Role of small GTP binding proteins in the growth-promoting and antiapoptotic actions of gastrin

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ALTHOUGH CHARACTERIZED AS a stimulant of gastric acid secretion, the peptide hormone gastrin also exerts growth-promoting effects on normal and malignant gastrointestinal tissues (12, 22, 29, 35, 40, 41, 53, 57). Gastrin is an important growth factor for the fetal pancreas (8, 49), and in the stomach, it is a potent stimulant for the growth of the gastric mucosa (52). In addition, gastrin has been shown to induce the growth of colonic, gastric, and pancreatic carcinomas both in vivo and in vitro, underscoring the importance of gastrin as a growth factor for gastrointestinal neoplasms (9, 12, 18, 19, 29, 35, 40, 52–54).

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 phosphatidylinositol 3-kinase (PI3-K), and it activates the ERKs or MAPKs (12, 14, 26, 47, 48, 57). In particular, it has been reported (14, 43, 48) that gastrin stimulates the growth of rat pancreatic adenocarcinoma cells (AR4–2J) through induction of the ERKs and of the early response gene c-fos through PKC-dependent and -independent mechanisms.

In addition to this well-established effect on the regulation of cellular growth and proliferation, gastrin has also been shown to be a potent inhibitor of cellular apoptosis. Indeed, work from our laboratory has demonstrated that gastrin inhibits AR4–2J cell apoptosis through a signal-transduction pathway that requires the activation of Akt (46), a protein kinase that is known to induce cellular growth and survival (1, 10, 11). In our studies, we have also reported that gastrin activates Akt through PI3-K-dependent, ERK-independent mechanisms, suggesting that MAPK and Akt belong to two separate signal-transduction cascades, which are activated by gastrin to regulate AR4–2J cell proliferation and apoptosis (46).

Interestingly, a recent report has indicated that inhibition of the proliferative and antiapoptotic actions of gastrin in the human pancreatic cancer cell line PAI1 leads to increased sensitivity of the cells to the action of cytotoxic agents, suggesting that the ability of gastrin to inhibit pancreatic cancer cell apoptosis might be of considerable clinical relevance and significance (53).

The small GTP binding proteins Ras, Rho, Rac, and Cdc42 are important molecular switches in the cellular activation process (6, 15, 23, 24, 30, 31, 33, 34, 36, 38, 43, 49a). These small GTP binding proteins have been shown to control numerous complex signal-transduction cascades such as those leading to the induction of the ERKs, the JNKs and the p38 kinase (55). Activation of Ras, Rho, Rac, and Cdc42 has been linked to the regulation of complex biological processes such as growth, survival, and apoptosis. (3, 6, 15, 24, 30, 31, 33, 34, 36, 38, 43).

In previous reports (43), it was demonstrated that incubation of the AR4–2J cells with either the Rho inhibitor Clostridium botulinum exoenzyme C3 or a dominant negative Rho gene blocks gastrin stimulation of both AR4–2J cell proliferation and c-fos gene transcription. In addition, gastrin has been shown to regulate the formation of stress fibers and the expression of PAI-2, a gene involved in the control of cellular invasiveness and apoptosis, through Rho-dependent mechanisms (45, 49). Thus Rho appears to be a crucial switch in the
signal-transduction cascades activated by gastrin to regulate adhesion, growth, and proliferation. It is currently unknown whether, in addition to Rho, gastrin also activates Ras, Rac, and Cdc42 and whether these small GTP binding proteins regulate the growth-promoting and antiapoptotic actions of gastrin. Accordingly, we undertook studies to investigate the role of Ras, Rac, Rho, and Cdc42 in gastrin induction of cellular growth and survival.

MATERIAL AND METHODS

Plasmids and adenoviral vectors. The replication defective adenoviral vectors expressing dominant negative, hemagglutinin (HA) tagged Akt under the control of the cytomegalovirus (CMV) promoter (Ad.dom.neg.Akt) was a gift of K. Walsh (Tufts University, Boston, MA) (16). The replication defective adenoviral vectors expressing green fluorescent protein (GFP)/dominant negative Rho, Rac, and Cdc42 under the control of the CMV promoter (Ad.dom.neg.Rho, Ad.dom.neg.Cdc42, Ad.dom.neg.Rac) were gifts of J. Bamburg (Colorado State University, Fort Collins, CO) (27). The replication defective adenoviral vectors expressing, under the control of the CMV promoter, either the β-galactosidase enzyme (Ad.CMV-β-gal) or dominant negative H-ras (Ad.dom.neg.Ras), were previously described (4, 33).

Cell culture and infection. For our experiments, we used the rat exocrine pancreatic cell line AR4–2J (obtained from American Type Culture Collection, Rockville, MD), which is known to express receptors for gastrin (G17) (12). The AR4–2J cells were grown at 37°C in DMEM supplemented with 10% FBS and 1% sodium pyruvate. The cells were maintained in 5% CO2-95% O2. For the apoptosis studies, the cells were cultured accordingly to previously reported methods (46). Briefly, the AR4–2J cells were starved for 24 h in serum-free medium [either DMEM or F-12 nutrient mixture (Ham’s; Gibco-BRL, Grand Island, NY)] transduced, when indicated, with 400 multiplicity of infection (moi) of either the adenoviral vectors expressing dominant negative Akt, H-ras, Rac, Cdc42, and Rac or of that expressing β-galactosidase for 16 h and then treated with or without gastrin for different time periods. Transduction of the AR4–2J cells with the adenoviral vectors expressing the GFP-tagged, dominant negative GTPases was monitored by examination of the cells before each experiment with an inverted fluorescence microscope. Control experiments were performed in nontransduced AR4–2J cells according to an identical protocol. For all other studies, the cells were starved for 24 h in serum-free medium and then treated with gastrin (10 nM) for different time periods. In some experiments, PD-98059 (50 μM, Cell Signaling, Beverly, MA) (2) was added 30 min before the addition of gastrin. In additional studies, the cells were preincubated for 24 h in 40 μg/ml exoenzyme C3 from C. botulinum before the addition of gastrin (43). PD-98059 was dissolved in DMSO (Sigma, 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.

GTPases activation assays. Activation of Ras, Rac, Cdc-42, and Rac was measured using GTPase activation assay kits from Upstate Biotechnology (Lake Placid, NY) according to the manufacturer’s instructions. Briefly, active, GTP-bound Ras, Rac, and Cdc42 in lysates from AR4–2J were measured by affinity precipitation using glutathione S-transferase (GST)-tagged fusion proteins containing the Ras binding domain of human c-Raf, the Rac binding domain of mouse Rhotekin, and the Rac and Cdc42 binding domains of human PAK-1, followed by Western blot analyses with specific anti-Ras, -Rac, -Rho, and Cdc42 antibodies. After Western blot analysis, the membranes were stained with the MemCode Reversible Protein Stain Kit (Pierce, Rockford, IL), according to the manufacturer’s instructions, to make sure that equal amounts of the GST-tagged fusion proteins were loaded on the gels.

Western blot analyses. AR4–2J cell lysates (80 μg) were loaded on a 10% SDS-polyacrylamide minigel and run at 20 A for 8 h. Protein concentrations were measured by the Bradford method (7). 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 blocked in 10 ml of 20 mM Tris, 0.15 M NaCl, and 0.3% Tween (TBST), 5% dry milk for 1 h, and then incubated for 16–18 h at 4°C in 10 ml TBST and 5% dry milk containing specific antibodies recognizing phosphorylated serine 473 of Akt kinase (1:1,000), phosphorylated threonine 202 and tyrosine 204 of ERK2 (1:1,000), and phosphorylated threonine 180 and tyrosine 182 of p38 kinase (1:1,000) (Cell Signaling). Control blots were incubated with the respective nonimmune sera.

Fig. 1. Effect of 10 nM gastrin on Rho, Ras, Rac, and Cdc-42 activation. Activation of Ras, Rac, Rho, and Cdc42 in lysates from AR4–2J cells stimulated with gastrin (G17) (10 nM) was measured by affinity precipitation using glutathione S-transferase (GST)-tagged fusion proteins containing the Ras binding domain of human c-Raf, the Rho binding domain of mouse Rhotekin, and the Rac and Cdc42 binding domains of human PAK-1, followed by Western blot analysis with specific anti-Ras, -Rac, -Rho, and -Cdc42 antibodies. Top: Western blot analyses depicting activated Ras (A), Rac (B), Rho (C), and Cdc42 (D). Bottom: stains of protein bands corresponding to Raf1 (1–149)-GST (A), Pak1 (67–150)-GST (B and D), and Rhotekin (7–89)-GST (C). Identical results were obtained in at least 1 other separate experiment.
were performed using antibodies recognizing Akt kinase, ERK2, and p38 kinase independent of their phosphorylation state (1:1,000; Cell Signaling). The membranes were washed in TBST for 30 min at room temperature and then exposed to the Amersham enhanced chemiluminescence (ECL) detection system according to the manufacturer’s instructions. For the H-ras Western blot analyses, the membranes were incubated for 16–18 h at 4°C in 10 ml TBST and 5% dry milk containing a specific anti-H-ras antibody (Santa Cruz Biotechnology, Santa Cruz, CA). 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 protein A directly conjugated to horseradish peroxidase (Amersham Life Science, Arlington Heights, IL) (1:2,500). The membranes were washed in TBST for 30 min at room temperature and then exposed to the Amersham ECL detection system according to the manufacturer’s instructions. Blots were quantitated by scanning densitometry.

Amplification and purification of adenoviral vectors. Briefly, recombinant adenoviruses were amplified as previously described using 293 cells (4). The viruses were subsequently concentrated and purified on a cesium chloride gradient (4). The concentration of the recombinant adenoviruses was assessed on the basis of the absorbency at 260 nm and on a limiting dilution plaque assay (49a).

ADP ribosylation of Rho in AR4–2J cells. Recombinant C3 was expressed in Escherichia coli using a plasmid encoding C. botulinum exoenzyme C3 (pET3a/C3) and purified as previously described (43). ADP ribosylation assays were carried out as previously described (43).

Terminal transferase-mediated dUTP nick end-labeling method. The AR4–2J 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 transferase-mediated dUTP (TdT) nick end-labeling (TUNEL) method as previously described (42, 46). The coverslips were washed with cold PBS and incubated with biotin-conjugated dUTP and terminal transferase enzyme for 1 h at 37°C. In some experiments, the cells were washed and then incubated with FITC-avidin for 1 h at room temperature (light protected). The specimens were mounted in slowfade containing 5 ug/ml propidium iodide and 0.05 mg/ml DNase-free RNase. The FITC-labeled DNA fragments in the apoptotic cells were visualized using a fluorescent microscope. For the experiments with the GFP-tagged dominant negative GTPases, AR4–2J cell apoptosis was measured by assessing the incorporation of digoxigenin-labeled nucleotides by the TdT enzyme using the ApopTag peroxidase kit (Serological, Norcross, GA), according to the manufacturer’s instructions. Two hundred cells were counted blindly,

Fig. 2. Transduction of the AR4–2J cells with adenoviral vectors expressing dominant negative forms of Rho, Rac, and Cdc42. Fluorescent and bright-field images of AR4–2J cells transduced with 400 multiplicity of infection (moi) of adenoviral vectors expressing GFP-tagged, dominant negative Rho (Ad.dom.neg.Rho), Rac (Ad.dom.neg.Rac), and Cdc42 (Ad.dom.neg.Cdc42). Objective magnification: ×40. Identical results were obtained in 1 other separate experiment.
and the positive cells were expressed as a percentage of total cells counted. Negative controls received only the label solution without terminal transferase.

$[^3]H$thymidine incorporation. These studies were conducted according to previously described techniques (39). Briefly, the AR4–2J cells were grown in 35-mm dishes in DMEM supplemented with 10% FBS in 5% CO$_2$ at 37°C. Subconfluent cells were cultured for 24 h in serum-free DMEM and then transduced with the adenoviral vectors. Sixteen hours after transduction, the cells were switched to serum-free medium containing 0.2 mM unlabeled thymidine. After being washed with serum-free medium, the cells were treated with 1 nM gastrin for an additional 24 h. Control experiments were performed by incubating the cells in incubation buffer. DNA synthesis was estimated by measurement of $[^3]H$thymidine incorporation into the trichloroacetic acid precipitable material according to the method of Seva et al. (39). $[^3]H$thymidine (0.1 μCi/ml, 10 Ci/mmol) was added during the last hour of the treatment period. Cells were washed with serum-free media to remove unincorporated $[^3]H$thymidine, and the DNA was precipitated with 5% trichloroacetic acid at 4°C for 15 min. Precipitates were washed twice with 95% ethanol, dissolved in 1 ml of 0.1 N NaOH, and analyzed in a liquid-scintillation counter (Beckman Instruments, Palo Alto, CA).

Cell growth. Cells were seeded onto six-well plates at a concentration of 10,000 cells/ml. After 24 h, the media were changed to serum-free media, and the cells were transduced with the adenoviral vectors. Gastrin (10 nM) was added 16 h after transduction. Media were changed every 24 h to new serum-free peptide-containing media, and the cells were counted using a Coulter Counter (Coulter Electronics, Hialeah, FL) after 4 days of stimulation.

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

Fig. 3. Transduction of the AR4–2J cells with the adenoviral vector expressing dominant negative H-ras. The AR4–2J cells were transduced with 400, 600, and 800 moi of the adenoviral vector expressing dominant negative H-ras (Ad.dom.neg.Ras). Control experiments were performed with the adenoviral vector expressing β-galactosidase. Expression of dominant negative Ras was monitored by Western blot analysis with an anti-H-Ras antibody. Identical results were obtained in 1 other separate experiment.

Fig. 4. Role of Ras, Rac, Rho, and Cdc42 on gastrin induction of AR4–2J cell proliferation. The AR4–2J cells were transduced with either Ad.CMV-β-gal or with adenoviral vectors expressing dominant negative Ras, Rac, Rho, and Cdc42. AR4–2J cell proliferation was measured by either cell counting (A) or by incorporation of tritiated thymidine into the cells (B). Data are expressed as fold induction over control (means ± SE). *$P$ < 0.05 compared with gastrin. Numbers on the bars indicate the number of times each experiment was repeated.

Fig. 5. Role of Akt on gastrin induction of AR4–2J cell proliferation. The AR4–2J cells were transduced with either Ad.CMV-β-gal or with the adenoviral vector expressing dominant negative Akt. AR4–2J cell proliferation was measured by either cell counting (A) or by incorporation of tritiated thymidine into the cells (B). Data are expressed as fold induction over control (means ± SE). *$P$ < 0.05. Numbers on the bars indicate the number of times each experiment was repeated.
RESULTS

The small GTP-binding proteins Ras, Rac, Rho, and Cdc42 are molecular switches that regulate important cellular functions such as growth, proliferation, and apoptosis through the activation of numerous signal-transduction pathways (6, 15, 23, 24, 30, 31, 33, 34, 36, 38, 43, 49a). Accordingly, we examined the effect of 10 nM gastrin on the activation of Ras, Rho, Rac, and Cdc42. For these studies, we took advantage of GST-tagged fusion proteins containing the Ras binding domain of human c-Raf, the Rho binding domain of mouse Rhotekin, and the Rac and Cdc42 binding domains of human PAK-1. GTP-bound, activated Ras, Rho, Rac, and Cdc42 were affinity precipitated using these agarose conjugated fusion proteins, separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with specific anti-Ras, -Rho, -Rac, and -Cdc42 antibodies. As shown in Fig. 1, gastrin induced the activation of Ras after 1 min and that of Rac after 5 min of incubation. Gastrin also induced the activation of Rho and Cdc42 after both 1 and 5 min of stimulation.

To examine the role of Rho, Rac, and Cdc42 in the growth-promoting and antiapoptotic actions of gastrin, the AR4–2J cells were transduced with 400 moi of adenoviral vectors...
expressing GFP-tagged, dominant negative forms of Rho, Rac, and Cdc42. Control experiments were performed with the adenoviral vector expressing β-galactosidase. Comparisons within the same field of fluorescent and bright-field images of cultured, transduced AR4–2J cells demonstrated that >90% of the cells were successfully transduced with the adenoviral vectors (Fig. 2).

To investigate the role of Ras in gastrin regulation of AR4–2J cells growth and apoptosis, the AR4–2J cells were transduced with 400, 600, and 800 moi of an adenoviral vector expressing a dominant negative H-ras gene. Control experiments were performed with the adenoviral vector expressing β-galactosidase. Expression of dominant negative Ras was monitored by Western blot analysis with an anti-H-ras antibody. As shown in Fig. 3, dominant negative Ras was successfully overexpressed in the AR4–2J cells.

We examined the role of Ras, Rho, Rac, and Cdc42 in gastrin induction of AR4–2J cell proliferation. For these studies, the AR4–2J cells were transduced with either the adenoviral vector expressing β-galactosidase or with adenoviral vectors expressing dominant negative Ras, Rac, Rho, and Cdc42. AR4–2 cell proliferation was measured by either cell counting or by incorporation of tritiated thymidine into the cells. The average number of AR4–2J cells was 16,046 ± 4,050 (mean ± SE, n = 8) in the control group and 31,801 ± 9,100 (mean ± SE, n = 8) after stimulation with 10 nM gastrin (P < 0.05). Similarly, in the tritiated thymidine incorporation assays, the average number of counts per minute was 1,274 ± 430 (mean ± SE, n = 7) in the control group and 2,773 ± 706 (mean ± SE, n = 7) in cells stimulated with 10 nM gastrin (P < 0.05). As shown in Fig. 4, dominant negative Ras, Rho, and Cdc42 but not dominant negative Rac effectively blocked the stimulatory action of gastrin on AR4–2J cell proliferation.

Protein kinase Akt is one of the signaling molecules that mediate the antiapoptotic action of gastrin in the AR4–2J cells (46). Thus we sought to investigate whether this kinase was also involved in the regulation of gastrin induction of AR4–2J cell proliferation. Transduction of the AR4–2J cells with an adenoviral vector expressing a HA-tagged dominant negative Akt gene inhibited gastrin stimulation of AR4–2J cell prolif-

Fig. 7. Role of exoenzyme C3 from Clostridium botulinum (C3) in G17 inhibition of serum withdrawal-induced apoptosis of AR4–2J cells. Representative photographs of AR4–J cells stained for apoptosis with the terminal transferase-mediated dUTP (TdT) nick end-labeling (TUNEL) method and FITC-labeled d-UTP (A). The AR4–2J cells were grown in serum-free medium in the absence or presence of 10 nM gastrin (G17) alone or in association with C3 (40 μg/ml). The means ± SE of % apoptosis in the AR4–2J cells are represented on the graphs (B). *P < 0.05. Numbers on the bars indicate the number of times each experiment was repeated.
eration assessed by both cell counting and by incorporation of tritiated thymidine into the cells (Fig. 5). Expression of HA-tagged dominant negative Akt was monitored by immunocytochemical staining of the transduced AR4–2J cells with an anti-HA mouse monoclonal antibody and with a donkey anti-mouse FITC-conjugated secondary antibody (46, and data not shown). Expression of dominant negative Ras, Rho, Cdc42, and Akt alone did not significantly affect AR4–2J cell proliferation (data not shown).

We investigated the role of Ras, Rho, Rac, and Cdc42 in the antiapoptotic action of gastrin. AR4–2J cells apoptosis was determined by quantitating DNA fragmentation with the TUNEL method. Culture of the AR4–2J cells in serum-free medium for 72 h induced AR4–2J cells apoptosis fourfold (46, and data not shown). As indicated in Fig. 6, gastrin (10 nM) significantly inhibited serum withdrawal-induced AR4–2J cell apoptosis, and this effect was blocked by expression of dominant negative Ras, Rho, and Cdc42 but not by dominant negative Rac. Expression of dominant negative Ras, Rho, and Cdc42 alone did not significantly affect serum withdrawal-induced AR4–2J cells apoptosis (data not shown).

To confirm the role of Rho in the antiapoptotic action of gastrin, we investigated the effect of recombinant exoenzyme C3 from C. botulinum, a toxin known to ADP ribosylate and inactivate Rho, on gastrin inhibition of AR4–2J cell apoptosis. The dose of toxin required to ADP ribosylate Rho in vivo was assessed by ADP-ribosylation assays, as previously reported (43). As shown in Fig. 7, in agreement with the observations generated with dominant negative Rho, C3 blocked the inhibitory effect of gastrin on AR4–2J cell apoptosis, underscoring the importance of Rho in the regulation of the antiapoptotic actions of gastrin.

The MAPks or ERKs are involved in the regulation of AR4–2J cells proliferation in response to gastrin stimulation (43, 48). We examined the role of these kinases in the antiapoptotic action of gastrin. Gastrin inhibited AR4–2J cell apoptosis induced by serum withdrawal, and this effect was blocked by PD-98059 a specific inhibitor of the upstream MAPK/ERK activator MEK1 (Fig. 8).
Because Ras, Rac, Rho, and Cdc42 mediate the growth-promoting and antiapoptotic actions of gastrin, we undertook studies to investigate the role of these small GTP-binding proteins in gastrin induction of Akt activation. Akt phosphorylation and activation were measured using Western blot analysis with a specific anti-phospho-Akt antibody directed against phospho-serine 473 of Akt. As shown in the Fig. 9, gastrin stimulated Akt phosphorylation, and this effect was inhibited by dominant negative Ras, Rho, and Cdc42 but not by dominant negative Rac. Expression of dominant negative Ras, Rho, and Cdc42 alone did not significantly affect the basal level of Akt phosphorylation (data not shown). Total Akt levels were monitored by Western blot analysis with an antibody recognizing Akt independent of its phosphorylation state.

Similarly, we investigated the role of Ras, Rac, Rho, and Cdc42 in gastrin induction of ERK2 activation. ERK2 phosphorylation and activation were measured using Western blot analyses with a specific anti-phospho-ERK2 antibody directed against phospho-threonine 202 and phospho-tyrosine 204 of ERK2. As depicted on Fig. 10, gastrin induced ERK2 activation, and this effect was inhibited by dominant Ras but not by dominant negative Rac, Rho, or Cdc42. Expression of dominant negative Ras, Rho, and Cdc42 alone did not significantly affect the basal level of ERK2 phosphorylation (data not shown). Total ERK2 levels were monitored by Western blot analysis with an antibody recognizing ERK2 independent of its phosphorylation state.

To confirm the specificity of the role of Rho in gastrin induction of Akt, we examined the effect of C3 on gastrin stimulation of both Akt and ERK2. As shown in the Western blot analyses of Fig. 11, whereas C3 failed to inhibit the stimulatory effect of gastrin on ERK2 phosphorylation and activation (Fig. 11A), it potently inhibited gastrin induction of Akt (Fig. 11B). Accordingly, Akt, but not ERK2, is downstream of Rho in a signal-transduction pathway activated by gastrin to modulate cellular apoptosis.

Because dominant negative Rac failed to affect the growth-promoting and antiapoptotic actions of gastrin, we undertook studies to confirm that the adenoviral vector expressing dominant negative Rac was biologically active. Rac is known to regulate the activation of p38 kinase, a member of the MAPK family of protein kinases, which is potently induced in response to osmotic shock (55). Accordingly, we investigated the effect of dominant negative Rac on p38 kinase activation in response to either gastrin or sorbitol stimulation. P38 kinase phosphorylation and activation were measured using Western blot analysis with a specific anti-phospho-p38 antibody directed against phospho-threonine 180 and phospho-tyrosine 182 of p38. As shown in the Fig. 12, dominant negative Rac inhibited p38 kinase activation induced by gastrin or sorbitol, confirming the specificity of Rac in the signal-transduction pathway activated by gastrin to modulate cellular apoptosis.

Fig. 9. Role of Ras, Rac, Rho, and Cdc42 in gastrin induction of Akt activation. A: Akt kinase phosphorylation and activation in lysates from AR4–2J cells stimulated for 5 min with gastrin (G17; 10 nM) after transduction of the cells with either Ad.CMV-β-gal or with dominant negative Ras, Rac, Rho, and Cdc42 were 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. B: graphs depicting densitometric analysis of the blots. Data are expressed as fold induction over control (means ± SE). *P < 0.05 compared with gastrin. Numbers on the bars indicate the number of times each experiment was repeated.

Fig. 10. Role of Ras, Rac, Rho, and Cdc42 in gastrin induction of ERK2 activation. A: ERK2 phosphorylation and activation in lysates from AR4–2J cells stimulated for 5 min with gastrin (G17; 10 nM) after transduction of the cells with either Ad.CMV-β-gal or with dominant negative Ras, Rac, Rho, and Cdc42 were studied by Western blot analysis using a specific anti-phospho-ERK2 antibody. Total ERK2 levels were monitored by Western blot analysis with an antibody recognizing ERK2 independent of its phosphorylation state. B: graphs depicting densitometric analysis of the blots. Data are expressed as fold induction over control (means ± SE). *P < 0.05. Numbers on the bars indicate the number of times each experiment was repeated.
blot analyses with a specific anti-phospho-p38 kinase antibody directed against phospho-threonine 180 and phospho-Tyrosine 182 of p38 kinase. As shown on Fig. 12, dominant negative Rac failed to affect gastrin induction of p38 kinase phosphorylation, whereas it significantly inhibited, by ~50%, the stimulatory effect of sorbitol. Total p38 kinase levels were monitored by Western blot analyses, with an antibody recognizing p38 independent of its phosphorylation state.

**DISCUSSION**

The peptide hormone gastrin regulates numerous, complex cellular functions such as growth, proliferation, apoptosis, migration, and secretion (12, 22, 29, 35, 40, 41, 46, 48–54, 56, 57). In this study, we analyzed the signal-transduction pathways that are activated by gastrin to stimulate cellular growth and survival.

The molecular mechanisms that regulate cellular proliferation and apoptosis have been the focus of numerous investigations. Several protein kinases and phosphatases have been shown to initiate complex programs of cellular activation that lead to either the induction or the inhibition of cellular growth, survival, and apoptosis (5, 10, 11, 13, 16, 17, 21, 24, 32, 34, 37, 38, 43, 55, 58). In this study, we report that gastrin induces AR4–2J cell proliferation and survival through the induction of the protein kinases ERK2 and Akt and the activation of several small GTP-binding proteins.

The antiapoptotic actions of Akt appear to rely on the ability of this protein kinase to phosphorylate the proapoptotic proteins Bad (10, 11, 13) and caspase-9 in vivo (10, 11) and to inactivate 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 proapoptotic molecules (11). In addition to these important antiapoptotic actions, Akt has also been shown to regulate the cell cycle machinery and to induce the expression of genes such as \(c-fos\) that play an important role in the regulation of cellular proliferation (11). Accordingly, the Akt signal-transduction pathway appears to function as a crucial point of convergence for the transmission of both mitogenic and survival signals in multiple cell types.

Several members of the MAPK family of protein kinases, such as the ERKs, the JNKs, and the p38 kinases, have also been shown to modulate, through the phosphorylation of downstream kinases and transcription factors, cellular proliferation, survival, and apoptosis (5, 24, 28, 31, 32, 37, 38, 43, 55, 58).

The ERKs, in particular, appear to regulate the expression of numerous proteins that are involved in the control of cellular proliferation and survival, such as \(c-Fos\), cyclin D1, Bc-X\(_L\), Bcl-2, and Mcl-1 (5). Accordingly, it is conceivable to speculate that, in the AR4–2J cells, gastrin might regulate the expression of cyclin D1, Bc-X\(_L\), Bcl-2, and Mcl-1 or the phosphorylation of forkhead/winged-helix transcription factors through both ERK2- and Akt-dependent mechanisms.

The small GTP binding protein Ras is an important switch in the cell-activation process. The role of Ras in gastrin signaling is currently poorly understood. Although some reports have suggested that gastrin does not activate Ras, others have indicated that gastrin induces the assembly of several adapter molecules involved in the process of Ras activation, such as...
She and Grb2 (14, 20, 26). In this study, we demonstrated that gastrin activates Ras and that this event leads to the induction of both the ERKs and Akt. In addition, we showed that gastrin stimulates AR4–2J cell proliferation and survival through a signal-transduction pathway that involves the activation of Ras, because the antiapoptotic and growth-promoting actions of gastrin were inhibited by an adenoviral vector expressing a dominant negative Ras gene. Accordingly, the ability of gastrin to activate Ras, appears to be a crucial event in the complex series of biochemical reactions that regulate gastrin induction of cellular proliferation and survival.

The complexity of the system is underscored by the observation that in addition to Ras, gastrin also activates Rho, Cdc42, and Rac. However, whereas Ras appears to be involved in gastrin induction of both ERK2 and Akt, Rho, Rac, and Cdc42 do not. In fact, dominant negative Rho, Rac, and Cdc42 failed to inhibit gastrin induction of ERK2. In contrast, dominant negative mutants of Rho and Cdc42 inhibited gastrin stimulation of Akt activation, suggesting that both Rho and Cdc42 participate in the signal-transduction cascade that regulates Akt in response to gastrin stimulation.

In contrast to these observations, dominant negative mutants of Ras, Rho, and Cdc42 exhibited complete inhibitory actions on gastrin stimulation of AR4–2J cell proliferation and survival. These findings suggest that gastrin might induce several protein kinases through the coordinated activation of specific small GTP-binding proteins to regulate AR4–2J cell proliferation and survival. It is possible that, in addition to Akt, Rho and Cdc42 might regulate the activation of protein kinases such as the p21-activated kinases, the Rho kinase, or the p38 kinases. In addition, Ras, Rho, and Cdc42 could be arranged in complex signaling cascades. Indeed, in some cell types, Ras is upstream of, and required for the activation of, Rho, Rac, and Cdc42 (6, 23, 24). Thus it is possible that gastrin might activate Akt through the sequential activation of Ras, Rho, and Cdc42.

One intriguing observation of our study is that whereas gastrin is able to induce Rac activation, a dominant negative mutant of this GTPase did not affect gastrin induction of both AR4–2J cell proliferation and survival. In addition, this construct was unable to modulate the stimulatory action of gastrin on the activation of both ERK2 and Akt. However, dominant negative Rac inhibited sorbitol-stimulated p38 kinase activation, indicating that this construct was biologically active.

These findings suggest that gastrin-mediated Rac activation is involved in the regulation of biological processes other than proliferation and survival. One of the best-characterized functions of Rac is that involving the regulation of cell motility and migration (6, 15, 23, 36). Interestingly, recent studies (25, 56) have shown that gastrin regulates cell migration. Thus it is possible that gastrin-mediated Rac activation of AR4–2J cells affects invasion of human renal cell carcinomas by up-regulating tissue bilirubin UDP-glucuronosyltransferase. Ann Intern Med 123: 478–484, 1995.

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