A Novel Mechanism for JAK2 Activation by a G Protein-coupled Receptor, the CCK2R

IMPLICATION OF THIS SIGNALING PATHWAY IN PANCREATIC TUMOR MODELS*

To date very few G protein-coupled receptors (GPCRs) have been shown to be connected to the Janus kinase (JAK)/STAT pathway. Thus our understanding of the mechanisms involved in the activation of this signaling pathway by GPCRs remains limited. In addition, little is known about the role of the JAK pathway in the physiological or pathophysiological functions of GPCRs. Here, we described a new mechanism of JAK activation that involves Goq proteins. Indeed, transfection of a constitutively activated mutant of Goq, (Q209L) in COS-7 cells demonstrated that Goq is able to associate and activate JAK2. In addition, we showed that this mechanism is used to activate JAK2 by a GPCR principally coupled to Goq, the CCK2 receptor (CCK2R), and involves a highly conserved sequence in GPCRs, the NPXXY motif. In a pancreatic tumor cell line expressing the endogenous CCK2R, we demonstrated the activation of the JAK2/STAT3 pathway by this receptor and the involvement of this signaling pathway in the proliferative effects of the CCK2R. In addition, we showed in vivo that the targeted CCK2R expression in pancreas of Elas-CCK2 mice leads to the activation of JAK2 and STAT3. This process may contribute to the increase of pancreas growth as well as the formation of preneoplastic lesions leading to pancreatic tumor development observed in these transgenic animals.

The Janus kinase/signal transducer and activator of transcription (JAK/STAT)1 pathway is well known to be activated by the family of cytokine receptors and to mediate a wide variety of biological effects, such as immune response, differentiation, cell survival, proliferation, or oncogenesis (1). The mechanism of JAKs activation is well known for the cytokine receptors. Ligand binding induces oligomerization of the receptor subunits, constitutively associated to JAKs, and a transphosphorylation of the tyrosine kinases. Activated JAKs in turn phosphorylate the receptor that recruits the STAT proteins (1). To date very few G protein-coupled receptors (GPCRs) have been shown to be connected to the JAK/STAT pathway (2–6). Thus, our understanding of the mechanisms involved in the activation of this signaling pathway by GPCRs remains limited. In particular it is not known whether a link exists between Goq protein and JAK family members. In addition, little is known about the role of the JAK pathway in the physiological or pathophysiological functions of GPCRs. The CCK2 receptor (CCK2R) is a seven-helix membrane-spanning receptor principally coupled to Goq proteins (7). Initially, this receptor was implicated in the secretory effects of a digestive peptide hormone, gastrin. However, the CCK2R is now recognized to mediate the mitogenic and anti-apoptotic effects of gastrin on gastrointestinal and pancreatic cells (8). In the transgenic mice Elas-CCK2, CCK2 receptor expression has been targeted in pancreatic acinar cells using transcriptional elements of the elastase-1 gene (9). Recently we have reported in this model (i) an increased pancreatic growth, (ii) an acinar to ductal trans-differentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis, (iii) and the development of tumors (10). Similar observations have been reported in two other transgenic models overexpressing transforming growth factor-α or a mutant Kras in exocrine pancreas (11, 12).

Among JAK members, JAK2 is widely described as being involved in cancer. Its inhibitor, AG490, has been shown to block constitutive STAT3 activation and suppress the proliferation of different cancer cell lines (13–16). Moreover, JAK2 can delay cell death by inducing Bcl-2 (17). The aim of this study was to analyze the mechanism for JAK2 activation by the CCK2R and to determine the putative role of this signaling pathway in the pathophysiological functions of this receptor in pancreatic tumor models.

EXPERIMENTAL PROCEDURES

Animals—Homozygous Elas-CCK2 mice used in this study have been described previously (10). At least three six-month-old homozygous Elas-CCK2 mice in a B6SJL1 background and three corresponding control littermate mice were used. Mice were reared in the 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.

Immunohistochemistry—Mice were killed by decapitation, and pancreases were excised, fixed in Bouin’s solution, and embedded in paraffin using standard techniques. Immunohistochemistry was performed as described previously (10). The antibodies used were anti-PY1007–1008 JAK2 antibodies (UBI) and anti-PY705 STAT3 (Cell Signaling). Sections were incubated with the appropriate secondary and tertiary peroxidase-labeled anti sera (Dako) at room temperature and exposed to a solution of diaminobenzidine for the anti-PY705-STAT3 staining and a solution of aminoethylcarbazole for the anti-PY1007–1008 JAK2, anti-JAK2, and anti-STAT3 staining. All dilutions and washes were
done in phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin.

**Western Blot Analysis on Isolated Acinar Cells or AR4–2J Cells**—Western blot analyses were performed on (i) dispersed acini from mice pancreas prepared as described previously (18) or on (ii) immunoprecipitates from AR4–2J 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 (19).

**Cell Culture and Proliferation Assay**—AR4–2J cells and COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with, respectively, 10 and 5% fetal calf serum at 37 °C in a 95% air, 5% CO₂ atmosphere. For proliferation assays, an optimal number of AR4–2J cells (4 × 10⁵ cells) were plated in 35-mm dishes, serum-starved for 24 h, then treated for 48 h with gastrin (10 nM). When indicated, cells were incubated with AG490 (10 μM). Cells were counted by using a Coulter electronic counter.

**JAK2 Kinase Assay**—After gastrin stimulation cells were lysed, and JAK2 was immunoprecipitated with specific antibodies (UBI). Kinase assays were performed and analyzed as described previously (20). Proteins were separated by SDS-PAGE, and the gel autoradiographed.

**Construction of Mutant Receptor cDNAs and Transient Transfection**—Mutant receptor cDNAs were constructed as described previously (7). Mutations were confirmed by DNA sequencing using an automated sequencer (Applied Biosystems). Plasmids coding for wild type or mutant CCK2R (6 μg) for JAK2 (2 μg) or for HA-tagged Q209L mutant (2 μg) were transiently transfected into COS-7 cells using the DEAE/dextran method as described previously (7). Murine JAK2 cDNA subcloned in pRK5 vector (pRK5-JAK2) was provided by Dr. James Ihle (Memphis, TN).

**Binding Studies and Measurement of Inositol Trisphosphate (IP₃) Accumulation**—24 h after transfection cells were transferred to 24-well culture plates and seeded at a density of 5,000 cells/well (binding studies) or 150,000 cells/well (IP₃ measurement). Binding studies and measurement of IP₃ accumulation were performed as described previously (7); assays were performed in duplicate in at least three separate experiments. Nonspecific binding was always less than 10% of total binding. Binding data were determined using the nonlinear least squares curve-fitting computer program LIGAND and GraphPad Prism Program (Software). EC₅₀ were calculated using GraphPad Prism Program Software.

**Immunofluorescence Staining**—COS-7 cells were grown for 24 h on

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**FIG. 1.** CCK2R expression in the acini of Elas-CCK2 mice induces the activation of JAK2. Immunohistochemistry analysis on paraffin-embedded pancreatic tissues or Western blots on lysates from isolated acinar cells were performed using antibodies specific for total JAK2 (JAK2) or detecting the active form of the protein, anti-PY1007–1008 JAK2 antibodies (PY-JAK2). Representative data from three experiments (three different animals in each group) are shown. A, original magnification ×40. B, blots were also probed with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading of proteins, and the results of Western blots (IB) were quantified and presented as the means ± S.E.

**FIG. 2.** CCK2R expression in the acini of Elas-CCK2 mice induces the activation of STAT3. Immunohistochemistry analysis on paraffin-embedded pancreatic tissues or Western blots (IB) on lysates from isolated acinar cells were performed using antibodies specific for total STAT3 (STAT3) or detecting the activated form of the protein, anti-PY705 STAT3 antibodies (PY-STAT3). Representative data from three experiments (three different animals in each group) are shown. A, original magnification ×40. B, blots were also probed with an antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to ensure equal loading of proteins, and results of Western blots were quantified and presented as means ± S.E.
plates containing cover slides. 24 h after transfection cells were fixed in 2% paraformaldehyde, permeabilized with methanol, blocked in 1% fetal calf serum-phosphate-buffered saline, and incubated with primary antibodies (anti-HA antibody (Berkeley Antibody Co., Covance), anti-PY1007–1008 JAK2 antibody (UBI) according to standard immunofluorescence methods. Secondary antibodies coupled to CY-3 or fluorescein isothiocyanate were purchased from Sigma and Jackson Immunoresearch Laboratories. Slides were mounted in fluorescent mounting medium (DAKO) and analyzed on a Nikon E400 microscope with a Sony DXC 950 camera and Visiolab 2000 software. Images were assembled using Adobe Photoshop software. For semiquantitative comparisons, identical volumes of antibody mix were used for all samples, and identical exposure times were taken.

**Statistical Analysis**—Means ± S.E. and Student tests were performed using GraphPad Prism. ***, p < 0.001; **, 0.001 < p < 0.01; *, 0.01 < p < 0.05; non significant, p > 0.05.

**RESULTS**

**CCK2R Expression in the Acini of Elas-CCK2 Mice Induces the Activation of JAK2**—We recently described the Elas-CCK2 mice expressing the human CCK2R in acini. These mice exhibit an increased pancreatic growth, an acinar to ductal differentiation, postulated to be a preneoplastic step in pancreatic carcinogenesis and tumor development (9). Among JAK family members, JAK2 is widely described to be involved in cancer, particularly in prostate and breast cancers. JAK2 activation was analyzed by immunohistochemical methods on pancreatic sections from control and Elas-CCK2R mice using antibodies specific for total JAK2 or by detecting the activated form of the protein phosphorylated on tyrosines 1007 and 1008.
Among the STAT family members (PY-JAK2). The tissues derived from Elas-CCK2 mice demonstrated higher levels of JAK2 activation as compared with control mice (Fig. 1A, upper panels). In contrast, total JAK2 protein expression was unchanged in the two mice models (Fig. 1A, lower panels).

To confirm and quantify JAK2 activation, Western blotting analyses were performed on lysates of acinar cells isolated from pancreas of control and Elas-CCK2 mice. JAK2 activation in Elas-CCK2 mice was significantly elevated (mean ± S.E.; 5.05-fold ± 0.08; 0.01 < p < 0.05; n = 3) as compared with controls (Fig. 1B). Thus, the expression of the CCK2R in mouse pancreatic acini induces JAK2 activation.

**CCK2R Expression in the Acini of Elas-CCK2 Mice Induces the Activation of STAT3—Among the STAT family members activated by the JAKs kinases, STAT3 is now recognized as an oncogene (21). We decided to study its activation in pancreas of control and Elas-CCK2 mice. JAK2 activation in Elas-CCK2 mice was significantly elevated (mean ± S.E.; 1.88-fold ± 0.08; 0.01 < p < 0.05; n = 3) as compared with controls (Fig. 1B). Thus, the expression of the CCK2R in mouse pancreatic acini induces JAK2 activation.

Activation of the JAK2/STAT3 Pathway by the CCK2R in Acinar Tumor Cells—JAK2 is not only involved in cell proliferation or survival but also regulates many processes like inflammation or erythropoiesis (1). Thus, treating Elas-CCK2 mice with the JAK2-specific inhibitor AG490 over weeks is not possible. Therefore, to address the role of JAK2 upstream of STAT3 activation induced by the CCK2R in acinar cells, we used the AR4–2J cells, the only pancreatic tumor cell line exhibiting an acinar phenotype, established after a chemically induced tumorigenesis by azaserine. AR4–2J cells were previously shown to express an endogenous CCK2R (22).

We first confirmed that the CCK2R was able to induce JAK2 activation in AR4–2J cells. Using JAK2 autophosphorylation ability, *in vitro* tyrosine kinase assays were performed in anti-JAK2 immunoprecipitates from cell lysates containing equal amounts of proteins. Gastrin (10 nM) significantly activates JAK2 phosphorylation at 1 min that was maximal after 1 h of stimulation by the CCK2R agonist (mean ± S.E.; 2.48-fold ± 0.04 compared with control; p < 0.001; n = 3). To investigate whether CCK2R induces STAT3 activation, serum-starved AR4–2J cells were treated with 10 nM gastrin for various times and lysed. Western blotting analysis performed with antibodies specific for the activated forms of STAT3 shows that CCK2R induces a significant activation of STAT3 from 15 to 120 min after gastrin treatment with a maximal activation after 60 min (mean ± S.E.; 3.04-fold ± 0.12 compared with control; p < 0.001; n = 3), whereas the amount of total STAT3 protein remains unchanged (Fig. 4). To determine the involvement of JAK2 in CCK2R-induced STAT3 ac-
We performed the corresponding mutation N386A on the human CCK2R, creating the N386A-CCK2R mutant, and studied its pharmacological characteristics (Table I). We first determined the affinity of the N386A-CCK2R mutant for its ligand by performing binding experiments as described under “Experimental Procedures.” Radioligand Scatchard analysis of binding to the WT-CCK2R and N386A-CCK2R mutant revealed that this CCK2R mutant possesses an affinity and a maximal binding capacity similar to the WT-CCK2R ($K_d$ 2.8 ± 0.5 nM and $B_{max}$ 8.5 ± 1.5 pmol/10⁶ cells $versus$ $K_d$ 1.8 ± 0.7 nM and $B_{max}$ 9.8 ± 4.0 pmol/10⁶ cells, respectively). These data indicate that the mutation of asparagine 386 into alanine neither affects the high affinity binding of CCK2R agonists nor the cell surface receptor expression. However, mutating the asparagine into alanine dramatically down-regulates the total IP$_3$ production (Fig. 7; mean ± S.E.; 83.04% ± 3.943, 0.01 < $p$ < 0.05; $n$ = 4). These data confirm that the N386A-CCK2R mutant highly decreased $G_q$-dependent signaling. Therefore, we tested its effect on JAK2 activation. Our results (Fig. 7, A and B) show that the N386A-CCK2R mutant cannot mediate JAK2 activation, in contrast to the WT-CCK2R. In addition, we demonstrate that the activation of the downstream effector of JAK2, STAT3, is also blocked when the CCK2R is mutated on the NPXYX motif (Fig. 7C). Thus, we demonstrate here the involvement of the conserved NPXYX motif within the receptor sequence in the activation of the JAK2/STAT3 pathway by the CCK2R and also the putative role of $G_q$ in this mechanism.

To our knowledge $G_q$ protein has never been demonstrated in JAK activation. To confirm the role of $G_q$ proteins in JAK2 activation, we transiently transfected the HA-tagged constitutively activated $\alpha_q$ mutant were grown for 24 h on plates containing cover slides, then similarly fixed and stained with both anti-HA and anti-PT1007–1008 JAK2 antibodies using standard immunofluorescence techniques (40X). B, Western blot (IB) analysis of the association between $G_q$ and JAK2 in COS-7 cells transfected (T) or not (NT) with cDNA coding for the HA-tagged (Q209L) constitutively activated $\alpha_q$ mutant. Cell lysates were immunoprecipitated (IP) with an anti-HA antibody and Western-blotted with the anti-JAK2 antibody. The blots were also probed with the antibody used for immunoprecipitation to ensure equal loading of the immunoprecipitated proteins.

tivation, we then tested the effect of a JAK2 inhibitor, AG490, on STAT3 activation in response to gastrin. The phosphorylation of STAT3 after gastrin stimulation was completely blocked by the JAK2 inhibitor, indicating that CCK2R-induced STAT3 activation is totally JAK2-dependent in this cellular model.

Role of JAK2 in Acinar Tumor Cell Proliferation Induced by CCK2R Activation—It has been previously reported that gastrin induces the proliferation of the AR4–2J cells through the CCK2R (22). To study the role of JAK2 in the proliferation of acinar tumor cells induced by the CCK2R, we measured AR4–2J proliferation in the presence or absence of the JAK2-specific inhibitor, 48 h after gastrin stimulation. CCK2R activation by gastrin induces a significant increase of cell proliferation (mean ± S.E.; 1.575-fold ± 0.18; $p$ < 0.05; $n$ = 3) (Fig. 5). Treatment of the cells with AG490 totally inhibits CCK2R-induced AR4–2J proliferation. This result confirms that JAK2 mediates CCK2R proliferative effects on AR4–2J cells.

Mechanism of CCK2R-induced JAK2 Activation—To study the molecular mechanism involved in JAK2 activation by the CCK2R, we used COS-7 cells transiently co-transfected with cDNAs coding for the human wild type CCK2R (WT-CCK2R) or mutant CCK2R and for JAK2. We first validated this model for CCK2R-induced JAK2 activation. Using JAK2 autophosphorylation ability, in vitro tyrosine kinase assays were performed in anti-JAK2 immunoprecipitates from cell lysates. Gastrin significantly activates JAK2 in this transiently transfected cell model. A rapid and significant activation of JAK2 (15 s), still detectable at 3 min, was found in response to gastrin (Fig. 6). Western blot analysis for JAK2 protein expression revealed an equal amount of the protein in transfected cells.

The CCK2R is known to be coupled to $G_q$ proteins (7). Recently, the NPXYX motif (X represents any amino acid), located at the end of the seventh transmembrane domain, has been shown to be involved in $G_q$-dependent signaling pathways induced by the rat CCK2R. Mutation of the asparagine of the NPXYX motif of the rat CCK2R into alanine inhibits $G_q$-dependent pathways such as IP$_3$ production or extracellular signal-regulated kinase activation induced by the receptor (7). This motif is conserved within the human CCK2R sequence.

**DISCUSSION**

Like cytokine receptors, several GPCRs have been shown to activate JAKs kinases and STAT family members. However, very little is known about the molecular mechanisms involved in the activation of the JAK/STAT pathway by this receptors family. Among the mechanisms of JAK activation by GPCR described previously, the requirement of both the highly conserved (E/D)RY motif within the receptor sequence and the heterotrimeric $G_q$ protein has been reported for the chemokine receptors (23). Regarding the angiotensin type 1-receptor, SHP2 has been shown to interact with the receptor via an immunoreceptor tyrosine-based inhibition motif-like motif (YIMP) and to play the role of an adaptor protein recruiting JAK2 and facilitating its activation (3), (24). We tested these different hypotheses in JAK2 activation by the CCK2R. Indeed, like the angiotensin type 1-receptor, the CCK2R has an ITIM-like motif within its C terminus tail (LSYTTI) capable to recruit SHP2 (data not shown). The CCK2R also presents an ITIM-like motif or the (E/D)RY motif within its C terminus tail (LSYTTI) capable to recruit SHP2 (data not shown). These data confirm that the N386A-CCK2R mutant highly decreased $G_q$-dependent signaling. Therefore, we tested its effect on JAK2 activation. Our results (Fig. 7, A and B) show that the N386A-CCK2R mutant cannot mediate JAK2 activation, in contrast to the WT-CCK2R. In addition, we demonstrate that the activation of the downstream effector of JAK2, STAT3, is also blocked when the CCK2R is mutated on the NPXYX motif (Fig. 7C). Thus, we demonstrate here the involvement of the conserved NPXYX motif within the receptor sequence in the activation of the JAK2/STAT3 pathway by the CCK2R and also the putative role of $G_q$ in this mechanism.

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**Mechanism of CCK2R-induced JAK2 Activation—**To study the molecular mechanism involved in JAK2 activation by the CCK2R, we used COS-7 cells transiently co-transfected with cDNAs coding for the human wild type CCK2R (WT-CCK2R) or mutant CCK2R and for JAK2. We first validated this model for CCK2R-induced JAK2 activation. Using JAK2 autophosphorylation ability, in vitro tyrosine kinase assays were performed in anti-JAK2 immunoprecipitates from cell lysates. Gastrin significantly activates JAK2 in this transiently transfected cell model. A rapid and significant activation of JAK2 (15 s), still detectable at 3 min, was found in response to gastrin (Fig. 6). Western blot analysis for JAK2 protein expression revealed an equal amount of the protein in transfected cells.

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form of $G_{q}$ associates and activates JAK2. In addition we demonstrate that CCK2R-induced JAK2 activation requires the NPXXY motif, located at the end of the seventh transmembrane domain and described in $G_{q}$-dependent signaling pathways. Our study also reveals that this motif is required for STAT3 activation by the CCK2R. Thus, the involvement of $G_{q}$ upstream of JAK2 could represent a new mechanism for the activation of this tyrosine kinase by GPCRs.

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 the cells. Here, we show that the JAK2/STAT3 pathway is activated by the CCK2R in pancreatic tumor cells and contributes to the proliferative effects mediated by this receptor.

Another important finding of this study is that the expression of a GPCR, namely the CCK2R, targeted in mouse pancreatic acinar tissue leads to the overactivation of the tyrosine kinase JAK2 and the transcription factor STAT3. These transgenic mice display an increased growth of the pancreas and develop neoplastic lesions, then pancreatic tumors, presenting a ductal phenotype similar to what is observed in human pancreatic cancers. The deregulation of the JAK2/STAT3 pathway by the CCK2R in these transgenic mice might represent an early step contributing to cell proliferation and pancreatic tumor development.

In summary, our study describes a new mechanism in JAK2 activation involving $G_{q}$ protein. We showed that this mechanism is used by the CCK2R to activate this tyrosine kinase and involves the NPXXY motif within the receptor sequence. Moreover, in pancreatic models we demonstrate in vitro and in vivo that the CCK2R activates the JAK2/STAT3 signaling pathway, a transduction cascade up-regulated during the tumor process in human.

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