Mechanism for Src activation by the CCK2 receptor: Pathophysiological functions of this receptor in pancreas

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Abstract

AIM: To investigate in vivo, whether CCK2 receptors (CCK2R) regulate proteins known to play a crucial role in cell proliferation and cancer development and analyse in vitro the molecular mechanisms that lead to Src activation; in particular, to identify the domains within the CCK2R sequence that are implicated in this activation.

METHODS: The expression and activation of Src and ERK were studied in vivo using immunofluorescence and western-blot techniques. We used pancreatic tissues derived from wild type or Elas-CCK2 mice that expressed the CCK2R in pancreatic acini, displayed an increased pancreatic growth and developed preneoplastic lesions. The pancreatic tumor cell line AR4-2J expressing the endogenous CCK2R or COS-7 cells transiently transfected with wild type or mutant CCK2R were used as in vitro models to study the mechanism of Src activation. Src activation was measured by in vitro kinase assays, ERK activation by western blot using anti-phospho-ERK antibodies and the involvement of Src in gastrin-induced cell proliferation by MTT test.

RESULTS: We showed in vivo that the targeted CCK2R expression in the pancreas of Elas-CCK2 mice, led to the activation of Src and the ERK pathway. Src was activated upstream of the ERK pathway by the CCK2R in pancreatic tumoral cells and contributed to the proliferative effects mediated by this receptor. In vitro results demonstrated that activation of the Src/ERK pathway by the CCK2R required the NPXXY motif, located within the CCK2R sequence at the end of the 7th transmembrane domain, and suggested the putative role of Gq in this mechanism.

CONCLUSION: Deregulation of the Src/ERK pathway by the CCK2R might represent an early step that contributes to cell proliferation, formation of preneoplastic lesions and pancreatic tumor development.

Key words: Gastrin; Src; Pancreas; CCK2 receptor

INTRODUCTION

The CCK2 receptor (CCK2R or CCKBR) is a G protein-coupled receptor, mainly coupled to Gq proteins[1]. Initially implicated in gastrin-mediated acid secretion CCK2R is now recognized to mediate mitogenic and anti-apoptotic effects on gastrointestinal and pancreatic cells[2]. In the transgenic mice, Elas-CCK2, CCK2R expression has been targeted in pancreatic acinar cells using transcriptional elements of the elastase-1 promoter[3]. Using this model, we have recently reported an increase in pancreatic growth as well as an acinar to ductal transdifferentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis that precedes the development of tumours[4]. Similar observations have been reported in two other transgenic models overexpressing TGF-α or a Kras mutant in exocrine pancreas[5].

Src family kinases are non receptor protein tyrosine kinases that mediate a wide variety of biological effects including cell survival, adhesion and proliferation. They are activated by many growth factor receptors. In addition, p60-Src is well established as an oncogene, and overexpression of Src tyrosine kinase has been described in human pancreatic adenocarcinomas[6]. Recently, Src inhibition by
AZM475271 demonstrated significant antitumorigenic and anti-metastatic activity in an orthotopic nude mouse model for human pancreatic cancer\(^9\). However, to our knowledge, there is currently little or no information regarding the deregulation of Src kinases in early stages of pancreatic carcinogenesis.

It is well established that numerous G protein-coupled receptors activate Src family kinases. However, very few publications have described the molecular mechanisms involved in Src activation by this receptor family. Molecular mechanisms of Src activation by CCK2R as well as the role of this kinase and related signalling pathways in the pathophysiological functions of CCK2R \textit{in vivo} remain largely unknown. This study had two main aims: First, to investigate \textit{in vivo}, using the Elas-CCK2 mouse model, whether CCK2R is involved in the regulation of proteins known to play a crucial role in cell proliferation and cancer development; Second, to analyse \textit{in vitro} the molecular mechanisms that lead to Src activation and in particular, to identify the domains within the CCK2R sequence implicated in this activation.

**MATERIALS AND METHODS**

**Animals**
Homozygous Elas-CCK2 mice used in this study have been previously described\(^9\). At least 3 homozygous Elas-CCK2 mice in a B6SJLF1 background and 3 corresponding control littermate mice were used (six months old). Mice were reared in routine animal facility of the IFR31 and maintained on a 12:12 h light-dark cycle. All the experiments were performed during the daytime. All procedures were approved by the IFR31 animal facility care committee.

**Antibodies and materials**
GAPDH was provided by Chemicon; phosho-tyr418-Src (IF and WB) by Biosource; ERK, Src (IF and WB) by Santa Cruz Biotechnology; phosho-ERK (IF and WB) by Cell Signaling; SRC (IP) by Oncogene Research Product; PP2, GP2A by Calbiochem.

**Immunofluorescence staining**
Mice were killed by decapitation and the pancreas was excised, fixed in Bouin’s solution and embedded in paraffin using standard techniques. Immunofluorescence staining was performed as previously described\(^9\). The detection was done using secondary antibodies coupled to Alexa Fluor 488. Slides were analyzed on a Nikon E400 microscope with a Sony DXC 950 camera and Visiolab 2000 software. For semi-quantitative comparisons, identical volumes of antibody were used for all samples and identical exposure times taken.

**Western-blot analysis**
Western-blot analyses were performed on dispersed acini from mouse pancreas prepared as previously described\(^9\), and on cell lysates or immunoprecipitates from AR4-2J or COS7 cells stimulated or not with gastrin. Fractions, containing identical levels of proteins, were separated by SDS-PAGE and analyzed by western-blot with the indicated antibodies as described previously\(^9\).

**Cell culture and proliferation assay**
AR4-2J cells and COS-7 cells were grown in DMEM supplemented with 10% and 5% fetal calf serum (FCS), respectively, at 37°C in a 95% air, 5 mL/L CO\(_2\) atmosphere. For proliferation assays, an optimal number of AR4-2J cells (4 × 10\(^4\) cells) were plated in 35-mm dishes, serum-starved for 24 h, then treated for 48 h with gastrin (10 nmol/L). When indicated, cells were incubated with PP2 (10 μmol/L). Cells were counted by using a Coulter electronic counter.

**Src kinase assay**
After gastrin stimulation, cells were lysed and Src immunoprecipitated with specific antibodies. Kinase assays were performed and analyzed as previously described\(^9\). Proteins were separated by SDS-PAGE and the gel autoradiographed.

**Construction of mutant receptor cDNAs and transient transfection**
Mutant CCK2R, N386A-CCK2R was previously described\(^9\). Plasmids coding for wild type or mutant CCK2R (6 μg) were transiently transfected into COS-7 cells using the DEAE/dextran method as described previously\(^9\).

**Statistical analysis**
Data were expressed as mean ± SE and Student’s \(t\) test was performed using “GraphPad Prism”. \(P < 0.05\) was taken as significant.

**RESULTS**

**Src status in Elas-CCK2 mice**
Src activation was analyzed by immunohistochemical methods on pancreatic sections from control and Elas-CCK2 mice of 6 month old using antibodies specific for total Src or detecting the activated form of the protein phosphorylated on tyrosine 418 (P-Src). Acinar tissues derived from Elas-CCK2 mice demonstrated higher levels of Src activation as compared to control mice (Figure 1A, upper panels). In contrast, total Src protein expression was unchanged in the two mouse models (Figure 1A, lower panels). To confirm and quantify Src activation, western-blot analyses were performed on lysates of acinar cells isolated from pancreas of control and Elas-CCK2 mice. Src activation in Elas-CCK2 mice was significantly elevated compared to controls (Figure 1B). Thus, the expression of the CCK2R in mouse pancreatic acini induced Src activation.

**The ERK pathways in Elas-CCK2 mice**
Activation of the ERK pathways by gastrin has previously been described \textit{in vitro}\(^10,11\). However, there is currently no \textit{in vivo} information about the status of the ERK pathways in gastrin signaling. Immunofluorescence analysis was per-
formed using antibodies specific for dually phosphorylated active ERK (P-ERK). Results demonstrated an increased immunoreactivity in acinar tissue of transgenic mice as compared to control mice (Figures 2A, upper panel). In addition, pancreatic acinar tissues of Elas-CCK2 mice also showed a higher level of total ERK expression as compared to control mice (Figure 2A, lower panel). These results were confirmed by western-blots performed on lysates of acinar cells isolated from pancreas of control and Elas-CCK2 mice (Figure 2B). Overall, these results were consistent with an upregulation of the ERK pathway in the pancreas of mice expressing the CCK2R.

**Src activation by CCK2R in tumour pancreatic acinar cells**

The pancreatic tumour cell line AR4-2J, that exhibits an acinar phenotype, was previously shown to express endogenous CCK2R[^12^]. We used this model to analyse the role of Src in the pathophysiological functions of the CCK2R and the molecular mechanisms potentially involved in Src activation by this G protein-coupled receptor.

We first confirmed that the CCK2R was able to induce Src activation. In *vitro* tyrosine-kinase assays were performed in anti-Src immunoprecipitates from cell lysates containing equal amounts of protein. We detected a rapid and significant increase in Src activation 15 s after stimulation of the CCK2R by gastrin (Figure 3A). In order to address the role of Src family kinases in proliferation of tumour acinar cells induced by CCK2R, we measured AR4-2J proliferation in the presence or absence of the Src specific inhibitor, PP2, 48 h after gastrin stimulation. CCK2R activation by gastrin induced a significant increase of cell proliferation. Treatment of cells with PP2 totally inhibited CCK2R-induced AR4-2J proliferation (Figure 3B).

We previously reported that CCK2R associates with the aq subunit of heterotrimeric G-proteins[^1^]. We therefore
Role of Src in proliferation of tumour pancreatic acinar cells induced by the CCK2R. AR4-2J cells were stimulated with Gastrin (10 nmol/L) for the times indicated (A) or 15 s (B). A: Src activity was determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. *P < 0.05 vs control. B: Serum-starved AR4-2J cells were treated with Gastrin for 48 h in the presence or absence of PP2 (10 μmol/L), and the proliferation determined as described in Methods. Data are presented as mean ± SE. *P < 0.001 vs control.

Serum-starved AR4-2J cells were treated with Gastrin (10 nmol/L) for 30 s and Src kinase activity determined as described in Methods. Immunoprecipitated proteins were also analyzed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. *P < 0.001 vs control.

**Mechanism of CCK2R-induced Src activation in COS-7 cells**

To further investigate the molecular mechanisms involved in Src activation by the CCK2R we used COS-7 cells transiently transfected with cDNAs coding for the human wild type CCK2R (WT-CCK2R) or mutant CCK2R. We first validated this model for CCK2R-induced Src activation. Lysates from cells stimulated or not with gastrin were immunoprecipitated with anti-Src antibodies and kinase assays performed as described in methods. A rapid activation of Src (15 s), still detectable at 1 and 5 min, was found in response to Gastrin (Figure 5A). Western-blot analysis for Src protein expression revealed an equal amount of the protein in transfected cells.

Recently, we reported that the NPXYY motif (X represents any amino acid), located at the end of the 7th transmembrane domain of the CCK2R, was involved in Gq-dependent signaling pathways induced by the CCK2R[13]. In particular, mutation of the asparagine (N) into alanine (A) inhibits Gq-dependent pathways such as inositol triphosphate (IP3) production. We therefore tested the hypothesis that Gqα might be involved in gastrin-induced Src activation.

Src proteins were immunoprecipitated with specific antibodies and their association with Gqα was analyzed by western blot with an anti-αq antibody. An increase in the amount of Src proteins coprecipitated with Gqα was detected in response to gastrin (Figure 4A). This effect was time-dependent and the kinetic correlated with that of Src activation in this cellular model. We then examined whether Src-like tyrosine kinase activity was in association with Gqα following gastrin stimulation. An increase in tyrosine kinase activity was detected in anti-Gqα immunoprecipitates after gastrin stimulation which was abolished when samples were treated with the specific Src inhibitor, PP2 (Figure 4B). In addition, pretreatment of the AR42J cells with a specific Gqα inhibitor, GP2A, completely blocked the activation of Src by gastrin (Figure 4C).

![Figure 3](image3.png)  
**Figure 3** Role of Src in proliferation of tumour pancreatic acinar cells induced by the CCK2R. AR4-2J cells were stimulated with Gastrin (10 nmol/L) for the times indicated (A) or 15 s (B). A: Src activity was determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. *P < 0.05 vs control. B: Serum-starved AR4-2J cells were treated with Gastrin for 48 h in the presence or absence of PP2 (10 μmol/L), and the proliferation determined as described in Methods. Data are presented as mean ± SE. *P < 0.001 vs control.

![Figure 4](image4.png)  
**Figure 4** Src activation by the CCK2R in tumour pancreatic acinar cells (A-C). AR4-2J cells were stimulated with Gastrin (10 nmol/L) for the times indicated (A) or for 15 s (B, C). When indicated, cells where pretreated with 10 μmol/L of PP2 or GP2A for 30 min. A: Cell lysates were immunoprecipitated (IP) with an anti-αq antibody and immunoblotted (IB) with the anti-Src antibody. The blots were also probed with the antibody used for immunoprecipitation to ensure equal loading of proteins; B, C: following immunoprecipitation with an anti-αq or an anti-Src antibody, Src activity was determined as described in Methods. Immunoprecipitated proteins were also analyzed by western blot using the anti-αq or anti-Src antibodies as indicated.

![Figure 5](image5.png)  
**Figure 5** Involvement of the NPXYY motif in Src activation by the CCK2R. A: COS-7 cells transfected with the human CCK2R were stimulated with Gastrin (10 nmol/L) for the time indicated. Src kinase activity was determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. *P < 0.05 vs control, †P < 0.01 vs control, ‡P < 0.001 vs control; B: COS-7 cells transfected with the WT or N386A mutant CCK2R were stimulated or not with Gastrin (10 nmol/L) for 30 s and Src kinase activity determined as described in Methods. Immunoprecipitated proteins were also analysed by western-blot using anti-Src antibodies. Results of autoradiography quantification are presented as mean ± SE. †P < 0.001 vs control.
Involvement of the NPXXY motif in ERK activation by the CCK2R.

It is well demonstrated that numerous G protein-coupled receptors activate Src family kinases. However, very few publications have described the molecular mechanisms involved in Src activation by this receptor family. In the present study our results demonstrate that CCK2R-induced Src activation requires the NPXXY motif, and suggest a putative role of Gq in this mechanism. Indeed, this motif was previously described in Gq-dependent signalling pathways[1,9]. In addition, we showed that Src and Gαq are within one protein complex and we observed a Src-like tyrosine activity associated with Gq. However, despite the appearance of Src and Gαq in the same immunocomplex, we cannot exclude the possibility that Src activation by Gαq requires other protein. Our study also reveals that downstream of Src, activation of the ERK pathway by the CCK2R also requires the NPXXY motif.

The direct interaction of the Src SH3 domain with proline-rich motifs has been reported for the β3 adrenergic and the purinergic P2Y2 receptors[15,16]. Src family kinases also possess an SH2 domain that could directly bind to tyrosine-phosphorylated β2 adrenergic receptor[16]. We tested these two hypotheses in Src activation by the CCK2R. Indeed, the CCK2R has two proline-rich sequences which might interact with SH3 domains. In addition, it also has an ITIM-like motif within the C-terminus tail (LSYTTI) that is potentially phosphorylated on tyrosine residues, in turn leading to the recruitment of PLCγ through its SH2 domain[10]. Mutation of the prolines into serines, as well as the replacement of the tyrosine 438 of the ITIM-like motif by a phenylalanine did not affect the activation of Src by the CCK2R in COS-7 cells (data not shown).

Transactivation of receptor tyrosine kinases is another mechanism by which G protein-coupled receptors activate Src family kinases. Recently, the CCK2R has been shown to transactivate the EGF receptor[17-19]. However, this mechanism seems to be dependent on the cellular model. In the present study, we have observed that inhibition of the EGF receptor by AG1487 did not abolish CCK2R-mediated Src activation in AR42J and COS7 cells (data not shown).

Our study also shows that Src is activated upstream of the ERK pathway by the CCK2R in pancreatic tumor cells and contributes to the proliferative effects mediated by this receptor.

The development of cancer is thought to be dependent on the deregulation of normal signaling pathways involved in cell proliferation, thus conferring a growth advantage to cells. The role of Src and the ERK pathway in the regulation of cell growth is well documented. These signalling molecules have been implicated in the proliferative effects induced by tyrosine kinase receptors, cytokine receptors and G protein-coupled receptors. Activation of Src and ERK proteins has been observed in several human cancers and may contribute to the neoplastic phenotype. However, there is currently very little information regarding the role of the SRC/ERK pathway in the early stages of pancreatic carcinogenesis.

Here we report that the expression of the CCK2R in mouse pancreatic acinar tissue leads to strong activation of the Src tyrosine kinase and the ERK pathway. These transgenic mice display an increased growth of the pancreas.

**DISCUSSION**

It is well demonstrated that numerous G protein-coupled...
and preneoplastic lesions. They also develop pancreatic tumors with a ductal phenotype similar to what is observed in human pancreatic cancers. Deregluation of the Src/ERK pathway by the CCK2R in these transgenic mouse tumors might represent an early step that contributes to cell proliferation, formation of preneoplastic lesions and pancreatic tumor development. In addition, while several studies have reported the in vitro activation of Src and ERKs by gastrin,[10,11,20,21] this study is the first to demonstrate in vitro the up-regulation of the Src/ERK pathway by CCK2R.

In summary, our study describes the mechanism by which the CCK2R, a GPRC mainly coupled to Gq, activates Src. Our results show the involvement of the NPXXY motif within the receptor sequence in this activation, and suggest the putative role of Gq in this mechanism. Moreover, in pancreatic tumoral models we demonstrate in vitro and in vivo that the CCK2R activates the Src/ERK signaling pathway, a transduction cascade upregulated during tumorigenesis in human.

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