Gastrin and Glycine-extended Progastrin Processing Intermediates Induce Different Programs of Early Gene Activation*

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We recently reported that gastrin and glycine-extended progastrin processing intermediates (G-Gly) exert growth-promoting effects on AR4–2J cells (derived from rat pancreas) via interaction with distinct receptors. In this study we sought to investigate the mechanisms by which gastrin and G-Gly stimulate cell proliferation. While gastrin increased [Ca2+]i in AR4–2J cells, G-Gly had no effect. Similarly, G-Gly had no effect either on basal and 10^{-7} M vasoactive intestinal polypeptide-stimulated cAMP generation, although gastrin is known to inhibit cAMP generation. Gastrin dose dependently stimulated AR4–2J cell mRNA content of both c-fos and c-jun, two genes known to function in regulating cell proliferation, but G-Gly had no effect. Gastrin also induced the expression of luciferase in AR4–2J cells transfected with a construct consisting of a luciferase reporter gene coupled to the serum response element of the c-fos gene promoter. In similar fashion, gastrin stimulated the activity of mitogen-activated protein kinase, an enzyme known to mediate the induction of the c-fos serum response element in response to growth factor stimulation. Although G-Gly had none of these effects of gastrin in AR4–2J cells, it stimulated activity of c-jun amino-terminal kinase, an enzyme known to phospho-rylate and transcriptionally activate c-jun. These data support the notion that gastrin stimulates cell proliferation by inducing c-fos and c-jun gene expression, while G-Gly acts by post-transcriptionally regulating early gene transcriptional activation. Our studies represent a novel model in which both the precursor and the product of a key processing reaction, peptide α-amidation, act cooperatively to stimulate cell proliferation via distinct receptors linked to different signal transduction pathways.

Although characterized as a stimulant of gastric acid secretion (1), the peptide hormone gastrin also exerts growth-promoting effects on normal and malignant gastrointestinal tissues (2–4). The structure of gastrin is similar to numerous other polypeptides of the brain and gut in that its carboxyl-terminal amino acid is amidated, and the amide moiety was thought to be an absolute requirement for biological activity (5–9). Recently, however, we have identified that glycine-extended intermediates of progastrin post-translational processing (G-Gly), the substrate for formation of carboxyl-terminally amidated gastrins, exert growth-promoting effects of their own via interaction with distinct receptors (10), introducing the novel concept that glycine-extended intermediates of prohormone processing reactions have independent and hitherto unrecognized important biological functions. While some of the intracellular events that are responsible for the growth-promoting effects of gastrin (11, 12) have been described, virtually nothing is known about the signal transduction pathways that are activated by G-Gly.

Growth factor stimulation of cell proliferation involves the activation of numerous protein kinases, which, in turn, regulate the expression and the transcriptional activation of the early response genes c-fos and c-jun (13). These genes take part in different programs of cell activation, and they play an important role in the propagation of mitogenic signals from the cell surface to the nucleus (13–16). Accordingly, we examined whether gastrin and G-Gly regulate the expression and the transcriptional activation of c-fos and c-jun. Our studies demonstrate that while gastrin stimulates c-fos and c-jun gene expression, G-Gly post-translationally regulates early gene transcriptional activation. These data support a novel model in which both the precursor and the product of a key processing reaction, peptide α-amidation, act in concert to stimulate cell proliferation via distinct receptors linked to different signal transduction pathways.

MATERIALS AND METHODS

Intracellular Calcium Measurement—[Ca^{2+}]i in AR4–2J cells was measured as described previously (17). The AR4–2J cells were plated at 20,000 cells/cm^{2} into 35-mm culture wells containing a single glass coverslip (7 × 22 mm). The cells were loaded with 4 mM Fura-2/AM for 45 min at 37 °C and transferred into a quartz cuvette into which the peptides under study were added directly. The fluorescence ratio (340/380 nm) was measured with a dual wavelength modular fluorometer (Spex Fluorolog 2, Spex Industries, Edison, NJ). Intracellular CAMP Measurement—AR4–2J cells (300,000 cells/ml) were cultured into 12-well plates as described previously (18). After 18 h in serum-free medium, the cells were incubated for 30 min in Earle's balanced salt solution containing 0.1% bovine serum albumin and 0.1 mM isobutylmethylxanthine with and without the peptides under study. After 30 min the cells were extracted with 1 ml of 100% ethanol. The supernatants were evaporated to dryness at 55 °C under a stream of nitrogen, and cAMP levels were measured using the “cyclic AMP-H- assay system” from Amersham Corp. according to the manufacturer's instructions.

Plasmids—Gal4-Δc un, Gal4-Δc un(AA), and 5×Gal-Luc (19) were gifts from M. Karin (San Diego, CA), SRE-Luc (20) was obtained from J.

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Pessin (Iowa City, IA), and pCMV-pGal was a gift from M. Uhler (Ann Arbor, MI). pGEX-KG-c-j un, a gift from J. Dixon (Ann Arbor, MI), was constructed by cloning amino acids 1–79 of c-j un into pGEX-KG (21) to generate GST-c-j un.

Cell Culture, Transient Transfection, and Luciferase Assays—For our experiments we used the rat exocrine pancreatic cell line AR4–2J, which is known to express receptors for both gastrin and G-CaR (10). The cells (obtained from American Type Culture Collection, Rockville, MD) were grown at 37 °C in 35-mm dishes in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum in 5% CO2, 95% O2. Subconfluent AR4–2J cells were transfected with 5 μg of the luciferase reporter plasmids and, where indicated, with 0.5 μg of the expression vectors. Transfections were carried out using Lipofectamine (Life Technologies, Inc.) as described previously (22). The day after transfection the medium was removed, and the cells were fed with serum-free medium (Dulbecco’s modified Eagle’s medium) for 24 h and then incubated for 6 h with or without various concentrations of human gastrin heptadecapeptide (G17-NH2; Bachem, Torrance, CA) and of human Leu15-glycine-extended gastrin-(2–17) (Leu15-G2–17-Gly, synthesized at the University of Michigan). At the end of the incubation period, the cells were washed and lysed, and luciferase assays were performed as described previously (22). Luciferase activity was expressed as relative light units and then normalized for protein content in the cell lysate to correct for differences in cell numbers among the different treatment groups. Protein concentrations were measured by the Bradford method (23). In some experiments the cells were cotransfected with the pCMV-pGal vector. β-Galactosidase activity was measured by the luminescent light derived from 10 μl of each sample incubated in 100 μl of Lumi-Gal 530 (Luminig, Southfield, MI) and used to normalize the luciferase assay data for transfection efficiency.

Normalizing of the data with either method yielded identical results.

Northern Blot Analysis—After 30 min of incubation with the test substances, the AR4–2J cells were lysed with TRIzol (Life Technologies, Inc.) according to the manufacturer’s instructions. Northern blot hybridization assays were performed as described previously (24). Equal amounts of each RNA sample, with ethidium bromide (10 mg/ml) in a final volume of 20 μl, were electrophoresed on a 1.2% agarose gel containing formaldehyde, and the RNA was transferred from the gel to nitrocellulose filters. The ethidium-stained ribosomal RNA bands in the gel were photographed before and after transfer to ensure that equivalent amounts of RNA were loaded onto each lane and that no residual RNA was left on the gel. The probes used for hybridization analysis were c-fos and glyceraldehyde-3-phosphate dehydrogenase cDNAs, obtained from American Type Culture Collection (Rockville, MD), and c-j un cDNA, kindly provided by D. Brenner (Chapel Hill, NC). The cDNAs were labeled with [32P]dCTP by the random priming procedure (25), and the nitrocellulose filters were hybridized to the [32P]-labeled cDNA probes as described previously (24).

Immunoprecipitations and In-gel MAPK Assay—Immunoprecipitations and in-gel mitogen-activated protein kinase (MAPK) assays were performed according to previously described techniques (26). Briefly, after 5 min of incubation with gastrin and G2–17-Gly the AR4–2J cells were lysed in 500 μl of lysis buffer (10 mM KPO4 (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM MgCl2, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate (Na3VO4), 2 mM dithiothreitol, 40 μg/ml phenylmethylsulfonyl fluoride, 10 mM edetic acid, 0.8 μg/ml leupeptin, 10 mg/ml p-nitrophenyl phosphate, and 10 μg/ml apronitin), transferred into microcentrifuge tubes, and spun at 16,000 × g for 20 min at 4 °C. Equal amounts of proteins from each treatment group (1000 μg) were incubated with a MAPK-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and mixed on a rotating platform for 3 h at 4 °C. Control experiments were conducted by mixing aliquots of the samples with identical volumes of preimmune serum. Aliquots of proteins were then resuspended in 30 μl of kinase buffer (20 mM HEPES at pH 7.6, 1 mM MgCl2, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 20 μg/ml p-nitrophenyl phosphate, 2 mM dithiothreitol, 20 μg/ml ATCase, and 5 μCi of [32P]ATP) and, after 20 min at 30 °C the reaction was terminated by washing with HEPES binding buffer. Phosphorylated proteins were eluted by boiling in 60 μl of electrophoresis buffer and applied to a 10% SDS-polyacrylamide gel followed by autoradiography.

Data Analysis—Data are presented as means ± S.E., where n is equal to the number of separate transfections performed with the AR4–2J cells. Statistical analysis was performed using Student’s t test. p values < 0.05 were considered to be significant.

RESULTS

Since mobilization of intracellular Ca2+ is an essential signal transduction mechanism that mediates the action of gastrin in AR4–2J cells (27), we investigated the effects of gastrin and G2–17-Gly on [Ca2+], mobilization. As depicted in Fig. 1A, in a fashion identical to our observations in canine gastric parietal cells (17), gastrin (10−8 M) increased [Ca2+], while G2–17-Gly had no effect at any of the doses tested (10−11–10−8 M). Similarly G2–17-Gly (10−10–10−7 M) had no effect on either basal or
10^-7 M vasoactive intestinal polypeptide-stimulated CAMP generation, while, in contrast, gastrin has been shown previously to inhibit CAMP generation in the AR4-2J cells (18). We then examined whether the growth stimulatory effects of gastrin and G2-17-Gly on AR4-2J cells are associated with induction of the early response genes c-fos and c-jun. As depicted in the Northern blot in Fig. 2, gastrin (10^-10^-8 M) induced c-fos-specific mRNA in a dose-dependent fashion, with a maximal effect detected at a dose of 10^-8 M. In contrast, no induction was noted when the cells were incubated with identical doses of G2-17-Gly. Similar results were obtained when the RNA was hybridized with a c-jun cDNA probe (not shown). Since growth factor-induced protein kinases are known to target a specific DNA regulatory element present in the promoter of the c-fos gene, known as the serum response element (SRE) (13), we investigated the effects of gastrin and G2-17-Gly on c-fos gene transcription regulated by the SRE. For these experiments we transfected the AR4-2J cells with luciferase reporter plasmids containing the c-fos SRE upstream of the thymidine kinase (TK) gene minimal promoter and the luciferase reporter gene. As depicted in Fig. 3, 10^-8 M gastrin induced SRE transcriptional activity 5-fold (5.0 ± 1.8-fold induction over control, mean ± S.E., n = 4), but G2-17-Gly did not, suggesting that only gastrin is able to regulate early gene expression. Cells transfected with a plasmid containing the TK gene minimal promoter but devoid of the SRE exhibited a 1.4-fold induction in response to treatment with gastrin (1.4 ± 0.1-fold induction over control, mean ± S.E., n = 11).

Since MAPK activation is required for induction of the SRE in response to growth factor stimulation (28), we tested the effect of G2-17-Gly on MAPK activity using in-gel kinase assay. As shown in Fig. 4, while gastrin induced MAPK activity, G2-17-Gly had no effect. JNK is known to phosphorylate the amino terminus of c-jun and to induce c-jun transcriptional activity in response to growth factor stimulation (29). Accordingly, we examined the effect of gastrin and G2-17-Gly on AR4-2J cell JNK activity. For these experiments we co-transfected the AR4-2J cells with the Gal4-c-jun expression vector and the 5xGal luciferase reporter plasmid. In this system, Gal4-c-jun can transactivate and stimulate luciferase activity only if the c-jun amino terminus is phosphorylated by JNK. G2-17-Gly dose dependently induced c-jun transcriptional activity with a maximal stimulatory effect achieved at a dose of 10^-8 M (4.4 ± 1.1-fold induction over control, mean ± S.E., n = 4). When we compared the effects of gastrin (10^-8 M) and G2-17-Gly (10^-8 M) using a larger number of observations (n = 20), we noted that JNK activity was also weakly stimulated by gastrin, although this effect was much less potent than the response observed with G2-17-Gly (1.8 ± 0.2 versus 3.2 ± 0.4-fold induction over control, in the presence of gastrin and G2-17-Gly, respectively) (Fig. 5B). The specificity of our luciferase assay data was confirmed by solid state JNK assays, in which we used AR4-2J cell extracts and GSH-agarose beads to which GST-c-jun was bound. In this system, 10^-8 M G2-17-Gly was able to stimulate JNK activity (Fig. 5C), confirming that G2-17-Gly is responsible for c-jun post-translational modification by phosphorylation and activation.

**DISCUSSION**

Although characterized primarily as a stimulant of gastric acid secretion (1), gastrin is a potent growth factor for both normal and malignant gastrointestinal tissues (2-4). Gastrin is initially synthesized as a large precursor that is post-translationally processed to form mature carboxyl-terminally amidated gastrin (5-9). Despite the observation that G-Gly achieves plasma levels roughly equivalent to those of gastrin (30), its physiological relevance, other than to serve as the immediate precursor for gastrin synthesis, has remained obscure. Recently, however, we reported that both G-Gly and gastrin acting on distinct receptors are equally potent in stimulating gastrin- and G2-17-Gly-induced MAPK activity in AR4-2J cells. Gastrin is a potent inducer of the H+K+-ATPase a-subunit gene expression, indicating that it could function to potentiate gastric acid secretagogue action by enhancing expression of the gene responsible for H+ generation (17). Thus, both the substrate and the product of the terminal progastrin processing reaction appear to have important biological functions through the activation of separate receptors. The elucidation of the signal transduction pathways that are responsible for the numerous physiological actions of gastrin has been the focus of intense...
investigation. In the AR4-2J cells, in particular, signaling through the gastrin/CCK receptor is linked to the simultaneous inhibition of cAMP generation and to the activation of membrane phospholipid turnover (27). In contrast, all that is known about G-Gly signaling is that, in the gastric parietal cell, it appears to activate cellular protein tyrosine kinases through a pathway that is independent of [Ca$^{2+}$]i mobilization (17). In this study, we have attempted to define further the signaling pathways that are responsible for the growth-promoting effect of G-Gly using AR4-2J cells as our model.

The process of cellular proliferation is under the control of a complex cascade of phosphorylation reactions that is triggered by the interaction of growth factors with their specific cellular receptors (13). One of the best characterized pathways linked to the control of cellular growth is known to involve the activation of the serine-threonine protein kinase Raf through its interaction with the small GTP-binding protein Ras (13). Raf is responsible for the phosphorylation and activation of a dual specificity protein kinase (mitogen-activated protein/extracellular signal-related kinase kinase (MEK)), which in turn phosphorylates both serine and tyrosine residues in a family of serine-threonine protein kinases known as MAPKs (13). The MAPKs phosphorylate numerous cellular proteins, including transcription factors such as p62-TCF or Elk-1 that play an important role in the transcriptional regulation of the promoter of the early response gene c-fos through the SRE (13, 28). These early response genes propagate mitogenic signals from second messengers to the nucleus and activate or repress the next set of genes in the biological programs initiated by the extracellular signals (13, 16). The activity of the best characterized members of this family of genes, c-fos and c-jun, is extensively regulated by phosphorylation catalyzed by specific kinases such as JNK, which appears to act in concert with the MAPK pathway in the complex regulation of early gene function (29, 31–33).

In our report we have demonstrated that gastrin can induce [Ca$^{2+}$] mobilization, early gene expression, and MAPK activation, confirming previous reports suggesting that its growth-promoting effects could be mediated at least in part by the activation of these signaling pathways (11, 12). In contrast, G-Gly had no effect on cAMP generation.
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