Molecular mechanisms for the antiapoptotic action of gastrin

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Although characterized as a stimulant of gastric acid secretion (10), the peptide hormone gastrin also exerts growth-promoting effects on normal and malignant gastrointestinal tissues (8, 10, 15, 18, 26, 30, 31, 33–36). Gastrin is an important growth factor for the fetal pancreas (5, 37), and in the stomach it is a potent stimulant for the growth of the gastric mucosa (10, 38). Studies (38) conducted in transgenic mice overexpressing amidated gastrin have indicated that gastrin leads to thickening of the fundic mucosa and to multifocal hyperplasia with an increased bromodeoxyuridine proliferation index. Together, these observations confirm the notion that gastrin plays an important role in the regulation of gastrointestinal mucosal growth. Reports (18, 26, 30, 38) have indicated that gastrin induces the growth of colonic and gastric carcinomas both in vivo and in vitro, underscoring the importance of gastrin as a growth factor for gastrointestinal neoplasms.

The intracellular signal transduction pathways activated by gastrin to induce cellular proliferation have been the focus of numerous investigations. Gastrin induces protein tyrosine kinase activity, stimulates phosphoinositide 3-kinase (PI 3-kinase), and activates the extracellular signal-regulated protein kinases (ERKs) or mitogen-activated protein kinases (MAPKs) (8, 10, 16, 40). Indeed, work from our (33–35) laboratory has demonstrated that gastrin stimulates the growth of rat pancreatic adenocarcinoma cells (AR42J) through induction of the ERKs and of the early response gene c-fos through PKC-dependent and -independent mechanisms.

Because growth factors are known to induce both cellular growth and survival (12), we have undertaken studies to examine the role of gastrin in the regulation of cellular apoptosis. We hypothesize that gastrin might be an important physiological inhibitor of apoptosis and that this effect might contribute to its ability to stimulate cellular growth and proliferation.

A signal transduction pathway involving PI 3-kinase and protein kinase B (PKB)/Akt has recently been shown (6, 7) to play an important role in the inhibition of cellular apoptosis. Akt is homologous to the PKA and PKC families of protein kinases (6, 7). In vivo, the
activity of Akt is regulated by growth factors and serum through the induction of PI 3-kinase (6, 7). Phosphorylation of Akt appears to be critical for Akt activation (6, 7). The major phosphorylation sites required for activation of Akt have been identified as threonine 308 and serine 473, which are the targets of the phosphoinositide-dependent kinases I (PDK1) and II (PDK2), respectively (6, 7). Recent investigations (6, 7) have suggested that growth factors induce the activation of PI 3-kinase, which in turn leads to the production of phosphatidylinositol-3,4-bisphosphate at the cell membrane. Akt binds to this lipid, dimerizes, and is stabilized in a partially active state (6, 7). The location at the membrane and the dimerization appear to enhance the ability of Akt to be phosphorylated and fully activated (6, 7).

Akt appears to send numerous survival signals in response to growth factors such as epidermal growth factor (EGF), nerve growth factor, platelet-derived growth factor, insulin growth factor-I, insulin, and interleukin-3 (6, 7). A recent study (22) has shown that ligands for seven transmembrane G protein-coupled receptors, such as carbamylcholine, can also activate Akt in both PC-12 and COS-7 cells, stably expressing either the M₁ or M₂ muscarinic receptor. The mechanisms that regulate the activation of Akt in response to growth factor stimulation have only been partially characterized. In addition, the intracellular signal transduction pathways that mediate the antiapoptotic actions of Akt are unknown. Accordingly, we undertook these studies to investigate the action of gastrin on cellular apoptosis induced by serum withdrawal and to dissect the signal transduction pathways responsible for this effect.

MATERIAL AND METHODS

Plasmids and adenoviral vectors. Bacteria transformed with the expression plasmid for glutathione-S-transferase-activated transcription factor-2 (1–109) [GST-ATF2 (1–109)] were a gift of J. Hän (Scripps Research Institute, La Jolla, CA). The replication-defective adenoviral vector expressing dominant-negative, hemagglutinin (HA)-tagged Akt (with serine 473 and threonine 308 mutated to alanine) under the control of the cytomegalovirus (CMV) promoter (Adeno-dAkt) was a gift of K. Walsh (Tufts University, Boston, MA) (11). The adenoviral vector expressing the β-galactosidase enzyme under the control of the CMV promoter (Ad.CMV-β-gal) was previously described (3).

Cell culture and infection. For our experiments, we used both the rat exocrine pancreatic cell line AR42J (obtained from American Type Culture Collection, Rockville, MD), which is known to express receptors for both gastrin (G17) and EGF (8), and CHO-K1 cells stably expressing the CCK-B receptor (CHO-K1/CCK-B cells) (a gift of I. Song, University of Michigan). The AR42J cells were grown at 37°C in DMEM supplemented with 10% fetal bovine serum (FBS) and 1% sodium pyruvate. The CHO-K1/CCK-B cells were grown at 37°C in DMEM supplemented with 10% FBS and 0.05% Geneticin (GIBCO BRL, Grand Island, NY). Both cell types were maintained in 5% CO₂-95%O₂. For the apoptosis studies, the cells were cultured in serum-free medium (either DMEM or Ham’s F-12 nutrient mixture; GIBCO BRL) for 72 h. In the experiments performed with CHO-K1/CCK-B cells, gastrin (G17) (10 nM) (Bachem, Torrance, CA) was added at the time of serum starvation. In the AR42J cell studies, the cells were serum starved, transduced when indicated with 600 multiplicities of infection (MOI) of either the adenoviral vector expressing dominant-negative Akt or that expressing β-galactosidase for 16 h, and then treated with or without gastrin for an additional 56 h. Control experiments were performed in nontransduced AR42J cells according to an identical protocol. For all other studies, the cells were starved for 24 h in serum-free medium and then treated for different time periods with either gastrin (1–100 nM) or EGF (10 nM). In some experiments, PD-98059 (50 μM; New England Biolabs, Beverly, MA) (2), SB-203580 (0.1–10 μM; Calbiochem, La Jolla, CA) (17, 39, LY-294002 (10 μM; Calbiochem), and wortmannin (200 nM; Calbiochem) were added 30 min before the addition of gastrin. PD-98059, SB-203580, LY-294002, and wortmannin were dissolved in DMSO (Sigma Chemical, St. Louis, MO). All other test substances were dissolved in water. Control experiments were performed by incubating the cells in either incubation buffer or vehicle without the test substances.

Immunoprecipitations and kinase assays. Immunoprecipitations and kinase assays were performed according to previously described techniques (25, 28, 35) with minor modifications. The AR42J cells were lysed in 500 μl of lysis buffer [50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1.5 mM MgCl₂, 1 mM Na₃VO₄, 10 mM NaF, 10 mM Na₃P₂O₇·10H₂O, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (ICN Biomedicals, Aurora, OH), 1 μg/ml leupeptin, and 1 μg/ml aprotinin]. The lysates were transferred into Microfuge tubes and spun at 16,000 g for 20 min at 4°C. Equal amounts of protein from each treatment group (300 μg) were incubated with either an anti-p38 kinase antibody (sc-535, Santa Cruz Biotechnology, Santa Cruz, CA) or with a monoclonal anti-Akt kinase antibody (New England Biolabs). Protein concentrations were measured by the Bradford method (4). Aliquots of protein A-Sepharose (50 μl) (Pharmacy Biotech, Piscataway, NJ) were then added, and the solutions were mixed for an additional hour. After centrifugation, the pellets were washed once with lysis buffer and twice with kinase buffer. Immunoprecipitated p38 kinase was used to phosphorylate 2 μg of GST-ATF2 (1–109). Kinase reactions were carried out in 20 μl of kinase buffer (18 mM HEPES, pH 7.4, 10 mM magnesium acetate, 50 μM ATP, and 2.5 μCi/sample [γ³²P]ATP) at 30°C for 30 min. Reactions were terminated by addition of 20 μl of 5 × electrophoresis buffer (for 5 ml: 2.5 ml glycerol, 1.25 ml 2-mercaptoethanol, 0.5 g SDS, 1.043 ml 1.5 M Tris, pH 6.8, and 1.25 mg bromophenol blue). The samples were then boiled for 5 min and applied to 10% SDS-polyacrylamide gels, followed by staining with Coomassie blue and destaining (25) to ensure that identical amounts of proteins were loaded on the gel. Labeled phosphoproteins were visualized by autoradiography and quantitated by scanning densitometry.

GST-ATF2 (1–109) was expressed and purified from Escherichia coli as previously described (25). Immunoprecipitated Akt kinase was used to phosphorylate glycogen synthase kinase-3α (GSK-3α). GSK-3α phosphorylation was measured by Western blot analysis with a specific anti-phospho-GSK-3α/β antibody. Reactions were carried out according to the instructions of the Akt kinase assay kit from New England Biolabs.

Western blots. AR42J cell lysates (80 μg) were loaded on 10% SDS-polyacrylamide minigels and run at 20 A for 8 h. The gels were transferred on an Immobilon-P transfer membrane (Millipore, Bedford, MA) in 25 mM Tris, 150 mM glycine, and 20% methanol. After transfer, the membranes were...
were blocked in 10 ml of TBST (20 mM Tris, 0.15M NaCl, and 0.3% Tween) and 5% dry milk for 1 h. The membranes were then incubated for 16–18 h at 4°C in 10 ml of TBST and 5% dry milk, containing either a specific anti-phospho-Akt antibody that recognizes phosphorylated serine 473 of Akt kinase (1:1,000) or a specific anti-phospho-p38 antibody that recognizes phosphorylated threonine 180 and phosphorylated tyrosine 182 of p38 kinase (1:1,000) (New England Biolabs). Control blots were performed using antibodies recognizing either Akt kinase or p38 kinase independent of their phosphorylation states (1:1,000) (New England Biolabs). For the GSK-3α phosphoblots, the membranes were incubated for 16–18 h at 4°C in 10 ml TBST and 5% BSA (Sigma Chemical) containing an anti-phospho-GSK-3α/β antibody (1:1,000) that recognizes phosphorylated serine 21 of GSK-3α and phosphorylated serine 9 of GSK-3β (New England Biolabs). At the end of the incubation period, the membranes were washed in TBST for 30 min at room temperature and then incubated for 1 h in TBST and 5% dry milk, containing either protein A, directly conjugated to horseradish peroxidase (HRP) (Amersham Life Science, Arlington Heights, IL) (1:2,500), for the Akt blots or an HRP-conjugated anti-rabbit secondary antibody (1:2,000) for the GSK-3α blots. The membranes were washed in TBST for 30 min at room temperature and then exposed to the Amersham enhanced chemiluminescence detection system according to the manufacturer's instructions.

**Amplification and purification of adenoviral vectors.** Briefly, recombinant adenoviruses expressing dominant negative Akt and β-galactosidase were amplified as described previously using 293 cells (3). The viruses were subsequently concentrated and purified on a cesium chloride gradient (3). The concentration of the recombinant adenovirus was assessed on the basis of the absorbance at 260 nm and on a limiting dilution plaque assay (3).

**Immunohistochemistry.** The AR42J cells were transduced with the adenoviral vector expressing HA-tagged dominant-negative Akt and cultured on slides for 24 h. At the end of the incubation period the cells were fixed in 4% formalin-PBS. The slides were blocked for 30 min with 20% donkey serum and incubated for 2 h with a mouse monoclonal anti-HA antibody (1:500) (Babco). The cells were rinsed with PBS, and a 1:150 dilution of a FITC-conjugated donkey anti-mouse IgG secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA) was added for 1 h. After being washed with PBS, the cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy. In control experiments the cells were incubated with the FITC-conjugated secondary antibody without the primary anti-HA antibody.

**Detection of adenoviral-delivered β-galactosidase.** For identification of AR42J cells transduced with the adenoviral vector expressing β-galactosidase, the cells were cultured on slides for 24 h and stained with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) after 24 h of infection. The cells were washed with PBS and then fixed in 0.5% glutaraldehyde at room temperature for 10 min. After two washes with 1 mM MgCl₂ in PBS, the cells were incubated overnight at 37°C in a solution consisting of 5 mM K₂Fe(CN)₆, 5 mM K₃Fe(CN)₅O₆, and 2 mM MgCl₂ in PBS with 0.1% X-gal. At the end of the incubation, the cells were rinsed with PBS and observed with a light transmission microscope.

**Terminal deoxynucleotidyltransferase-mediated dUTP-FITC nick end-labeling method.** The AR42J cells were cultured on coverslips and fixed in 4% neutral-buffered formalin for 15 min followed by methanol for 10 min. The cells were stored at −20°C for 24 h. Apoptosis was detected using the terminal deoxynucleotidyltransferase-mediated dUTP-FITC nick end-labeling (TUNEL) method as previously described (32). The coverslips were washed with cold PBS and incubated with biotin-conjugated dUTP and terminal transferase enzyme for 1 h at 37°C. After washing, the cells were incubated with FITC-avidin for 1 h at room temperature (light protected). The specimens were mounted in SlowFade containing 5 μg/ml propidium iodide and 0.05 mg/ml DNase-free RNase. The FITC-labeled DNA fragments in the apoptotic cells were visualized using a fluorescence microscope. Two hundred cells were counted blindly, and the positive cells were expressed as a percentage of the total cells counted. This included adherent early necrotic cells that stained with terminal deoxynucleotidyltransferase-mediated dUTP-FITC nick end-labeling (TUNEL) method. The AR42J cells were grown in either 10% fetal bovine serum (FBS) (a) or in serum-free medium in the absence (b) or presence of 10 nM G17 (c). B: means ± SE of %apoptosis in AR42J cells is depicted. *P < 0.05, no. given in parentheses in bars indicates n.
propidium iodide but not with FITC. The negative control received only the label solution without terminal transferase.

Flow cytometric assays. DNA fragmentation was determined by DNA staining of isolated nuclei by flow cytometry (21). The cells were harvested by gentle scraping, washed, and lysed in a buffer containing 0.1% sodium citrate, 0.01% Triton X-100, and 0.1 mg/ml propidium iodide. After incubation at 4°C, the nuclei were analyzed for DNA content by flow cytometry. Nonapoptotic nuclei were distinguished from apoptotic nuclei on the basis of their tight scatter profile. Flow cytometry was performed by the Biomedical Flow Cytometry Core Facility of the Cancer Center of the University of Michigan.

Cell viability assay. After 72 of culture in either 10% FBS or in serum-free medium, AR42J and CHO-B cell viability was measured by exposing the cells to 0.2% trypan blue (Sigma Chemical) for 10 min and counting the number of blue-positive cells per 200 cells in a blinded manner (32).

[3H]Thymidine incorporation. These studies were conducted according to previously described techniques (29). Briefly, the AR42J cells were grown in 35-mm dishes in DMEM supplemented with 10% FBS in 5% CO₂ at 37°C. Subconfluent cells were cultured for 24 h in serum-free DMEM containing 0.2 mM unlabeled thymidine. After washing with serum-free medium, the cells were treated with 1 nM gastrin for an additional 24 h. Control experiments were

Fig. 2. Effect of G17 on serum withdrawal-induced apoptosis of CHO-K1/CCK-B cells. CHO-K1/CCK-B cells were grown in either 10% FBS or in serum-free medium in the absence or presence of 10 nM G17. A: the % of cells showing DNA degradation was quantified by flow cytometry, as shown by the sub-G peak labeled as C. B: means ± SE of %apoptosis in CHO-K1/CCK-B cells is depicted.

Fig. 3. Effect of gastrin on Akt phosphorylation. Phosphorylation of Akt kinase in lysates from AR42J cells stimulated for 5, 30, and 60 min with 10 nM G17 was studied by Western blot analysis using a specific anti-phospho-Akt antibody. Total Akt kinase levels were monitored by Western blot analysis with an antibody recognizing Akt kinase independent of its phosphorylation state. Identical results were obtained in at least 3 other separate experiments.

Fig. 4. Effect of gastrin on Akt activity. Akt kinase in lysates from AR42J cells stimulated for 5 min with 10 nM G17 was immunoprecipitated, and its activity was measured by kinase assays using glycogen synthase kinase-3β (GSK-3β) as substrate. GSK-3β phosphorylation was measured by Western blot analysis with a specific anti-phospho-GSK-3a/β antibody. Levels of immunoprecipitated Akt kinase were monitored by Western blot analysis with an antibody recognizing Akt kinase independent of its phosphorylation state. Identical results were obtained in 1 other separate experiment.
tical analysis was performed using Student’s t-test.

Data analysis. Data are presented as means ± SE. Statistical analysis was performed using Student’s t-test. P < 0.05 was considered significant.

RESULTS

We investigated the effect of gastrin on apoptosis induced by serum withdrawal. For these studies, AR42J cell apoptosis was measured by quantitating DNA fragmentation with the TUNEL method. As shown in Fig. 1, culture of the AR42J cells in serum-free medium for 72 h increased AR42J cell apoptosis by fourfold. Incubation of the cells in serum-free medium containing 10 nM gastrin significantly inhibited this effect. Measurement of AR42J cell apoptosis by flow cytometry yielded similar results (data not shown). To confirm the validity of these observations and to demonstrate the specific involvement of CCK-B receptors in the antiapoptotic action of gastrin, we performed experiments using CHO-K1 cells stably expressing the CCK-B receptor (CHO-K1/CCK-B). CHO-K1/CCK-B cell apoptosis was measured using flow cytometry. Serum withdrawal for 72 h induced CHO-K1/CCK-B cell apoptosis, whereas addition of 10 nM gastrin potently reversed this effect (Fig. 2). The specificity of this finding was confirmed by the observation that gastrin had no effect on the survival of wild-type CHO-K1 cells, which do not express CCK-B receptors (data not shown). As indicated by the trypan blue exclusion assays, serum starvation for 72 h did not significantly affect both AR42J and CHO-K1/CCK-B cell viability. In particular, AR42J cell viability was 94% in the presence of 10% FBS and 93% in serum-free conditions. Similarly, CHO-K1/CCK-B cell viability was 97% and 96% in the presence and absence of 10% FBS, respectively. Thus gastrin inhibits serum withdrawal-induced apoptosis of both AR42J and CHO-K1/CCK-B cells.

To examine the signal transduction pathways activated by gastrin to suppress AR42J cell apoptosis, we investigated the effect of gastrin on Akt kinase, a signaling molecule known to play an important role in the inhibition of cellular apoptosis (6, 7). First, we studied the effect of gastrin on Akt phosphorylation using Western blots with a specific anti-phospho-Akt antibody directed against phosphoserine 473 of Akt. Gastrin (10 nM) stimulated Akt phosphorylation, with a maximal effect detected between 5 and 30 min of incubation (Fig. 3). Similar results were observed in the presence of 100 nM gastrin, indicating that maximal phosphorylation of Akt by gastrin is achieved at doses ranging between 10 and 100 nM (data not shown). Total Akt levels were monitored by Western blot analysis with an antibody recognizing Akt independent of its phosphorylation state (Fig. 3).

To confirm that phosphorylation of Akt correlates with Akt activation, we conducted studies in which we examined the effect of gastrin on Akt activity. For these experiments, Akt was immunoprecipitated with a specific anti-Akt antibody and its activity was measured in an in vitro kinase assay using GSK-3α as substrate. GSK-3α phosphorylation was measured by Western blot analysis with a specific anti-phospho-GSK-3α/β antibody. As shown in Fig. 4, 10 nM gastrin induced Akt activation, confirming the notion that

![Fig. 5. Effect of LY-294002 and wortmannin on gastrin-induced phosphorylation of Akt. Phosphorylation of Akt kinase in lysates from AR42J cells stimulated for 5 min with 10 nM G17 alone or in association with either 10 μM LY-294002 or 200 nM wortmannin was studied by Western blot analysis using a specific anti-phospho-Akt antibody. Total Akt kinase levels were monitored by Western blot analysis with an antibody recognizing Akt kinase independent of its phosphorylation state. Identical results were obtained in at least 3 other separate experiments.](http://ajpgi.physiology.org/)
phosphorylation of Akt on serine 473 correlates with Akt activation. Levels of immunoprecipitated Akt were monitored by Western blot analysis with an antibody recognizing Akt independent of its phosphorylation state (Fig. 4).

Gastrin is known to induce PI 3-kinase in AR42J cells (16). Because activation of PI 3-kinase is an important step in the signaling pathway that leads to Akt activation in response to growth factor stimulation (6, 7), we undertook studies to examine whether gastrin induction of Akt phosphorylation requires activation of PI 3-kinase. As shown in Fig. 5, gastrin stimulated Akt phosphorylation, and this effect was inhibited by both 10 \( \mu \)M LY-294002 and 200 nM wortmannin, two specific and well-characterized inhibitors of PI 3-kinase, indicating that gastrin targets Akt through PI 3-kinase-dependent signaling pathways.

![Phosphorylation of Akt and P38 kinase](image)

**Fig. 7.** Effect of SB-203580 (0.1–10 \( \mu \)M) on gastrin-induced phosphorylation of Akt and p38 kinase. Phosphorylation of Akt (A) and p38 kinase (B) in lysates from AR42J cells stimulated for 5 min with G17 (10 nM) alone or in association with SB-203580 (0.1–10 \( \mu \)M) was studied by Western blot analysis using specific anti-phospho-Akt and anti-phospho-p38 kinase antibodies. Total Akt and p38 kinase levels were monitored by Western blot analysis with antibodies recognizing Akt and p38 kinase independent of their phosphorylation states. Identical results were obtained in at least 3 other separate experiments.

We further dissected the signal transduction pathways that regulate Akt activation in response to gastrin stimulation. In particular, we investigated the role of p38 kinase, a signaling molecule involved in the regulation of cellular growth and survival (39), in gastrin induction of Akt phosphorylation. First, we examined the effect of gastrin on p38 kinase activity. p38 was immunoprecipitated from AR42J cell lysates with a specific anti-p38 kinase antibody, and its activity was measured in vitro kinase assay using transcription factor ATF2 as substrate. As shown in Fig. 6, gastrin potently induced p38 kinase activity in a time- and dose-dependent fashion (Fig. 6) with a maximal stimulatory effect detected between 10 and 100 nM after 5 min of incubation. EGF (10 nM) failed to induce p38 kinase activity in AR42J cells (data not shown).

To define the role of p38 kinase in gastrin signaling to Akt, we examined the effect of the p38 kinase inhibitor SB-203580 on gastrin induction of Akt phosphorylation. Although SB-203580 is considered to be a specific inhibitor of p38 kinase, a recent study (19) has indicated that this agent can also inhibit the activity of PDK1. Thus we conducted a detailed dose-response analysis of the effects of SB-203580 (0.1–10 \( \mu \)M) on the phosphorylation of both Akt and p38 kinase. As shown in Fig. 7, 1 \( \mu \)M SB-203580 completely inhibited p38 kinase phosphorylation. In contrast, a 10-fold higher concentration of SB-203580 (10 \( \mu \)M) was required to completely inhibit Akt phosphorylation.

![Effect of SB-203580 on gastrin-induced phosphorylation of Akt](image)

**Fig. 8.** Effect of SB-203580 on gastrin- and epidermal growth factor (EGF)-induced phosphorylation of Akt. Phosphorylation of Akt kinase in lysates from AR42J cells stimulated for 5 min with either 10 nM G17 or 10 nM EGF, alone or in association with 10 \( \mu \)M SB-203580, was studied by Western blot analysis using a specific anti-phospho-Akt antibody. Total Akt kinase levels were monitored by Western blot analysis with an antibody recognizing Akt kinase independent of its phosphorylation state. Identical results were obtained in at least 4 other separate experiments.
Fig. 10. Infection of the AR42J cells with adenoviral vectors. Histochemical staining for β-galactosidase in nontransduced AR42J cells (A) and AR42J cells transduced with the adenoviral vector expressing the β-galactosidase enzyme under the control of the cytomegalovirus (CMV) promoter (AD-CMV-β-gal) (B). C: AR42J cells transduced with the hemoagglutinin (HA)-tagged dominant-negative Akt expressing adenoviral vector (Adeno-dnAkt) and stained with a mouse monoclonal primary anti-HA antibody and a donkey anti-mouse FITC-conjugated secondary antibody. D: control AR42J cells transduced with the adenoviral vector AD.CMV-β-gal and incubated with both the primary anti-HA antibody and the donkey anti-mouse FITC-conjugated secondary antibody. Magnification, ×60. Identical results were obtained in 1 other separate experiment.

achieve complete inhibition of Akt phosphorylation. Identical results were observed when the effects of similar doses of SB-203580 were tested on the kinase activities of both Akt and p38 kinase (data not shown). These data indicate that SB-203580-sensitive kinase activities other than p38 kinase might be involved in gastrin induction of Akt phosphorylation.

To further establish the specificity of the effect of SB-203580 on Akt phosphorylation, we investigated whether this agent would also inhibit the stimulatory action of EGF (10 nM). As indicated in Fig. 8, EGF induction of Akt phosphorylation was unaffected by SB-203580 (10 μM). This suggests that gastrin, but not EGF, activates SB-203580-sensitive kinase activities in the AR42J cells.

Gastrin induces MAPK in the AR42J cells (35, 40). Because MAPK is known to be involved in the regulation of apoptosis, we investigated the role of this kinase in gastrin induction of Akt phosphorylation. As shown in Fig. 9, gastrin (10 nM) induction of Akt phosphorylation was completely inhibited by SB-203580 (10 μM) but not by the highly specific MAPK kinase (MEK) inhibitor PD-98059 (50 μM), suggesting that gastrin targets Akt via MEK-independent mechanisms.

To confirm the role of Akt in the antiapoptotic action of gastrin, we conducted studies in which we transduced the AR42J cells with the AD.CMV-β-gal or Adeno-dnAkt adenoviral vector. First, we performed experiments to demonstrate that AR-2J cells can be successfully transduced with these adenoviral vectors. Figure 10B shows histochemical staining of the AR42J cells for β-galactosidase after infection of the cells with 600 MOI of the adenoviral vector AD.CMV-β-gal for 16 h. Examination of 100 cells in three separate slides revealed that that 67 ± 0.88% (n = 3) of the cells were successfully infected with AD.CMV-β-gal. No staining was detected in control, noninfected AR42J cells that were subjected to the same staining protocol, as shown in Fig. 10A. Immunocytochemical staining of Adeno-dnAkt-transduced AR42J cells with an anti-HA mouse monoclonal antibody and a donkey anti-mouse FITC-conjugated secondary antibody indicated that ~70% of the AR42J cells were transduced by this adenoviral vector (Fig. 10C). Control experiments conducted with AR42J cells transduced with the adenoviral vector AD.CMV-β-gal and incubated with both the primary anti-HA antibody and the donkey anti-mouse FITC-conjugated secondary antibody demonstrated only faint background fluorescence (Fig. 10D). Furthermore, infection with AD.CMV-β-gal did not alter the function of the AR42J cells, because both infected and noninfected cells showed identical growth responses to gastrin stimulation. In particular, gastrin induced [3H]thymidine incorporation into noninfected AR42J cells by 1.52 ± 0.1-fold (n = 3) over unstimulated control cells and by 1.65 ± 0.07-fold (n = 3) into AR42J cells that were infected with 600 MOI of the AD.CMV-β-gal adenoviral vector.

Using the TUNEL method, we observed that transduction of the AR42J cells with the adenoviral vector expressing dominant-negative Akt, but not with that expressing β-galactosidase, reversed the antiapoptotic action of gastrin, underscoring the functional importance of Akt in gastrin-mediated inhibition of AR42J cell apoptosis (Fig. 11). Serum starvation-induced apoptosis of AD.CMV-β-gal-transduced AR42J cells was identical to that of nontransduced cells, indicating that AD.CMV-β-gal does not affect AR42J cell apoptosis (Figs. 1 and 11). Similarly, transduction of the AR42J cells with AD.CMV-β-gal failed to affect AR42J cell viability (data not shown).

DISCUSSION

The peptide hormone gastrin regulates numerous complex cellular functions such as growth, proliferation, and secretion (8, 10, 15, 18, 26, 30, 31, 33–36). In
In this study, we have demonstrated that gastrin is also a potent inhibitor of cellular apoptosis.

Apoptotic cell death plays an important role in the process of regulated growth and development of multicellular organisms (12, 13). Cells are targeted by both death-inducing and survival-promoting agents. The ultimate fate of a cell is therefore determined by the integration of these complex and opposing signals. Cells undergoing apoptosis exhibit specific morphological changes such as DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage, and disassembly (12, 13).

The molecular mechanisms that regulate cellular apoptosis have been the focus of numerous investigations. In particular, phosphorylation and dephosphorylation of cellular proteins appear to be important mechanisms for the transmission and integration of both death-inducing and survival-promoting signals. Numerous protein kinases and phosphatases have been shown to initiate complex programs of cellular activation that lead to either the induction or inhibition of cellular apoptosis (12, 14, 20, 23, 27, 39, 41).

PKB/Akt, in particular, is known to induce cell survival (6, 7, 9). Akt phosphorylates several cellular proteins that are known to play an important role in the induction of apoptosis. Akt phosphorylates the pro-apoptotic proteins Bad (6, 7, 9) and caspase 9 in vivo (6, 7). Akt also phosphorylates and inactivates nuclear proteins such as those belonging to the family of forkhead/winged-helix transcription factors that appear to be important for the transcription and expression of pro-apoptotic molecules such as Fas ligand (7). Accordingly, the Akt signaling pathway appears to function as a crucial element for the transmission of survival signals in multiple cell types.

In addition to Akt, some of the best characterized signal transduction pathways known to modulate cellular apoptosis are the MAPK, c-Jun NH2-terminal kinase, and p38 kinase signaling cascades (39). The specific intracellular targets of these kinases have been only partially characterized. The role of p38 kinase in the regulation of cellular apoptosis appears to be complex. Although in some systems activation of p38 kinase is linked to induction of apoptosis (39), in others, such as NIH/3T3 cells (27), Jurkat cells (23), and cardiac myocytes (41), induction of p38 kinase appears to have a protective effect. Furthermore, the kinetics of p38 kinase activation appear to play an important role in the regulation of cell survival. A recent report (27) has suggested that in some systems tumor necrosis factor-α (TNF-α) induces p38 kinase activity with biphasic kinetics. Although the first phase of activation appears to be transient and to protect cells from TNF-α-induced apoptosis, the second phase has no effect on cell survival.

We have demonstrated that gastrin stimulates both the activity and phosphorylation of p38 kinase with kinetics similar to those observed in systems in which activation of p38 kinase is linked to induction of cell survival (27). Furthermore, we have reported that gastrin induction of Akt phosphorylation could be effec-

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**Fig. 11.** Effect of Adeno-dnAkt on G17 inhibition of serum withdrawal-induced apoptosis of AR42J cells. A: representative photograph of AR42J cells stained for apoptosis with the TUNEL method. The AR42J cells were grown in either 10% FBS (a) or in serum-free medium after infection with the adenoviral vector AD.CMV-β-gal in the absence (b) or presence of 10 nM G17 (c). Cells transduced with the vector Adeno-dnAkt and grown in serum-free medium in the presence or absence of G17 are shown in d and e, respectively. B: means ± SE of % apoptosis in AR42J cells is depicted. MOI, multiplicity of infection. *P < 0.05.
Effect of gastrin on cellular apoptosis

Gastrin inhibits cellular apoptosis induced by se-


tivation of Akt stimulated by the small GTP-binding protein Rac (24). However, in our study, we have also observed that the concentration of SB-203580 necessary to affect gastrin stimulation of Akt phosphorylation is 10-fold higher than that required to block the stimulatory action of gastrin on p38 kinase activity. Accordingly, these findings raise the possibility that SB-203580-sensitive kinases other than p38 kinase could be activated by gastrin to induce Akt in AR42J cells. Interestingly, similar results were observed in a recent study (19) conducted in a murine T cell line in which SB-203580 was found to block interleukin-2-stimu-

lated Akt activation through inhibition of PDK1, independently of p38 kinase. Thus SB-203580 might inhibit the stimulatory effect of gastrin on Akt through its ability to block PDK1 activation.

Alternatively, the difference in the concentrations of SB-203580 required to inhibit Akt and p38 kinase might reflect the presence of several isoforms of p38 kinase in the AR42J cells. Mammalian cells are known to express multiple isoforms of p38 kinase (p38α, p38β, p38γ, and δ) (17, 39). Whereas some of these isoforms are highly sensitive to SB-203580 (p38α and p38β2), others are either partially (p38β) or completely resistant to inhibition by SB-203580 (p38γ and δ) (17). The antibodies used in our studies predominantly recognize p38 kinase-α, commonly referred to as p38 kinase. In addition, although p38 kinase-α and -β appear to share numerous similarities, one study (23) has indicated that p38 kinase-β might play a greater role in the inhibition of cellular apoptosis than the other isoforms of p38 kinase. Accordingly, it is possible that in our system gastrin might activate p38 kinase-β, which is only partially inhibited by SB-203580, to stimulate Akt and inhibit AR42J cell apoptosis. This possibility would explain the fact that a higher concentration of SB-203580 was required to inhibit the stimulatory effect of gastrin on Akt activation. It is clear that additional studies are necessary to characterize in more detail the specific isoforms of p38 kinase that are activated by gastrin in the AR42J cells and to gain more insight into the role of PDK1 in the antiapoptotic action of gastrin.

We have also demonstrated that MEK1 does not play any role in either the phosphorylation or activation of Akt. This observation is in agreement with studies (9) demonstrating that the MAPK pathway is not involved in the activation of Akt in other cellular systems.

Ligands for seven transmembrane G protein-coupled receptors, such as carbamylcholine, can activate Akt in PC-12 and COS-7 cells stably expressing either the M1 or M2 muscarinic receptor through signaling pathways that involve PI 3-kinase and βγ-subunits of GTP-bind-

ing proteins (22). In our study, we have demonstrated that gastrin inhibits cellular apoptosis induced by se-

rum withdrawal through the activation of specific gas-

trin/CCK-B receptors. We have also dissected the sig-

nal transduction pathways responsible for this effect using AR42J cells, which express endogenous gastrin/ CCK-B receptors, as our model. In this system we have demonstrated that gastrin induces Akt phosphoryla-

tion through the activation of PI 3-kinase and SB-

203580-sensitive kinase activities that need to be fur-

ther characterized. In addition, we have demonstrated that gastrin inhibits AR42J cell apoptosis through a signal transduction pathway that involves the activation of Akt, because the antiapoptotic action of gastrin was inhibited by an adenoviral vector expressing a dominant-negative Akt gene.

In conclusion, gastrin is a potent inhibitor of cellular apoptosis through its ability to induce PKB/Akt. This novel action of gastrin is likely to represent an impor-

tant mechanism responsible for the growth-promoting effect of the hormone on both normal and neoplastic gastrointestinal tissues.

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