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Gastrin stimulates the formation of a $p60^{Src}$/$p125^{FAK}$ complex upstream of the phosphatidylinositol 3-kinase signaling pathway

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Abstract The molecular events whereby gastrin occupancy of G/CCK_B receptors leads to phosphatidylinositol (PI) 3-kinase activation have been examined. We report here that this peptide promotes the association between two non-receptor tyrosine kinases, $p60^{Src}$ and $p125^{FAK}$, and elicits a parallel increase in tyrosine phosphorylation and activity of both kinases. Gastrin-induced PI 3-kinase activity was coprecipitated with $p60^{Src}$ and $p125^{FAK}$ and was inhibited by herbimycin A, the selective Src inhibitor PP-2 or cytochalasin D, which disrupts the actin cytoskeleton and prevents $p125^{FAK}$ activity. These results indicate, for the first time, that a $p60^{Src}$/$p125^{FAK}$ complex acts upstream of the gastrin-stimulated PI 3-kinase pathway.

Key words: Gastrin; G protein-coupled receptor; Phosphatidylinositol 3-kinase; Src-family kinases; Focal adhesion kinase; Cellular proliferation

1. Introduction

Although characterized as a stimulant of gastric acid secretion, the peptide hormone gastrin exerts growth-promoting effects on normal and neoplastic gastrointestinal tissues. This regulatory peptide has been reported to stimulate the growth of normal gastrointestinal mucosa [1]. It also induces the proliferation of colon, gastric and pancreatic cancer cell lines established in vitro [2–4], as well as tumor cells transplanted in vivo [5]. These effects have been shown to be mediated by a specific transmembrane G protein-coupled receptor, the G/CCK_B receptor, which is known to be linked to the phospholipase C/protein kinase C pathway [4,6]. Like many growth factors involved in cell proliferation, gastrin activates the mitogen-activated protein kinases (MAPK) cascade [7,8] and enhances subsequently the expression of early genes known to function in regulating cell growth, such as the proto-oncogenes c-*fos* or c-*jun* [9]. Phosphatidylinositol 3-kinases (PI 3-kinases), lipid kinases which catalyze the 3′-phosphorylation of inositol phospholipids, are known to play an important role in mitogenesis. This family of kinases is activated by a variety of cell receptors. Receptors with intrinsic tyrosine kinase activity recruit heterodimeric PI 3-kinase consisting of a 110-kDa catalytic subunit (p110) and an 85-kDa regulatory subunit (p85). Activation of PI 3-kinase by tyrosine kinase receptors, such as platelet-derived growth factor receptor or epidermal growth factor receptor, is well documented and is mediated by binding of autophosphorylated tyrosine residues of the tyrosine kinases receptors to the SH2 (for Src homology 2) domain of p85 [10,11]. In contrast, the mechanism leading to PI 3-kinase activation by insulin [12] or insulin-like growth factor I [13] involves the phosphorylation of an intermediate protein, the insulin receptor substrate-1 (IRS-1), which then associates the SH2 domain of p85. In addition to the tyrosine phosphorylation-dependent activation of PI 3-kinase, it has been reported that the βγ subunits of G proteins also stimulate the lipid kinase activity of a novel PI 3-kinase that do not interact with p85, designated p110γ [14]. Only few ligands acting via G protein-coupled receptors, such as angiotensin or noradrenaline, have been shown to increase the activity of the p85/p110 PI 3-kinase [15,16]. We have recently demonstrated that gastrin induced PI 3-kinase activity in anti-p85 precipitates [17]. However, the mechanism leading to the activation of the classical p85/p110 form of PI 3-kinase by seven transmembrane spanning receptors is still poorly defined. Tyrosine phosphorylation has been involved in the intracellular signaling of peptides that act as potent growth factors through G protein-coupled receptors. Since the G/CCK_B receptor does not possess intrinsic tyrosine kinase activity, the tyrosine phosphorylations induced by gastrin could be mediated by activation of one or more non-receptor tyrosine kinases. Several lines of evidence suggest a role for the ubiquitous non-receptor Src-family tyrosine kinases in signal transduction leading to cell proliferation via G protein-coupled receptors. Indeed, inhibition of Src-related kinases has been shown to block angiotensin II-stimulated Ras activation [18] as well as lysophosphatidic acid and α2 adrenergic receptor-stimulated MAPK pathway [19]. The catalytic activity of $p60^{Src}$ is tightly regulated in vivo by phosphorylation: phosphorylation of tyrosine 527 in the C-terminal tail is inhibitory whereas the kinase domain phosphorylation (at tyrosine 416) is stimulatory [20]. Although it is known that gastrin stimulates the phosphorylation of Src-family kinases [21,22], whether this peptide activates or not $p60^{Src}$ has not yet been established.

Since we have previously reported that gastrin exerts trophic effects on the tumor-derived pancreatic acinar cell line AR4-2J [4], we analyzed, in this cellular model, the possibility that this peptide might stimulate the activity and the association of $p60^{Src}$ with another cytosolic tyrosine kinase, the focal adhesion kinase ($p125^{FAK}$). $P125^{FAK}$ is phosphorylated on tyrosine residues following activation by integrins and oncogenes [23], addition of growth factors [24] or stimulation by neuropeptides, such as bombesin, vasopressin or gastrin [6,25]. We also assessed the role of the $p60^{Src}$/$p125^{FAK}$ complex in the gastrin-stimulated PI 3-kinase signaling pathway.

2. Materials and methods

2.1. Cell culture

AR4-2J cells, originally obtained by Jessop and Hay [26] from a rat exocrine pancreatic tumor (azaserine induced), were a gift from Dr. C.

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Logsdon (Ann Arbor, MI). The cells, plated at 75 000 cells/ml, were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum. The medium was changed every 2 days.

2.2. Immunoprecipitation

AR4-2J cells were serum starved in DMEM for 18 h before peptide addition. After stimulation, the cells were washed with ice cold buffer A (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 100 mM NaF, 2 mM orthovanadate, pH 7.5) and homogenized in 500 μl lysis buffer (buffer A containing 1% Triton X-100 or 1% NP-40 for kinase activities, 0.5 mM phenylsulfonyl fluoride, 20 μM leupeptin, 100 IU/ml trasylol) for 15 min at 4°C. The solubilizates were immunoprecipitated with the indicated antibodies preadsorbed on protein-A or protein-G Sepharose.

2.3. Western blotting analysis

Immunoprecipitates were washed twice with 30 mM HEPES, pH 7.5 containing 30 mM NaCl and 0.1% Triton X-100, resuspended in SDS sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon PVDF Millipore). Membranes were blocked with saline buffer (10 mM Tris, 140 mM NaCl, pH 7.4) containing 5% bovine serum albumin (BSA) or non-fat dried milk and incubated overnight with the indicated antibodies. Proteins were visualized using ^{125}I-protein-A followed by autoradiography.

2.4. Immune complex kinase assays

Immunoprecipitates were washed twice with lysis buffer and four times with TBS (25 mM Tris, pH 7.5, 150 mM NaCl). Kinase assays were carried out at 30°C for 10 min in 40 μl of kinase buffer (20 mM HEPES, pH 7.5, 10 mM $MnCl_2$, 1 mM dithiothreitol, 3.75 μM cold ATP, 5–10 μCi of [γ-^{32}P]ATP). In Src-kinase assays, 5 μM of enolase was added as an exogenous substrate. Samples were analyzed by SDS-PAGE under reducing conditions. The gels were then treated with 1 N KOH at 60°C for 1 h after electrophoresis and autoradiographed.

2.5. PI 3-kinase assay

After immunoprecipitation with the indicated antibodies, the pellets were assayed for the PI 3-kinase activity. After washing, the pellets were resuspended in 30 μl 20 mM HEPES, 0.4 mM EDTA, 0.4 mM Na_2HPO_4. The kinase reaction was started by addition of phosphatidylinositol (PtdIns) at a final concentration of 0.2 mg/ml, 10 mM $MgCl_2$ and 50 μM [γ-^{32}P]ATP. After 15 min, the reaction was stopped by addition of 4 M HCl and the phosphoinositol lipids were extracted with chloroform/methanol (1:1). Phospholipids contained in the organic phase were separated by thin layer chromatography. The plate was analyzed by autoradiography.

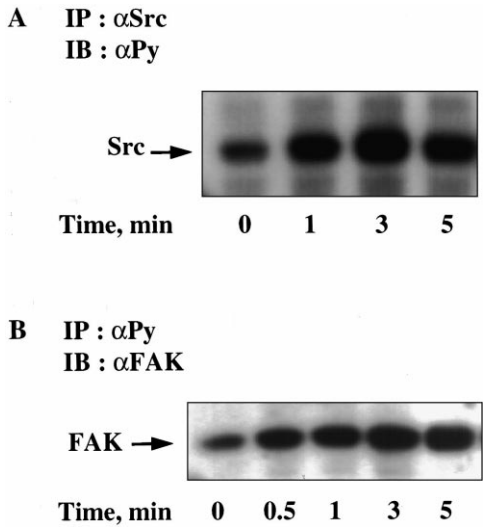

Fig. 1. Tyrosine phosphorylation of p60^{Src} and p125^{FAK} in response to gastrin. AR4-2J cells were incubated for the times indicated with 10 nM of gastrin. Cellular proteins were immunoprecipitated (IP) with (A) anti-p60^{Src} or (B) anti-phosphotyrosine (Py) antibodies and immunoprecipitates were immunoblotted (IB) with (A) anti-phosphotyrosine or (B) anti-p125^{FAK} antibodies. The migration of phosphorylated p60^{Src} and p125^{FAK} is indicated by the arrow. The data presented are representative of three independent experiments with similar results.

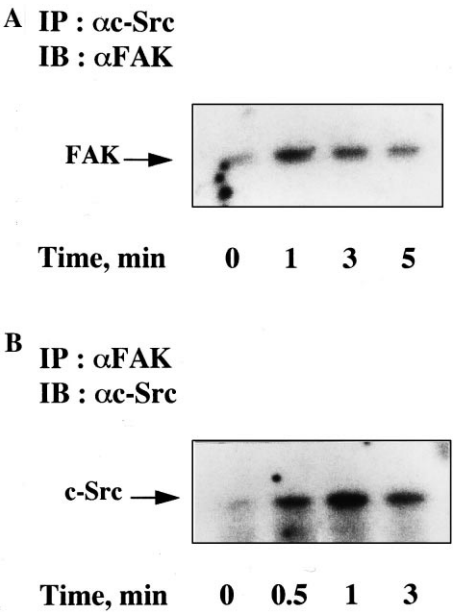

Fig. 2. Gastrin induces association of p60^{Src} with p125^{FAK}. AR4-2J cells were stimulated for the indicated time periods with 10 nM gastrin. A: Cell extracts were immunoprecipitated (IP) with an antibody to p60^{Src} and immunoblotted (IB) using anti-p125^{FAK} antibodies. The migration of p125^{FAK} is indicated by the arrow. B: Cellular proteins were immunoprecipitated (IP) with an anti-p125^{FAK} antibody and immunoprecipitates were immunoblotted (IB) with an anti-p60^{Src} antibody. The association between p60^{Src} and p125^{FAK} is indicated by the arrow. Similar results were obtained in three independent experiments.

2.6. Materials

Human gastrin$_{2-17ns}$ was purchased from Bachem (Switzerland). ^{125}I-Na (100 mCi/ml) was obtained from Amersham and [γ-^{32}P]ATP (7000 Ci/mmol) from ICN (France). PtdIns from bovine liver, cytochalasin D, enolase and wortmannin were purchased from Sigma (France). Herbimycin A, PP-2 and LY294002 were from Calbiochem (France). Anti-p125^{FAK} and anti-phosphotyrosine antibodies were from Santa Cruz (Tebu, France). Monoclonal anti-p60^{Src} antibodies were obtained from Oncogene Science (Genzyme, France). Rabbit polyclonal antibodies specific to p85 were kindly provided by Drs. Y. Le Marchand-Brustel and J.F. Tanti (INSERM U.145, Nice, France).

3. Results

3.1. Tyrosine phosphorylation of p60^{Src} and p125^{FAK} in response to gastrin

We first investigated whether gastrin could induce tyrosine phosphorylation of p60^{Src} and p125^{FAK} in pancreatic tumor cells. AR4-2J cells were stimulated with 10 nM gastrin for varying lengths of time (Fig. 1). Cell lysates were subjected to immunoprecipitation with anti-p60^{Src} (A) or anti-phosphotyrosine (B) antibodies and precipitates were analyzed by immunoblotting using anti-phosphotyrosine (A) or anti-p125^{FAK} (B) antibodies. An increase in tyrosine phosphorylation of both p60^{Src} and p125^{FAK} was detected within 1 min and reached a maximum at 3 min (p60^{Src}: 2.53-fold ±0.2,

p125^{FAK}: 2.0-fold ±0.2, $n=3$). The magnitude of tyrosine phosphorylation of p60^{Src} decreased after 3 min, whereas for p125^{FAK} it remained stable until 5 min.

3.2. Gastrin induces association of p60^{Src} with p125^{FAK}

To determine whether p125^{FAK} associates p60^{Src} following gastrin stimulation, immunoprecipitates from AR4-2J cell lysates obtained with an anti-p60^{Src} antibody were immunoblotted with an anti-p125^{FAK} antibody (Fig. 2A). The amount of a 125-kDa protein recognized by anti-p125^{FAK} antibody was increased in the anti-p60^{Src} precipitates after gastrin stimulation. The association between p60^{Src} and p125^{FAK} reached a maximum at 1 min (3.4-fold ±0.1, $n=3$) and decreased thereafter. This association was also confirmed by immunoblot using an anti-p60^{Src} antibody in anti-p125^{FAK} immunoprecipitates (Fig. 2B).

3.3. Activation of p60^{Src} and p125^{FAK} tyrosine kinases by gastrin

We then examined the ability of gastrin to regulate kinase activity of p60^{Src} in AR4-2J cells. Src kinase assays were performed in anti-p60^{Src} immunoprecipitates after treatment of the cells with 10 nM of gastrin (as described in Section 2). The phosphorylation of the exogenous substrate enolase was measured. The p60^{Src} kinase activation by gastrin was increased within 30 s, reached a peak value at 1 min (2.2-fold ±0.2, $n=3$) and decreased thereafter (Fig. 3A). Kinase activity of p125^{FAK} has been shown to correlate with its phosphorylation. Since gastrin increased tyrosine phosphorylation of this protein, we examined whether activation of p125^{FAK} was detected following gastrin stimulation. In vitro autophosphorylation assays were carried out on anti-p125^{FAK} immunoprecipitates. As shown in Fig. 3B, we observed a rapid and transient increase (maximum at 1 min: 2.5-fold ±0.4, $n=3$) in p125^{FAK} activity. The rapid kinetics of p60^{Src} and p125^{FAK} activation could be correlated to the phosphorylation of both proteins in response to gastrin.

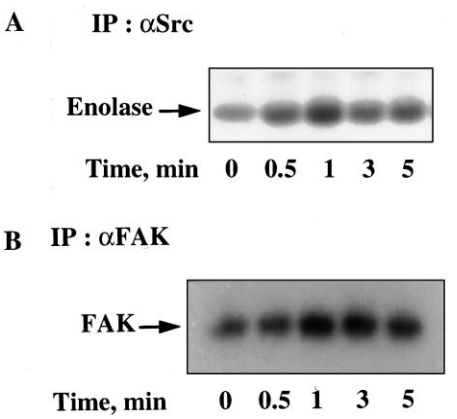

Fig. 3. Activation of p60^{Src} and p125^{FAK} tyrosine kinases by gastrin. AR4-2J cells were stimulated with 10 nM gastrin for varying lenghts of time. A: P60^{Src} was immunoprecipitated (IP) with a specific anti-p60^{Src} antibody and its kinase activity was assayed as described in Section 2. The arrow indicates the phosphorylated exogenous substrate enolase. B: P125^{FAK} activity was measured in anti-p125^{FAK} immunoprecipitates as described in Section 2. The autophosphorylation of p125^{FAK} protein is indicated by the arrow. The data presented are representative of three independent experiments with similar results.

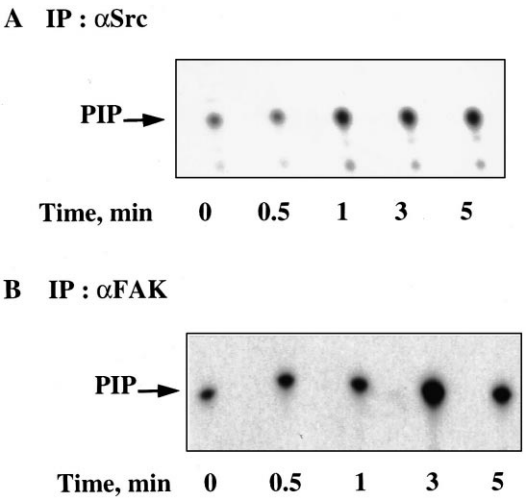

Fig. 4. Involvement of p60^{Src} and p125^{FAK} in gastrin-induced PI 3-kinase activation. AR4-2J cells were treated for the times indicated with 10 nM gastrin. Cell lysates were immunoprecipitated (IP) with an antibody against p60^{Src} (A) or p125^{FAK} (B). Immunoprecipitates were assayed for PI 3-kinase activity using PtdIns as substrate. The phospholipids were resolved on thin layer chromatography plates. The arrow indicates the phosphorylated substrate, PtdIns-P (PIP). Similar results were obtained in three independent experiments.

3.4. Activation of PI 3-kinase in anti-p60^{Src} and anti-p125^{FAK} precipitates following gastrin stimulation

We have recently demonstrated that gastrin activates the PI 3-kinase in AR4-2J cells [17]. To go further into the mechanism that relay the signal from the G/CCK$_B$ receptor to this enzyme, we examined whether PI 3-kinase activity was detected in association with p60^{Src} after gastrin stimulation. PI 3-kinase activity was measured in anti-p60^{Src} immunoprecipitates from cells treated with 10 nM of gastrin (Fig. 4A). The activation reached a maximum 1 min (3.0-fold ±0.4, $n=3$) after peptide addition. We also analyzed the possibility that p125^{FAK} could play a role in the PI 3-kinase pathway induced by gastrin. PI 3-kinase assays were performed in anti-p125^{FAK} precipitates from gastrin-treated AR4-2J cells. An increase in PI 3-kinase activity was detectable at 30 s (Fig. 4B). The stimulation reached a peak value within 3 min of treatment (3.1-fold ±0.7, $n=3$) and decreased at 5 min. Thus, a time-dependent increase in PI 3-kinase activity was observed in anti-p60^{Src} and anti-p125^{FAK} precipitates after gastrin stimulation, suggesting that p60^{Src} and p125^{FAK} are potentials mediators of PI 3-kinase activation by gastrin.

3.5. p60^{Src} and p125^{FAK} act upstream of PI 3-kinase activation by gastrin

To determine the contribution of Src-family tyrosine kinases in the PI 3-kinase activation induced by gastrin, we examined the effect of herbimycin A or the novel specific Src-protein tyrosine kinase inhibitor PP-2 [27]. Preincubation of the cells with 1 μM of herbimycin A or 50 μM of PP-2 respectively diminished by 91% ±1 and 102% ±1 ($n=3$) the PI 3-kinase activity in anti-p85 precipitates (Fig. 5A), confirming the role of Src-related kinases in this signaling cascade. The specificity of herbimycin A or PP-2 was assessed in vitro. Both inhibitors completely abolished basal and gastrin-stimulated p60^{Src} kinase activity (Fig. 5B, left) without affecting the autophosphorylation of p125^{FAK} (data not shown). We then determined the effect of cytochalasin D, known to depolymer-

ize the actin cytoskeleton and prevent the activation of p125^{FAK} by neuropeptides [28,29]. Pretreatment of cells with 2.5 μM of cytochalasin D blocked p125^{FAK} autophosphorylation (Fig. 5B, right) and greatly diminished PI 3-kinase activity (88% ±4, $n=3$) assessed in anti-p85 precipitates in response to gastrin (Fig. 5A).

To support the conclusion that p60^{Src} and p125^{FAK} are upstream of PI 3-kinase activation rather than in a downstream pathway, we examined the effects of two commonly used PI 3-kinase inhibitors, wortmannin and LY294002, on p60^{Src} and p125^{FAK} activation stimulated by gastrin. We did not observed any inhibition of gastrin-induced tyrosine kinase activities in cells pretreated with wortmannin or LY294002 (Fig. 5D) at drug concentrations (10 nM and 10 μM respectively) which totally abolish the activation of the PI 3-kinase by gastrin (Fig. 5C).

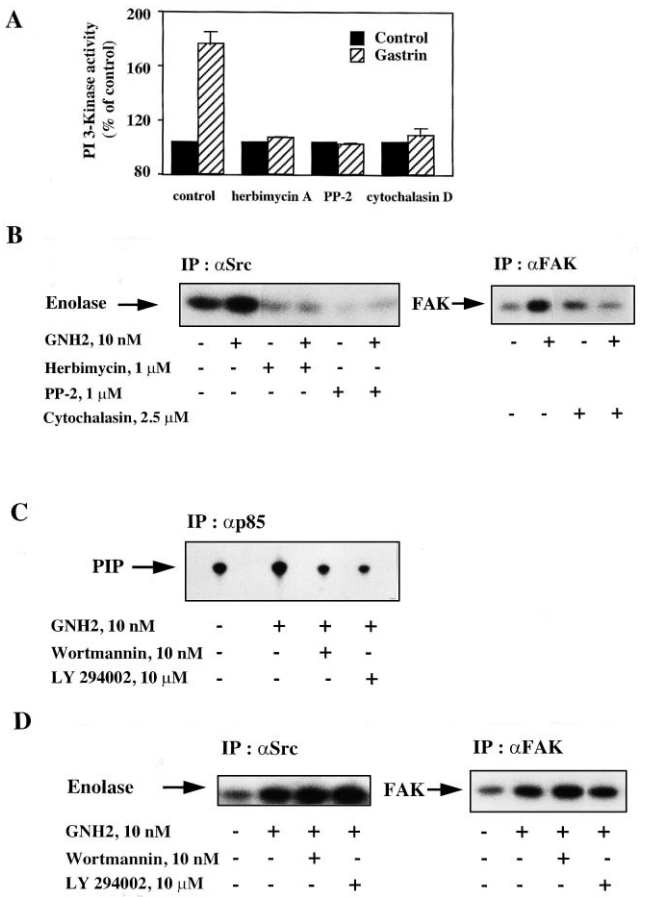

Fig. 5. p60^{Src} and p125^{FAK} act upstream of PI 3-kinase activation by gastrin. A: AR4-2J cell lysates were pretreated with the vehicle, 1 μM herbimycin A for 24 h, 50 μM PP-2 for 30 min or 2.5 μM cytochalasin D for 2 h in the absence (black bars) or presence (hatched bars) of 10 nM gastrin. PI 3-kinase activity was measured in anti-p85 immunoprecipitates using PtdIns as substrate. The data were plotted as percentages of the control values. Data from three separate experiments are presented as means ± S.E.M. (bars). B: Left: AR4-2J cells were stimulated with 10 nM gastrin; p60^{Src} was immunoprecipitated and in vitro kinase reactions were performed in presence or absence of 1 μM herbimycin A or 1 μM PP-2. Right: Cells were pretreated with 2.5 μM cytochalasin D for 2 h and then treated with 10 nM gastrin. The extracts were immunoprecipitated with an anti-p125^{FAK} antibody and in vitro kinase reactions were carried out as described in Section 2. C: AR4-2J cell lysates were pretreated with the vehicle, 10 nM wortmannin or 10 μM LY294002 for 1 h, in the presence or not of 10 nM gastrin. PI 3-kinase activity was measured in anti-p85 immunoprecipitates using PtdIns as substrate. The phospholipids were resolved on thin layer chromatography plates. The arrow indicates the phosphorylated substrate, PtdIns-P (PIP). D: AR4-2J cell lysates were pretreated with the vehicle, 10 nM wortmannin or 10 μM LY294002 for 1 h, in the presence or not of 10 nM gastrin. The extracts were immunoprecipitated with an anti-p60^{Src} or an anti-p125^{FAK} antibody and in vitro kinase reactions were carried out as described in Section 2.

4. Discussion

G protein-coupled receptors have been shown to mediate rapid tyrosine phosphorylations of several proteins that participate in mitogenic signal transduction. Since these receptors lack intrinsic tyrosine kinase activity, recruitment of non-receptor tyrosine kinases might play an important role in G protein-coupled receptor signaling. Several reports describe the implication of candidate tyrosine kinases linking this class of receptors to the Ras/MAPK cascade, a pathway known to be involved in cell proliferation. These include proto-oncogenes like p60^{Src} and Src-family kinases such as Fyn, Lyn and Yes or the more distantly related Syk [30,31]. Inhibitors of Src-related kinases or transfection of cells with Csk (for C-terminal Src kinase), a negative regulator of Src, demonstrate that Src-like kinases link the βγ subunit of the heterotrimeric G protein to activation of the MAPK pathway through phosphorylation of Shc and the recruitment of Grb2 and Sos [19]. In addition, p125^{FAK} and the novel Ca^{2+}- and PKC-dependent protein tyrosine kinase PYK2 [32] define a new subfamily of non-receptor tyrosine kinases which can also be activated by neuropeptides [25]. PYK2 can acts to link Gi and Gq-coupled receptors with Grb2 and Sos to activate the MAPK signaling pathway [33]. Although it is becoming increasingly clear that a number of non-receptor tyrosine kinases can act upstream of the Ras/MAPK cascade, the contribution of these kinases to the activation of the PI 3-kinase pathway by G protein-coupled receptors is still poorly understood. Since we have recently reported that gastrin stimulates the p85/p110 PI 3-kinase in AR4-2J cells [17], we undertook the present study to further characterize the upstream events that can link the G/CCK$_B$ receptor to the PI 3-kinase.

In the first part of this work, we demonstrate that gastrin potently stimulates the enzymatic activity of p60^{Src} and p125^{FAK}. This activation is in accordance with enhanced phosphorylation of these proteins by this peptide. In addition, we have shown an association of p60^{Src} with p125^{FAK} upon gastrin stimulation. This interaction might regulate p60^{Src} and/or p125^{FAK} activities. In the second part of this work, we have examined the involvement of these two tyrosine kinases in the PI 3-kinase pathway induced by gastrin. We have detected an increased PI 3-kinase activity in anti-p60^{Src} and anti-p125^{FAK} precipitates in response to gastrin, with kinetics correlated to those observed for the activation of p60^{Src} and p125^{FAK}. Inhibitors of Src kinases family members, herbimycin A or the more recent and specific PP-2 [27], greatly diminishes PI 3-kinase activity, at drug concentrations similar to those reported to inhibit activation of these enzymes by other growth factors [34,35], suggesting that p60^{Src} could lie upstream of the PI 3-kinase in gastrin-treated cells. Agonist-mediated increase in p125^{FAK} activation has been previously shown to be accompanied by profound alterations in the organization of the actin cytoskeleton and in the assembly of focal adhesions, the areas of the plasma membrane where

$p125^{FAK}$ is localized [24,36,37]. In the present study, we also demonstrate that cytochalasin D, which disrupts the network of actin microfilaments, prevents autophosphorylation of $p125^{FAK}$ and decreases the activity of the PI 3-kinase by gastrin. Thus, we identified a gastrin-dependent PI 3-kinase pathway that requires the integrity of the actin cytoskeleton and the activation of $p125^{FAK}$ and $p60^{Src}$. In addition, $p125^{FAK}$ and $p60^{Src}$ were not blocked by PI 3-kinase inhibitors, supporting the conclusion that both kinases act upstream of PI 3-kinase rather than in a downstream pathway.

In summary, our results demonstrate for the first time that activation of a $p60^{Src}/p125^{FAK}$ complex, that might be responsible for the proliferative effects of gastrin, is one mechanism by which the G protein-coupled receptor G/CCK_B stimulates the PI 3-kinase pathway.

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