Gastrin-induced DNA synthesis requires p38-MAPK activation via PKC/Ca^{2+} and Src-dependent mechanisms

Stephanie Dehez, Laurence Daulhac, Aline Kowalski-Chauvel, Daniel Fourmy, Lucien Pradayrol, Catherine Seva*

Groupe de Recherche de Biologie et Pathologie Digestives, INSERM U.531, CHU Rangueil, 1 avenue J. Poulhes, Institut Louis Bugnard, Bat. L3, 31403 Toulouse, France

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Abstract We present evidence that gastrin, binding to a G protein-coupled receptor, activates the p38-mitogen-activated protein kinase (MAPK) pathway. Blockage of protein kinase C (PKC) by GF109203X, depletion of intracellular calcium by thapsigargin or inhibition of Src family kinases by PP2 prevented p38-MAPK activation and the Src kinase activity stimulated by gastrin. Inhibition of the PI 3-kinase by wortmannin or LY294002 did not affect these responses. In addition, the p38-MAPK inhibitor, SB203580, repressed gastrin-induced [^{3}H]thymidine incorporation, indicating a major role of p38-MAPK in the growth-promoting effect of gastrin. Our results demonstrate that gastrin-induced DNA synthesis requires p38-MAPK activation through mechanisms that involve calcium mobilization, PKC and Src family kinases. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Gastrin; Mitogen-activated protein kinase; Tyrosine kinase; Protein kinase C; Calcium; Cellular proliferation

1. Introduction

Gastrin, originally identified as a potent stimulant of gastric acid secretion, has also been shown to be a growth factor for normal and malignant gastrointestinal tissues. Its trophic effects, mediated through a G protein-coupled receptor (GPCR), the CCKB receptor (also known as CCK-2), have been observed on normal gastrointestinal mucosa [1] as well as on colon, gastric and pancreatic cancer cell lines in vitro or transplanted in vivo [2–4]. More recent studies on transgenic mice overexpressing gastrin or deficient for this gene have confirmed the role of this hormone in cell proliferation [5,6]. Mitogen-activated protein kinase (MAPK) pathways are important signal transduction cascades linking extracellular stimuli to intracellular responses. Among the different MAPK pathways described in the literature (extracellular signal-regulated kinase (ERK) pathway, stress-activated protein kinase/Jun-N-terminal kinase (JNK) pathway and p38-MAPK pathway), the ERK pathway has been clearly involved in the proliferative effects induced by receptor tyrosine kinases, cytokine receptors as well as GPCRs. Several laboratories, including ours, have reported the activation of the ERK pathway by the CCKB receptor and the contribution of this signaling cascade in growth-promoting effects mediated by this receptor [7–9].

The p38-MAPK was initially identified as a signaling pathway mediating cellular stress induced by UV, proinflammatory cytokines, heat and osmotic shocks [10]. More recent studies have shown that p38-MAPK can also play a critical role in the regulation of cell proliferation by growth factors, such as fibroblast growth factor and hepatocyte growth factor [11,12] or cytokines such as granulocyte colony stimulated factor, interleukin (IL)-2 or IL-7 [13,14]. p38-MAPK has also been shown to be activated by GPCRs [15,16]. However, the contribution of this signaling pathway in the proliferative effects mediated by GPCR remains largely unknown.

In particular, activation of the p38-MAPK pathway by gastrin and its role in the proliferative effects mediated by the CCKB receptor have never been studied. Here, we have examined if the human CCKB receptor expressed in Chinese hamster ovary (CHO) cells would induce the p38-MAPK activation and whether this effect would be involved in the growth factor action of gastrin. We have also analyzed the transduction mechanisms that may link the CCKB receptor to the p38-MAPK pathway. Gastrin-dependent activation of the CCKB receptor has been shown to induce the rapid hydrolysis of phosphatidylinositol-biphosphates by phospholipase C (PLC) to generate inositol triphosphates and diacyl-glycerol which respectively mobilizes intracellular calcium and stimulates protein kinase C (PKC). We therefore analyzed the contribution of these early signals, generated by activation of PLC, to the activation of the p38-MAPK by gastrin.

Since we have previously reported that ERK activation by gastrin is mediated by a signaling cascade including the phosphorylation of Shc proteins by Src-like tyrosine kinases [7], we have also analyzed the possibility that Src family kinases could serve as intermediates between the CCKB receptor and the activation of the p38-MAPK pathway.

Here, we report the activation of p38-MAPK by gastrin through a mechanism that involves PKC, calcium mobilization and Src family kinases. Our data also demonstrate an essential role for the p38-MAPK pathway in transducing the proliferative activity of gastrin.

*Corresponding author. Fax: (33)-561322403.
E-mail: sevac@rangueil.inserm.fr

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2. Materials and methods

2.1. Cell line

CHO cells, stably transfected with an expression plasmid encoding the human CCKB receptor ($B_{max} = 2.87 \pm 0.1$ pmol/10^6 cells), were obtained from C. Escrieut (Toulouse, France).

2.2. Immunoprecipitations and Western blotting analysis

Cells were serum-starved for 18 h before peptide addition. After stimulation, the cells were washed and the soluble fractions, containing identical amounts of proteins, were immunoprecipitated with the indicated antibodies, as described previously [17]. Proteins from immunoprecipitates or whole cell lysates were separated by SDS–PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blotted with the indicated antibodies, as described previously [17]. Proteins were visualized using ^{125}I-protein A (p38-MAPK detection) or horseradish peroxidase-chemoluminescent reagent and peroxide (phospho-ERK detection). The autoradiograms were densitometrically analyzed, and the data from at least three separate experiments plotted as fold stimulation and presented as means ± S.E.M.

2.3. Kinase assays

For p38-MAPK or JNK assays, anti-p38-MAPK or anti-JNK1 immunoprecipitates were washed twice with lysis buffer containing 1% Triton X-100 and three times in buffer B (50 mM HEPES–150 mM NaCl–0.1% Triton–10% glycerol) and then incubated for 30 min in buffer B containing 10 mM Mg acetate, 3 μg GST-ATF2 (activating transcription factor-2) (1–96) or 2 μg GST-c-Jun (1–79), 5 μM ATP and 0.3 μCi [γ-^{32}P]ATP. Reaction was stopped by addition of Laemmli sample buffer and boiling. Samples were analyzed by SDS–PAGE and the gels were autoradiographed.

C-Src kinase assays were performed as described previously [7]. The autoradiograms were densitometrically analyzed, and the data from at least three separate experiments plotted as fold stimulation and presented as means ± S.E.M.

2.4. [^{3}H]Thymidine incorporation

DNA synthesis was analyzed by measurement of [^{3}H]thymidine incorporation as described previously [7]. The results are means ± S.E.M. of four experiments performed in triplicate.

2.5. Materials

[^{3}H]Thymidine (18 Ci/mmol) was obtained from Amersham Pharmacia Biotech. [γ-^{32}P]ATP (7000 Ci/mmol) was from ICN. Anti-JNK1 and anti-p38α-MAPK, GST-c-Jun and GST-ATF2 were from Santa Cruz, anti-p60-Src antibodies from Oncogene Science, anti-phosphotyrosine from Transduction Laboratories, anti-phospho-ERK antibodies from New England Biolabs.

3. Results and discussion

3.1. Gastrin stimulates the phosphorylation and the activity of p38-MAPK

We first investigated whether gastrin could induce the tyrosine phosphorylation of p38α-MAPK in CHO cells stably expressing the human CCKB receptor.

Cells were treated with 10 nM of gastrin for various times and lysates were immunoprecipitated with anti-phosphotyrosine antibodies. Western Blot with a specific p38α-MAPK antibody (Fig. 1A) revealed an increased phosphorylation of p38α-MAPK in response to gastrin maximal at 5 min (8.8 ± 1.9-fold) that decreased at 10 min.

We then analyzed the effect of gastrin on the kinase activity of p38α-MAPK. The p38α-MAPK activity was measured by phosphorylation of the exogenous substrate GST-ATF2, after immunoprecipitation of the cell lysates with an anti-p38α-MAPK antibody. Results in Fig. 1B indicate that gastrin treatment resulted in activation of the kinase with a time course correlated to that observed for the phosphorylation of the enzyme. The maximal stimulation, 5.3 ± 0.8-fold, observed at 5 min, decreased at 10 min. Fig. 1C shows a late kinetic of p38α-MAPK phosphorylation by gastrin. After 15 min of gastrin treatment, a stimulation of 2-fold over basal is observed, that increased to 3-fold at 30 min. This phosphorylation remained elevated for at least 2 h.

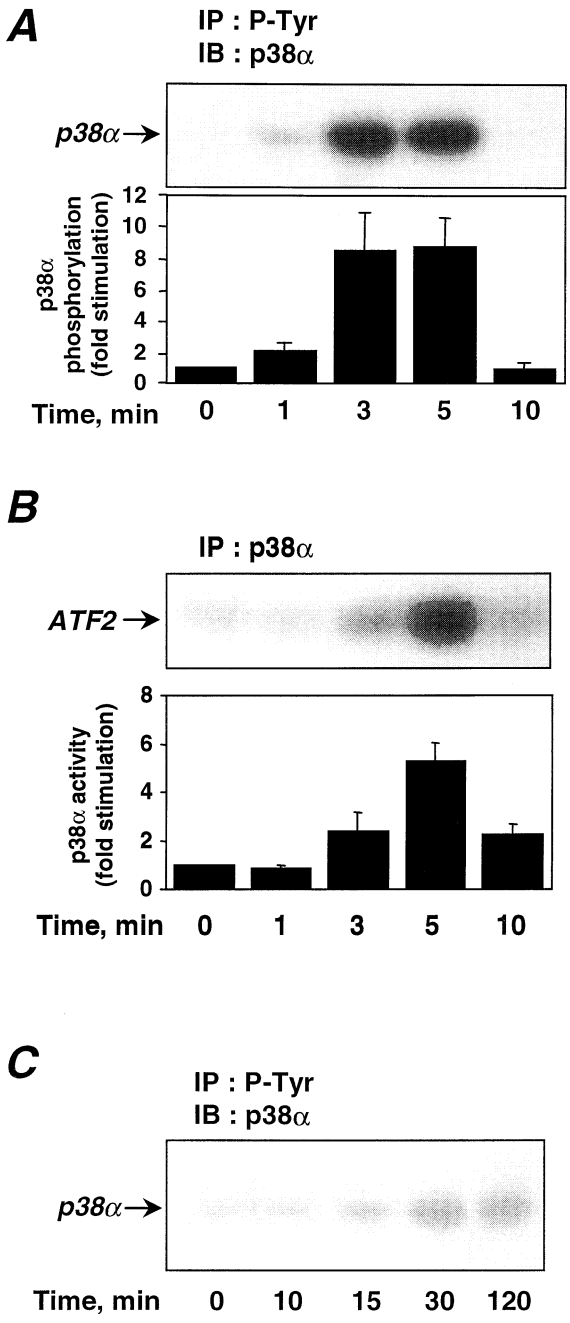

Fig. 1. Gastrin stimulates the phosphorylation and the activity of p38-MAPK. Cells were stimulated for the times indicated with 10 nM of gastrin. Proteins from cell lysates were prepared and immunoprecipitated (IP) with anti-phosphotyrosine (P-Tyr) or anti-p38α-MAPK antibodies then subjected to immunoblotting (IB) (A,C) or p38α-MAPK assay (B) as described under Section 2. Comparable amounts of proteins were detected in whole cell lysates or immunoprecipitates of all the above experiments (data not shown).

3.2. Role of PKC and calcium in the activation of p38-MAPK by gastrin

Since the CCKB receptor is known to be linked to the PLC/

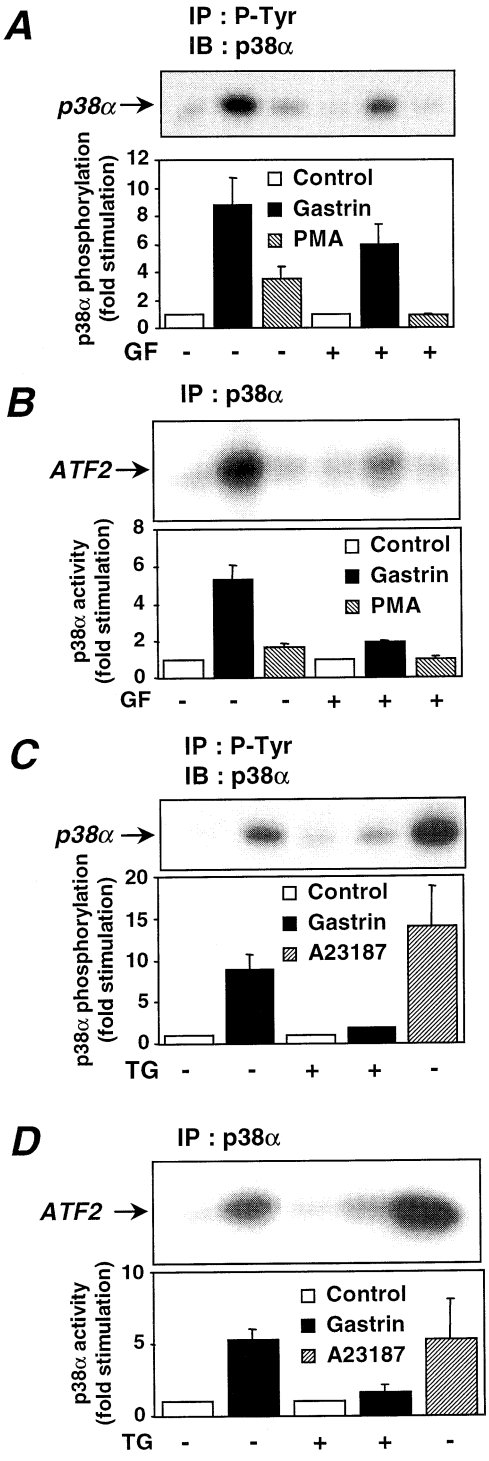

Fig. 2. Role of PKC and calcium in the activation of p38-MAPK by gastrin. After 30 min pre-incubation with (+) or without (−) 5 μM of GF109203X (GF), or 1 μM of thapsigargin (TG), cells were stimulated for 5 min with 10 nM of gastrin, for 20 min with 100 nM of PMA or for 45 min with 10 μM of A23187. Proteins from cell lysates were immunoprecipitated with anti-phosphotyrosine or anti-p38α-MAPK antibodies then subjected to immunoblotting (A,C) or to p38α-MAPK assay (B,D) as described under Section 2. Comparable amounts of proteins were detected in whole cell lysates or immunoprecipitates of all the above experiments (data not shown).

PKC pathway, we investigated whether PKC and calcium mobilization contribute to gastrin-induced p38-MAPK activation.

In Fig. 2A,B, direct activation of phorbol ester-sensitive PKC isoforms by 100 nM of phorbol 12-myristate 13-acetate (PMA) for 20 min induced an increase in the phosphorylation and activity of p38α-MAPK. However, this activation was moderately stimulated in response to PMA compared to gastrin (1.7 ± 0.2-fold).

Pretreatment of the cells with 5 μM of a potent and specific PKC inhibitor, GF109203X, prevented the PMA-stimulated p38α-MAPK activation (Fig. 2A,B). In contrast, p38α-MAPK phosphorylation and activity induced by gastrin were only partially inhibited by GF109203X (respectively 37.2 ± 9.6% and 79.1 ± 1.6%). These results indicate that maximal activation of p38α-MAPK by gastrin involves a PKC-dependent mechanism but suggest the existence of an additional signaling pathway independent of PKCs. Our results are in good correlation with a recent study that has shown that Gαq, which is known to be coupled to the CCKB receptor, can stimulate p38-MAPK by a mechanism involving PKCs [18].

Very little is known about the contribution of intracellular calcium mobilization by GPCR to the activation of the p38-MAPK pathway. To assess whether this early event participates to p38-MAPK activation, cells were treated for 45 min with the calcium ionophore A23187. Both, p38α-MAPK phosphorylation and activity (Fig. 2C,D) were increased in response to 10 μM of A23187 (respectively 13.9 ± 5-fold and 5.3 ± 2.7-fold). In addition, gastrin-induced p38-MAPK activation was prevented by pretreatment of the cells with 1 μM of thapsigargin, a selective inhibitor of the endoplasmic reticulum ATPase which is responsible for maintaining intracellular calcium stores. These results demonstrate that intracellular calcium plays a critical role in the activation of the p38-MAPK pathway by the CCKB receptor.

3.3. Role of Src family tyrosine kinases in p38-MAPK activation induced by gastrin

Expression of the v-Src oncoprotein has been shown to strongly stimulate p38-MAPK in different cell types [19,20]. However little is known about the contribution of Src family kinases to the signaling pathways linking GPCR to p38-MAPK activation. We have previously reported that gastrin can stimulate a Src kinase activity in CHO cells transfected with the CCKB receptor [7]. In this study, we have analyzed the role of intracellular calcium and certain PKC isoforms in gastrin-induced Src kinase activity and determined whether Src family tyrosine kinases are involved in the gastrin-induced activation of p38-MAPK.

In Fig. 3A, activation of Src was measured in anti-Src immunoprecipitates using enolase as a substrate. A similar increase in Src activity was observed after treatment with PMA or gastrin (respectively 2.0 ± 0.2-fold and 2.6 ± 0.5-fold). Pre-incubation of the cells with the PKC inhibitor, GF109203X, completely blocked the maximal increase of Src kinase activity obtained with both PMA and gastrin. Similarly, depletion of the intracellular calcium pools with thapsigargin abolished gastrin-induced Src kinase activity (Fig. 3B).

To determine the contribution of Src family kinases to the activation of p38-MAPK by gastrin, we measured the phosphorylation and the activity of the kinase in CHO cells pre-

treated with a specific Src-like inhibitor PP2. In the presence of 10 μM of PP2, that abolished the gastrin-stimulated Src kinase activity (Fig. 3C), gastrin stimulation of p38α-MAPK phosphorylation and activity were reduced by respectively 84.6 ± 0.1% and 86.0 ± 6.0% compared to control cells (Fig. 4A,B). The results indicate that p38-MAPK activation by gastrin is mainly mediated by Src family kinases although a Src-independent signaling pathway may also be involved.

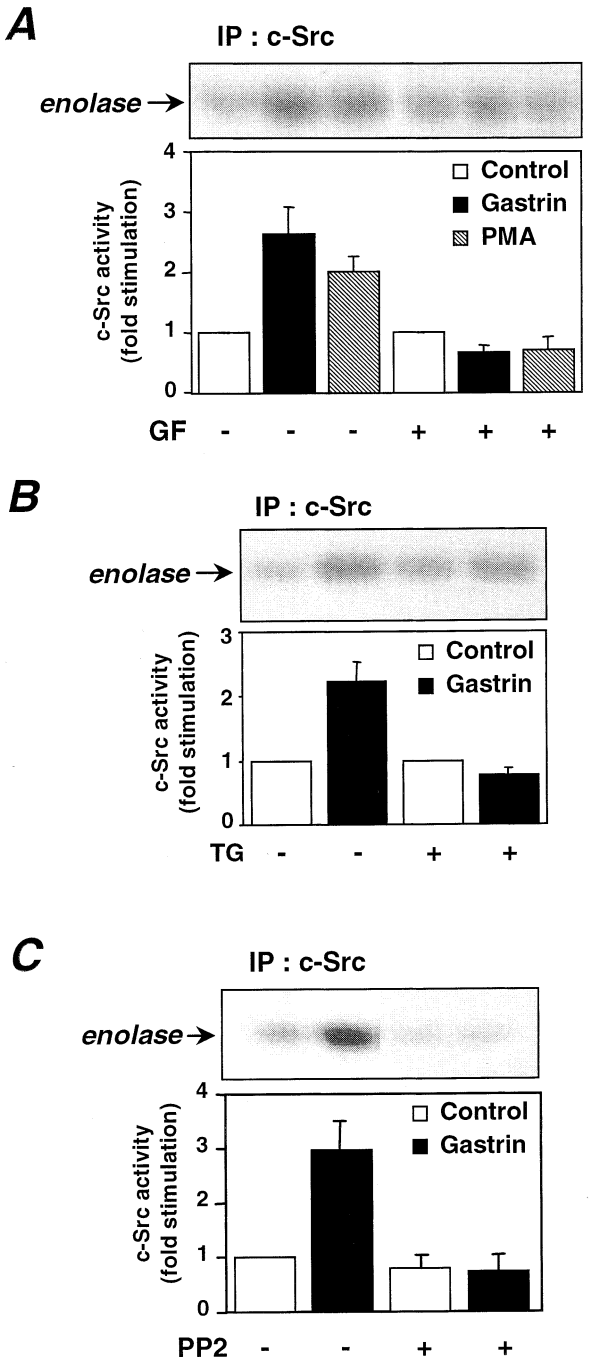

Fig. 3. Role of PKC and calcium in c-Src tyrosine kinases activation induced by gastrin. After 30 min pre-incubation with (+) or without (−) 5 μM of GF109203X (GF), 1 μM of thapsigargin (TG) or 10 μM of PP2, cells were stimulated for 1 min with 10 nM of gastrin or for 20 min with 100 nM of PMA. Proteins from cell lysates were prepared and subjected to Src kinase activity as described under Section 2. Comparable amounts of proteins were detected in whole cell lysates or immunoprecipitates of all the above experiments (data not shown).

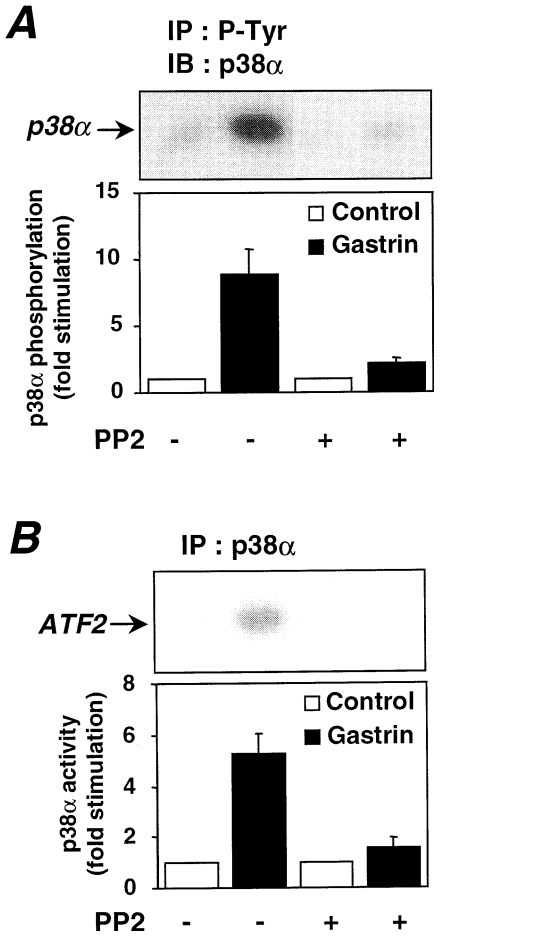

Fig. 4. Role of Src family tyrosine kinases in p38-MAPK activation induced by gastrin. After 30 min pre-incubation with (+) or without (−) 10 μM of PP2, cells were stimulated for 5 min with 10 nM of gastrin. Proteins from cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody phosphotyrosine or anti-p38α-MAPK antibodies then subjected to immunoblotting (A) or to p38α-MAPK assay (B) as described under Section 2. Comparable amounts of proteins were detected in whole cell lysates or immunoprecipitates of all the above experiments (data not shown).

3.4. *Effect of wortmannin and LY294002, two PI 3-kinase inhibitors, on gastrin-induced p38-MAPK activation*

We have previously reported the activation of the p85/p110 PI 3-kinase by the CCKB receptor [17] and its role upstream of the ERK pathway induced by gastrin [21]. We therefore examined whether the PI 3-kinase pathway would be involved in the activation of p38-MAPK by gastrin. For this purpose, cells were treated with increasing an concentration of PI 3-kinase inhibitors, wortmannin (1–1000 nM) and LY294002 (0.01–100 μM) prior to gastrin stimulation. In contrast to what we previously observed for the ERK pathway, neither phosphorylation of p38α-MAPK nor its activity induced by gastrin was affected by the PI 3-kinase inhibitors (Fig. 5).

3.5. *Role of the p38-MAPK pathway in gastrin-induced DNA synthesis*

The importance of the p38-MAPK pathway in gastrin-

stimulated CHO cells proliferation was investigated by using the p38-MAPK inhibitor, SB203580.

Fig. 6A shows that pretreatment of the cells with 10 μM of SB203580 completely blocked the activation of p38α-MAPK by gastrin. At this concentration, the activation by gastrin of the two other MAPKs, JNK and ERK was not significantly inhibited (Fig. 6B,C). We therefore analyzed the effect of SB203580 on [^{3}H]thymidine incorporation stimulated by gastrin (Fig. 6D). We observed a 86.4 ± 12.7% decrease of gastrin-induced DNA synthesis in pretreated cells compared to control cells whereas SB203580 alone had no effect on basal [^{3}H]thymidine incorporation. The p38-MAPK pathway has previously been shown to be activated by GPCR. However, the understanding is just beginning to emerge about the role of p38-MAPK in the wide biological effects of these receptors. The p38-MAPK pathway has been involved in cell migration mediated by the angiotensin receptor [22], cell scattering [23] and inhibition of cell proliferation [24] mediated by the α1B-adrenergic receptor and organization of the actin cytoskeleton mediated by the CCKA receptor [25]. Here we show that the p38-MAPK pathway can play a central role in growth-promoting effects mediated by the GPCR, CCKB.

In summary, our study reports the activation of p38α-MAPK by the CCKB through a mechanism that involves PKC, intracellular calcium mobilization and Src family kinases. Our data also demonstrate a critical role for the p38-MAPK pathway in transducing the proliferative activity of gastrin.

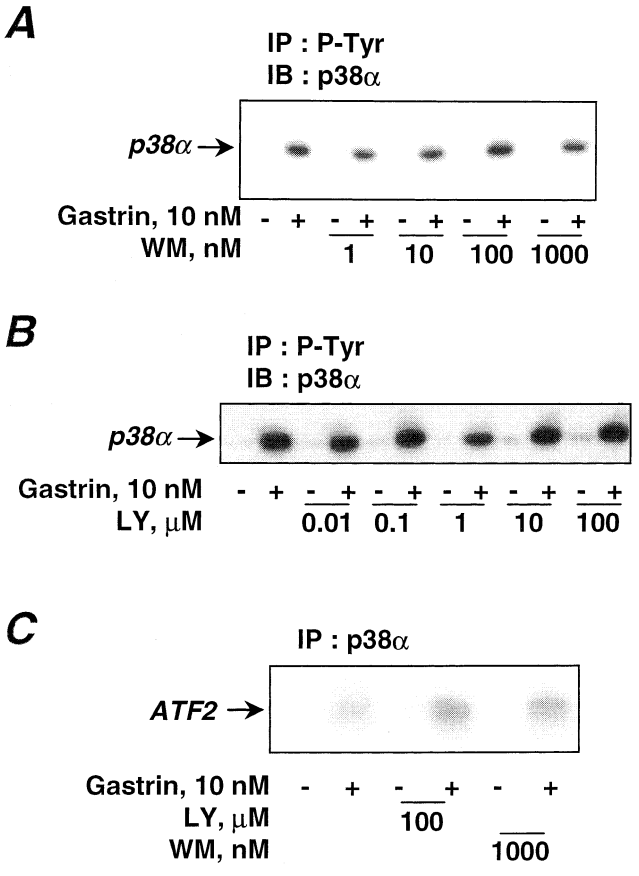

Fig. 5. Effect of wortmannin and LY294002 on gastrin-induced p38-MAPK activation. After 60 min pre-incubation with the indicated concentrations of wortmannin (WM) or LY294002 (LY), cells were incubated for 5 min with (+) or without (−) 10 nM of gastrin. Proteins from cell lysates were immunoprecipitated with anti-phosphotyrosine or anti-p38α-MAPK antibodies then subjected to immunoblotting (A) or p38α-MAPK assay (B) as described under Section 2. Comparable amounts of proteins were detected in whole cell lysates or immunoprecipitates of all the above experiments (data not shown). The autoradiograms are representative of three separate experiments.

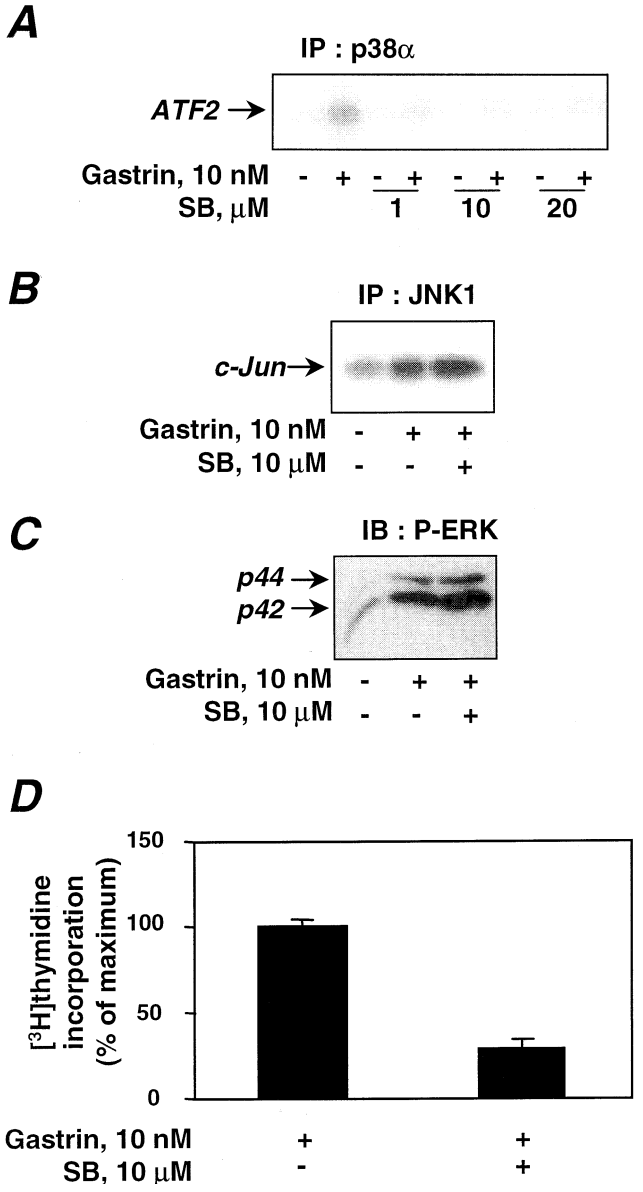

Fig. 6. Role of p38-MAPK pathway in gastrin-induced DNA synthesis. A: Lysates from cells treated (+) or not (−) with 10 nM of gastrin were prepared and subjected to p38α-MAPK assay in presence of the indicated concentrations of SB203580. B,C: After 30 min pre-incubation with (+) or without (−) 10 μM of SB203580 (SB), cells were incubated for 5 min with (+) or without (−) 10 nM of gastrin. Cell lysates were prepared and subjected to ERK and JNK assays as described under Section 2. D: After 30 min pre-incubation with (+) or without (−) 10 μM of SB203580 (SB), cells were stimulated for 24 h with 100 nM of gastrin. DNA synthesis was measured by [^{3}H]thymidine incorporation under serum-free conditions as described under Section 2. Maximal [^{3}H]thymidine incorporation induced by gastrin averaged 140 ± 6%.

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