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Wortmannin-Sensitive Activation of p70S6-Kinase and MAP-Kinase by the G Protein-Coupled Receptor, G/CCK_B

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The gastrin/CCK_B (G/CCK_B) G protein-coupled receptor has been shown to mediate the proliferative effects of gastrin on normal and neoplastic gastro-intestinal tissues. In the present study, we examined the signal transduction mechanisms coupled to this receptor. We report here that phosphorylation and activity of the $p70^{S6K}$, whose major substrate is the ribosomal S6 protein, are enhanced in response to gastrin. These effects were completely reversed by a commonly used PI-3-kinase inhibitor, wortmannin, suggesting that $p70^{S6K}$ may be a downstream target of PI-3-kinase in a signaling cascade induced by gastrin. In addition, blocking PI-3-kinase activity by wortmannin partially decreased gastrin-induced MAPK activation (42% ± 3) as well as the tyrosine phosphorylation of Shc (50% ± 6), an upstream regulator of the Ras-dependent MAPK pathway. These results indicate that at least two signaling pathways lead to MAPK activation by gastrin, only one of which is sensitive to PI-3-kinase inhibitors.

Gastrin, a peptide hormone produced by antral G cells, is a potent stimulant of gastric acid secretion (1). Gastrin is also known to function as a growth factor stimulating proliferation of normal and neoplastic gastrointestinal cells (2-6). Trophic effects of gastrin have been reported on colon, gastric and pancreatic cancer cell lines *in vitro* (3, 4) or transplanted *in vivo* (5, 6). We have previously reported that gastrin, acting via a specific transmembrane G protein-coupled receptor (G/CCK_B), stimulates the tyrosine phosphorylation of several proteins including the adaptor protein Shc (7). Phosphorylated Shc subsequently associates with the Grb2/Sos complex and initiates the MAP Kinases (MAPK) cascade, a pathway known to play an important role in cell proliferation. We recently demonstrated that gastrin also stimulates the PI-3-kinase (8), a lipid kinase known to be activated by a large number of hormones and growth factors (9).

The Ser/Thr kinase p70S6-Kinase ($p70^{S6K}$), whose major substrate is the ribosomal S6 protein, appears to be an important element in the regulation of proteins synthesis and cell proliferation by mitogens (10). The mechanisms leading to $p70^{S6K}$ activation are still poorly understood, however, several recent studies suggest that PI-3-kinase lies upstream of $p70^{S6K}$. Indeed, mutant forms of PI-3-kinase that are constitutively active indirectly stimulate $p70^{S6K}$ phosphorylation and activation (11). In contrast, blocking PI-3-kinase activity by receptors mutations or PI-3-kinase inhibitors, prevents the activation of $p70^{S6K}$ by growth factors (12). We have previously reported that gastrin exerts growth-promoting effects on a tumor-derived pancreatic acinar cell line (AR4-2J) through the G/CCK_B G protein-coupled receptor (3, 13). We therefore investigated whether this peptide could regulate the $p70^{S6K}$ in this cellular model and we examined the involvement of PI-3-kinase activation in this pathway. We also assessed the role of PI-3-kinase in gastrin-mediated MAPK activation.

We report that gastrin regulates the activation of $p70^{S6K}$ by a pathway sensitive to PI-3-kinase inhibitors. In addition, we demonstrate that PI-3-kinase activity could be involved in gastrin-mediated MAPK signaling pathway at a point upstream of Shc phosphorylation.

EXPERIMENTAL PROCEDURES

Cell culture. AR4-2J cells, originally obtained by Jessop and Hay (14) from a rat exocrine pancreatic tumor were a gift from Dr. C. Logsdon (Ann Arbor, MI). The cells were grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal calf serum.

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Immunoprecipitation. AR4-2J cells were serum starved in DMEM for 18h 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, 0.5 mM phenylmethylsulfonylfluoride, 20 μM leupeptin, 100 UI/ml Trasylol) for 15 min at 4°C. The solubilizates were immunoprecipitated with the indicated antibodies preadsorbed on protein-A sepharose.

Western blotting analysis. Immunoprecipitates, prepared as described above, were separated by SDS-PAGE and proteins were 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 by using ^{125}I-protein-A followed by autoradiography.

P70S6-kinase assay. After immunoprecipitation with a specific anti-$p70^{S6K}$ antibody, immune-complexes were incubated for 30 min at 37°C in reaction buffer (50 mM MOPS, 1 mM dithiothreitol, 30 μM ATP, 5mM $MgCl_2$, 10 mM 4-nitrophenylphosphate, pH 7.2), containing [γ-^{32}P]-ATP (0.3 μCi) and S6 protein derived from 40S ribosomes. Proteins were then separated by SDS-PAGE and revealed by autoradiography.

Immune complex assays for MAPK activation. After immunoprecipitation with a specific anti-p44-MAPK antibody, immune-complexes were incubated for 30 min at room temperature in reaction buffer (50 mM Hepes, pH 7.5 containing 150 mM NaCl, 200 μM orthovanadate, 0.1% Triton X-100, 10% glycerol, 0.5 mM phenylmethylsulfonylfluoride, 20 μM leupeptin, 100 UI/ml Trasylol) with Myelin Basic Protein (MBP) at 0.2 mg/ml, 10 mM Mg Acetate and [γ-^{33}P]-ATP (3000 Ci/mmole). The reaction was stopped by depositing the samples on P81 Whatmann chromatography paper immersed in 1% orthophosphoric acid. After 3 washes in 1% orthophosphoric acid, the radioactivity was counted.

Materials. Human $gastrin_{2\text{-}17ns}$ was purchased from Bachem (Switzerland). ^{125}I-Na (100 mCi/ml) was obtained from Amersham and [γ^{33}P]-ATP (3000 Ci/mmol) from Isotopchim. PtdIns from bovine liver and wortmannin were purchased from Sigma (France). Anti-phosphotyrosine and anti-Shc antibodies were from Upstate Biotechnology (Euromedex, France). Anti-$p70^{S6K}$ and anti-p44-MAPK antibodies were obtained from Santa Cruz (Tebu, France).

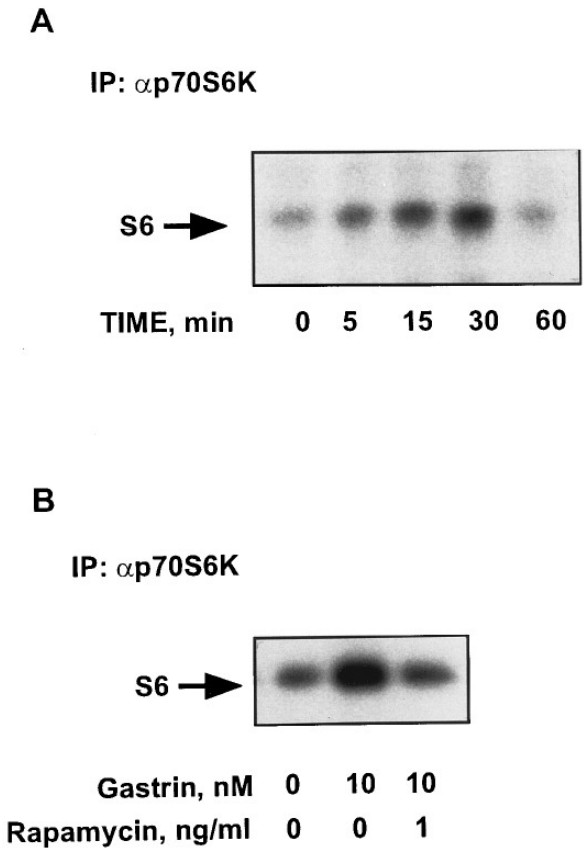

FIG. 1. $p70^{S6K}$ activation in gastrin-treated AR4-2J cells. (A) AR4-2J cells were incubated for the times indicated with 10 nM of gastrin. (B) Cells pretreated for 1h in the absence or presence of 1 ng/ml of rapamycin were subsequently stimulated or not with 10 nM of gastrin for 30 min as indicated. $P70^{S6K}$ was immunoprecipitated (IP) with a specific anti- $p70^{S6K}$ antibody and its kinase activity was assayed on ribosomal S6 protein as described under "Experimental procedures". The arrow indicates the phosphorylated S6 protein. The data presented are representative of five independent experiments with similar results.

RESULTS

$p70^{S6K}$ activation in gastrin-treated AR4-2J cells. Given the role of $p70^{S6K}$ in the mitogenic response (10), we first analysed the ability of gastrin to activate the $p70^{S6K}$ in AR4-2J cells. $P70^{S6K}$ activity was measured in an immunocomplex kinase assay using a specific $p70^{S6K}$ anti-antibody. As shown in figure 1A, treatment of the cells with 10 nM of gastrin caused a potent increase in $p70^{S6K}$ activity detectable at 5 min. The activation was transient, reached a maximum (2.58 fold $\pm$ 0.16, n = 5) 15-30 min after peptide addition and declined thereafter. Pretreatment of the cells with 1 ng/ml of the immunosuppressant rapamycin, which selectively blocks the activation of $p70^{S6K}$ by a number of mitogens, also abolished the activation of this enzyme by gastrin (fig. 1B).

Inhibition by wortmannin of gastrin-induced activation of $p70^{S6K}$. It has been recently reported that PI 3-kinase may play a critical role in the signaling pathway leading to $p70^{S6K}$ activation (11, 12). In addition, we have previously demonstrated that gastrin activates the PI 3-kinase in AR4-2J cells (8). We therefore investigated the role of this enzyme in gastrin-induced activation of $p70^{S6K}$ by using wortmannin, a commonly used PI 3-kinase inhibitor. Pretreatment of the cells with increasing concentrations of wortmannin led to a dose-dependent inhibition of the $p70^{S6K}$ activity (fig. 2) precipitated from gastrin-stimulated cells, suggesting that PI 3-kinase lies upstream of $p70^{S6K}$ in a signaling cascade induced by gastrin. The wortmannin concentration required for a 50% inhibition was approximately 5 nM and complete inhibition of gastrin-induced $p70^{S6K}$ activity was observed at 100 nM.

Inhibition by rapamycin and wortmannin of gastrin-induced $p70^{S6K}$ phosphorylation. Activation of $p70^{S6K}$ by growth factors has been shown to correlate with

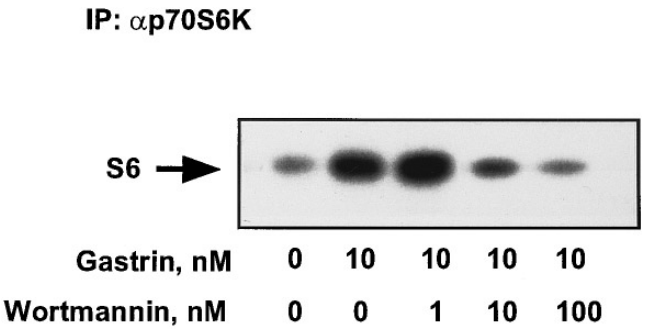

FIG. 2. Inhibition by Wortmannin of gastrin-induced activation of $p70^{S6K}$. AR4-2J cells were pretreated for 1h in the absence or presence of increasing concentrations of wortmannin. Cells were subsequently incubated or not with 10 nM of gastrin for 30 min as indicated. S6 kinase activity was measured in anti-$p70^{S6K}$ immunoprecipitates (IP) as described under "Experimental Procedures". The arrow indicates the phosphorylated S6 protein. The data presented are representative of three independent experiments with similar results.

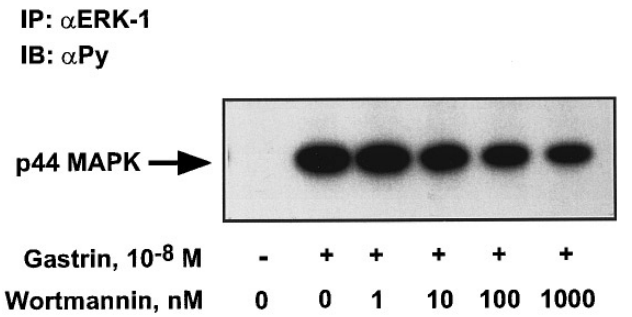

FIG. 4. Inhibition of gastrin-induced p44-MAPK tyrosine phosphorylation by the PI 3-kinase inhibitor, wortmannin. AR4-2J cells were pretreated for 1h in the absence or presence of increasing concentrations of wortmannin. Cells were subsequently incubated or not with 10 nM of gastrin for 3 min as indicated. Cellular proteins were immunoprecipitated with an anti-p44-MAPK antibody and western blotted with an anti-phosphotyrosine antibody. The arrows indicate the migration of precipitated p44-MAPK. The data presented are representative of three independent experiments with similar results.

its phosphorylation on Ser/Thr residues (15, 16). We therefore examined whether $p70^{S6K}$ could be phosphorylated in response to gastrin by measuring the reduction in the electrophoretic mobility of the protein on SDS polyacrylamide gel. A slower migrating form of $p70^{S6K}$ was detected by immunoblot in gastrin-treated cells (fig. 3) indicating that gastrin activation of $p70^{S6K}$ is correlated with increased phosphorylation of the protein in AR4-2J cells. A total inhibition of gastrin-induced $p70^{S6K}$ phosphorylation was observed in cells pretreated with rapamycin or wortmannin at drug concentrations which also abolish the activation of the enzyme by gastrin.

Inhibition of gastrin-induced MAPK activity by the PI 3-kinase inhibitor, wortmannin. We then examined the role of PI 3-kinase in gastrin-mediated MAPK signaling pathway. Tyrosine phosphorylated p44-MAPK was visualized by immunoprecipitation with an anti-p44-MAPK antibody and western blotting with an anti-phosphotyrosine antibody. Preincubation of the cells with wortmannin led to a dose-dependent attenuation of p44-MAPK tyrosine phosphorylation (fig. 4). Maximal inhibition of 40% ± 4 (n = 3) was obtained with wortmannin 100 nM. Inhibition obtained with a higher concentration (1000 nM) was not significantly different.

To directly assess wortmannin effects on gastrin-induced MAPK activation, we assayed MAPK catalytic activity *in vitro* using Myelin Basic Protein as substrate. Pretreatment of AR4-2J cells with increasing concentrations of wortmannin partially inhibited MAPK activation in response to gastrin (fig. 5). The maximal inhibition achieved with 100 nM wortmannin

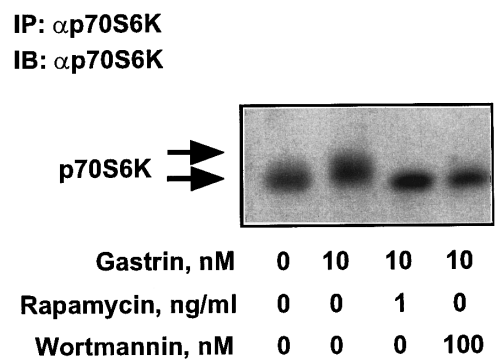

FIG. 3. Inhibition by Rapamycin and Wortmannin of gastrin-induced $p70^{S6K}$ phosphorylation. AR4-2J cells were pretreated for 1h in the absence or presence of 1 ng/ml of rapamycin or 100 nM of wortmannin. Cells were subsequently incubated or not with 10 nM of gastrin for 30 min as indicated. Cellular proteins were immunoprecipitated with an anti-$p70^{S6K}$ antibody and western blotted with the same antibody. The arrows indicate the positions of $p70^{S6K}$ at various phosphorylation states. The data presented are representative of three independent experiments with similar results.

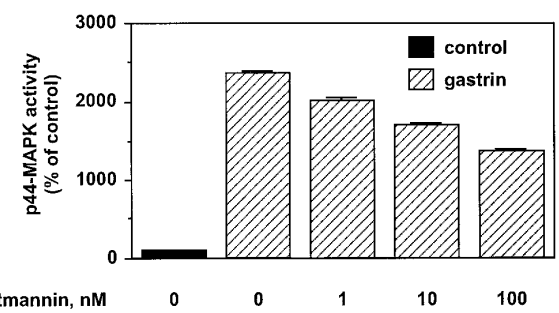

FIG. 5. Inhibition of gastrin-induced MAPK activity by the PI 3-kinase inhibitor, wortmannin. AR4-2J cells were pretreated for 1h in the absence or presence of increasing concentrations of wortmannin. Cells were subsequently incubated or not with 10 nM of gastrin for 3 min as indicated. Immune complex assays for MAP kinase activity were performed as described under "Experimental Procedures".

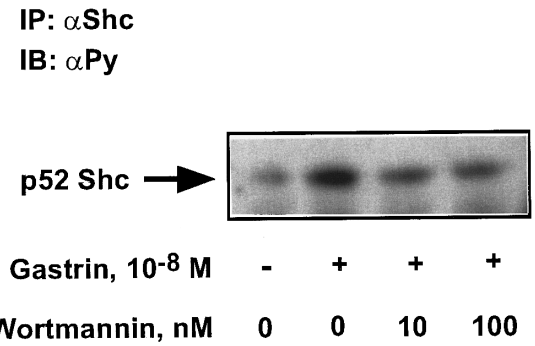

FIG. 6. Inhibition of gastrin-induced Shc phosphorylation by the PI 3-kinase inhibitor, wortmannin. AR4-2J cells were pretreated for 1h in the absence or presence of different concentrations of wortmannin as indicated. Cells were subsequently incubated or not with 10 nM of gastrin for 3 min. Cellular proteins were immunoprecipitated with an anti-Shc antibody and western blotted with an anti-phosphotyrosine antibody. The arrows indicate the migration of precipitated p52-kDa Shc isoform. The data presented are representative of three independent experiments with similar results.

(42% ± 3, n = 3) was similar to that obtained with the inhibitor on gastrin-induced MAPK tyrosine phosphorylation. The basal MAPK phosphorylation and activity was not significantly affected by treatment of the cells with wortmannin alone. In addition, to demonstrate that MAPK inhibition was not due to non specific effects of the drug on MAPK itself, PI 3-kinase inhibitor was included in the immunocomplex kinase assay in vitro. No inhibitory effect was observed on the basal MAPK activity (data not shown).

Inhibition of gastrin-induced Shc phosphorylation by the PI 3-kinase inhibitor, wortmannin. Tyrosine phosphorylation of Shc proteins and subsequent complex formation with Grb2 and Sos appear to be a common mechanism by which tyrosine kinase receptors and gastrin receptors initiate the activation of MAPK (7, 17). We therefore examined whether gastrin-induced Shc phosphorylation was altered by wortmannin pretreatments. Lysates from cells pretreated with PI 3-kinase inhibitor prior peptide stimulation were immunoprecipitated with an anti-Shc antibody and analysed by western blot using an anti-phosphotyrosine antibody. As shown in figure 6, the tyrosine phosphorylation of p52-kDa Shc isoform in response to gastrin was maximally decreased by 50% ± 6 (n = 3) in the presence of 100 nM wortmannin, indicating that PI 3-kinase is required upstream of Shc proteins in the gastrin-mediated MAPK activation signaling pathway.

DISCUSSION

Activation of the Ser/Thr kinase $p70^{S6K}$, whose major substrate is the ribosomal S6 protein, appears to be one mechanism by which mitogens regulate synthesis of proteins required for cell proliferation (10). This enzyme has been reported to be activated by a large number of hormones and growth factors that stimulate cell proliferation (18-22). In addition, inhibition of $p70^{S6K}$ activity by specific inhibitors or antibodies raised against $p70^{S6K}$ represses protein synthesis and cell proliferation induced by serum or growth factors (18, 21, 23, 24). $P70^{S6K}$ function has also been shown to be essential for cell cycle progression as well as for the expression of immediate early gene products involved in cell growth (23).

In the present study, we demonstrate that gastrin, acting through a G protein-coupled receptor, potently stimulates the enzymatic activity of $p70^{S6K}$. This activation correlated with enhanced phosphorylation of the protein. Both phosphorylation and activity of the S6 kinase induced by gastrin were completely blocked by rapamycin at drug concentrations similar to those reported to inhibit activation of the enzyme by other growth factors (25, 26).

Results recently obtained in our laboratory demonstrated that activation of PI 3-kinase is a common intracellular event initiated by tyrosine kinase receptors and the G/CCK_B G protein-coupled receptor (8). Here we report that gastrin-induced $p70^{S6K}$ activity was completely reversed by the commonly used PI 3-kinase inhibitor, wortmannin, suggesting in accordance with other reports that $p70^{S6K}$ could be a downstream target of the PI 3-kinase pathway. Wortmannin also prevented the full activation of MAPK by gastrin, indicating that PI 3-kinase forms a possible link between the gastrin receptor and the MAPK pathway. However, the drug did not completely inhibit the increase in MAPK activity induced by gastrin even at the maximal concentrations of wortmannin utilized. This partial inhibition indicates that at least two signaling pathways lead to MAPK activation by gastrin, only one of which is sensitive to PI 3-kinase inhibitors. Additional work will be required to identify the wortmannin-insensitive pathway and determine its contribution to gastrin-induced MAPK activation.

To go further into the mechanism that relay the signal from the PI 3-kinase to MAPK, we investigated the effect of wortmannin on gastrin-induced Shc phosphorylation. Indeed, tyrosine phosphorylation of Shc and its subsequent association with the Grb2/Sos complex appear to be common mechanisms by which tyrosine kinase receptors and G/CCK_B receptors stimulate the Ras-dependent MAPK pathway. Gastrin-induced Shc phosphorylation was partially decreased when cells were pretreated with wortmannin indicating that PI 3-kinase activity could be involved in gastrin-mediated MAPK signaling pathway at a point upstream of Shc phosphorylation. To confirm these results, we also tested on gastrin action the effects of LY-294002, another PI 3-kinase inhibitor structurally unrelated to wortmannin. The maximal inhibitions of Shc phosphorylation and MAPK

activation achieved with LY-294002 (100 μM) were similar to those achieved by wortmannin.

In summary, our results demonstrate that gastrin regulates the activation of $p70^{S6K}$ by a pathway sensitive to PI-3-kinase inhibitors. They also suggest that PI-3-kinase activity could be involved in gastrin-mediated MAPK signaling pathway at a point upstream of Shc phosphorylation.

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