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Glycine-Extended Gastrin Promotes the Invasiveness of Human Colon Cancer Cells

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Colorectal cancers express significant amounts of immature glycine-extended gastrin (G-Gly) and G-Gly is able to stimulate cell proliferation in colonic cell lines and mucosa. Here we wished to investigate whether G17-Gly promote the invasiveness of LoVo human colonic cancer cells, a process which requires degradation of extracellular matrix by proteases and concomitant induction of cell migration. We confirmed that LoVo cells express gastrin and gastrin/CCK-B receptor mRNAs. We showed that these cells secrete matrix metalloproteinase (MMP)-1, -2, and -9. The function of MMP being to degrade components of extracellular matrix, they may thus favor cell migration. As compared to controls, G17-Gly (10^{-7} to 10^{-12} M) significantly enhanced about two to three times the LoVo cell migration through Matrigel, an artificial basement matrix barrier. Moreover, G17-Gly increased and gastrin/CCK-B receptor antagonists decreased MMP secretion in conditioned culture media of LoVo cells. Our findings show that physiological doses of incompletely processed form of gastrin induce the invasiveness of tumor cells *in vitro* and suggest a novel potential role for this peptide in the metastatic process of colonic cancers *in vivo*. © 2001 Academic Press

Key Words: **glycine-extended gastrin; matrix metalloproteinase; colon cancer cell; CCK-B receptor antagonist; zymography; cell invasion.**

Gastrin is a peptidic hormone essentially secreted by gastric antrum and proximal duodenum, which belongs to the same family as cholecystokinin (CCK), and shares with it the same membranous gastrin/CCK B receptor. It is synthesized as a progastrin precursor that undergoes proteolytic cleavages to a mature 17-amino acid C-terminally amidated form of gastrin (G-17). Posttranslational processing intermediates of gastrin, specifically glycine-extended gastrins (G-Gly), are produced in significant amounts in colonic cancers which, often, do not contain fully processed amidated G17 (1–4). Recently, widespread expression of progastrin and G-Gly immature forms has also been found in colonic polyps (5).

For a long time, amidation of gastrin was thought to be an essential prerequisite for biological activity. We and others have shown that, whether endogenous or exogenous, amidated G17 exerts growth-promoting effects on normal human gastrointestinal mucosae (6–9). However, the role of gastrin in the development of colorectal cancers has been controversial for many years (6, 10, 11). Although nonamidated gastrin precursors were initially thought to be biologically inactive, G-Gly, in 1994, has been reported to have a proliferative effect on tumor pancreatic cells (12). Since that time, other studies have shown that non-amidated gastrins can stimulate the normal and neoplastic gastrointestinal cell proliferation both *in vitro* (13–16) and *in vivo* (17–19).

These findings prompted us to explore whether immature G-Gly may also play a role in colon cancer cell invasion. We chose to work on the LoVo human colon cancer cell line because it expresses gastrin and secretes small amounts of the glycine-extended heptapeptide G17-Gly (16, 20) and because G17-Gly has been found to exert growth promoting effects on LoVo cells (15, 16). Since the tumoral invasion is dependent mainly on the degradation by proteases of extracellular matrices and concomitant induction of cell movement (21–23), we investigated also whether LoVo cells produce some matrix metalloproteinases (MMP) that may favor cell migration. The present study is the first to show that G17-Gly is able to stimulate the invasiveness of colon cancer cells *in vitro.*

MATERIAL AND METHODS

Cell culture and reagents. LoVo cell line, derived from a human colon adenocarcinoma metastase, was purchased from the European Collection of Cell Culture. Cells from passages 5–20 were grown in

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Ham's F12 medium without phenol red (Eurobio) supplemented with 10% fetal calf serum (FCS) (GIBCO BRL, Eragny, France) in a humidified atmosphere containing 5% CO_2 at 37°C. Medium was changed 3 times weekly.

Human G-17-Gly was purchased from Neosystem (France). Two selective gastrin/CCK-B receptor antagonists were used: CI-988 (or PD 134308) (12, 16) purchased from RBI (Sigma) and YM022 (15, 24, 25) (gift of Yamanouchi Pharmaceutical Research, Japan).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was extracted from harvested LoVo cells using Trizol-Reagent (GIBCO BRL). First-strand cDNA was synthesized from 1.6 μg of total RNA using murine reverse transcriptase and the first strand cDNA synthesis kit from Pharmacia Biotech (Uppsala, Sweden). Specific primers for human MMP-1, MMP-2 and MMP-9 (26) produced DNA amplicons of 786-, 605- and 243-bp, respectively. Human gastrin cDNA was amplified from base 8 to base 317 (GenBank Accession No. EO1795) with the following specific primers: sense, 5′-CAGCGACTATGT-GTGTATGTGCTG-3′; antisense, 5′-TGCTCGAGGATTGTTAGTTCTCATC-3′, the reaction generating a 309-bp amplicon. Specific primers for gastrin/CCK-B receptors were: sense, 5′-ATGCTCATCATCGTGGTCCTG-3′; antisense, 5′-AGTGTACACGGGGTAGGG-CAC-3′ (15), the reaction generating a DNA amplicon of 362-bp. The cDNA mixture was amplified by RT-PCR in a final volume of 50 μl, with 25 pmoles of each primer. Forty amplification cycles were performed consisting of denaturation at 95°C for 1 min (MMPs) or 3 min (gastrin and gastrin/CCK B receptor), annealing at 60°C (MMP and gastrin/CCK B receptor) or at 55°C (gastrin) for 1 min and polymerization at 72°C for 2 min. The amplification was terminated by a final extension at 72°C for 10 min. The PCR products (10 μl samples) were subjected to electrophoresis in 1% agarose gel previously stained with ethidium bromide and the bands were visualized under UV illumination. For negative controls, the reaction was performed without reverse transcriptase and, for positive controls, it was performed with β-actin specific primers.

In vitro cell invasion assays. Assays were performed as previously described (22, 23). Briefly, 8-μm pored Transwell filter chambers (Corning-Costar, Cambridge, MA) were used. The upper side of polycarbonate membranes was coated with Matrigel, a reconstituted basement membrane matrix (Becton Dickinson, Bedford, MA), diluted to 5 mg/ml. Cells (5×10^4) were seeded in the upper compartment containing FCS-free medium with 0.1% BSA (Sigma). G17-Gly was added to the same medium in the lower compartment at different concentrations, 0 (control) or 10^{-7} to 10^{-12} M. The chambers were incubated for 6 or 24 h at 37°C to allow the cells to migrate from the upper chamber towards the lower chamber. At the end of the experiment, the nonmigratory cells on the upper surface of the membranes were removed with a cotton swab without touching the lower surface. The cells which had migrated through the Matrigel + membrane barrier and were attached to the lower surface of the membrane were fixed in methanol and stained with toluidine blue. They were counted at high-power magnification (×400), using a calibrated ocular grid, in 10 different representative areas on each filter membrane. The mean value for each invasion assay was obtained by averaging mean values from 3 to 6 wells. Several independent sets of experiments were performed.

Zymography. Serum-free conditioned media were collected from normal culture or from upper Transwell compartments after invasion assays. Zymographies were performed as reported (22, 23). The absence of phenol red in the culture medium allowed measurement of protein concentration using the Biorad protein assay (Biorad, Hercules, CA). Proteins (40 μg loaded onto each lane) were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis containing 1 mg/ml of gelatin or casein. After protein migration, gels were stained for 1 h in 30% methanol/10% glacial acetic acid solution containing 1.5% (w/v) Coomassie blue then destained in the same solution in the absence of dye. Unstained areas corresponded to zones of MMP proteolytic activities. For some experiments, the digested bands were scanned, then their surface and densitometry were measured with the NIH Image 1.61/ppc program.

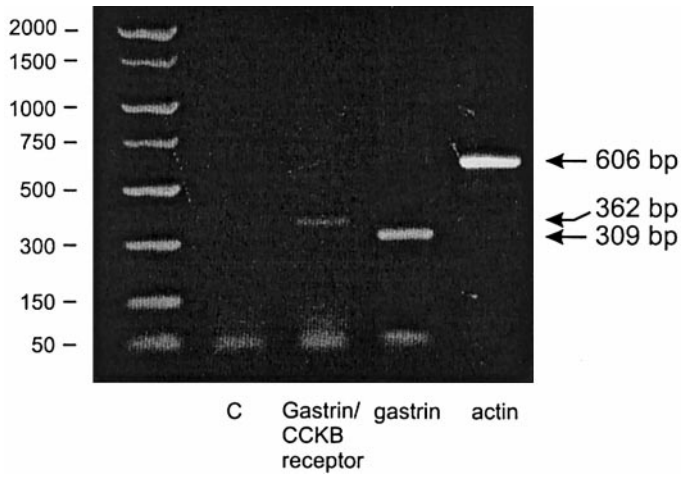

FIG. 1. RT-PCR from LoVo cell extracted RNA showing DNA amplified sequences with the expected size of 309 bp for gastrin and 362 bp for gastrin/CCK B receptor. Molecular weight markers are indicated on the left. The amplification by RT-PCR of a β-actin cDNA sequence generating a 606-bp DNA amplicon served as positive control. C, negative control performed without reverse transcriptase.

Statistical analysis. Values were expressed as means ± 1 SEM. Multiple comparisons were analysed by one-way ANOVA followed by the *t* test for comparison between two groups. The level of statistical significance was set at $P < 0.05$.

RESULTS

LoVo human colonic cells express gastrin, gastrin/CCK-B receptor, and MMP mRNAs and secrete MMP. Results of RT-PCR showed that LoVo cells expressed gastrin and gastrin/CCK B receptor mRNAs (Fig. 1).

Since the function of MMPs is to degrade the extracellular matrix and basement membrane, which may help cell migration through Matrigel, we analyzed the potential ability of LoVo cells to secrete MMPs. It was found that LoVo cells expressed mRNAs of MMP-1 collagenase, MMP-2 and MMP-9 gelatinases (Fig. 2A). Analysis of zymograms from their conditioned culture media confirmed the secretion of proteases by LoVo cells. Gelatin zymograms revealed two bands of proteolytic activities, one at 92 kDa, molecular weight corresponding to that of the latent form of MMP-9, the other at 62 kDa, which corresponds to the molecular weight of the active form of MMP-2. One proteolytic band was detected on casein zymograms at about 50 kDa which could correspond, among other MMPs of similar molecular weight, to MMP-1 (Fig. 2B). These 3 MMPs are known to be involved in colonic carcinogenesis; no attempt was made to detect the presence of other MMPs.

Gastrin 17-Gly promotes LoVo cell invasiveness. We then investigated the capability of G17-Gly of stimulating the invasiveness of LoVo cells *in vitro.* Representative photographs of Transwell membranes after

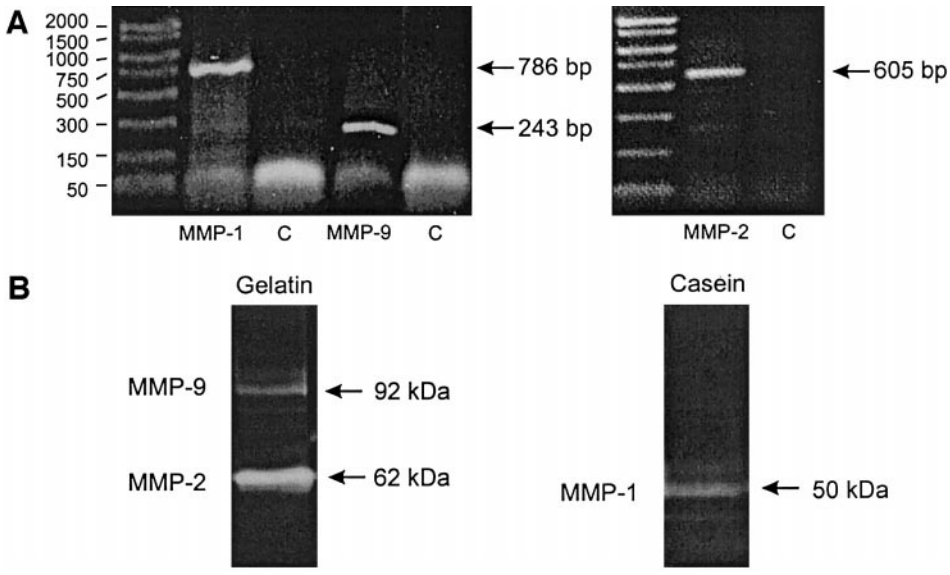

FIG. 2. Detection of MMPs. (A) RT-PCR from LoVo cell extracted RNA showing DNA amplicons of expected 786 bp for MMP-1, 605 bp for MMP-2, and 243 bp for MMP-9. Molecular weight markers are indicated on the left. C, control without reverse transcriptase. (B) Zymographies from conditioned media. Cells were FCS-deprived for 24 h and cultured in medium lacking phenol red. Forty micrograms of proteins were loaded onto each lane. Two bands on the gelatin gel indicated the presence of the latent form of MMP-9 at 92 kDa and the active form of MMP-2 at 62 kDa. One band at 50 kDa was detected on the casein gel, which could correspond to MMP-1.

different conditions of incubation are shown in Figs. 3A and 3B. Control LoVo cells cultured in FCS-free medium were spontaneously invasive and the numbers of invading cells increased with time, from 4.7 ± 1.5 cells to 30.4 ± 4.6 cells per ocular grid after 6 and 24 h incubation, respectively. When G17-Gly was added into the medium of the lower compartment, the invasiveness of LoVo cells was significantly enhanced ($P < 0.05$ to $P < 0.01$ in comparison with control cells), whatever the duration of invasion assay or the peptide concentration (Fig. 4). Thus, invading cells were about 3 times and 2.25 times more numerous after 6 and 24 h incubation in the presence of G17-Gly, respectively. In those assays, it was checked by careful microscopic examination that invading cells were all isolated (see Fig. 3A for instance). This assured us that results could indeed be attributed to an invasive-promoting effect of G17-Gly on LoVo cells and not, if hypothetically equal numbers of control and gastrin-treated cells had crossed the barrier, to a proliferative effect of G17-Gly.

MMP secretion is influenced by gastrin. Afterwards, we wished to investigate whether G17-Gly was able to modulate MMP secretion. In a first time, in order to test this hypothesis, we performed zymographies from some conditioned culture media collected at the end of invasion assays. There was an increase in the gelatinase expression in media of LoVo cells incubated in the presence of G17-Gly, as compared to media of control cells. This increase was confirmed by densitometric analysis and was concomitant to the stimulation of cell invasiveness (Fig. 5A). Second, cells were

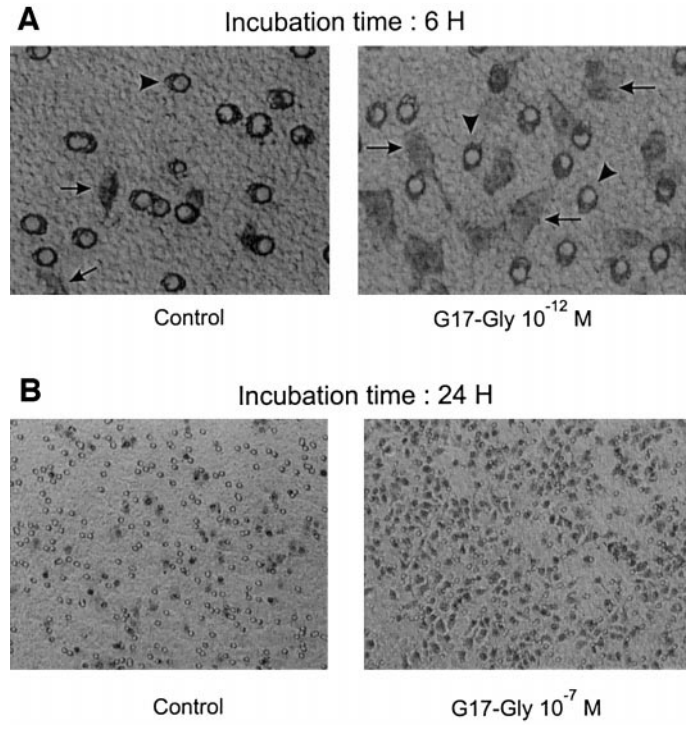

FIG. 3. Effect of 6-h incubation (A) or 24-h incubation (B) with G17-Gly on the invasiveness of LoVo cells in invasion chamber. Cells were seeded on Matrigel coating the upper side of the Transwell porous membrane and cultured in FCS-free medium. G17-Gly was added in the lower compartment. Each panel is a representative photograph of the lower side of membranes showing pores (arrow heads) and invading cells having migrated across Matrigel + membrane, some retaining elongated shape (arrows). (A) High magnification; (B) low magnification.

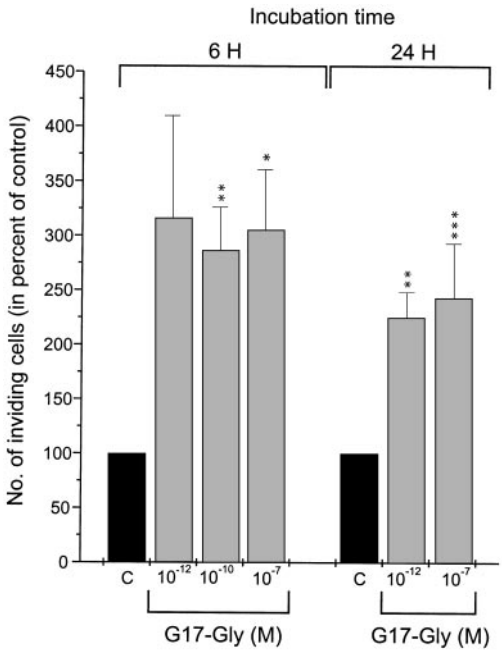

FIG. 4. Estimation of the number of invading cells after incubation of LoVo cells in the presence of G17-Gly for 6 or 24 h. Cell counts (mean 10 per well) were performed at 400 magnification. Each column represents the mean of assays performed in triplicate or sextuplicate. Results are expressed in percent of control values. Invading cells were 2.25 to 3 times more numerous in the presence of G17-Gly. C, control cells. * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$ as compared with control cells.

normally cultured on plastic flasks for 3–4 days in medium supplemented with FCS, then for 24 h in FCS-free medium. At the end of this period, two CCK-B receptor antagonists, CI-988 or YM222 (10^{-8} M), were added to freshly renewed FCS-free medium. After 24 h of culture, media were collected and zymographies then densitometric analysis of the digested bands obtained were performed. Figure 5B shows that the two molecules decreased the expression of MMP-2 and MMP-9 in media, indicating a diminution in the production of MMPs by the cells and, consequently, some degree of endogenous gastrin implication in that production.

DISCUSSION

Since the report of the growth-promoting effect of G-Gly on pancreatic cells by Seva *et al.* (12), the interest in studying the potential biological activities of incompletely processed forms of gastrin and their pathophysiological consequences has been renewed. That these forms may function as autocrine growth factors has emerged as a new concept. Our present findings provide the first evidence that the glycine-extended processing intermediate G17-Gly is capable of activating the invasion of extracellular matrix by human colonic cancer cells *in vitro.*

First of all, we found that LoVo cells expressed and released a specific subset of MMPs in their culture media. MMPs are key enzymes in tumoral invasion; their function being to degrade components of the extracellular matrix, the migration of LoVo cells across Matrigel, an artificial reconstituted basement membrane matrix, became theoretically possible. Then, we confirmed that LoVo cells express both the genes of gastrin (16, 20) and of gastrin/CCK B receptor (15, 20). Recently, some investigators detected noticeable amounts of G17-Gly in media of LoVo cells (16). In the current study, exogenous G17-Gly, originally tested in our model, stimulated the spontaneous invasiveness of

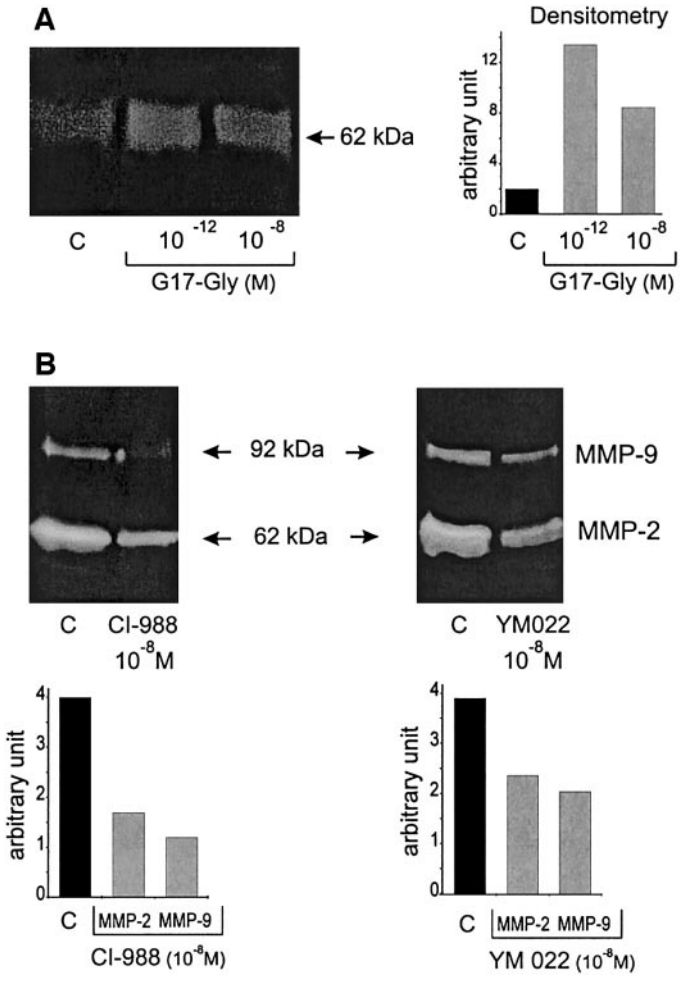

FIG. 5. Factors influencing MMP production. (A) Zymograms from conditioned culture media of Transwell upper compartments collected at the end of invasion assay. When G17-Gly was introduced into the FCS-free culture medium, increased cell invasion was associated with increased MMP-2 expression in this medium. This was confirmed by densitometric analysis of the digested bands. C, control cells. (B) Effect of CCK-B receptor antagonists. Cells were FCS-deprived for 24 h, then cultured again for 24 h in fresh FCS-free medium in presence of YM022 or CI-988 at 10^{-8} M and gelatin zymographies performed at the end of the experiment. The expression of MMP-2 and MMP-9 was decreased by the two antagonists. This was confirmed by densitometric analysis. C, control.

LoVo cells. Very interestingly, this promoting effect on the cell invasiveness was observed as early as 6 h after incubation of cells in the presence of G17-Gly, and at concentrations of the peptide within the physiological range (10^{-10} to 10^{-12} M). Also very interesting is to note that the maximal stimulating effect of G17-Gly on the proliferation of LoVo cells has been found to be about 140 (16) or 165% (15) of control values, whereas the promoting effect on the cell invasiveness was stronger, reaching 225–316% of control values. These findings suggest that the significant amounts of G-Gly secreted by some epithelial colonic tumors may have potential implications in their malignant progression.

Furthermore we found that, when LoVo cells were cultured onto Matrigel in the presence of G17-Gly, the increased number of invading cells was associated with an increase in gelatinase expression in conditioned media at the end of invasion assay. Finally, in normal culture conditions, incubation of LoVo cells with two selective gastrin/CCK-B receptor antagonists resulted in decreased MMP activity in the culture media. These two latter results would suggest that the small endogenous production of G17-Gly form by LoVo cells is able to influence the MMP production and that action appears mediated by gastrin/CCK-B receptors. However, until now, the precise receptors mediating, for instance, the trophic actions of progastrin and G-Gly remain uncertain and it is not clear if there is one or several (13, 15, 16, 29). As recently proposed by Dockray (30), it is conceivable that a modified version of the gastrin-CCK B receptor, perhaps generated by alternative splicing, mediates the effects of these immature forms of gastrin, but at present this idea is largely speculative. In the present work we used a very potent gastrin/CCK-B receptor antagonist, YM022. This compound displays a binding affinity in the nanomolar range for the gastrin/CCK B receptor and a 900 times higher selectivity for this receptor over the CCK A receptor (25). Moreover, it has been shown that YM022 inhibited the proliferative effect of G17-Gly on LoVo cells (15). Future studies should be carried out to try of determining which receptors mediate the new actions of G-Gly described here.

Focusing on the colon, G-Gly has been reported to exert growth-promoting effects on nontransformed and cancerous colonic cell lines *in vitro* (13, 15, 16) and human colonic cancer *in vivo* (19). Overexpression of either progastrin or G-Gly in transgenic mice has been found to result in increased cell proliferation in the colonic mucosa (17, 18). Recently, mice overexpressing progastrin have been shown to be predisposed for developing aberrant colonic crypt foci, adenomas and carcinomas in response to a chemical carcinogen (27, 28). Our current study extends the role of G17-Gly to colon cancer invasion.

In conclusion, we have shown that immature G17-Gly is involved in tumor cell invasiveness *in vitro*. Our results strongly suggest a novel potential physiological role for incompletely processed forms of gastrin in colonic cancer progression *in vivo*.

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