Glycine-extended gastrin regulates HEK cell growth

VINZENZ M. STEPAN,1,2 DIETER F. KRAMETTER,1 MASASHI MATSUSHIMA,2 ANDREA TODISCO,2 JOHN DELVALLE,2 AND CHRIS J. DICKINSON1

Departments of 1Pediatrics and 2Internal Medicine, University of Michigan, Medical Center, Ann Arbor, Michigan 48109

Glycine-extended gastrin regulates HEK cell growth. Am. J. Physiol. 277 (Regulatory Integrative Comp. Physiol. 46): R572–R581, 1999.—Posttranslational processing of progastrin to a carboxy terminally amidated form (G-NH2) is essential for its effect on gastric acid secretion and other biological effects mediated by gastrin/CCK-B receptors. The immediate biosynthetic precursor of G-NH2, glycine-extended gastrin (G-Gly), does not stimulate gastric acid secretion at physiological concentrations but is found in high concentrations during development. G-NH2 and G-Gly have potent growth stimulatory effects on gastrointestinal tissues, and G-NH2 can stimulate proliferation of human kidney cells. Thus we sought to explore the actions of G-NH2 and G-Gly on the human embryonic kidney cell line HEK 293. HEK 293 cells showed specific binding sites for 125I-labeled Leu15-G17-NH2 and 125I-Leu15-G17-Gly. Both G-NH2 and G-Gly induced a dose-dependent increase in [3H]thymidine incorporation, and both peptides together significantly increased [3H]thymidine incorporation above the level of either peptide alone. G-NH2 and G-Gly were detected by radioimmunounassay in serum-free conditioned media. Antibodies directed against G-NH2 and G-Gly lead to a significant reduction in [3H]thymidine incorporation. G-NH2 but not G-Gly increased intracellular Ca2+ concentration. We conclude that G-NH2 and G-Gly act cooperatively via distinct receptors to stimulate the growth of a nongastrointestinal cell line (HEK 293) in an autocrine fashion.

cholecystokinin-B receptor; human embryonic kidney cell; cellular proliferation

THE CARBOXY TERMINALLY amidated gastrointestinal peptide hormone gastrin (G-NH2) is a well-known stimulant of gastric acid secretion that also enhances the growth of gastrointestinal tissues (33). G-NH2 stimulates growth of parietal (24) and enterochromaffin-like cells of the stomach (13), colonic epithelial cells (38, 40, 46), and the pancreas (8). Recently, G-NH2 has been implicated in the growth of digestive tract tumors from the stomach, colon, and pancreas (10, 17, 30, 37). There are few reports regarding a possible functional role of G-NH2 on cells of nongastrointestinal origin. Expression of gastrin is seen in sperm (35) and some bronchogenic (31) and ovarian (45) carcinomas. G-NH2 also stimulates growth of a pituitary cell line in a paracrine fashion via gastrin/CCK-B receptors (51). Blackmore et al. (1) have shown that the growth of Wilms tumors derived from renal tissue is stimulated by G-NH2 in an autocrine fashion, and de Weerth et al. (9) demonstrated the growth stimulatory effects of G-NH2 on the kidney mediated by gastrin/CCK-B receptors. Although Reubi and coworkers (34) failed to detect gastrin/CCK-B receptors on renal cell tumors, a recent report indicates that gastrin/CCK-B receptors are present in the kidney and mediate the effects of G-NH2 on urine sodium excretion (29).

The primary gastrin mRNA translation product, preprogastrin, requires extensive posttranslational processing before it assumes its biologically relevant structure. Previously, it was felt that processing intermediates were not physiologically relevant. Indeed, removal of the carboxy-terminal amide of G-NH2 completely abolishes its acid stimulatory effects mediated by standard gastrin/CCK-B receptors and the immediate precursor of G-NH2, glycine-extended gastrin (G-Gly), is at least four orders of magnitude less potent than G-NH2 in the acute stimulation of acid secretion from gastric parietal cells (16, 26). However, other evidence suggests a physiological role for G-Gly. G-Gly is stored in brain and gut tissues (6, 32), secreted with G-NH2 from antral G cells into the circulation (42), and achieves concentrations in plasma roughly equivalent to those of G-NH2 (7, 14, 18). Moreover, G-Gly is seen in greater concentrations than G-NH2 during development and in some malignant tissues that express gastrin (15, 20, 28). G-Gly also stimulates the growth of an exocrine pancreatic cell line (AR4–2J; 37) and different colon cancer cell lines (41) via receptors distinct from gastrin/CCK-B receptors that mediate the effects of G-NH2. Finally, transgenic mice overexpressing G-Gly have an increased colonic proliferative index (21).

Thus the purpose of the present study was to determine the effect of amidated and glycine-extended gastrins on the growth of a nongastrointestinal human embryonic kidney cell line, HEK 293. Additionally, we wished to determine if these cells were capable of synthesizing and secreting these peptides, thus providing a mechanism for autocrine growth regulation.

METHODS

Cell Culture

HEK 293 and Cos 7 cells were purchased from the American Type Culture Collection. Cells were grown in monolayer cultures in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum and 1% sodium pyruvate in an atmosphere of 95% air and 5% CO2 at 37°C. Cultures were passaged at 4- to 6-day intervals to maintain the cells at subconfluent densities.

Peptides

Human G17-NH2 and Leu15-G17-NH2 were purchased from Research Plus (Bayonne, NJ). Human Leu15-G2–17-Gly...
was synthesized in the protein core facility of the University of Michigan (Ann Arbor, MI) and purified by HPLC (purity >95%). Sequence of the Leu$^{15}$G$^{-2}_{17}$Gly was confirmed by amino acid analysis and mass spectroscopy.

**Binding Studies**

Human Leu$^{15}$G$^{-2}_{17}$NH$_2$ and Leu$^{15}$G$^{-2}_{17}$Gly were 125I-labeled on Tyr$^{12}$ with an adaptation of the chloramine-T method and purified by HPLC as described previously (37). The specific activities of the labels were ~1.5 µCi/pmol. Binding assays were performed on isolated cells that were detached in PBS containing 0.02% EDTA. Cells (2 x 10$^6$) were incubated with 125I-labeled Leu$^{15}$G$^{-2}_{17}$NH$_2$ or Leu$^{15}$G$^{-2}_{17}$Gly with or without 1 µM of unlabeled G$^{-2}_{17}$NH$_2$ or Leu$^{15}$G$^{-2}_{17}$Gly in a Krebs-HEPES buffer supplemented with 0.5% bovine serum albumin, 0.03% soybean trypsin inhibitor, and 0.05% bacitracin in a total volume of 1 ml at 37°C until equilibrium. Specific binding was calculated as the difference between the total amount of label bound and the amount of label remaining bound in the presence of 1 µM cold peptide. For competitive binding studies, cells were incubated with 120 pM 125I-Leu$^{15}$G$^{-2}_{17}$Gly or 120 pM 125I-Leu$^{15}$G$^{-2}_{17}$NH$_2$ without (control) or with 1 µM of Leu$^{15}$G$^{-2}_{17}$Gly, G$^{-2}_{17}$NH$_2$, or one of the gastrin/ CCK-B receptor antagonists, PD-134308 (Parke Davis Pharmaceutical Research, Ann Arbor, MI) (37), JB-93182 (J ans Black Foundation, London, UK) (19), or D2 (a gift from Dr. Yoshi Goto, Daiichi Pharmaceuticals, Tokyo, Japan) (37), or the CCK-A receptor antagonist L-364718 (Merck) (43) in a total volume of 1 ml until equilibrium.

**Proliferation Studies**

[3H]thymidine incorporation. Cells were grown in media supplemented with 10% fetal bovine serum, plated, allowed to attach overnight, and then cultured in serum-free media containing 0.2 mM unlabeled thymidine. After being washed with serum-free media, cells were treated with increasing concentrations of G$^{-2}_{17}$NH$_2$ or Leu$^{15}$G$^{-2}_{17}$Gly. DNA synthesis was estimated by measurement of [3H]thymidine incorporation into the TCA precipitable material. [3H]thymidine (0.1 µCi/ml; 18 Ci/pmol) was added 2 h before the end of a 24-h treatment period. Cells were then washed twice with serum-free medium to remove unincorporated [3H]thymidine, and DNA was precipitated with 5% TCA 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. Results are expressed as percent control unstimulated [3H]thymidine incorporation (mean ± SD of 5 different experiments, each performed 6 times). For the detection of growth inhibition, cells were incubated with the specific antibodies [AB 5135 (25) for G$^{-2}_{17}$NH$_2$ and AB R5B5 (12) for glycine-extended gastrin] for 48 h. Antibody specificities were determined in radioimmunoassay cross-reactivity studies using radiolabeled Leu$^{15}$G$^{-2}_{17}$NH$_2$ and G$^{-2}_{17}$Gly for ABS 5135 and R5B5, respectively. Antibodies were used in a 1:1,000 and a 1:2,000 dilution of AB 5135 1:1,000 and a 1:500 dilution of AB R5B5 was used as control at 1:500 dilution.

**Gastrin Gene Expression**

Total cellular RNA was isolated from HEK 293 and gastrin-producing G cells from the canine antrum with TRIzol Reagent (Life Technologies). A reverse transcription reaction was performed using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim). One microgram of total RNA was reverse transcribed using a random primer (dN)6. For the PCR, reaction primers were synthesized on a DNA synthesizer (Applied Biosystems) according to published sequences (2). Primer 1 was complementary to nucleotides 9–30 of the human gastrin cDNA (2), and primer 2 was complementary to nucleotides 314–294, covering 313 bp of the human gastrin cDNA that corresponds to portions of exons 2 and 3 of the human gastrin gene (48). PCR reactions with the appropriate primers were performed with a Perkin Elmer Cetus thermocycler under the following conditions: 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C for 40 cycles. To control for contamination, the procedure was also performed in one tube without any input RNA. The amplified products were separated by gel electrophoresis on a 1.2% agarose gel in 1X TBE buffer and then, after denaturing (0.25 N HCl for 20 min, 0.5 M NaOH with 1.5 M NaCl for 30 min and 1 M ammonium acetate with 0.1 M NaOH for 30 min), blotted to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and ultraviolet crosslinked. After a 2-h prehybridization, the filter was hybridized with a 32P-labeled (Rediprime, Amershams, Arlington Heights, IL) human gastrin cDNA probe in a 0.1 M HEPES (pH 7.5), 5 X SSC (1 X SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5 X Denhardt’s solution with 100 µg/ml salmon sperm DNA for 3 h at 60°C. The membrane was washed two times in 2 X SSC, 20 min each, at 62°C, another 20 min in 1 X SSC at 62°C, 10 min in 1 X SSC at 75°C, and exposed at –80°C for 2 h.

For determination of gastrin peptide production, 2 x 10$^6$ cells were plated in 6-cm dishes and grown for 24 h, media was then changed to serum free for another 24 h, cells were collected, centrifuged at 12,000 rpm for 5 min, and the supernatant stored at –20°C until assay. Media were analyzed by radioimmunoassay using AB 5135 that is specific for amidated forms of gastrin and cross-reacts ~1% with glycine-extended gastrins or progastrins extended beyond the carboxy-terminal glycine residue (25). G-Gly was quantified with AB 8237 that cross-reacts ~5% with amidated gastrins or progastrins extended beyond the carboxy-terminal glycine residue (25).

**Gastrin/ CCK-B Receptor Identification**

For immunoprecipitation cells were lysed in 500 µl of lysis buffer [0.05 M HEPES (pH 7.4), 0.15 M NaCl, 1% Triton X, 0.0015 M MgCl$_2$, 0.001 M EDTA, 0.001 M NaVO$_4$, 0.01 M Na-pyrophosphate, 0.01 M NaF, 10 µg/ml apronin, 10 µg/ml leupeptin, 10 µg/ml 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride], transferred into microfuge tubes, and spun at 12,000 g for 10 min. Equal amounts of protein were incubated with the human gastrin/ CCK-B receptor AB 95163 (kindly provided by Helen Wong and John Walsh, Los Angeles, CA) and mixed on a rotary shaker overnight at 4°C. Control experiments were conducted by incubating aliquots of the cell lysate with rabbit serum. Aliquots of protein A sepharose (Pharmacia Biotech) were then added, and the solutions were mixed for one additional hour. After centrifugation, pellets were washed two times with lysis buffer, resuspended in 20 µl of electrophoresis buffer (for 0.1 µl 1 M glycerol, 0.5 µl 2-mercaptoethanol, 3 ml 10% SDS, 1.25 ml 1 M Tris buffer, 2 ml 0.1% bromphenol blue, 0.6 g urea), boiled for 5 min, applied to an 8% SDS-
polyacrylamide gel, and transferred to a nitrocellulose membrane. Membranes were incubated in Tris-buffered saline (0.15 M NaCl) containing 0.3% tween and 5% nonfat dry milk for 1 h at room temperature to block nonspecific binding sites. Blots were then incubated with the primary antibody (gastrin/CCK-B) at a final dilution of 1:1,000 in Tris-buffered saline for 2 h, washed twice, and incubated with a peroxidase linked secondary antibody (Zymed rabbit anti-mouse horseradish peroxidase, 1:250) for 60 min. Immunoreactive bands were visualized with the use of a standardized enhanced chemoluminescence immunoblotting detection system (Amersham).

Measurement of Ca²⁺ Concentration

HEK 293 cells were cultured for 24 h on 22-mm round microscope coverslips and subsequently loaded with fura 2-acetoxymethyl ester (1 µM) for 20 min. Cells were washed twice with washing buffer, consisting of 1× Earle's balanced salt solution, 10 mM HEPES, 25 mM NaHCO₃, and 0.1% FBS, and coverslips were placed in an aluminum superfusion chamber maintained at 37°C. For measurement of fura 2 fluorescence, a Zeiss Axiosvert inverted microscope and Attofluor digital imaging system were used. Intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) was calculated from the ratios of the fluorescence intensities of fura 2 at 334 and 380 nm with an emission wavelength of 500 nm. One microscopic field was examined per coverslip. Cells were judged to have responded to a ligand if [Ca²⁺]ᵢ increased by at least 100% over baseline.

Detection of G-Gly Receptors on HEK 293 Cells by Photomulsion

HEK 293 (10⁵) or Cos 7 (as a negative control) were plated onto slide flasks (18 × 50 mm, Nunc International) for 24 h and then incubated with [¹²⁵I]-Leu¹⁵-G₁₇-Gly (400,000 cpm) in 2 ml binding buffer at 37°C for 2 h. Cells were washed twice and then fixed with 2.5% glutaraldehyde in PBS at room temperature for 15 min. After washing with PBS, the upper part of the slide flask was detached and the slide was dipped in 0.5% gelatin and dried. Slides were then coated with Kodak NTB-2 photoemulsion (Kodak, Rochester, NY) and exposed at a temperature for 15 min. After washing with PBS, the upper part of the slide flask was detached and the slide was dipped in 0.5% gelatin and dried. Slides were then coated with Kodak NTB-2 photoemulsion (Kodak, Rochester, NY) and exposed for 4 days at 4°C. After being developed and fixed the slides were examined by dark-field microscopy.

Statistics

Data were compared using the Student’s t-test to determine statistical significance. Differences with P values of <0.05 were considered significant.

RESULTS

G-NH₂ and G-Gly Receptor Binding

The presence of G-NH₂ and G-Gly specific binding sites on HEK 293 cells was investigated using [¹²⁵I]-labeled human Leu¹⁵-G₁₇-NH₂ and human Leu¹⁵-G₂₋₁₇-Gly as described in Methods. Specific binding sites for both ligands were detected on HEK cells with a percent specific binding of 50 ± 6% (n = 6) for [¹²⁵I]-Leu¹⁵-G₁₇-NH₂ and 51 ± 9% (n = 6) for [¹²⁵I]-Leu¹⁵-G₂₋₁₇-Gly. Competitive binding studies showed that unlabeled G₁₇-NH₂, as well as three different gastrin/CCK-B receptor antagonists (PD-134308, JB-93182, and D2), completely displaced [¹²⁵I]-labeled Leu¹⁵-G₁₇-NH₂ but not Leu¹⁵-G₂₋₁₇-Gly (Fig. 1A). [¹²⁵I]-Leu¹⁵-G₂₋₁₇-Gly binding was completely displaced by Leu¹⁵-G₂₋₁₇-Gly, but not by G₁₇-NH₂ or the gastrin/CCK-B receptor antago-

nists in concentrations as high as 1 µM (Fig. 1B). The CCK-A receptor antagonist L-364718 had no effect on binding of either ligand (Fig. 1). G₁₇-NH₂ and Leu¹⁵-G₂₋₁₇-Gly dose dependently inhibited [¹²⁵I]-Leu¹⁵-G₁₇-NH₂ and [¹²⁵I]-Leu¹⁵-G₂₋₁₇-Gly specific binding, respectively, with an IC₅₀ of ~10⁻⁹ M for G₁₇-NH₂ and ~5 × 10⁻⁷ M for Leu¹⁵-G₂₋₁₇-Gly (Fig. 2).

Proliferation Studies

The growth stimulatory abilities of G₁₇-NH₂ and Leu¹⁵-G₂₋₁₇-Gly were investigated by measuring the peptide-induced stimulation of [³H]thymidine incorporation. G₁₇-NH₂ and Leu¹⁵-G₂₋₁₇-Gly stimulated [³H]thymidine incorporation in a dose-dependent manner, with maximal stimulatory effects seen at 10⁻⁹ M (144 ± 10% for G₁₇-NH₂ and 145 ± 10% for Leu¹⁵-G₂₋₁₇-Gly, n = 6; Fig. 3, A and B). The gastrin/CCK-B receptor antagonist PD-134308 (10⁻⁸ M) completely inhibited the growth stimulatory effects of G₁₇-NH₂ but had no influence on Leu¹⁵-G₂₋₁₇-Gly-stimulated [³H]thymidine incorporation (Fig. 4). When HEK 293 cells were simultaneously stimulated with G₁₇-NH₂ and Leu¹⁵-G₂₋₁₇-Gly at maximal doses (10⁻⁹ M), [³H]thymidine incorporation increased above the maximal level of either peptide alone (166 ± 4%, n = 6; Fig. 5). To confirm that the increases in [³H]thymidine incorporation reflected cellular growth, we noted that a 4-day stimulation with G₁₇-NH₂ (10⁻⁹ M) and Leu¹⁵-G₂₋₁₇-Gly (10⁻⁹ M) increased total cell number by 143 ± 11 and 146 ± 8%, respectively (mean ± SD, n = 4, P < 0.01 vs. unstimulated control).

Incubation of HEK 293 cells with specific antisera against amidated (AB 5135) and glycine-extended (AB 5825) gastrin revealed a dramatic reduction in [³H]thymidine incorporation, with a maximal inhibitory effect at a dilution of 1:1,000 for AB 5135 and 1:500 for AB 5825 (65 ± 6 and 62 ± 5%, respectively; mean ± SD, n = 4). When cells were incubated with both antibodies at the same time the inhibitory level exceeded those of either antibody alone (Fig. 6).

Gastrin Gene Expression

The expression of gastrin mRNA in HEK 293 cells was determined by RT-PCR and Southern analysis. Amplification with primers 1 and 2 revealed a specific PCR product of 313 bp in HEK 293 cells and canine antral G cells (Fig. 7). No PCR product was detected in the sample without any input RNA. Amidated and glycine-extended gastrin in the cell media was quantified by radioimmunoassay with the use of ABs 5135 and 8237. HEK cells released both G₁₇-NH₂ (2.2 ± 0.3 pmol/l) and G-Gly (188 ± 48 pmol/l) into serum-free cell medium (mean ± SE, n = 4).

Detection of the Gastrin/CCK-B Receptor by Immunoblotting

Immunoprecipitation and Western blotting of cell lysates with AB 95163 directed against the amino-terminal end of the gastrin/CCK-B receptor showed one specific band corresponding to an apparent molecular mass of 80 kDa (Fig. 8). Cos 7 cells, transiently
transfected with the human gastrin/CCK-B receptor were used as a positive control. Immunoprecipitation was done with the same samples in the presence of rabbit serum to identify possible nonspecific bands.

Detection of G-Gly Specific Binding Sites by Photoemulsion

The presence of G-Gly receptors on HEK 293 cells was confirmed by photoemulsion. Cells were incubated with $^{125}$I-labeled Leu$^{15}$-G$\text{2}_{-17}$-Gly and then exposed and developed as described in METHODS. Figure 9 shows a representative dark-field photomicrograph with three positive cells in a $\times$200 magnification. Similar results were obtained in at least four different experiments. To rule out nonspecific binding effects, Cos 7 cells were used as a negative control and showed no binding (data not shown).

[Ca$^{2+}$] Measurements

G17-NH$_2$ (10$^{-7}$ M) evoked a significant increase of [Ca$^{2+}$]$_i$ in 46 $\pm$ 7% of HEK 293 cells ($n = 6$, mean $\pm$ SD, 142 single cell tracings), whereas G-Gly in concentrations up to 10$^{-7}$ M had no effect (Fig. 10A). The gastrin/CCK-B receptor antagonist PD-134308 (10$^{-7}$ M) completely inhibited the G17-NH$_2$ (10$^{-7}$ M)-induced increases in [Ca$^{2+}$]. This inhibition could be reversed.
after subsequent washing and stimulation of cells with $10^{-7}$ M of G17-NH$_2$ in 45 ± 13% of the cells (n = 3, mean ± SD, 55 single cell tracings, Fig. 10B).

**DISCUSSION**

In addition to its role in the regulation of gastric acid secretion, gastrin is also a potent stimulant of gastrointestinal cell proliferation and differentiation (33). Gastrin is expressed in tissues outside the gut but its physiological effects in these tissues remain largely unknown. Recently, investigators have demonstrated the growth stimulatory effects of G-NH$_2$ on tissues derived from the kidney mediated by gastrin/CCK-B receptors (9) and effects on urine sodium excretion mediated by gastrin/CCK-B receptors (29). Thus we sought to determine if G-NH$_2$ had growth-promoting effects on a human embryonic kidney cell line (HEK 293). Our data (Figs. 1–3) demonstrate that G-NH$_2$ is capable of binding to specific cell surface receptors and enhancing growth of HEK 293 cells as measured by [H$^3$]thymidine incorporation and cell number. Because these effects were reversed by several specific gastrin/CCK-B receptor antagonists, it suggests that the proliferative actions of G-NH$_2$ are mediated via these receptors. The presence of gastrin/CCK-B receptors on HEK 293 cells was also confirmed by the Western blot (Fig. 4).
8). Other investigators have noted gastrin receptors capable of mediating the trophic actions of G-NH₂ that are not inhibited by either gastrin/CCK-B or CCK-A receptor antagonists (3, 39). These non-A/non-B gastrin/CCK receptors do not appear to be present on HEK 293 cells, because the trophic actions of G-NH₂ and its binding were completely reversed with gastrin/CCK-B receptor antagonists.

As is the case with other peptide hormones, progastrin must undergo several important processing steps to yield the mature carboxy terminally amidated peptide gastrin (G-NH₂). The immediate biosynthetic precursor of G-NH₂, G-Gly, was previously thought to be devoid of biological activity. In fact, G-Gly is at least four orders of magnitude less potent than G-NH₂ in the acute stimulation of acid secretion from gastric parietal cells (16, 26) and in displacing G-NH₂ from gastrin/CCK-B receptors. However, recent evidence suggests that G-Gly and perhaps progastrin have proliferative actions in the pancreas and colon mediated via receptors distinct from gastrin/CCK-B receptors (17, 21, 37, 41, 46, 50). As was the case with G-NH₂, G-Gly also dose dependently stimulates growth of HEK 293 cells as measured by increases in [³H]thymidine incorporation and cell number (Fig. 3). Moreover, the potency and efficacy of the effects of G-Gly were similar to those of G-NH₂ and occurred at physiological concentrations of plasma G-Gly (7, 14, 18). In contrast to G-NH₂ binding, binding of G-Gly to HEK 293 cells was not inhibited by either gastrin/CCK-B or CCK-A receptor antagonists (Fig. 2). This suggests that the trophic actions of G-Gly on HEK 293 cells are mediated by receptors distinct from those for G-NH₂.

If the proliferative actions of G-NH₂ and G-Gly are mediated by distinct receptors, then we surmised that they would use distinct signal transduction mechanisms. In other systems, G-NH₂ binding to the gastrin/CCK-B receptor results in phospholipase C activation, formation of inositol triphosphate, and release of calcium from intracellular stores (23). As shown in Fig. 10,
G-NH₂ stimulated an increase in \([\text{Ca}^{2+}]_i\) that was inhibited by a gastrin/CCK-B receptor antagonist. In contrast, G-Gly had no effect on intracellular calcium concentrations. In previous studies using the rat exocrine pancreatic cell line AR4–2J we noted similar results (44). In AR4–2J cells G-NH₂, but not G-Gly, induced mitogen-activated protein kinase activation and expression of the early response genes c-fos and c-jun (44). Conversely, G-Gly, but not G-NH₂, induced activation of c-jun amino-terminal kinase (jun kinase) leading to bioactivation of c-jun. Although G-Gly may activate jun kinase in HEK 293 cells, basal jun kinase activity was higher than the maximally stimulated activity seen in other cell lines and we were unable to observe a consistent significant increase in jun kinase activity with G-Gly (data not shown). Thus it is possible that the trophic actions of G-Gly on HEK 293 cells were mediated by jun kinase or perhaps another signal transduction mechanism that does not alter intracellular calcium concentrations such as PI-3-kinase (22). Nevertheless, it does appear that proliferative effects of G-NH₂ and G-Gly are complementary, because effects of both peptides together exceeded the maximal effects of either peptide alone (Fig. 5).

Finally, we wished to determine if the trophic actions of gastrin were of an autocrine nature. As shown in Fig. 7, we were able to detect gastrin gene expression in HEK 293 cells. We also noted significant quantities of G-NH₂ and G-Gly in conditioned media of these cells, suggesting the gastrin gene product was translated and processed into bioactive peptides. Finally, incubation of HEK cells in media containing antibodies directed against G-NH₂ and G-Gly led to a marked decrease in \([\text{H}]\text{thymidine incorporation (Fig. 6), suggesting that both gastrin gene products enhance cell proliferation via an autocrine mechanism. Furthermore, this autocrine stimulation of HEK 293 cell growth by G-Gly may partially explain the high levels of basal jun kinase activity found in these cells. The autocrine stimulation of tumor cell growth via gastrin also occurs in colon cancer cell lines (27, 40).}

Although amidated and glycine-extended gastrins were detected in conditioned media from HEK 293 cells, we were unable to detect significant amounts of gastrin peptide in cell extracts. These findings are consistent with earlier reports examining the storage and processing of gastrin or other peptide hormone precursors in nonendocrine cells (5, 11). Nonendocrine cells such as HEK 293 and most native cells of renal origin do not possess a well-defined regulated pathway of secretion that allows for efficient processing and
storage of peptide precursors within secretory granules. The enzymes within neuroendocrine cells contain secretory granules wherein prohormones are processed and mature peptides are stored until the cell is stimulated to release granular contents into plasma (4). Thus, when progastrin is expressed in nonendocrine cells, such as HEK 293, it is not efficiently processed and large amounts of progastrin are quickly released into the media with smaller amounts of mature peptide (11). It is important to note that although the concentrations of G-NH₂ and G-Gly found in media are small, they are within the range necessary to stimulate cell growth. Because progastrin processing is inefficient in these cells, it is possible that other progastrin processing intermediates extended beyond the carboxy-terminal glycine (including progastrin itself) are probably also secreted by these cells. A recent report suggests that there may be receptors for progastrin distinct from those for G-Gly or G-NH₂ (50). Although not specifically addressed in this report it is possible that HEK 293 cells express progastrin receptors that mediate its potential trophic effects.

It should be noted that the EC₅₀ (10⁻¹¹ M) for G-Gly stimulation of proliferation occurs at much lower doses than the IC₅₀ (5 × 10⁻⁷ M) for the displacement of ¹²⁵I-Leu¹⁵-G₂—¹⁷-Gly binding. There are several potential explanations for these results. We feel that the most likely explanation is that occupancy of relatively few cell surface G-Gly receptors is necessary for growth stimulation. Thus low doses of G-Gly provide sufficient receptor occupancy to stimulate growth but there are a greater number of “spare” receptors present on the cell surface that continue to bind G-Gly (52). Thus higher concentrations of G-Gly are necessary to displace ¹²⁵I-Leu¹⁵-G₂—¹⁷-Gly from these “spare” receptors. A second possibility is that there is more than one G-Gly receptor or multiple affinity states of a single receptor. Indeed, in cross-linking studies with the rat exocrine pancreatic cell line AR4-2J we noted two distinct bands that specifically bind ¹²⁵I-Leu¹⁵-G₂—¹⁷-Gly (36). Another possibility is that ¹²⁵I-Leu¹⁵-G₂—¹⁷-Gly binds to a high-affinity G-Gly receptors that are displaced to other receptors (perhaps gastrin/CCK-B receptors) with low doses of cold G-Gly. In this case, ¹²⁵I-Leu¹⁵-G₂—¹⁷-Gly bound to other receptors would then be displaced by higher concentrations of G-Gly. To rule out this possibility, we performed G-Gly displacement binding studies in the presence of a gastrin/CCK-B receptor antagonist (10⁻⁸ M PD-134308), which did not alter the G-Gly displacement binding curve (data not shown).

To place the present study into perspective it is useful to understand the nature of HEK 293 cells. HEK 293 cells are an H5 transformed cell line derived from human renal cortical cells (38) that demonstrate the formation of apical zonae occludentes. HEK 293 cells do not have a prominent brush border (38). These cells do produce other growth factors, including growth hormone factor-β, and express cell surface receptors for epidermal growth factor factor (49). Gastrin/CCK-B receptors are not generally expressed on human renal cell carcinomas (34) but are expressed on Wilms' tumor cells derived from the kidney and do mediate the effects of gastrin on urinary sodium excretion (29). There are no data on the presence of G-Gly or progastrin receptor tissues of renal origin other than the present report.

Perspectives

In our studies we have shown that HEK 293 cells express the gastrin gene and secrete small but significant quantities of G-NH₂ and G-Gly into media. HEK 293 cells also express distinct cell surface receptors for G-NH₂ and G-Gly that mediate the complementary proliferative actions of the peptides via distinct signal transduction mechanisms. The concentrations of G-NH₂ and G-Gly secreted into the media are sufficient to stimulate growth of HEK 293 cells in an autocrine manner. Because the concentrations of G-Gly often exceed those of G-NH₂ during development it is possible that these gastrin gene products may play a role in the growth and development of the kidney. Moreover, because other prohormone precursors are often poorly processed during development, this study suggests that other prohormone processing intermediates may also possess distinct biological activities yet to be defined.

This work was supported in part by funds from National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grants R01-DK-34006 and R01-DK-47938 and by funds from the University of Michigan Gastrointestinal Peptide Research Center (NIDDK Grant P30-DK-34933). V. Stepan received funding from a Max Kade Postdoctoral Research Grant (Max Kade Foundation, New York, NY).

Address for reprint requests and other correspondence: C. J. Dickinson, Pediatric Gastroenterology, Univ. of Michigan, Medical
REFERENCES


