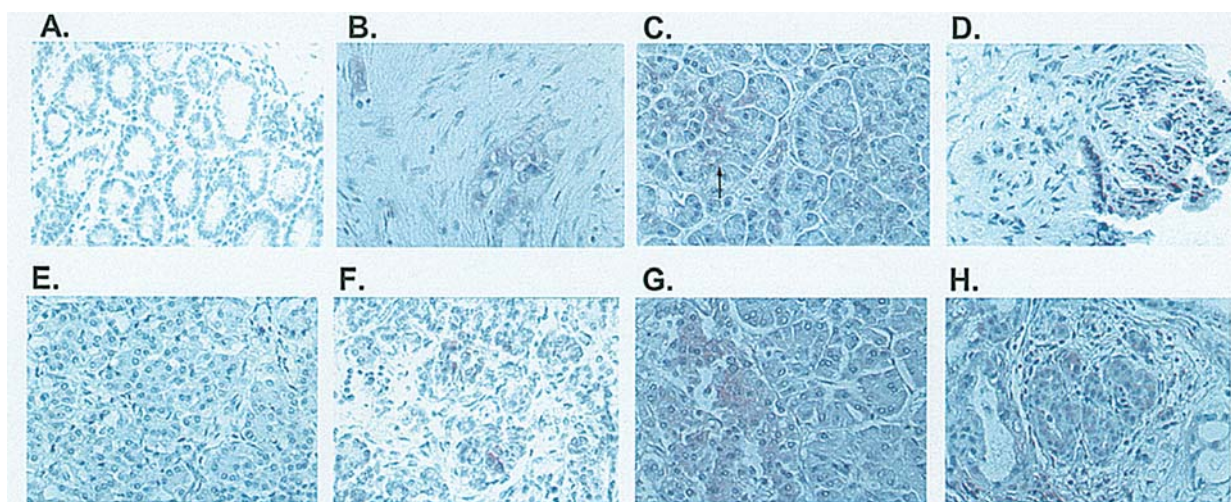
Figure 1. Expression of CHK in primary pancreatic tumors. Immunohistochemical staining was performed on paraffin-embedded sections of pancreatic carcinomas and normal control pancreatic tissues using anti-CHK antibodies. A and E, normal pancreatic controls; B-D and F-H, pancreatic cancer samples; obtained from different patients. The expression of CHK was observed in pancreatic tumors but not in normal pancreatic tissues.

GFP-positive cells within 48 h following infection. The m.o.i. value was 10 for the PANC-1 cells.

Cell growth assay. PANC-1 cells (10^5 cells/well) were spread in 24-well plates and starved in serum-free medium. Cells were then grown in serum-free medium alone or transfected with a GFP-CHK pCDNA3 expression construct or with the GFP-pCDNA3 control vector. Viable cells were stained with 0.1% crystal violet. Staining was performed with 2% deoxycholate and quantitated by spectrophotometry (490 nm).

Cell invasion assay. The Matrigel invasion assay was performed using 6.5-mm Transwell chambers (8-μm pore size, Costar). Matrigel was diluted in cold distilled water (2 μg/ml) that was added to the upper wells of the Transwell chambers. The Matrigel was reconstituted with medium for 1 h at 37°C before the addition of cells. Cells were starved in serum-free medium, then resuspended at a concentration of 1-2x10^6 cells/ml in serum-free medium containing 0.1% bovine serum albumin. Cell suspension (100 μl) was added to each well. After 18 h, the cells that had not invaded were removed from the upper surface of the filters using cotton swabs. The cells that had invaded to the lower surface of the filters were fixed with methanol, stained with 0.2% crystal violet, and counted.

Results and discussion

Expression of CHK in pancreatic cancer tissues. To examine CHK expression in the pancreas and in pancreatic tumors, immunohistochemical studies were performed on 6 pancreatic cancer specimens and on 4 control normal pancreatic tissues. The antibodies used for these studies were specific anti-CHK polyclonal antibodies generated in our laboratory, and raised against the C-terminal fragment of CHK. Analyses of CHK expression were performed using immunohistochemistry on paraffin sections. All of the pancreatic cancer specimens obtained from 10 patients stained positive for CHK (Fig. 1). None of the control normal pancreatic tissues revealed
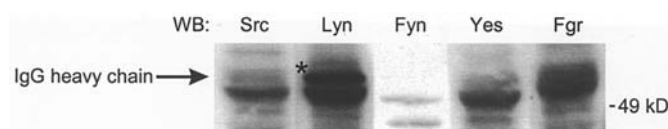

Figure 2. Expression of Src-family tyrosine kinases in PANC-1 cells. The expression levels of Src, Lyn, Fyn, Yes and Fgr were determined by immuno-precipitation with the respective antibodies against the Src-family tyrosine kinases, followed by SDS-PAGE and Western blotting with the specified antibodies. *The band which corresponds to the Lyn kinase.

binding to the antibodies. We also observed a similar pattern of staining with anti-ErbB-2 antibodies (data not shown). These data suggest, similar to our previous observations in breast carcinoma (32,44), that there is induction of CHK expression in pancreatic cancer.

Expression of Src-family kinases in pancreatic cancer cell lines. It has previously been demonstrated that pancreatic cancer cell lines express numerous members of the Src-family tyrosine kinases (46). We performed a series of immunoprecipitation experiments with monoclonal antibodies against Src, Fyn, Lyn, Yes and Fgr, and found that the expression of Lyn kinase was abundant in these cells (Fig. 2).

Association of CHK with ErbB-2 in pancreatic cancer cell lines. To further investigate the potential binding of CHK to ErbB-2, we performed a series of precipitation studies with the SH2 domain of CHK as a GST-fusion protein. Pancreatic cancer cells (PANC-1) were starved overnight and stimulated with 50 ng/ml of recombinant human EGF (Fig. 3). The results of these experiments revealed that the SH2 domain of CHK bound to ErbB-2 after 10 min of stimulation with EGF.

Effects of CHK on Lyn activity and PANC-1 cell proliferation and invasion. We evaluated the modulation of Lyn kinase activity by CHK kinase in PANC-1 cells. PANC-1 cells were infected with a recombinant adenoviral CHK construct (CHK-
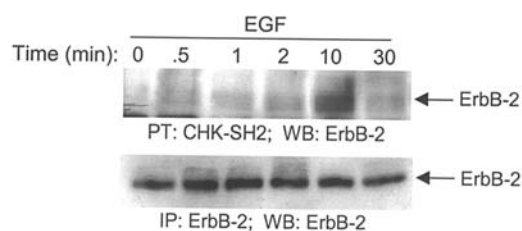
Figure 3. Association of the SH2 domain of CHK with ErbB-2 in pancreatic cancer cells upon stimulation with EGF. PANC-1 cells ($5x10^6$ cells/plate) were serum-starved and then stimulated with 50 ng/ml of recombinant human EGF at room temperature for the indicated time-points. The lysates were precipitated (PT) with a CHK-SH2 GST fusion protein (10 μg for 90 min at 4˚C) (upper panel) or immunoprecipitated (IP) with the c-neu monoclonal antibody, ErbB-2 (Calbiochem) (lower panel). After washing, the precipitates were separated by 7% SDS-PAGE and Western blotted (WB) with polyclonal anti-ErbB-2 antibodies.
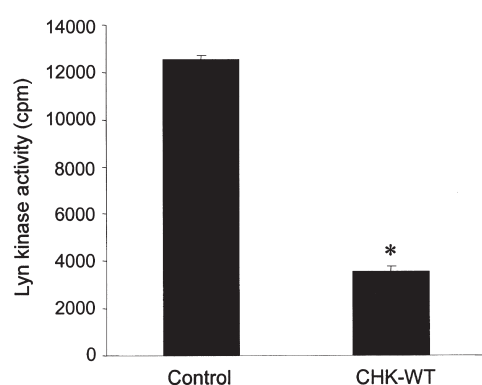

Figure 4. Effects of the adenoviral CHK construct on Lyn kinase activity in PANC-1 cells. Cells were infected with a recombinant adenoviral CHK construct (CHK-WT) or with control adenoviral vector at an m.o.i. of 10. Twenty-four hours following infection with the adenoviruses, total cell lysates were immunoprecipitated with Lyn specific antibodies and analyzed for Lyn kinase activity as determined by an *in vitro* kinase assay (cpm). The data shown are the mean values ± SD of two experiments performed in triplicate. *$p \leq 0.001$.
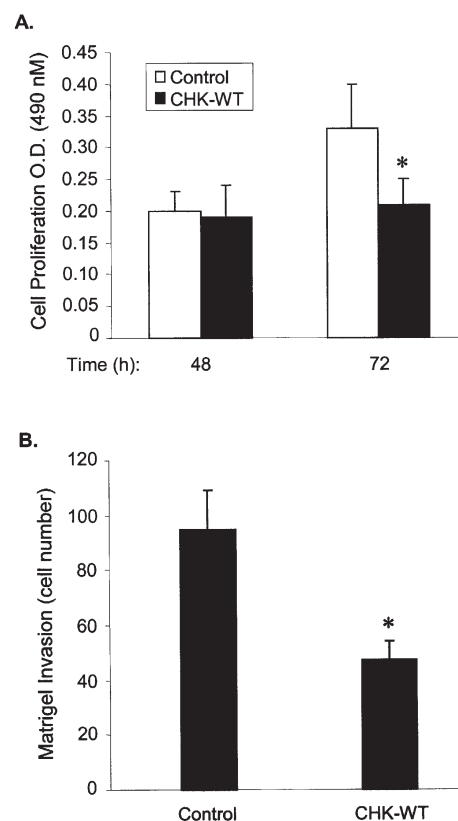

Figure 5. Effects of CHK on the proliferation and invasion of PANC-1 cells. A, PANC-1 cells were transfected with the GFP-pCDNA3 vector (control) or with the GFP-CHK pCDNA3 construct (CHK-WT). After 24 h, cells were sorted and analyzed for proliferation using the MTT assay, as indicated. The data shown are the mean values ± SD of 3 experiments performed in triplicate. *$p \leq 0.05$. B, PANC-1 cells were infected with control adenovirus or CHK-adenoviral vector (CHK-WT) for 24 h at an m.o.i. of 10. Cells were then tested for their ability to invade the Matrigel by using a cell invasion assay as described in Materials and methods. The data shown are the mean values ± SD of 3 experiments performed in triplicate. *$p \leq 0.01$.

WT) or with control adenoviral vector for 24 h. Cells were then lysed and assayed for Lyn kinase activity. As shown in Fig. 4, Lyn kinase activity was significantly inhibited (by ~65%) in the PANC-1 cells overexpressing CHK.

Next, PANC-1 cells were transfected with either the GFP-pCDNA3 control vector or GFP-CHK pCDNA3 construct (CHK-WT). The transfected cells were sorted after 24 h and were analyzed for proliferation. As shown in Fig. 5A, the PANC-1 cells overexpressing CHK exhibited reduced proliferation as compared to the control PANC-1 cells 72 h following transfection. In addition, the invasion of PANC-1 cells overexpressing CHK was significantly inhibited (by 52%) as compared to the control cells (Fig. 5B). These results indicate that CHK acts to inhibit pancreatic cancer cell growth and invasion.

Pancreatic cancer is one of the most aggressive forms of cancer, partly due to its insensitivity to most treatment modalities. This resistance towards cytotoxic therapy is thought to be caused, to some degree, by a general resistance of the pancreatic cancer cells towards apoptosis, which is mediated by intrinsic or extrinsic factors. In addition, pancreatic

cancers overexpress receptors of the EGF-R family including EGF-R and ErbB-2, and Src-family kinases. In this study, we observed and characterized the expression of CHK in normal and malignant human pancreatic tissues as well as human pancreatic tumor cell lines. We also found that CHK associated with ErbB-2 in PANC-1 cells and downregulated Lyn kinases upon EGF stimulation. The association of CHK with ErbB-2 resulted in the close proximity of CHK to Lyn kinase which also associated with ErbB-2, leading to the C-terminal phosphorylation of Lyn and to inactivation of its kinase activity. CHK also inhibited the invasion of PANC-1 cells. Thus, CHK is involved in the negative regulation of ErbB-2 and Src-family kinases.

In conclusion, this study demonstrates that CHK is involved in the negative regulation of ErbB-2 and Lyn kinases in pancreatic cells.

Acknowledgements

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