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Original Articles

Glycine-Extended Gastrin Exerts Growth-Promoting Effects on Human Colon Cancer Cells

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Abstract

Background: Since human colon cancers often contain significant quantities of progastrin-processing intermediates, we sought to explore the possibility that the biosynthetic precursor of fully processed amidated gastrin, glycine-extended gastrin, may exert trophic effects on human colonic cancer cells.

Materials and Methods: Binding of radiolabeled glycine-extended and amidated gastrins was assessed on five human cancer cell lines: LoVo, HT 29, HCT 116, Colo 320DM, and T 84. Trophic actions of the peptides were assessed by increases in [^{3}H]thymidine incorporation and cell number. Gastrin expression was determined by northern blot and radioimmunoassay.

Results: Amidated gastrin did not bind to or stimulate the growth of any of the five cell lines. In contrast, saturable binding of radiolabeled glycine-extended gastrin was seen on LoVo and HT 29 cells that was not inhibited by amidated gastrin (10^{-6} M) nor by a gastrin/CCK_B receptor antagonist (PD 134308). Glycine-extended gastrin induced a dose-dependent increase in [^{3}H]thymidine uptake in LoVo (143 ± 8% versus control at 10^{-10} M) and HT 29 (151 ± 11% versus control at 10^{-10} M) cells that was not inhibited by PD 134308 or by a mitogen-activated protein (MAP) or ERK kinase (MEK) inhibitor (PD 98509). Glycine-extended gastrin did stimulate jun-kinase activity in LoVo and HT 29 cells. The two cell lines expressed the gastrin gene at low levels and secreted small amounts of amidated gastrin and glycine-extended gastrin into the media.

Conclusions: Glycine-extended gastrin receptors are present on human colon cancer cells that mediate glycine-extended gastrin's trophic effects via a MEK-independent mechanism. This suggests that glycine-extended gastrin and its novel receptors may play a role in colon cancer cell growth.

Introduction

In addition to its role in the regulation of gastric acid secretion, gastrin is also a potent stimulant of gastrointestinal cell proliferation and differentiation (1). However, the role of gastrin in the development of colorectal cancer has been controversial for many years (2). Several studies demonstrated a growth-stimulatory effect of gastrin or pentagastrin on colon cancers in vivo and in vitro (3–5) whereas others have failed to confirm this effect (6,7). Other human studies have been performed with conflicting results, but a recent epidemiological study suggests that prolonged hypergastrinemia may play a role in the

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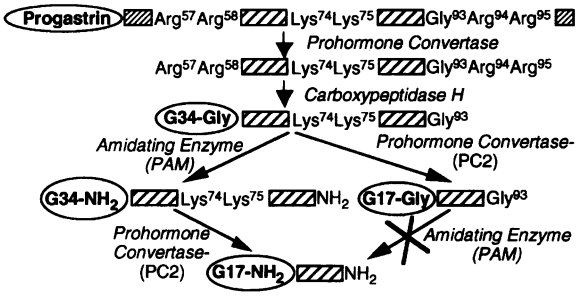

Fig. 1. Gastrin post-translational processing. Gastrin is synthesized on the endoplasmic reticulum and then transported to the Golgi. In well-differentiated neuroendocrine cells, progastrin is sorted in the *trans*-Golgi network to the regulated pathway of secretion and into secretory granules that contain the enzymes necessary to complete progastrin processing. In these granules, the amino- and carboxy-terminal flanking regions are cleaved by a prohormone convertase at dibasic arginine residues (ArgArg) and the carboxy-terminal arginines removed by carboxypeptidase H revealing G34-Gly. G34-Gly can then be amidated by the peptidyl-glycine alpha-amidating monooxygenase (PAM) to form G34-NH_2 or cleaved at internal lysine/lysine (LysLys) residues resulting in the production of G17-Gly. Biosynthetic studies (64) suggest that although G34-NH_2 can be cleaved at the LysLys site by a prohormone convertase to yield G17-NH_2, G17-Gly is not amidated by PAM. Thus, G17-Gly may be a distinct end-product of progastrin processing in antral G-cells.

development of some human colonic and gastric tumors (8,9). Adding to the controversy is the fact that some investigators have found substantial quantities of gastrin within some human colon cancers whereas others have found little, if any, gastrin (2). Recent studies have begun to resolve some of these conflicting data. Although gastrin gene expression can be found in normal human colonic tissues and many colon cancers, the 101 amino acid primary gastrin mRNA translation product, progastrin, requires extensive post-translational processing before it assumes its biologically relevant structure (10). However, most "gastrin" antisera recognize only the fully mature 17 amino acid carboxyl-terminally amidated form of gastrin (G-NH_2, Fig. 1). Since progastrin is often poorly processed in nonendocrine cells, such as those found in the colon, it explains why these "gastrin" antisera fail to detect significant quantities of peptide. Consistent with this notion is investigators' recent finding that colon cancers contain large amounts of progastrin and other progastrin-processing intermediates such as glycine-extended gastrin (G-Gly) but little fully processed G-NH_2 (11–14).

To explore the possibility that progastrin-processing intermediates play a role in gastrointestinal cell growth, we noted that G17-Gly stimulates the growth of an exocrine pancreatic cell line, AR4-2J, via receptors distinct from gastrin/cholecystokinin-B (gastrin/CCK_B) receptors (15). We (11) and others (12) have detected progastrin-processing intermediates such as G-Gly in concentrations similar to that of G-NH_2 in some colon cancers. Also, when the human gastrin gene is heterologously expressed in the liver of transgenic mice, there is an overproduction of unprocessed, but not processed, gastrin gene products (16). This progastrin overproduction is associated with an increase in colonic mucosal proliferation, suggesting that progastrins play a role in colonic cell growth. Thus, in the present studies we sought to determine if human colon cancer cell lines express receptors for G-Gly that mediate its trophic effects. We also examined the proliferative effects of G-NH_2 and G-Gly in combination on cells that express both receptors.

Materials and Methods

Cell Culture

The human colon cancer cell lines LoVo, HT 29, HCT 116, Colo 320DM, and T 84 and the rat pancreatic tumor cell line AR4-2J were purchased from the American Type Culture Collection and grown in monolayer cultures in their appropriate growth media in an atmosphere of 95% air and 5% CO_2 at 37°C. Cultures were passaged at 4- to 6-day intervals to maintain the cells at subconfluent densities.

Binding Studies

Human Leu^{15}-G17-NH_2 (Research Plus, Bayonne, NJ) and Leu^{15}-$G_{(2\text{-}17)}$-Gly (University of Michigan, protein core facility; peptide purity >95%, by HPLC and mass spectometry) were ^{125}I-labeled on Tyr^{12} with an adaptation of the chloramine T method and purified by high-pressure liquid chromatography (HPLC) as described previously (15,17). The specific activities of the labels were ≈1.5 μCi/pmol. Binding assays were done on isolated cells that were detached in phosphate-buffered saline (PBS) containing 0.02% EDTA. Cells (2×10^6) were incubated with ^{125}I-labeled Leu^{15}-G17-NH_2 or Leu^{15}-$G_{(2\text{-}17)}$-Gly with or without 1 μM of unlabeled G17-NH_2 or Leu^{15}-$G_{(2\text{-}17)}$-Gly in a Krebs-*N*-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid

buffer supplemented with 0.5% bovine serum albumin (BSA), 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. Saturation-binding studies with increasing concentrations of ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly (0–0.5 nM) were performed on HT 29 and LoVo cells. For competitive binding studies, cells were incubated with 120 pM ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly without (control) or with 1 μM of Leu^{15}-G$_{(2-17)}$-Gly, G17-NH$_2$, or the gastrin/CCK$_B$ receptor antagonist PD 134308 (Parke Davis Research, Ann Arbor, MI) in a total volume of 1 ml until equilibrium.

Proliferation Studies

[^{3}H]THYMIDINE INCORPORATION. Cells grown in their appropriate media supplemented with 10% (HT 29, HCT 116, Colo 320DM, T 84, AR4-2J) or 20% (LoVo) fetal bovine serum (FBS) were plated, allowed to attach overnight, and then cultured in serum-free media containing 0.2 mM unlabeled thymidine. After washing cells with serum-free media, cells were treated with increasing concentrations of G17-NH$_2$ or Leu^{15}-G$_{(2-17)}$-Gly or 100 nM 12-O-tetradecanoyl-phorbol-13-acetate (TPA; Sigma, St. Louis, MO). In some experiments cells were treated with the gastrin/CCK$_B$ receptor antagonist PD 134308 (100 nM) or the MEK [mitogen-activated protein (MAP) and ERK kinase] inhibitor PD 98059 (25 μM) (New England Biolabs, Beverley, MA) 30 min prior to stimulation. DNA synthesis was estimated by measurement of [^{3}H]thymidine incorporation into the trichloroacetic acid (TCA)-precipitable material. [^{3}H]thymidine (0.1 μCi/ml; 18 Ci/pmol) was added 2 hr before the end of an 8-hr treatment period for the colon cancer cell lines and a 24-hr treatment period for the AR4-2J cells. Cells were then washed twice with serum-free medium to remove unincorporated [^{3}H]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.

CELL COUNTING. To ensure that the incorporation of [^{3}H]thymidine into HT 29 and LoVo cells represented cell growth, we also performed proliferative assays by cell counting. HT 29 and LoVo cells were seeded onto 6-well plates in their appropriate media at a concentration of 10,000 cells/ml. After 24 hr, media were changed to serum-free media containing 10^{-9} M Leu^{15}-G$_{(2-17)}$-Gly. Media were changed every 24 hr to new serum-free, peptide-containing media and cells were counted in a Coulter cell counter (Coulter Corporation, Miami, FL) after 4 days. Results were expressed as percentage increase in cell number over unstimulated controls.

Analysis of Signal Transduction

ELK 1 ACTIVITY. Subconfluent LoVo and HT 29 cells were cotransfected with 2 mg of the chimeric GAL-4-Elk-1 expression vector and 2 mg of the 5Xgal luciferase reporter plasmid and 0.25 mg of β-galactosidase. Transfections were carried out using Lipofectamine (Gibco BRL, Grand Island, NY) as previously described (18). The day after transfection, media were removed and cells were placed in serum-free media for 24 hr and then incubated for 5 hr with or without 10 nM of Leu^{15}G$_{(2-17)}$-Gly or 100 nM TPA. In some experiments cells were pretreated for 30 min with the MEK inhibitor PD 98059 (25 μM). At the end of the incubation period, cells were washed and lysed, and luciferase assays were performed as previously described (18). Luciferase activity was expressed as RLU (relative light units) and normalized for β-galactosidase activity. β-galactosidase activity was measured by the luminescent light derived from 10 μl of each sample incubated in 100 μl of Lumi-Gal 530 (Lumigen, Southfield, MI) and used to correct the luciferase assay data for transfection efficiency.

JUN KINASE ASSAY. Immunoprecipitations and the in-gel *c*-jun amino-terminal kinase (JNK) assays were performed as described (19). Briefly, after a 30-min incubation with G-Gly (10^{-8} M), cells were lysed in 500 μl of lysis buffer [10 mM KPO_4 (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate (Na_3VO_4), 2 mM dithiothreitol, 40 μg/ml phenylmethylsulfonyl fluoride, 10 nM okadaic acid, 0.8 μg/ml leupeptin, 10 mg/ml p-nitrophenylphosphate, and 10 μg/ml aprotinin], transferred into microfuge tubes, and spun at 16,000 × *g* for 10 min at 4°C. Equal amounts of protein from each treatment group (1000 μg) were incubated with an anti-c-jun kinase 1–specific antibody (Santa Cruz Biotech-

nology, Santa Cruz, CA) and mixed on a rotating platform for 15 hr at 4°C. Aliquots of protein A sepharose (Pharmacia, Piscataway, NJ) were then added and the solutions mixed for 1 additional hr. After centrifugation, pellets were washed two times with lysis buffer and resuspended in 20 μl of electrophoresis buffer [for 10 ml: 1ml glycerol, 0.5 ml 2-mercaptoethanol, 3 ml 10% sodium dodecyl sulfate (SDS), 1.25 ml 1 M Tris buffer, 2 ml 0.1% bromophenol blue, 0.6 g urea], boiled for 5 min, and applied to a 10% SDS-polyacrylamide gel containing 0.5 mg/ml GST-*c*-Jun (Sigma) (20). After electrophoresis the gel was washed with two changes of 20% 2-propranol in 50 mM Tris (pH 8.0) for 1 hr and then with two changes of 50 mM Tris (pH 8.0) containing 5 mM 2-mercaptoethanol for 1 hr. The enzyme was denatured by incubating the gel with two changes of 6 M guanidine-HCl for 1 hr and then renatured with five changes of 50 mM Tris (pH 8.0) containing 0.04 % TWEEN 40 and 5 mM 2-mercaptoethanol for 1 hr. The kinase reaction was performed in conditions inhibitory to cyclic nucleotide–dependent protein kinase and Ca^{2+}-dependent protein kinases by incubating the gel at 25°C for 1 hr with 40 mM HEPES (pH 8.0) containing 0.5 mM EGTA, 10 mM $MgCl_2$, 2 μM protein kinase inhibitor peptide, 20 μM ATP, and 2.5 μCi/ml of [γ^{32}P]ATP (6000 Ci/mmol). After incubation, the gel was washed with a 5% (w/v) TCA solution containing 1% (w/v) sodium pyrophosphate, dried, and subjected to autoradiography.

Gastrin Expression in Cell Lines

GENE EXPRESSION. Total cellular RNA was isolated from HT 29 and LoVo cells using TRIzol Reagent (Gibco BRL). RNA was also extracted from canine antral G cells for use as a positive control. Primer 1 for the polymerase chain reaction (PCR) was complementary to nucleotides 9–30 of the human gastrin cDNA (21) and primer 2 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 (22). One microgram of total RNA was reverse transcribed using a random primer $p(dN)_6$. The PCR reaction was performed under the following conditions: 1 min at 94°C, 2 min at 55°C, and 2 min at 72°C for 40 cycles. As a control for contamination, the procedure was also performed using one tube without any input RNA. The amplified products were separated on a 1% agarose gel in 1× TBE buffer, and then after denaturing (0.25 N HCl for 20 min, 0.5 M NaOH and 1.5 M NaCl for 30 min, and 1 M ammonium acetate and 0.1 M NaOH for 30 min), blotted to a nitrocellulose membrane and UV cross-linked. After a 2-hr prehybridization, the filter was hybridized with a ^{32}P-labeled human gastrin cDNA probe in a 0.1 M HEPES (pH 7.5), 5× SSC, 5× Denhardt's solution with 100 μg/ml salmon sperm DNA for 3 hr at 60°C. The membrane was washed two times in 2× SSC, 20 min each at 62°C, another 20 min in 1× SSC at 62°C, and finally for 10 min in 1× SSC at 75°C, and exposed to X-ray film at −80°C for 2 hr.

GASTRIN PEPTIDE ANALYSIS. For analysis of gastrin peptide expression, 2×10^6 cells (LoVo and HT 29) were plated in 6-cm dishes in their appropriate media and grown for 24 hr, then media were changed to serum-free media and after another 24 hr the serum-free media were collected. Media were centrifuged at 12,000 rpm for 5 min and stored at −20°C until assay. Media were analyzed by radioimmunoassay using antibody 5135, which is specific for amidated forms of gastrin and cross-reacts <1% with glycine-extended gastrins or progastrins extended beyond the carboxyl-terminal glycine residue (23). G-Gly was quantified with antibody 8237, which cross-reacts <5% with amidated gastrins or progastrins extended beyond the carboxyl-terminal glycine residue (23).

Statistics

Where appropriate, results are expressed as the mean ± SD, except where otherwise stated. Comparisons between groups were made by the Student's *t*-test. Differences with p values of <0.05 were considered significant.

Results

G-Gly and G-NH_2 Receptor Binding

The presence of G-Gly- and G-NH_2-selective binding sites on the different cell lines was investigated with ^{125}I-labeled human Leu^{15}-$G_{(2\text{-}17)}$-Gly and human Leu^{15}-G17-NH_2 as described in Materials and Methods. AR4-2J cells were used as a positive control for both peptides in all experiments. None of the five different colon cancer cell lines showed significant specific binding sites for ^{125}I-Leu^{15}-G17-NH_2 (Fig. 2A). ^{125}I-Leu^{15}-$G_{(2\text{-}17)}$-Gly specific binding was detected

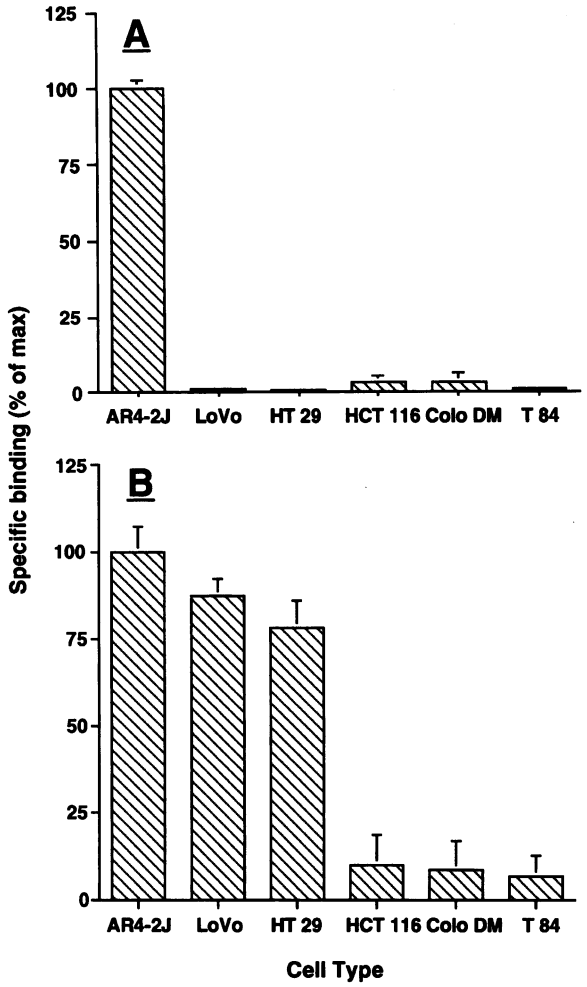

Fig. 2. ^{125}I-Leu^{15}-G17-NH$_2$ and ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly binding. Percent of maximal specific binding of ^{125}I-Leu^{15}-G17-NH$_2$ (A) and ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly (B) on the five human colon cancer cell lines compared to AR4-2J (rat pancreatic acinar) cells. Specific binding of ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly to AR4-2J cells was 65 ± 8% and to LoVo and HT 29 cells 57 ± 8% and 51 ± 5%, respectively. Specific binding of ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly to HCT 116, Colo 320 DM, and T84 cells was not significant. Mean ± SD of seven different experiments, each performed in duplicate.

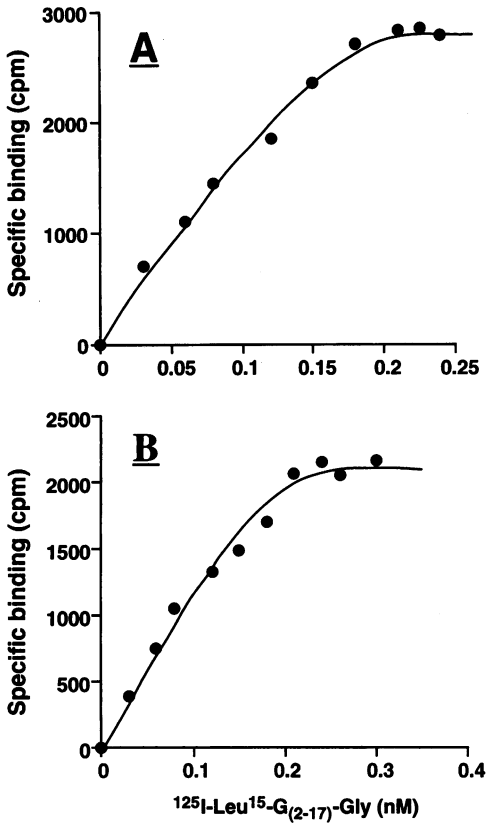

Fig. 3. ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly saturation binding. Specific binding of increasing concentrations of ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly on LoVo (A) and HT 29 (B) cells. The results are representative of similar data in four additional experiments. Scatchard analysis of the five results revealed a Kd of 1.2 ± 0.3 × 10^{-10} M for LoVo and 1.8 ± 0.4 × 10^{-10} M for HT 29 cells. The B_{max} values were 2 ± 1.5 × 10^{-12} M for the LoVo and 3 ± 2 × 10^{-12} M for the HT 29 cells.

on LoVo and HT 29 cells but not on the HCT 116, Colo 320DM, or T 84 cells (Fig. 2B). The percent specific binding was 57 ± 8% (n = 7) on LoVo and 51 ± 5% (n = 7) on HT 29 cells. For comparison, the percent specific binding of ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly to AR4-2J cells was 65 ± 8% (n = 7).

The specific binding of ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly increased in a dose-dependent manner. Scatchard analysis revealed a Kd value of 1.2 ± 0.3 × 10^{-10} M (n = 5) for the LoVo cells and 1.8 ± 0.4 × 10^{-10} M (n = 5) for HT 29 cells (Fig. 3). The binding capacity (B_{max}) was 2 ± 1.5 × 10^{-12} M (n = 5) for the LoVo cells and 3 ± 2 × 10^{-12} M (n = 5) for HT 29 cells. Competitive binding studies showed that unlabeled Leu^{15}-G$_{(2-17)}$-Gly displaced ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly from specific binding sites on LoVo and HT 29 cells, but that G17-NH$_2$ and the gastrin/CCK$_B$ receptor antagonist (PD 134308) had no effect, even in concentrations as high as 1 μM (Fig. 4). As shown previously, unlabeled 1 μM G17-NH$_2$ and PD 134308 completely inhibited the binding of ^{125}I-Leu^{15}-G17-NH$_2$ to AR4-2J cells (15).

Proliferation Studies

Detailed proliferation studies were performed on the two cell lines (LoVo and HT 29) that expressed G-Gly-receptors. Leu^{15}-G$_{(2-17)}$-Gly stim-

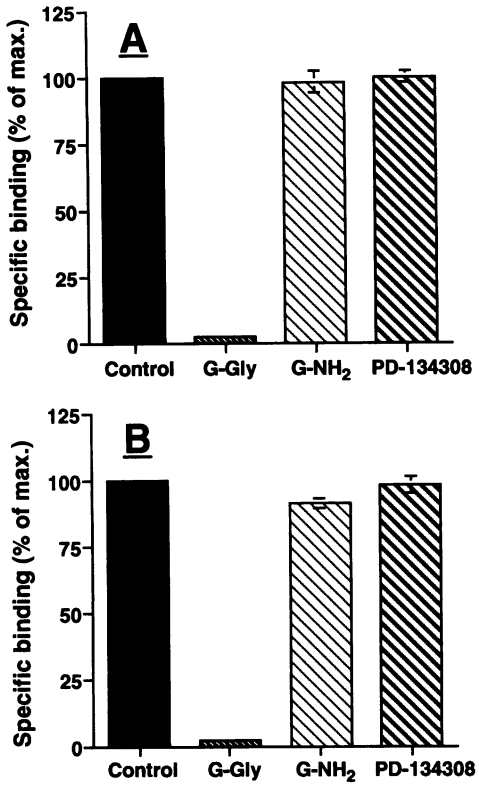

Fig. 4. ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly binding specificity. Displacement of ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly bound to LoVo (A) and HT 29 (B) cells with unlabeled Leu^{15}-G$_{(2-17)}$-Gly (10^{-6} M), G17-NH$_2$ (10^{-6} M), and the gastrin/CCK$_B$ receptor antagonist PD 134308 (10^{-6} M). Mean ± SD of three different experiments, each performed in triplicate.

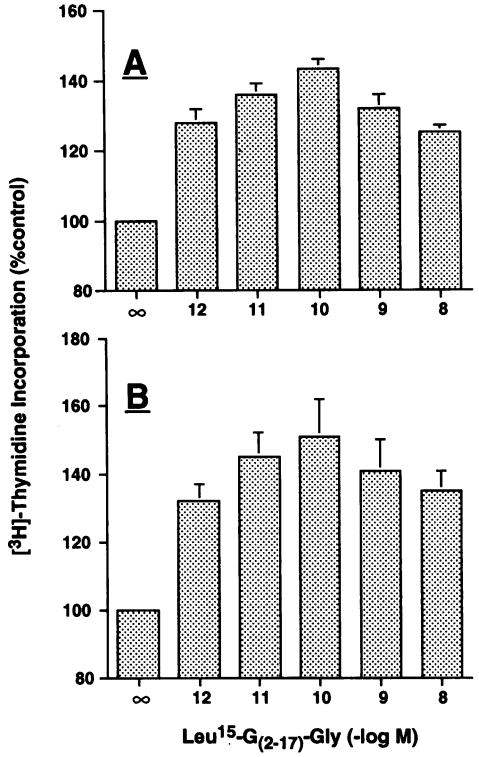

Fig. 5. Effect of G-Gly on [^{3}H]thymidine incorporation. Leu^{15}-G$_{(2-17)}$-Gly stimulated [^{3}H]thymidine incorporation into LoVo (A) and HT 29 (B) cells. Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation. Mean ± SE of seven different experiments, each performed in sextuplicates.

ulated [^{3}H]thymidine incorporation in a dose-dependent fashion in both cell lines with a maximal stimulatory effect seen at 10^{-10} M (151 ± 11% for HT 29 and 143 ± 8% for LoVo cells compared to unstimulated controls, mean ± SE, n = 7) (Fig. 5). The stimulatory effect of G-Gly on HT 29 and LoVo cells was not altered by the presence of the gastrin/CCK$_B$ receptor antagonist PD 134308 (10^{-8} M). Leu^{15}-G$_{(2-17)}$-Gly (10^{-9} M) did not stimulate [^{3}H]thymidine incorporation into the three colon cancer cell lines that did not demonstrate ^{125}I-Leu^{15}-G$_{(2-17)}$-Gly binding. G17-NH$_2$ did not stimulate [^{3}H]thymidine incorporation into HT 29 or LoVo cells. To correlate the increases in [^{3}H]thymidine incorporation with cellular growth, HT 29 or LoVo cells were counted after a 4-day stimulation period with 10^{-9} M Leu^{15}-G$_{(2-17)}$-Gly. Total cell number increased in LoVo and HT 29 cells by 152 ± 7% and 160 ± 8%, respectively (mean ± SE, n = 7) (Fig. 6).

G-Gly Signal Transduction Mechanisms

To investigate the potential signal transduction mechanisms that mediate G-Gly's trophic actions, we noted that G17-NH$_2$, but not G-Gly, enhanced expression of c-fos and c-jun in AR4-2J cells (18). G17-NH$_2$ enhanced the expression of these early response genes, which have been linked to cell growth via activation of protein kinase C (PKC) and MAP kinase as well as the upstream activator of MAP kinase, MAP and ERK kinase (MEK) (24). MAP kinase activation results in phosphorylation of ERK2, which phosphorylates a nuclear transcription factor (Elk-1) that enhances c-fos expression by binding to the c-fos serum response element (SRE). In contrast to G17-NH$_2$, G-Gly did not induce MAP kinase activation or enhance phosphorylation of Elk-1, but did stimulate phosphorylation and thus bioactivation of c-jun by jun-kinase (18). To investigate if these mechanisms were operative in the human colon cancer cell lines we used a yeast hybrid system involving cotrans-

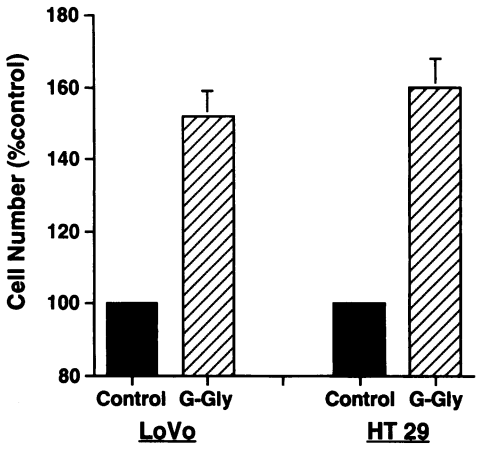

Fig. 6. Effect of G-Gly on proliferation. Increase in cell number after a 4-day stimulation period with 10^{-9} M Leu^{15}-$G_{(2-17)}$-Gly in LoVo and HT 29 cells. Results are expressed as percent of unstimulated control. Mean ± SE of seven different experiments, each performed in triplicates.

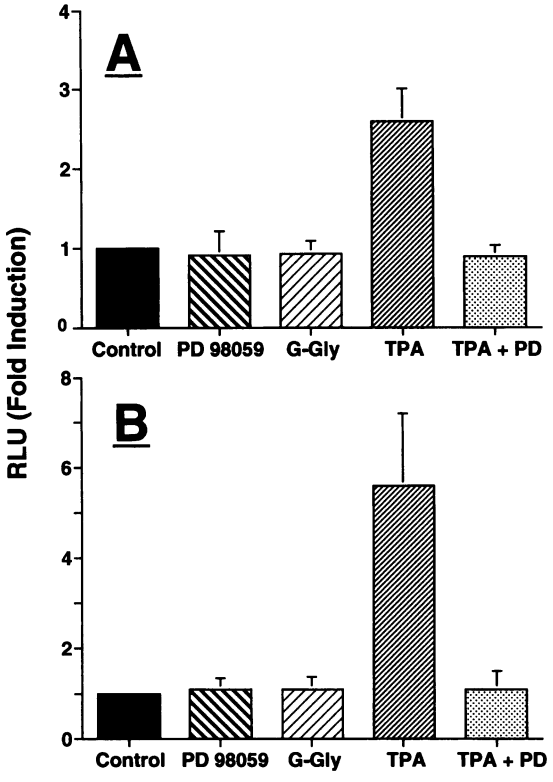

Fig. 7. SRE transcriptional activity in LoVo (A) and HT 29 (B) cells. Cells were cotransfected with the GAL-4-Elk-1 expression vector and the 5Xgal luciferase reporter plasmid and treated with 10^{-8} M Leu^{15}-$G_{(2-17)}$-Gly or 100 nM TPA with or without the MEK inhibitor (PD 98059, 25 μM). Results are normalized by β-gal activity and expressed as n-fold induction over control. Mean ± SD of four different experiments, each performed in duplicate.

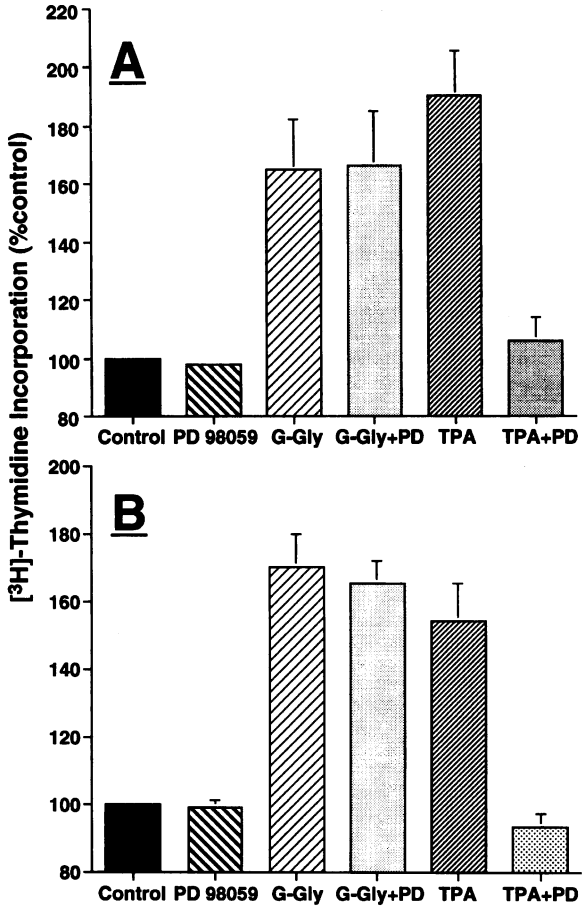

Fig. 8. Effect of the MEK inhibitor (PD 98059) on G-Gly- and TPA induced proliferation. LoVo (A) and HT 29 (B) cells were stimulated with 10^{-9} M Leu^{15}-$G_{(2-17)}$-Gly or 100 nM TPA with or without preincubation with 25 μM of PD 98059. Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation. Mean ± SE of four different experiments, each performed in sextuplicates.

fection of the cells with a chimeric GAL4-Elk-1 expression vector and the 5Xgal-luciferase reporter plasmid. In this system GAL4-Elk-1 transactivates and stimulates luciferase activity only if the carboxyl terminus of Elk-1 is phosphorylated by ERK2. We also wished to determine if G-Gly might also stimulate jun-kinase activity in these cells (19). As shown in Figure 7, 10 nM G-Gly did not significantly increase luciferase activity in LoVo and HT 29 cells (0.96 ± 0.2 and 1.1 ± 0.2 fold induction, respectively). However, when these cells were stimulated with TPA (100 nM), a 2.6 ± 0.4 (LoVo) and a 5.6 ± 1.6 (HT 29) fold induction in 5Xgal-luciferase activity was seen, which was blocked by pretreatment of cells with the MEK inhibitor PD 98059 (LoVo, 0.9 ± 0.15

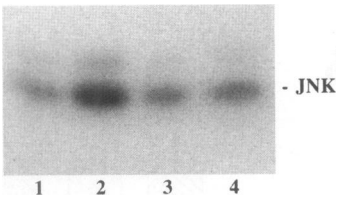

Fig. 9. Effect of G-Gly on JNK activity. Cells were lysed after a 30-min treatment with Leu^{15}-$G_{(2-17)}$-Gly (10^{-8} M). Lysates (lane 1, LoVo control; lane 2, LoVo with Leu^{15}-$G_{(2-17)}$-Gly; lane 3, HT 29 control; lane 4, HT 29 with Leu^{15}-$G_{(2-17)}$-Gly) were then immunoprecipitated and an in-gel JNK assay was performed as described in Materials and Methods. The gel shown above is representative of at least three others with similar results. Densitometric measurement revealed an average 2.3 ± 0.5 fold induction in LoVo cells and a 1.3 ± 0.1 fold induction in HT 29 cells. Mean ± SD of three different experiments, $p < 0.05$ versus control.

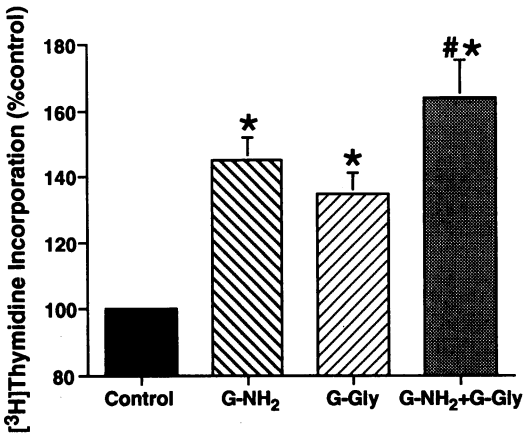

Fig. 10. Effect of G-Gly and $G\text{-}NH_2$ on cell growth. Effect of $G17\text{-}NH_2$ (10^{-9} M), Leu^{15}-$G_{(2-17)}$-Gly (10^{-9} M), and both peptides together on [^{3}H]thymidine incorporation in AR4-2J cells. Results are expressed as percent of control, unstimulated [^{3}H]thymidine incorporation. Mean ± SD of six different experiments, each performed in sextuplicates. $^*p < 0.05$ versus control and $^\#p < 0.05$ versus $G\text{-}NH_2$.

and HT 29, 1.1 ± 0.3). Next, we sought to determine if these effects on signal transduction were related to cell growth. As depicted in Figure 8, the stimulatory effects of TPA (10^{-7} M) on [^{3}H]thymidine incorporation into LoVo and HT 29 cells were blocked with the MEK inhibitor PD 98059 (25 mM). In contrast, the effects of 10^{-9} M Leu^{15}-$G_{(2-17)}$-Gly on [^{3}H]thymidine incorporation into these cells was not blocked with PD 98059 (Fig. 8). However, an in-gel jun-kinase assay demonstrated that Leu^{15}-$G_{(2-17)}$-Gly (10^{-8} M) did stimulate jun-kinase activity in HT 29 and LoVo cells (Fig. 9).

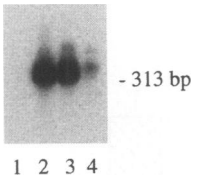

Fig. 11. Gastrin gene expression in LoVo and HT 29 cells. PCR products (lane 1, water negative control; lane 2, antral G-cells as a positive control; lane 3, LoVo; lane 4, HT 29) were analyzed on a 1% agarose gel and probed with a ^{32}P-labeled human gastrin cDNA.

We hypothesized that the enhancement of c-fos and c-jun expression by $G17\text{-}NH_2$ and the G-Gly induced bioactivation of c-jun via jun-kinase were complementary in their biological actions. Thus, we incubated AR4-2J cells that express receptors for both $G17\text{-}NH_2$ and G-Gly with these ligands. Both $G17\text{-}NH_2$ (10^{-9} M, 145 ± 11%, $n = 6$) and Leu^{15}-$G_{(2-17)}$-Gly (10^{-9} M, 132 ± 8%, $n = 6$) stimulated increases in [^{3}H]thymidine incorporation. However, both peptides together at 10^{-9} M significantly increased [^{3}H]thymidine incorporation above the maximum level of either peptide alone (161 ± 13%, $n = 6$) (Fig. 10).

GASTRIN GENE EXPRESSION. We were not able to detect gastrin mRNA expression by northern blotting, but the expression of gastrin mRNA in LoVo and HT 29 cells could be determined by reverse transcribed (RT) PCR and Southern analysis. In both cell lines, amplification with primer 1 and primer 2 revealed a specific PCR product of 313 bp, with a much higher expression level in LoVo than in HT 29 cells (Fig. 11). No PCR product was detected in the sample without any input RNA. $G\text{-}NH_2$ and G-Gly were quantified by radioimmunoassay in serum-free LoVo and HT 29 media. Small amounts of $G\text{-}NH_2$ were detected in media conditioned by both cell lines (LoVo, 5.6 ± 1.5 pmol/l; HT 29, 2.6 ± 0.6 pmol/l; mean ± SE), but G-Gly was detected in much higher concentrations (LoVo, 56 ± 18 pmol/l; HT 29, 79 ± 24 pmol/l; mean ± SE).

Discussion

The trophic effects of gastrin on gastrointestinal tissues have been known for quite some time

(1,25). However, the role of gastrin in the development of colorectal cancer has been controversial (3–7,9,26–29). One problem in many of these studies is that they generally examine the proliferative actions of the fully processed peptide amide. However, recent studies have noted that human colon cancers contain unprocessed gastrin precursors in amounts that exceed those of $G\text{-}NH_2$ (11–14). Moreover, transgenic mice with elevated plasma levels of progastrin but not $G\text{-}NH_2$ have an increased colonic proliferative index (16).

Thus, we (30) and others (31) have sought to characterize the cellular and molecular mechanisms that may mediate the trophic effects of progastrin-processing intermediates on colonic mucosa. We have chosen to study extensively the physiologic effects of one gastrin-processing product, G-Gly. Interest in G-Gly has been fueled by the observations that G-Gly is stored in brain and gut tissues (32–34) and secreted with $G\text{-}NH_2$ from antral G-cells into the circulation (35), and achieves concentrations in plasma roughly equivalent to those of $G\text{-}NH_2$ (36–38). Moreover, G-Gly is seen in greater concentrations than $G\text{-}NH_2$ in some colon cancers (10). Previously, we also identified G-Gly receptors, distinct from the gastrin/CCK_B receptor that mediated the tropic effects of G-Gly on an exocrine pancreatic cell line, AR4-2J (15). Thus, we sought to determine if human colon cancers might produce G-Gly and express specific G-Gly receptors that mediate its trophic effects.

We noted a dose-dependent increase in [^{3}H]thymidine incorporation in two (LoVo and HT 29) of the five human colonic cell lines with an EC_{50} of approximately 10^{-10} M (Fig. 5). LoVo and HT 29 cells also demonstrated specific binding sites for ^{125}I-Leu^{15}-$G_{(2\text{-}17)}$-Gly (Figs. 2 and 4). Scatchard analysis revealed a Kd that was approximately 2×10^{-10} M for both cell lines, which is similar to the EC_{50} for growth (Fig. 3). This suggests that these G-Gly binding sites mediate the proliferative effects of G-Gly. This notion is supported by the fact that G-Gly did not bind to or stimulate the growth of the three other tested cell lines. Additionally, the concentrations needed for cell growth are within the physiological range of G-Gly found in human plasma (39,40) and similar to the Kd for rat pancreatic acinar cells, AR4-2J (15). Finally, Koh et al. (41) have recently reported that G-Gly can act as a trophic agent in the mouse colon. In transgenic mice overexpressing G-Gly but not $G\text{-}NH_2$ or progastrin they noted colonic cell hyperplasia.

To rule out the possibility that the G-Gly effects may be mediated by gastrin/CCK_B receptors, we noted that ^{125}I-Leu^{15}-$G_{(2\text{-}17)}$-Gly binding could be displaced by neither $G17\text{-}NH_2$ (10^{-6} M) nor the gastrin/CCK_B receptor antagonist PD-134308 (10^{-6} M) (Fig. 4). Additionally, PD-134308 (10^{-6} M) did not inhibit the proliferative actions of G-Gly. Finally, $G\text{-}NH_2$ did not bind to, nor stimulate the growth of, any of the five cell lines, suggesting that the actions of G-Gly are mediated by a receptor distinct from the gastrin/CCK_B receptor.

Identification of the contribution of gastrin to colon cancer cell growth has been difficult because a variety of gastrin receptors have been identified that appear to mediate gastrin's trophic effects (27,31,39,40). $G\text{-}NH_2$ requires its carboxyl-terminal amide moiety for full biological activity mediated by gastrin/CCK_B receptors. Indeed, removal of the carboxyl-terminal amide of $G\text{-}NH_2$ completely abolishes its acid stimulatory effects mediated by standard gastrin/CCK_B receptors and the immediate precursor of $G\text{-}NH_2$, G-Gly, is at least four orders of magnitude less potent than $G\text{-}NH_2$ in stimulating acid secretion from gastric parietal cells (42,43). However, gastrin/CCK_B or CCK_A receptor antagonists do not always inhibit the trophic actions of $G\text{-}NH_2$, suggesting that these $G\text{-}NH_2$ proliferative effects are mediated by non-A/non-B gastrin/CCK receptors (31,39,40). The fact that we could not identify ^{125}I-Leu^{15}-$G17\text{-}NH_2$ binding sites (Fig. 2) or demonstrate an enhancement in [^{3}H]thymidine incorporation with $G17\text{-}NH_2$ on any of the five cell lines suggests that they do not express functional non-A/non-B CCK receptors. Moreover, it also suggests that the G-Gly receptor is distinct from previously described non-A/non-B gastrin/CCK receptors (31,39,40). It has also been demonstrated that antisense gastrin mRNA treatment of Colo 320 DM and HCT 116 cells results in an inhibition of cell proliferation (44). Since we were unable to detect receptors for either amidated or glycine-extended gastrin on these cells, it is possible that receptors for progastrin peptides may be present on these cells.

Although we did not detect ^{125}I-Leu^{15}-$G17\text{-}NH_2$ binding to any of the cell lines, other investigators have identified gastrin/CCK_B antagonist (L-365-918) binding sites on HT 29 cells (27). Others have demonstrated $G17\text{-}NH_2$-stimulated growth of LoVo cells (26) and detected a splice variant of the gastrin/CCK_B receptor expressed in these cells (45). On the other hand, Hoosein et al. (46) and Blackmore et al. (47) reported the

absence of high-affinity receptors for G17-NH_2 on HCT 116 and HT 29 cells, respectively. To ensure that our ^{125}I-Leu^{15}-G17-NH_2 ligand was capable of binding to the gastrin/CCK_B receptor, we utilized a cell line (AR4-2J) with a well-characterized gastrin/CCK_B receptor (15,48) as a positive control. In side-by-side experiments we identified ^{125}I-Leu^{15}-G17-NH_2 binding to AR4-2J cells, but could not consistently detect significant binding to any of the five colon cancer cell lines (Fig. 2). As expected, we were also able to demonstrate the proliferative effects of G17-NH_2 on the AR4-2J cells without being able to detect an enhancement in [^{3}H]thymidine incorporation with G17-NH_2 in any of the five cell lines. One possible explanation for the differences between the results of others and our findings is that we may be examining different subclones of LoVo and HT 29 cells.

In previous studies we had sought to examine the nuclear mechanisms that mediate the proliferative actions of G-NH_2 and G-Gly (18,24). In AR4-2J cells, G-NH_2, but not G-Gly, induces expression of the early response genes c-*fos* and c-*jun* (18). c-fos activation is mediated via activation of MAP kinase and induction of SRE transcriptional activity via the MEK/MAP kinase pathway and the nuclear transcription factor Elk-1 (49–51). To determine whether the G-Gly-induced proliferative effect in our cell lines depended on this pathway, we used the highly specific MEK inhibitor, PD 98509 (52). G-Gly-induced increases in [^{3}H]thymidine incorporation were not altered by the presence of the MEK inhibitor. Furthermore, G-Gly showed no increase in luciferase activity in cotransfection studies with a chimeric GAL4-Elk-1 expression vector and the 5Xgal-luciferase reporter plasmid, suggesting that G-Gly was not involved in the activation of Elk-1. Taken together, these studies demonstrate that the trophic effects of G-Gly are not mediated by the MAP kinase pathway. To eliminate the possibility that LoVo and HT 29 cells might not be capable of MAP kinase pathway activation, we stimulated these cells with the PKC activator TPA. PKC has been shown to activate Raf directly and stimulate MAP kinase via activation of MEK (53). In our cells, TPA induced proliferation and increases in luciferase activity in cotransfection studies with chimeric GAL4-Elk-1 expression vector and the 5Xgal-luciferase reporter plasmid. Since the MEK inhibitor PD 98509 reversed both TPA-induced effects, it demonstrated that HT 29 and LoVo cells are capable of MAP kinase pathway activation.

Since activation of JNK has been linked to Ras-dependent malignant transformation (54), we wished to determine if G-Gly might stimulate JNK activity, especially in view of the fact that G-Gly was not involved in MAP kinase pathway activation. As shown in Figure 9, G-Gly markedly enhanced JNK activity in LoVo cells with a much smaller but nevertheless distinct effect in HT 29 cells. Although the level of JNK induction in HT 29 cells is less than that observed in LoVo cells, previous investigators have demonstrated different levels of JNK induction with the same ligand in different cell lines (55). It is interesting to note that we did not observe Elk-1 activation in these cells, even though JNK has been shown to activate Elk-1 in some cells (56,57). In other cellular systems, however, JNK does not appear to activate Elk-1, as is the case in our human colon cancer cell lines (58,59). Finally, since others have noted induction of JNK activity during colonic tumorigenesis (60), it is interesting to speculate on the role that G-Gly may play in this effect.

Although other mechanisms remain to be elucidated, our data suggest that G-NH_2 and G-Gly stimulate growth via distinct yet complementary mechanisms. To determine if the events described above result in a complementary effect on growth, we incubated AR4-2J cells with G-NH_2, G-Gly, and both peptides together. As previously reported (15), G-NH_2 and G-Gly enhanced [^{3}H]thymidine incorporation, but the use of the two peptides together at maximal doses resulted in a stimulation of [^{3}H]thymidine incorporation significantly greater than the maximal effect of either peptide alone (Fig. 10). There are other data to support this notion that G-Gly works in a complementary fashion to enhance the biological activity of G-NH_2 and other gastrointestinal peptides. Although acute administration of G-Gly has no effect on gastric acid secretion (42,43), chronic administration of G-Gly markedly enhances G-NH_2- and histamine-stimulated acid secretion in vivo (61) and that from isolated parietal cells via an increase in the expression of the proton pump H^+,K^+-ATPase (62).

It is interesting to speculate on the source of G-Gly responsible for cell growth in vivo. We noted that LoVo and HT 29 cells expressed the gastrin gene (Fig. 8), as previously described by van Solinge et al. (14). In contrast to these studies (14), we were able to detect small amounts of G-NH_2 and G-Gly that were secreted from these cells. These findings suggest that human colon cancers produce gastrin-processing intermediates capable of stimulating their growth in an auto-

crine fashion via cell surface receptors distinct from those that recognize G-NH_2 (63). This notion of an autocrine effect of gastrins on growth is supported by the observation that colonic cancer cell lines transfected with an antisense gastrin probe have reduced growth (44). Conversely, it is also possible that growth could be stimulated by G-Gly secreted from other tissues since the Kd for G-Gly binding and the EC_{50} for its proliferative effects are well within the physiologic levels of G-Gly found in plasma (37,38).

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