The C-terminal flanking peptide of progastrin induces gastric cell apoptosis and stimulates colonic cell division in vivo

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Abstract:
Progastrin (PG) is processed into a number of smaller peptides including amidated gastrin (Gamide), non-amidated glycine-extended gastrin (Ggly) and the C-Terminal Flanking Peptide (CTFP). Several groups have reported that PG, Gamide and Ggly are biologically active in vitro and in vivo, and are involved in the development of gastrointestinal cancers. CTFP is bioactive in vitro but little is known of its effects in vivo. This study investigated the bioactivity of CTFP in vivo in normal tissues using gastrin deficient (GASKO) mice and in two mouse models of cancer (SCID mice bearing xenograft tumors expressing normal or knocked-down levels of gastrin and a mouse model of hepatic metastasis). As with Ggly, CTFP treatment stimulated colonic proliferation in GASKO mice compared to control. CTFP also significantly increased apoptosis in the gastric mucosa of male GASKO mice. CTFP did not appear to effect xenograft growth or the incidence of liver metastases. This is the first demonstration that CTFP has specific biological activity in vivo in the colon and stomach.

Keywords: C-terminal flanking peptide, progastrin, Ggly, mouse, CRC, cancer

Abbreviations: CTFP, C-terminal flanking peptide; Ggly, glycine extended gastrin; Gamide, amidated gastrin; PG, progastrin; GASKO, gastrin knockout; AS, antisense; VO, vector only;
ECL, enterochromaffin-like; RPMI, Roswell Park Memorial Institute medium; DMEM, Dulbecco's Modified Eagle Medium; FBS, Fetal bovine serum; PBS, Phosphate buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Gy, Gray; Ci, curie; SEM, standard error of the mean

**Glossary:** INS-GAS, mice with elevated levels of amidated gastrin; MTI/G-gly, mice with elevated levels of glycine extended gastrin; hGAS, mice with elevated levels of progastrin; GASKO, mice with no forms of progastrin derived peptides; AGS, human gastric cancer cell line; LIM1839, human gastric cancer cell line; IMGE-5, non-transformed mouse gastric epithelial cell line; DLD-1, human colorectal cancer cell line; SW1222, human colorectal cancer cell line; HCT-15, human colorectal cancer cell line; MoCR, mouse colorectal cancer cell line
1. Introduction

Amidated gastrin (Gamide), which is synthesized predominantly in the G cells of the gastric antrum, is the fully processed gastric hormone derived from preprogastrin (Figure 1) [8]. Gamide was originally assumed to be the only biologically relevant and active form of progastrin (PG), as it acts on the cholecystokinin 2 receptor (CCK2R) to stimulate gastric acid secretion and proliferation in the stomach and proximal small intestine [8, 19, 22]. However, PG and several PG-processing intermediates, including glycine-extended gastrin (Ggly) and the C-Terminal Flanking Peptide (CTFP) of progastrin, are now known to also be biologically active. These non-amidated PG-derived peptides have negligible affinity for the CCK2R [1, 19, 32]. Putative receptors have been reported for PG [34], and glycine extended gastrin (Ggly) [39], but not yet for CTFP.

PG, Gamide, Ggly and CTFP were identified in the gastric antrum, plasma and colorectal cancer extracts [25, 35, 36]. Pauwels et al. reported that circulating levels of CTFP were similar to those of Gamide [31], however Smith et al. later demonstrated that CTFP was the predominant peptide in the antrum and circulation, with concentrations between 4- to 60-fold higher than Gamide, respectively [35]. A correlation between circulating Gamide and cancer was observed, with high Gamide associated with a 3.9 fold increased risk for developing colorectal carcinomas [36]. Immature gastrin peptides (non-amidated forms) were also identified in primary tumors and resected colorectal cancer [25, 35]. Ciccotosto et al. found elevated concentrations of PG-derived peptides in resected colorectal tumors and in the circulation of patients with colorectal carcinoma [3]. Many colorectal carcinomas express the gastrin gene; however they lack the enzymes to fully convert PG to Gamide, resulting in immature precursors [25]. CTFP was found to be the main PG form in resected colorectal tumors, but was not necessarily elevated in the plasma from patients with colorectal cancer compared to disease-free patients [35].

PG-derived peptides are linked to the development of cancer [10]. In vitro evidence demonstrates PG, Gamide, Ggly and CTFP act independently as growth factors in gastrointestinal cancer cell lines [1, 35], and CRC cells expressing antisense gastrin can lose their tumorigenic potential, which can be restored with exogenous PG [17]. PG stimulates proliferation [23, 33], while Gamide and Ggly activate both cell proliferation and migration [16, 22, 27, 28]. Ggly inhibits apoptosis, whereas Gamide may be anti- [2] or pro-apoptotic [6, 24], depending on the cell line studied. CTFP activates MAP-kinase signaling and stimulates both proliferation and migration in non-transformed gastric epithelial cells (IMGE-5) as well as stimulating proliferation in gastric (LIM1839) and colonic carcinoma cell lines (SW1222, HCT-15) [35]. Patel et al. showed that inhibition of apoptosis by CTFP in human gastric cancer (AGS) cells was dependent on the PI3-kinase pathway [30].

Mice over-expressing different forms of PG have significantly different phenotypes compared to wild type littermates. Mice with elevated Gamide levels (INS-GAS mice) have increased acid secretion, thickened oxyntic mucosa, and decreased apoptosis and increased proliferation in the gastric mucosa [24, 38], and are prone to develop gastric cancer.
spontaneously after reaching 20 months in age [12, 37]. Mice with increased circulating Ggly (MTI/G-gly [5, 20]) or PG (hGAS [38]) concentrations provide evidence that non-amidated forms of gastrin primarily act in the colon to stimulate mitosis and inhibit apoptosis [20, 38]. Experiments with mice over-expressing PG-derived peptides either endogenously or exogenously or with wild type mice treated with excess peptides has increased understanding of the potential roles PG-derived peptides may play in vivo. However, such mice are unable to define exactly which forms are responsible for specific activities, as different processing intermediates can also be found in the over-expressing mice. There are currently no mice that overexpress CTFP without overexpressing other PG peptides also.

Gastrin knockout (GASKO) mice provide an important model, since infusion of individual PG fragments in vivo allows comparisons with results identified in overexpressing mice [14, 20]. In the stomach, mice deficient in gastrin displayed decreased parietal and enterochromaffin-like (ECL) cells, and had a thinner fundic mucosa and impaired acid secretion. In the colon, mucosal height and proliferation were also decreased. The GASKO phenotype could be fully or partially rescued when the mice were crossed with mice over-expressing different forms of PG or treated with exogenous peptides [10, 20]. Gamide infusion was able to restore the altered gastric phenotype [6, 14, 20], while PG and Ggly appeared to predominately act in the colon [14].

Ottewell et al. compared the effects of PG, Ggly, PG55-80, PG72-80 and CTFP (PG75-80) treatment on colonic mitosis. When gastrin-deficient mice were treated with peptides 30 min prior to exposing them to 8 Gy of γ-irradiation, PG or PG55-80 (but not Ggly, PG72-80 or CTFP) could stimulate colonic mitosis 4.5 h post irradiation [29]. The contrast between this study and previous findings where Ggly injection alone could significantly increase colonic proliferation in GASKO mice [20] suggests that longer-term infusion of CTFP may be needed to see a biologically relevant response. The long term effects of CTFP on the stomach and colon of GASKO mice have not been determined.

While the in vitro work has provided strong evidence that CTFP has growth factor activities similar to other bioactive PG-derived peptides, the preliminary in vivo data was inconclusive in establishing whether or not CTFP was also active in animals. Here, we have examined the in vivo effects of CTFP on normal tissue using GASKO mice and two mouse models of cancer (xenograft and metastasis). Xenograft mouse models are useful to determine if any cell line has the ability to develop into a tumor in vivo. Ferrand et al. found that the human CRC cell line DLD-1 transfected with antisense gastrin (AS), in contrast to DLD-1 cells transfected with vector only (VO), could not establish tumors in vivo [9]. Metastases models aid the study of cancer spreading from a primary site to a secondary site, via the blood or lymph. Kuruppu et al. developed a hepatic metastasis mouse model using CBA mice injected with MoCR CRC cells to study cell migration to the liver and subsequent development into hepatic tumors [21]. In both models mutated and scrambled forms of CTFP were studied in parallel to determine off target non-specific activities for CTFP and to establish its specificity. Here we have resolved the question of whether CTFP has biological relevance in vivo as we provide the first evidence of CTFP promoting
apoptosis in the stomach and proliferation in the colon in mice. However, the observation that CTFP was unable to restore xenograft development in mice injected with DLD-1 AS cells, or to modify the metastatic potential of CRC cells to the liver, indicates that CTFP activity may be more pronounced in normal mucosa.
2. Material and Methods

2.1 Reagents

Amidated human gastrin (Gamide) and human glycine extended gastrin (Ggly) were synthesized by Auspep (Melbourne, AUS). All other PG-derived peptides used in this study were synthesized by Mimotopes (Melbourne, AUS) (see Table 1). All consumables were purchased from Sigma Aldrich (Castle Hill, AUS) unless otherwise noted.

2.2 Cell culture

The human colon cancer, DLD-1, and human stomach cancer, AGS, cell lines were obtained from ATCC. DLD-1 cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 7.5% fetal bovine serum (FBS). AGS cells were grown in Roswell Park Memorial Institute medium (RPMI) containing 7.5% FBS and 20 mM HEPES. Cells were grown at 37°C in 5% CO₂. The mouse colon cancer cell line, MoCR, was kindly provided by Prof C Christophi (Department of Surgery, Melbourne, AUS) and maintained through serial passaging in vivo as described [21]. For in vitro work, MoCR cells were grown at 37°C in 5% CO₂ in RPMI containing 10% FBS.

2.3 Proliferation

Cell proliferation was determined using ³H-thymidine incorporation as previously reported [35]. Cells were seeded (5 x 10^3/well) in a 96 well plate in 100 μl full medium. The following day, cells were washed with serum free medium and incubated for a further 24 h in serum free medium containing treatments and 10 μCi/mL [methyl-³H]-thymidine. Cells were harvested using a NUNC cell harvester and ³H-thymidine incorporation determined using a β-counter (Packard, Meriden, USA).

2.4 Cell migration assays

The wound healing assay was performed as described previously [35]. MoCR cells were grown to 90% confluence on a 12 well plate in full medium. Cells were incubated for 24 h in serum free medium and a straight line was scratched through the monolayer of cells with a 20 μl pipette tip. The cell were washed twice with PBS and then treated with Gamide or CTFP in RPMI medium with 1% FBS. 12 well plates were marked to allow photographs of the same wound regions to be taken at 0, 17, 24 and 42 hours post treatment using a Nikon Coolpix 5000 with a microscope coupler (Nikon, Lidcombe, AUS). Wound size was calculated by averaging 6 different measurements across the wound site.

The cell migration/invasion assay was adapted from [18]. Cell culture insert membranes (8 μm pore size, Becton Dickinson, North Ryde, AUS) were coated on the underside with 3 μg/ 30 μl of human fibronectin and placed in a 24-well plate. The top side of these membranes were seeded with 5 x 10^4 cells in 100 μl of 0.1% bovine serum albumin
(BSA) in serum free DMEM (DLD-1) or RPMI (AGS). Bottom chambers were filled with 600 μl of matching media and incubated at 37°C in 5% CO₂ for an additional 24 h in the presence or absence of different treatments. Membranes were collected after 24 h, first wiping cells from the top membrane layer with a cotton swab and fixing and staining the underside with Quick-Dip (Fronine, Sydney, AUS). Cells on the lower surface of the membrane were mounted onto cover slips and imaged as 24 pictures/ membrane with a Nikon Coolscope 2 (Nikon, Lidcombe, AUS) at 20x. Cells were then counted using Image ProPlus (Media Cybernetics Inc., Silver Spring, USA).

2.6 Cell survival

Cells were seeded in full medium into 10 cm Petri dishes (1 x 10⁶/dish) and grown overnight at 37°C in 5% CO₂. Cells were washed with serum free medium and replaced with serum free medium containing different PG-derived peptides for an additional 48 h. All non-attached cells were discarded after 48 h and adherent cells were harvested with trypsin and re-suspended in phospho-buffered saline (PBS) (1 x 10⁶/mL). Cells were stained with propidium iodine and run through a BD FACSCanto II with BD FACSDiva software (BD Biosciences, San Jose, USA) to measure positive stained cells as an indication of cell death.

2.7 Murine models

All animal studies were approved by the Austin Health Animal Research Ethics Committee. Gastrin knockout (GASKO) mice [13] were bred and housed at the Austin Health BioResource Facility. Male and female mice aged 10-12 weeks old were injected daily for 20 days (5 days a week for 4 weeks) with 100 μl/injection of PBS or PG derived peptides in PBS. Treatment groups were 1 x day PBS (control), 1 x day Ggly (75 nmoles), and 2 x day CTFP, mutant CTFP or scrambled CTFP (50 nmoles per injection). At the end of the experiment animals were sacrificed and blood and tissues removed. Stomach and colon tissues were fixed in 10% formalin, embedded and stained according to the following manufacturer’s protocols. In brief, crypt height was determined from H&E stained sections, proliferation using anti-ki67 staining (Neomarkers, ThermoScientific, Scoresby, AUS), and apoptosis using anti-caspase 3 staining (R&D Systems, Minneapolis, USA). Secondary staining used the DAKO anti-rabbit secondary antibody kit (DAKO Australia Pty. Ltd., Campbellfield, AUS). Stained sliced sections of tissue were imaged using a Nikon Coolscope (Nikon) and analysed on ImageProPlus 6.0 (Media Cybernetics Inc.). Height, proliferation and apoptosis are given as average ratio of positive cells (stained) to negative cells (unstained) per crypt in each group.

Xenograft tumors were grown on the side flanks of SCID mice (6-8 weeks old) following subcutaneous injection of DLD-1 cells (3-5 x 10⁶ in 50 µl RPMI with 10% FBS) stably transfected with control (VO) or antisense gastrin (AS) as described previously [9, 17]. Mice were injected intra-peritoneally with PBS, Ggly (75 nmoles) or CTFP (100 nmoles) daily for 21 days. Callipers were used to measure tumor volume (= (length x width²)/2) every second
Liver metastases were induced as described previously [21]. Male CBA mice (6-8 weeks) were anaesthetized and an incision made below the left rib cage to expose the spleen. Mice were given an intra-splenic injection of 50 µl MoCR cell suspension (0.5 x 10^6 cells/ml). A splenectomy was performed 2 min post injection and the wound was sutured. Mini-osmotic pumps (Model 1002, Alzet, CA, USA) were used to infuse Gamide, Ggly or CTFP for 11 days at a rate of 2.5 nmol/kg/h (Gamide and Ggly) or 10 nmol/kg/h (CTFP). Peptides were suspended in PBS containing 0.1% BSA. Primed pumps were inserted into mice 10 days following the induction of liver metastasis, at a time that corresponded to the onset of exponential growth of liver metastases in this model [21]. 6-7 mice per group were sacrificed 21 days post induction and livers excised and fixed in 10% formalin for 48 h and preserved in 50% ethanol prior to processing. Quantitative stereology was used to measure liver metastases. The liver was sliced into 1.5 mm transverse sections using a tissue fractionator and every second slice was imaged with a digital camera (Nikon Coolpix 5000) for stereological analyses using Image Pro Plus (MediaCybernetics). Liver metastases and total volumes were determined.

2.8 Statistical Analysis

Values are given as the average ± standard error of the mean (SEM). Results were analysed using one-way analysis of variance (ANOVA) and significance determined with a Bonferroni post-hoc test using SigmaStat software (SPSS, Chicago, USA). Values were deemed significant when p < 0.05.

3. Results

3.1 CTFP stimulates AGS cell proliferation

A ^3^H thymidine incorporation assay was used to determine if PG-derived peptides could stimulate mitosis in human gastrointestinal carcinoma cell lines. Proliferation rates in the human gastric carcinoma cell line AGS were significantly increased by CTFP at 100 nM (Figure 2A). The observation that mutant and scrambled forms of CTFP did not enhance proliferation up to 100 nM suggested that the stimulation was a specific effect. As AGS cells do not have a CCK2 receptor the absence of any effect of Gamide (1 nM) was not unexpected. Ggly did not significantly stimulate proliferation of AGS cells, but did significantly stimulate proliferation in the human colon carcinoma cell line (DLD-1) at 10 nM (Figure 2B). CTFP, and its mutant and scrambled forms, did not stimulate proliferation of DLD-1 cells. Previously, Copps et al. compared binding and proliferation rates of DLD-1 cells treated with truncated Gamide/Ggly peptides and found the N-terminal region to be most important for activity [4].

3.2 CTFP stimulates AGS and DLD-1 cell migration/invasion
The effect of PG-derived peptides on cell migration and invasion was determined in a modified Boyden chamber with the underside of the membrane coated with fibronectin. With the human gastric carcinoma cell line AGS, CTFP enhanced cell migration at both 10 nM and 100 nM (Figure 3A). Mutant and scrambled CTFP, Gamide and Ggly had no effect. Previously Noble et al. showed that Gamide stimulated AGS cell migration using a wound healing assay [26], while Smith et al. found that both CTFP and Ggly could enhance wound healing in IMGE-5 gastric cells [35]. With the human colonic carcinoma cell line DLD-1, CTFP and Ggly both significantly increased cell migration at 10 nM and 100 nM, respectively (Figure 3B). No significant increase was observed with mutant or scrambled forms of CTFP or with Gamide.

3.3 CTFP protects against serum-starvation induced AGS cell death

CTFP has previously been reported to inhibit apoptosis in AGS cells at 10 nM and 100 nM [30]. Mutant and scrambled forms of CTFP were used to determine if this activity was specific to CTFP, or was a non-specific effect. While CTFP (100 nM) decreased cell death, mutant and scrambled CTFP had no effect at the same concentration (Figure 4A). None of the PG-derived peptides tested were able to protect DLD-1 cells from cell death induced by serum starvation (Figure 4B).

3.4 CTFP increased gastric apoptosis in male GASKO mice

The effects of CTFP, mutant CTFP, scrambled CTFP and Ggly on gastric mucosal thickness, proliferation and apoptotic rates were examined in gastrin knockout mice. No significant difference in crypt height (Figure 5A), or in cell proliferation as measured by Ki67 staining (Figure 5B), was found in the gastric mucosa between animals treated with CTFP, mutant CTFP, scrambled CTFP or Ggly. CTFP treatment significantly increased gastric apoptosis as measured by caspase 3 staining in male (Figure 5C), but not in female (Figure 5D), GASKO mice. A sex difference has previously been reported by Ottewell et al. for colonic apoptosis 24 h post AOM treatment in GASKO mice crossed with hGAS mice, with female mice having significantly less colonic apoptosis compared to male mice [29]. Additionally, Helicobacter pylori infected male INS-GAS mice over-expressing Gamide had significantly higher rates of gastric cancer than female mice [11].

3.5 CTFP stimulated colonic proliferation but not apoptosis

The effects of CTFP, mutant CTFP, scrambled CTFP and Ggly on colonic mucosal thickness, proliferation and apoptotic rates were examined in gastrin knockout mice. Crypt height (Figure 6A) was unchanged by treatment. In contrast, a significant increase in proliferation was observed in the colons from GASKO mice infused with CTFP or Ggly compared to saline control (Figure 6B, D). This finding, which was not observed for mutant or scrambled forms of CTFP, is the first demonstration that CTFP is significantly biologically active in vivo, and that its activity is not a small molecule off target effect. Although GASKO mice injected with Ggly had a significant decrease in apoptotic indices (Figure 6C, E), there
was no significant decrease in rates of apoptosis in animals treated with CTFP, mutant CTFP or scrambled CTFP.

3.6 **CTFP could not rescue tumorigenicity in DLD-1 AS cells**

Previously, DLD-1 cells transfected with antisense gastrin (AS) were unable to develop into xenograft tumors [9]. When exogenous PG was used in vitro, cells regained similar phenotypes as their wild type or vector only (VO) controls [17]. The ability of CTFP or Ggly to rescue the lack of growth of DLD-1 AS cells in vivo was determined in a mouse xenograft model where DLD-1 AS tumor growth was compared to DLD-1 VO control. Only VO cells developed into tumors, and neither Ggly nor CTFP treatment could restore tumor growth with AS cells. In addition, no difference in size (Figure 7A), proliferation rates (Figure 7B) or apoptotic rates (Figure 7C) were observed in the VO tumors treated with these peptides compared to control.

3.7 **CTFP stimulated mouse CRC cell line (MoCR) migration in vitro**

The ability to enhance the migration of MoCR cells was examined in vitro using the wound healing assay. A wound was scratched through a monolayer of MoCR cells followed by treatment with CTFP and Gamide over 42 hours. Both Gamide and CTFP significantly increased the distance the cells had migrated into the gap at 17, 24 and 42 h compared to the untreated control (Figure 8). This observation indicated that these cells were sensitive to these PG-derived peptides and could possibly increase their migration in vivo.

3.8 **CTFP did not enhance MoCR metastasis in vivo**

A hepatic metastasis model was used to determine the effects of PG-derived peptides on CRC-derived liver metastases. Mice were infused with PBS, Gamide, Ggly or CTFP. Stereological analyses were performed to compare tumor volume to normal liver volume and to calculate per cent liver metastases (Table 2). All groups had similar metastatic rates, and there was no significant difference between tumor load and liver sizes for groups treated with PG-derived peptides compared to control.

4. **Discussion**

The progression from normal tissue to cancer requires cells to undergo several changes that include overcoming a finite number of cell divisions and contact inhibition as well as subsequently gaining the ability to migrate and the capacity to prevent programmed cell death. Growth factors can stimulate these activities in cells, resulting in pro-carcinogenic growth [15]. In the present study we examined the specific activity of CTFP as a growth factor in vitro and in vivo by comparing it to the PG-derived peptides Gamide and Ggly, and to mutated and scrambled forms of CTFP (Table 1).

In vitro assays were used to initially characterize the growth factor activities of PG-derived peptides. CTFP was able to stimulate proliferation in the gastric carcinoma cell line
AGS (Figure 2A), but not in the colonic carcinoma cell line DLD-1 (Figure 2B), while enhancing migration in both AGS and DLD-1 cells (Figure 3). Patel et al. showed that inhibition of apoptosis by CTFP in AGS cells was dependent on the PI3-kinase pathway [30]. CTFP and Ggly both decreased cell death in AGS cells (Figure 4A), but did not significantly affect survival of DLD-1 cells (Figure 4B). The differences observed in these cell lines derived from different parts of the gastrointestinal tract may be useful to later explore the various pathways by which CTFP and Ggly activate their biological activities. Importantly, mutant and scrambled forms of CTFP were inactive in vitro in the proliferation (Figure 2), migration (Figure 3) and cell death (Figure 4) assays. Their lack of activity strongly indicates that the observed effects of CTFP in vitro are specific and not due to a small molecule off target effect, which has been a criticism of similar studies with other unrelated small molecules.

Limited experiments have reported effects of CTFP in vivo. While Gamide stimulated gastric histamine release in male Wistar rat stomachs ex vivo, CTFP and PG72-80 could not, indicating that neither peptide acted through the CCK2 receptor [7, 32]. Short term infusion of human forms of Ggly, CTFP, and PG72-80 also failed to enhance mitotic proliferation rates in mice 4.5 h post γ-irradiation, while PG and PG55-80 stimulated proliferation in the same experiment [29]. This result may be due to the short duration of treatment, as proliferative effects of Ggly were observed in colons from mice over-expressing Ggly (MTI/Ggly) or when GASKO mice were infused with Ggly for 2 weeks [20]. It is possible that the stimulatory effect observed for PG55-80 resulted from the combination of both Ggly and CTFP and that longer term treatment may reflect this synergy as both Ggly and CTFP alone may need more time or higher doses to produce an effect. Therefore we treated GASKO mice with CTFP, Ggly or PBS for 4 weeks to determine if apoptosis or proliferation rates in normal tissues would change. CTFP significantly enhanced gastric apoptosis in male mice (Figure 5C), but not in female mice (Figure 5D). This result was unexpected as in vitro data demonstrated CTFP possessed pro-survival activity in human gastric cancer cells (Figure 4A; [30]). However, similar findings have been observed for Gamide, where anti- [2], no [24] or pro-apoptotic [6, 24] effects have been reported in different cell lines. Cui et al. found Gamide, like CTFP, was pro-apoptotic in gastric epithelial cells in GASKO mice [6]. The difference between in vitro and in vivo results may be due to the degree of differentiation found in normal versus cancer cell types or the complexity that whole organisms introduce compared to simple cell culture systems. It is possible Gamide and CTFP exert the pro-apoptotic activity on a shared downstream pathway. Unlike Ggly, CTFP had no effect on apoptosis in the GASKO mouse colon (Figure 6C). The observation that Ggly significantly decreased colonic crypt apoptosis in GASKO mice (Figure 6C) supports previous work indicating Ggly could protect from induced cell death (trend in Figure 4B; [27]).

Gamide, but not Ggly, stimulated gastric proliferation in GASKO mice [20]. Conversely, although the observation that CTFP stimulated proliferation in AGS cells (Figure 2A) suggested that it might act on gastric proliferation in vivo, CTFP had no effect on gastric proliferation in mice (Figure 6C). AGS cells do not express the CCK2R and as expected did not respond to Gamide treatment in this study. CTFP and Ggly both significantly increased
colonic crypt proliferation rates in GASKO mice (Figure 6B), consistent with previous work in CRC cell lines [35]. Mutant and scrambled forms of CTFP had no activity in any of the in vitro or in vivo assays. Our GASKO mice data provides the first evidence that CTFP has growth factor activities in vivo.

After establishing that CTFP had specific activity in normal mouse mucosa, two mouse cancer models were used to determine if CTFP could alter the growth parameters of xenograft tumors or CRC liver metastasis in vivo. Clones of the CRC cell line DLD-1 transfected with antisense gastrin (AS) or vector only (VO) control were grown as xenografts in immune deficient mice to test if CTFP could restore the lack of growth in the AS cells and if CTFP could alter the tumor growth in the VO tumor xenograft. While both CTFP and Ggly have growth factor activities, these peptides alone were not sufficient to restore tumorigenicity of DLD-1 AS cells in vivo. CTFP and Ggly treatments also did not affect the size, proliferation or apoptotic rates in DLD-1 VO xenograft tumors compared to control mice. The hepatic metastasis model allowed us to determine if the increased mouse CRC (MoCR) migration observed in vitro in response to Gamide and CTFP (Figure 8) correlated with enhanced MoCR migration in vivo. Neither Gamide, Ggly nor CTFP had any significant effect compared to control (Table 2). While active individually in vitro and in GASKO mice, it is possible that these peptides alone are not sufficient to further alter growth parameters in transformed cancer cells. It is also possible that the endogenous levels of PG-derived peptides circulating in the immune deficient and CBA mice would be sufficient to mask potential changes that may be more pronounced in mice lacking these peptides. Further work to explore this possibility will be required.

5. Conclusion

CTFP had significant stimulatory effects on apoptosis in the stomach and on proliferation in the colon in GASKO mice. This is the first demonstration of biological activity of CTFP in vivo. Increased colonic proliferation is an early step in the progression of normal tissue to cancer [15], and hence CTFP may contribute to an environment that favours CRC development in vivo. However, no significant CTFP activity was observed in two cancer models, representative of the growth and metastasis of established tumors. Determining how CTFP increases gastric apoptosis in male GASKO mice and enhances proliferation in both male and female GASKO mice is ongoing work in our laboratory and should lead to identification of the CTFP receptor and of independent and shared signaling pathways for the different PG-derived peptides.
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Figure Legends

Figure 1. Processing of human progastrin in the antral G cell. Preprogastrin, the precursor to progastrin, is 101 amino acids long. Progastrin translocates to the trans-Golgi network after its signal sequence (-21 to 0) is cleaved in the endoplasmic reticulum. Progastrin undergoes modifications (sulfation or phosphorylation) and cleavage of the modified peptide by the endopeptidase prohormone convertase (PC) releases CTFP. Carboxypeptidase E removes the C-terminal arginines from the immature gastrins. PC further cleaves glycine extended gastrin-34 (G34-gly) to Ggly (G17-Ggly), both of which can be amidated by peptidyl α-amidating mono-oxygenase (PAM) to give Gamide (17 or 34).

Figure 2. CTFP stimulates proliferation of AGS cells. Proliferation of human gastric (AGS, A) and colonic (DLD-1, B) cancer cell lines was measured in vitro using the 3H thymidine incorporation assay for 24 h. Cells were treated with 5% FBS or the indicated concentrations of the human (h) forms of the following progastrin-derived peptides: Gamide (hGA), Ggly (hGG), C-terminal flanking peptide (hCTFP), mutated CTFP (hMut), scrambled CTFP (hScr). Data are the mean ± SEM from 3 or more experiments, each in triplicate: p < 0.05 *, p < 0.01 **.

Figure 3. CTFP stimulates migration of AGS and DLD-1 cells. Cell migration of human gastric (AGS, A) and colonic (DLD-1, B) cancer cell lines in vitro was measured with a transwell assay. Cells were treated in serum-free medium without or with the indicated concentrations of the following progastrin-derived peptides: Gamide (hGA), Ggly (hGG), C-terminal flanking peptide (hCTFP), mutated CTFP (hMut), scrambled CTFP (hScr). Data are the mean ± SEM from 3 or more experiments, each in triplicate: p < 0.05*, p < 0.01**. The
right panel (C) shows representative pictures of transwell membranes for the AGS cell line. Images of migrating DLD-1 cells were similar.

Figure 4. CTFP reduces the induction of cell death by serum starvation. Human gastric (AGS, A) and colonic (DLD-1, B) cancer cell lines were serum starved and treated with peptides in vitro. Treatment groups contained serum starved medium without or with the indicated concentrations of the following progastrin-derived peptides: Gamide (hGA), Ggly (hGG), C-terminal flanking peptide (hCTFP), mutated CTFP (hMut) and scrambled CTFP (hScr). Data are the mean ± SEM from 3 or more experiments and were normalized so that untreated control equalled 1, p < 0.05*.

Figure 5. Effect of progastrin-derived mouse (m) peptides on the stomachs of gastrin KO mice. Mice were treated daily for 4 weeks with PBS, 75 nmoles mGgly (mGG), 100 nmoles mCTFP, 100 nmoles mutant mCTFP (mMut), or 100 nmoles scrambled mCTFP (mScr). (A) Gastric mucosal crypt height was measured for male and female gastrin KO mice after staining with H&E. Crypt heights were not significantly changed. (B) Stomach crypt proliferation rates were determined using anti-ki67 staining. (C, D) Apoptosis was determined in gastric crypts from male (C) and female (D) mice using anti-caspase 3 staining and normalized to the value for PBS-treated mice of the appropriate gender. Cells positively stained with Ki67 or caspase 3 antibodies were compared to the number of haematoxylin counter-stained cells in each crypt. Data are shown as average of stained cells per crypt + SEM, p < 0.05*.

Figure 6. Effects of progastrin-derived mouse (m) peptides on the colons of gastrin KO mice. Mice were treated daily for 4 weeks with PBS, 75 nmoles mGgly (mGG), 100 nmoles mCTFP, 100 nmoles mutant mCTFP (mMut), or 100 nmoles scrambled mCTFP (mScr). (A) Colonic crypt height was measured after staining with H&E. Crypt heights were not significantly changed. (B) Colonic crypt proliferation rates were determined using anti-ki67 staining. (C) Apoptosis was determined in colonic crypts using anti-caspase 3 staining and normalized to the value for PBS-treated mice. The results are shown for male and female animals combined. Data are shown as average of normalized stained cells per crypt ± SEM, p < 0.5*, p < 0.01**. (D) Representative images of colonic crypts after ki-67 staining (brown), counterstained with haematoxylin (blue). (E) Representative images of colonic crypts after caspase-3 staining (brown), counterstained with haematoxylin (blue).

Figure 7. Effect of progastrin-derived peptides in a xenograft model. Mice were xenografted with DLD-1 cells expressing normal (vector only, VO) or decreased levels of progastrin (antisense, AS). Only VO tumors were able to grow into xenografts. Mice were treated daily for 4 weeks with PBS, 75 nmoles mGgly (mGG) or 100 nmoles mCTFP. (A) Tumor growth was measured for 21 days, but no significant difference in size was observed. (B) Tumor proliferation rates were determined using anti-ki67 staining. (C) Tumor apoptosis was determined using anti-caspase 3 staining. (D) Representative pictures of tumor sections stained with ki67, caspase 3 or IgG (control). No significant difference was observed compared to the PBS treated control.

Figure 8. Effect of progastrin-derived peptides on migration of the MoCR cell line. A monolayer of MoCR mouse CRC cells was scratched to create a wound. Cells were treated
without and with 10 nM Gamide or 10 nM mCTFP to determine if these PG-derived peptides could modify migration into the wound. (A) Wound sizes were significantly smaller after 17, 24 and 42 h treatment with GA or CTFP compared to control. Data are the mean ± SEM from 3 experiments, each in triplicate: p < 0.01**. (B) Representative images depicting wound closing.
The C-terminal flanking peptide of progastrin induces gastric cell apoptosis and stimulates colonic cell division in vivo

Highlights:
- The C-terminal flanking peptide (CTFP) of progastrin is bioactive in vitro.
- CTFP increases colon proliferation in vivo.
- CTFP is pro-apoptotic in male mouse stomachs.
- CTFP did not enhance xenograft or metastatic tumor growth in vivo.
Figure 1

Endoplasmic reticulum

Trans-Golgi network and immature secretory vesicles

Secretory granules

PREPROGASTRIN

-21 0 1

Signal peptidase

Tyrosyl-protein sulfotransferase

Casein-like kinase

PROGASTRIN

1 36 37

PC

37 54 55

G34-GLY

55 71

PC

G17-GLY

37 54 55

G34-AMIDE

NH2

G17-AMIDE

NH2

PC

PAM

Gly

Gly

Gly

Gly

Casein-like kinase

Tyrosyl-protein sulfotransferase

Signal peptidase
Figure 2

A  AGS

B  DLD-1

\[ \text{\textsuperscript{3}H} \text{Thymidine Incorporation (%)} \]

<table>
<thead>
<tr>
<th></th>
<th>AGS</th>
<th>DLD-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>FBS (5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hGA (1nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hGG (10nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hCTPP (100nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hMuK (100nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>hScr (100nM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

** indicates statistical significance.
Figure 3

A  AGS

![Bar graph showing cell migration percentages for different treatments in AGS cells.]

B  DLD-1

![Bar graph showing cell migration percentages for different treatments in DLD-1 cells.]

C  Control and treated cells under microscope.

- **: p < 0.01
- *: p < 0.05

Treatments:
- Control
- hGA (1nM)
- hGG (10nM)
- hCTFP (100nM)
- hMut (100nM)
- hScr (100nM)
Figure 3 black and white

A

AGS

B

DLD-1

C

Control

hGA (1nM)

hGG (10nM)

hCTFP (100nM)

hMut (100nM)

hScr (100nM)
Figure 4

(A) AGS

(B) DLD-1
Figure 5

A  Crypt Heights

B  Proliferation

C  Apoptosis (Male)

D  Apoptosis (Female)
**Figure 6**

**A** Crypt Heights

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Crypt Height (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>160</td>
</tr>
<tr>
<td>mGG</td>
<td>180</td>
</tr>
<tr>
<td>mCTFP</td>
<td>200</td>
</tr>
<tr>
<td>mMut</td>
<td></td>
</tr>
<tr>
<td>mScr</td>
<td></td>
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</tbody>
</table>

**B** Proliferation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ki67 Stained Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>mGG</td>
<td><strong>20</strong></td>
</tr>
<tr>
<td>mCTFP</td>
<td><strong>20</strong></td>
</tr>
<tr>
<td>mMut</td>
<td></td>
</tr>
<tr>
<td>mScr</td>
<td></td>
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</table>

**C** Apoptosis

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caspase 3 Stained Cells (%)</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>mGG</td>
<td><strong>1.2</strong></td>
</tr>
<tr>
<td>mCTFP</td>
<td><strong>1.4</strong></td>
</tr>
<tr>
<td>mMut</td>
<td></td>
</tr>
<tr>
<td>mScr</td>
<td></td>
</tr>
</tbody>
</table>

**D** Ki67 colon

- PBS
- mGgly
- mCTFP
- mMut
- mScr

**E** Caspase 3 colon

- PBS
- mGgly
- mCTFP
- mMut
- mScr

* denotes statistical significance compared to PBS.
Figure 6 black and white

**A** Crypt Heights

<table>
<thead>
<tr>
<th></th>
<th>Crypt Height (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>mGG</td>
<td></td>
</tr>
<tr>
<td>mCTFP</td>
<td></td>
</tr>
<tr>
<td>mMut</td>
<td></td>
</tr>
<tr>
<td>mScr</td>
<td></td>
</tr>
</tbody>
</table>

**B** Proliferation

<table>
<thead>
<tr>
<th></th>
<th>Ki67 Stained Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>mGG</td>
<td>**</td>
</tr>
<tr>
<td>mCTFP</td>
<td></td>
</tr>
<tr>
<td>mMut</td>
<td></td>
</tr>
<tr>
<td>mScr</td>
<td></td>
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</tbody>
</table>

**C** Apoptosis

<table>
<thead>
<tr>
<th></th>
<th>Caspase 3 Stained Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td></td>
</tr>
<tr>
<td>mGG</td>
<td>**</td>
</tr>
<tr>
<td>mCTFP</td>
<td></td>
</tr>
<tr>
<td>mMut</td>
<td></td>
</tr>
<tr>
<td>mScr</td>
<td></td>
</tr>
</tbody>
</table>

**D** Ki67 colon

- PBS
- mGgly
- mCTFP
- mMut
- mScr

**E** Caspase 3 colon

- PBS
- mGgly
- mCTFP
- mMut
- mScr

Figure 6 shows the effects of different treatments on crypt heights, proliferation, and apoptosis in the colon. The graphs indicate significant differences in these parameters across various conditions, with asterisks marking statistical significance.
Figure 7

A. Tumour Growth

B. Proliferation

C. Apoptosis

D. Immunohistochemistry
Figure 7 black and white

**A** Tumour Growth

![Graph showing tumour growth over time withPBS, mGG, and mCTFP as treatments.]

- **PBS**: Open circle with a line and error bars.
- **mGG**: Black triangle with a line and error bars.
- **mCTFP**: Dark grey X with a line and error bars.

Day: 3, 4, 7, 9, 11, 14, 16, 18, 21

**B** Proliferation

![Bar chart comparing ki67 stained cells (%).]

- **PBS**: White bar with a line and error bars.
- **Ggly**: Black bar with a line and error bars.
- **CTFP**: Grey bar with a line and error bars.

**C** Apoptosis

![Bar chart comparing Caspase 3 stained cells (%).]

- **PBS**: White bar with a line and error bars.
- **Ggly**: Black bar with a line and error bars.
- **CTFP**: Grey bar with a line and error bars.

**D** Immunohistochemistry images

- **IgG**: White and grey images.
- **ki67**: White and grey images.
- **Caspase 3**: White and grey images.
Figure 8

A

Wound Healing Assay

<table>
<thead>
<tr>
<th>Wound size (%)</th>
<th>Control</th>
<th>Gamide</th>
<th>CTFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>42 hr</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Control | Gamide | CTFP

0 hr | 17 hr | 24 hr | 42 hr
Table 1. Progastrin-derived peptides used in this study, excluding PG55-80 and PG72-80, which were included as a reference to previous work. All peptides had free N- and C-termini unless otherwise noted. The sequences of human or mouse amidated gastrin (Gamide), glycine-extended gastrin (Ggly), the C-terminal flanking peptide (CTFP), and mutant (Mut) and scrambled (Scr) forms of the CTFP are listed below together with their synthetic purity.

<table>
<thead>
<tr>
<th>HUMAN SEQUENCE</th>
<th>MOUSE SEQUENCE</th>
<th>OTHER NAMES</th>
<th>REF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamide</td>
<td>QGPWLEEEEEEAYGWMDF-amide (88%)</td>
<td>Gastrin-17; G17; PG55-71</td>
<td></td>
</tr>
<tr>
<td>Ggly</td>
<td>QGPWLEEEEEEAYGWMDFG (93%)</td>
<td>QRPRMEEEEEEAYGWMDFG (87%)</td>
<td></td>
</tr>
<tr>
<td>CTFP</td>
<td>SAEDEN (94%)</td>
<td>SAEDQ (90%)</td>
<td></td>
</tr>
<tr>
<td>Mutant CTFP</td>
<td>SAAAAN (95%)</td>
<td>SAAAAN (99%)</td>
<td>Mut</td>
</tr>
<tr>
<td>Scrambled CTFP</td>
<td>ENADSE (96%)</td>
<td>EQAESD (98%)</td>
<td>Scr</td>
</tr>
<tr>
<td>PG55-80</td>
<td>QGPWLEEEEEEAYGWMDFGRRSAEDEN</td>
<td>CFP/ gastrin-17; PG55-80</td>
<td>[28]</td>
</tr>
<tr>
<td>PG72-80</td>
<td>Y-GRRSAEDEN</td>
<td>Y-PG72-80</td>
<td>[28]</td>
</tr>
<tr>
<td>PG</td>
<td>SWKPRSQQPDAPLGTGANKRLELPWLEQQGPASHHRRLQGPQPPHLQVDPSKKQGPWLEEEEEEAYGWMDFGRRSAEDEN</td>
<td>PG1-80</td>
<td>[28]</td>
</tr>
</tbody>
</table>

Table 2. Stereological analyses of livers taken from mice bearing colon cancer liver metastases. The volume and metastases values are given as mean ± SEM for mice infused with PBS, Gamide, Ggly or mCTFP. No statistically significant difference compared with control was observed.

<table>
<thead>
<tr>
<th>PARAMETER</th>
<th>TREATMENT</th>
<th>Control</th>
<th>Gamide</th>
<th>Ggly</th>
<th>CTFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metastasis (%)</td>
<td></td>
<td>70 ± 3</td>
<td>76 ± 2</td>
<td>74 ± 2</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Normal Liver Volume (mm³)</td>
<td></td>
<td>1460 ± 160</td>
<td>1320 ± 60</td>
<td>1220 ± 80</td>
<td>1220 ± 70</td>
</tr>
<tr>
<td>Tumour Volume (mm³)</td>
<td></td>
<td>3650 ± 500</td>
<td>4360 ± 430</td>
<td>3560 ± 210</td>
<td>3170 ± 450</td>
</tr>
<tr>
<td>Total Liver Volume (mm³)</td>
<td></td>
<td>5110 ± 540</td>
<td>5690 ± 430</td>
<td>4780 ± 220</td>
<td>4390 ± 420</td>
</tr>
</tbody>
</table>
Author/s:
Marshall, KM; Patel, O; Bramante, G; Laval, M; Yim, M; Baldwin, GS; Shulkes, A

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Date:
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