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The Production and Role of Gastrin-17 and Gastrin-17-Gly in Gastrointestinal Cancers

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Abstract

The gastrointestinal peptide hormone gastrin is responsible for initiating the release of gastric acid in the stomach in response to the presence of food and/or humoral factors such as gastrin releasing peptide. However, it has a role in the growth and maintenance of the gastric epithelium, and has been implicated in the formation and growth of gastric cancers. Hypergastrinemia resulting from atrophic gastritis and pernicious anemia leads to hyperplasia and carcinoid formation in rats, and contributes to tumor formation in humans. Additionally, gastrin has been suspected to play a role in the formation and growth of cancers of the colon, but recent studies have instead implicated gastrin processing intermediates, such as gastrin-17-Gly, acting upon a putative, non-cholecystokinin receptor. This review summarizes the production and chemical structures of gastrin and the processing intermediate gastrin-17-Gly, as well as their activities in the gastrointestinal tract, particularly the promotion of colon cancers.

1. Gastrin processing in the GI tract

John Sydney Edkins postulated in 1905 [1–2] that the acid secretory activity of the stomach could be attributed to the agent gastrin. The gastrin gene is expressed and gastrin secreted in a variety of cells in the body, among them those of the small and large intestine (duodenal, jejunal, ileal, and colonic mucosas), the pancreas, neuroendocrine tissue (the pituitary and hypothalamus, cerebellum, vagal nerve, and adrenal medulla), the genitals and the respiratory tract, where it serves a variety of purposes [3–18]. However, “mature” gastrin is predominantly manufactured by the antral mucosa of the stomach, where G endocrine cells secrete the peptide in response to the presence of amino acids, dietary amines, and calcium in the stomach, for the purpose of stimulating gastric acid secretion [19–25, reviewed in 26]. Neutralization of acid or inhibition of acid secretion also stimulates gastrin release [27–33]. In addition to luminal stimuli, basolateral stimuli of the G cells by GRP or Ach from nerve fibers, or by humoral factors such as EGF, also cause the expression and secretion of gastrin in some species [34–35]. In response to increased acid levels or VIP, D endocrine cells, in turn, secrete somatostatin, which acts to inhibit the secretion of gastrin.

The expression of the gastrin gene and the processing of its polypeptide product in the gastrointestinal tract have been gradually revealed through extraction from gastrin-producing tissues and characterization of its processing intermediates with antibodies, chromatography and peptide analysis tools such as mass spectrometry. Genomic DNA information revealed the gastrin gene structure. The mRNA of the 101-residue gastrin precursor preprogastrin is translated at the endoplasmic reticulum, where an N-terminal signaling sequence is cleaved

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from the peptide between an alanyl and a seryl residue to yield the 80-residue progastrin [36–38] (Figure 1). Progastrin then passes through the Golgi complex and the trans-Golgi network, where it can be sulfated on Tyr^{86} and/or phosphorylated on Ser^{96}. It is further packaged into secretory vesicles, in which the dibasic sites Arg^{57}-Arg^{58} and Arg^{94}-Arg^{95} are probably cleaved on the C-terminal side by the prohormone convertases PC1/3 and PC2, and the remaining C-terminal basic residues Arg^{94}-Arg^{95} are removed by carboxypeptidase E to give the peptide G34-Gly [39–41; reviewed 42–43]. Another cleavage by prohormone convertases at Lys^{74}-Lys^{75} gives G17-Gly [39–41]. Various factors within the secretory vesicles modify these cleavages, including the differential expression of PC1/3 and PC2 and the pH of the vesicle interior (pH > 5.5 may inhibit the maturation of prohormone convertases) [41,44–46].

In addition, the sulfation of Tyr^{86} and the phosphorylation of Ser^{96} may affect the cleavage of local dibasic sites, though they have not been shown to affect the biological activity of gastrin, unlike the closely related peptide CCK, which requires sulfation of tyrosine for activity. Sulfation and phosphorylation may both increase processing of progastrin [44,46], while phosphorylation may also affect the conversion of glycine-extended gastrin intermediates to mature gastrins [47].

The C-terminal glycyl residue of G34-Gly is finally converted to a terminating amide group by the PAM enzyme to give the mature G34, which is additionally sometimes cleaved to give mature G17 [39–40] (Figure 2). G17-Gly is not generally converted directly to G17 by PAM [39]. G17 and G34 are the “classical” gastrins, those that were originally found to stimulate gastric acid secretion, while G17-Gly, G34-Gly, and progastrin are the “non-classical” gastrins, or “processing intermediates,” those peptides were not known to have any biological activity until recently. The ratio of amidated gastrins to processing intermediates varies considerably across the tissues in which the gastrin gene is expressed. Processing intermediates are quite scarce in the gastric antrum, making up only about 5% of gastrin gene products, while in the duodenum the value is 20% [48]. In the colonic mucosa, amidated gastrins are virtually unknown [48]. Additionally, probably due to variable cleavage of the Lys^{74}-Lys^{75} bond, the amount of G34 compared to G17 varies in different tissues. In the GI tract, region-specific antisera have shown that G17 predominates in the antral mucosa, while G34 is more prevalent in the duodenum [26].

2. Physiological roles of gastrin in the stomach

Ultimately, gastrin has two major roles in the GI tract, the first of which is the well- known stimulation of gastric acid secretion in the stomach. The mature gastrins (as well as a small percentage of non-classical gastrins) are secreted via Ca^{2+}-dependent release in the regulated secretory pathway from G cells in the gastric antrum and, to a lesser extent, the duodenum [49,50]. These mature gastrins act upon ECL cells of the gastric fundus, stimulating the release of histamine, again dependent on intracellular calcium from stores or from the activation of calcium channels [51–59]. Histamine then, via paracrine diffusion, interacts with parietal (oxytinic) cells, stimulating the upregulation of surface H^+/K^+ proton pumps and thus the secretion of acid [51,60]. Gastrin itself and acetylcholine also act directly on parietal cells through surface receptors to stimulate acid secretion; in this case, histamine potentiates the activity of gastrin, but this effect is still secondary to the stimulation of acid secretion by histamine [61–65]. Gastrin may also sensitize parietal cells to other acid secretagogues [66].

As acid secretion increases, D endocrine cells release somatostatin, which acts on parietal cells to inhibit acid secretion, and on ECL cells to inhibit histamine secretion, providing a negative feedback control mechanism [30–31,67–68]. In the duodenum, secretin (through bicarbonate release) and CCK (through the potentiation of secretin) also inhibit acid secretion as stomach contents pass into the small intestine.

Additionally, gastrin promotes the maintenance and proliferation of the gastric epithelium [reviewed in [26],42–43,48,69]. It does this by promoting the proliferation of gastrin receptor-expressing ECL cells, as well as mucous neck cells in the gastric mucosas, that can differentiate into parietal cells, though gastrin does not cause proliferation of parietal cells themselves [70–76]. H_2 receptor antagonists and proton pump inhibitors, as well as resection of the acid-secreting mucosa, suppress gastric acid secretion, which promotes elevated gastrin secretion. This promotes increased ECL proliferation [77–80]. Resection of the antral mucosa inhibits this ECL cell hyperplasia, additionally indicating that gastrin is responsible for mucosal proliferation [81]. ECL cells, in turn, may indirectly promote the proliferation of other gastric epithelial cells that do not express the gastrin receptor through the secretion of growth factors such as TGF-α and HB-EGF [82–85, reviewed in 86]. Patients with increased levels of gastrin in circulation show a greater thickness of the gastric and colonic mucosa, as well as a larger mass of parietal and ECL cells [75].

The acceptance of gastrin as a growth factor for the gastric mucosa naturally led to the suggestion that it might play a role in gastrointestinal cancers. Along with the ability to stimulate proliferation, gastrin has been found to have other biological activities, including inhibition of apoptosis [87] and the stimulation of invasion and migration of epithelial cells [88–90]. Conditions such as atrophic gastritis and pernicious anemia, as well as the aforementioned administration of proton pump inhibitors or H_2 receptor antagonists, reduce gastric acid secretion significantly (achlorhydria), leading to hypergastrinemia [91–92, reviewed in 48]. The presence of gastrin-secreting neoplasms (gastrinomas), associated with the Zollinger-Ellison syndrome, in the pancreas, duodenum, and the gastric antrum, may also lead to hypergastrinemia [95–100]. The resulting stimulation of ECL cell proliferation from hypergastrinemia frequently leads to ECL cell hyperplasia and carcinoids in rats [91–94]. Similar hypergastrinemia in humans generally leads to hyperplasia only, and progression to tumor formation seems to be condition-specific and require other factors in addition to gastrin [101]. Zollinger-Ellison multiple endocrine neoplasia-1 syndrome patients, for instance, are considerably more likely to have ECL cell carcinoids (up to 30% of those with the disease) than those with atrophic gastritis/pernicious anemia (5%) [101–102]. Colonization of the stomach by the bacteria *Helicobacter pylori*, a significant factor in the development of atrophic gastritis, causes a decrease in acid secretion, leading to an increase in gastrin secretion. This increase in gastrin stimulates the proliferation of *H. pylori* that in turn, leads to a further decrease in gastric acid secretion and eventual achlorhydria and hypergastrinemia, leading to gastric atrophy and cancer, as well as the formation of ulcers [103–104]. The small percentage of patients who actually develop cancer, as noted above, indicates that other factors must be involved, including environmental or inherited factors or the inflammatory response [105–107].

It has been suggested that gastrin is itself not a factor in tumor formation, but merely a marker of achlorhydria, which is the actual factor in causing infection and inflammation that leads to carcinogenesis [108–110]. Regardless, gastrin is probably involved in the acceleration of malignant tumor formation [104] and promotion of the growth of gastric tumors [111–115, reviewed in 48].

3. Gastrin and CCK_2-R

The actions of the hormones CCK are mediated by two receptors, CCK_1-R and CCK_2-R (formerly CCK-A and CCK-B). Both belong to the family of G-protein-coupled receptors, (GPCRs) which are characterized by seven trans-plasma membrane helical domains. CCK_1-R is distributed widely in the peripheral nervous system and in the upper gastrointestinal tract, and less often in the central nervous system. In the nervous system, CCK_1-R is present on vagal afferent neurons and may mediate the physiological response of satiety [116–117]. It also

regulates dopamine release that may affect neuropsychiatric disorders such as schizophrenia [118, reviewed in 119]. In the gut, CCK_1-R is present in the gastric mucosa (mostly in the basal region of the fundic mucosa) and gallbladder and GI smooth muscle cells, and is probably expressed by pancreatic acini and islets, as well as vagal nerve cells [120–121, reviewed in 122–124]. It mediates the release of pepsinogen from gastric chief cells and somatostatin from D cells to inhibit gastric acid secretion [125–126], gall bladder contraction [127–128], delay of gastric emptying [129–131], the relaxation of the sphincter of Oddi and the lower esophageal sphincter [132], and the secretion of enzymes and hormones from the pancreas [133–138], as well as pancreatic proliferation [139–142].

CCK_2-R, on the other hand, is dominant in the central nervous system, where it may be involved in inhibiting dopamine release and provoking the anxiety response [119,143]. CCK_2-R is also found in the upper GI tract in the gastric mucosa (mostly in the midglandular region of the fundic mucosa, where parietal cells are present), where in non-cancerous cells it mediates the activities linked to gastrin above: proliferation of the gastric mucosa, stimulation of histamine from ECL cells, stimulation of acid from parietal cells, as well as inhibition of somatostatin release from D cells [62,144–145, reviewed in 122–124]. It is also expressed in the pancreas and probably mediates glucagon secretion there in humans [146]. CCK_2-R is also expressed in gastric and pancreatic tumor cell lines where it may mediate tumor growth [147–156], while CCK_1-R may be present in some pancreatic tumor cell lines but role of the receptor there is unclear [157–159]. Ultimately, the cloning of cDNAs for brain CCK_2-R and for the gastrin parietal cell receptor indicated that the two receptors are identical, and thus, that CCK_2-R is bound by gastrin with high affinity and mediates its effects in the gastric mucosa [160–162].

Like gastrin, CCK is produced from the processing of a 115-amino acid residue preprohormone into mature C-terminally amidated peptides, occurring as 58- and 8-residue forms [163–164]. The primary structure of gastrin and CCK are related by their identical C-terminal tetrapeptide sequences (Trp-Met-Asp-Phe) and amidation. This sequence is essential for biological activity at both receptor subtypes. Maximum biological activity at the CCK_1-R receptor requires at least the seven C-terminal residues of the CCK sequence, with the N-terminal tyrosyl residue sulfated (Tyr^{52} of CCK-58). Sulfated CCK-8 meets this requirement. G17 (sulfated or not) and non-sulfated CCK-8 bind with 500–1000-fold less affinity, while CCK-4 binds with 10000-fold less affinity [165–166, reviewed in 122]. Binding at CCK_2-R is less exclusive. Sulfated and non-sulfated gastrin and CCK-8 bind with nearly equal (sub-nanomolar) affinity to CCK_2-R, and CCK-4 binds with micromolar affinity [167, reviewed in 122]. Despite the importance of sulfation to gastrin processing, it has seemingly no role in the biological activity mediated by CCK_2-R, in contrast to CCK_1-R. C-terminal amidation is, however, essential to high binding affinity, as the processing intermediate G17-Gly has less affinity for CCK_2-R than CCK-4 [168–169].

The signal transduction pathways of CCK_2-R leading to gastric acid secretion and factors promoting cancer (such as motility, invasion, inflammation, proliferation, and anti-apoptotic action) are varied, with some being typical pathways of other GPCRs, and others novel [reviewed in 69,122,124]. Notably, the phosphatidyl inositol pathway has been shown to be triggered in gastric parietal [170–171] and ECL cells [171], AR4-2J pancreatic cells [172–173], normal colonic epithelial cells [174–178] and HT-29 colon cancer cells [179]. Binding of CCK or gastrin activates a pertussis toxin insensitive G_q protein, which dissociates into the $G_{\alpha q}$ and $G_{\beta\gamma}$ subunits. $G_{\alpha q}$ is bound to a GDP molecule in the inactive form; this GDP is exchanged for a GTP molecule upon dissociation of the G_q protein [170,180–181]. G_q then activates the PLC-isoform (among other phospholipases in different cell types), which hydrolyzes plasma membrane-bound PIP_2 into the second messengers IP_3 and DG [170,173, 175,177,179,182]. IP_3 binds to a ligand-gated Ca^{2+} channel in the ER and mobilizes Ca^{2+} from internal stores, while DG activates PKC [176–178,183–186]. PKC and/or Ca^{2+} mobilization

in turn probably activate a cascade of MAPKs, including ERK1/2 and JNKs in several studied cell types [90,171–172,187–189] that have been shown to be involved in cell growth, differentiation, survival, and apoptosis. Using ERK kinase inhibitors, the ERK1/2 pathway activated by CCK receptors has been shown to control cell proliferation, migration, and regulation of gastrin-sensitive gene transcription [88,187,190]. CCK_2-R has also been shown to initiate MAPK cascades through the activation of tyrosine kinases [reviewed in 124,191]. In pancreatic AR42J cells and in human gastric cancer cells, CCK_2-R activates Src family kinases that in turn, and dependent on PKC, phosphorylate Shc, enabling it to complex with Grb2 and Sos [172,188,192–193]. This complex then activates the small GTPase Ras and the protein kinase Raf, which activate the ERK cascade. The ERK cascade might also be initiated by a direct, Ras-independent activation of Raf by PKC [194–195].

Another signaling pathway through the Src kinases is the PI3-K pathway. Activation of the pathway in response to gastrin in cell lines expressing CCK_2-R has been reported [190,196–198]. PI3-K is activated by tyrosine kinases through a complex with the adaptor protein IRS-1, and activates the downstream effector protein Akt [190,198]. The PI3-K/Akt pathway, as activated by gastrin in pancreatic AR4-2J cells, mediates cell proliferation, anti-apoptotic actions, and cell migration and adhesion [87,196–197,199]. Finally, the JAK2/STAT3 pathway has been shown to be directly activated by CCK_2-R in rat pancreatic acinar cells, though few GPCRs do so (the pathway is typically initiated by cytokine receptors) [197,200]. JAK2 is a tyrosine kinase that activates the transcription factor STAT3, which then binds to certain genes. The JAK/STAT pathway is known to mediate anti-apoptosis, cell proliferation, and carcinogenesis.

As discussed previously, cell proliferation may also be stimulated by gastrin in gastric epithelial cells that do not express CCK_2-R by transactivation of the EGF receptor. Gastrin stimulates the secretion of HB-EGF, which binds to the EGF receptor on the surface of epithelial cells. This causes tyrosine phosphorylation of the EGF receptor and eventual downstream initiation of the ERK cascade [82,85].

In addition to the signaling pathways described above that mediate acid secretion, cell proliferation, migration, invasion, and survival, in gastric and colonic cancer cells and intestinal epithelial cells, the PI3-K and MAPK pathways also increase the expression of COX-2 [201–203]. As well as the inflammation mediated by COX-2-formed prostaglandins being a major risk factor for cancer, COX-2 has also been linked to cell proliferation, transformation, invasion, and angiogenesis, all major factors in carcinogenesis. Similarly, the transcription factor NF-κB is activated by CCK_2-R leading to the expression of interleukin-8 and other proinflammatory gene products [204].

4. The conformation of gastrin in aqueous and membrane-mimicking solutions

While the C-terminal tetrapeptide fragment of G17 and CCK-8 is essential for biological activity at CCK_2-R [205] the secondary and tertiary structure of G17 that affects binding and activation of CCK_2-R has been repeatedly investigated using NMR, CD spectroscopy, and computational analysis. ECD as well as NMR spectroscopic analysis of G17 and various C-terminal fragments of G17 up to [Nle^{15}]G17(5–17) (replacement of Met^{15} with Nle does not significantly affect the conformation or biological activities of the peptide [206]) have shown that the peptide has no secondary and tertiary structure in aqueous solution or in DMSO [207–210] However, subsequent studies in TFE, aqueous solutions containing phospholipids, and aqueous solutions containing SDS micelles have shown G17 and G17 C-terminal analogs adopt a similar, ordered structure in these solvents [207–209,211–212]. Whether or not this

structure represents an active conformation of G17 in the presence of CCK_2-R has been the subject of considerable study.

Results of CD and computational studies of the C-terminal tetrapeptide G17(14–17) indicate that the peptide is partially folded in TFE into a C_7 structure, with a hydrogen bond between the carbonyl oxygen of Met^{15} and the amide proton of Phe^{17} [208–209,212]. While, in the short gastrin fragments the aromatic side chain of the C-terminal tetrapeptide appear to not be subject to any conformational constraints, extending the chain to [Nle^{15}]G17(10–17) seems to cause a change in the C-terminal environment and rigidity of the Trp^{14} side chain [208–209]. Extending the N-terminus further to [Nle^{15}]G17(6–17), and then to [Nle^{15}]G17(5–17) strongly enhances the α-helical signature of the CD spectrum, suggesting that a portion of the peptide folds into an α-helix in TFE [208]. While chain extension increases the α-helicity of the peptide, it does not significantly change the local conformation of the C-terminal region [208].

On the basis of the above results and on the propensity of the various amino acid residues to form secondary structure [214], Peggion and associates suggested a model for the conformation of G17 in TFE and membrane-mimicking environments [208]. They concluded that the C-terminal amino acids are unlikely to form α-helical structure, but that since elongation of the peptide through the pentaglutamyl sequence clearly causes conformational changes in the C-terminal region, an interaction must exist between the "head" and the "tail" of the peptide. This implies a β-bend or β-turn as an additional structure in the central portion of the peptide; they suggested Ala-Tyr-Gly-Trp as the most likely sequence to form a turn. The existence of α-helical structure including the residues of the N-terminal portion of the peptide, on the other hand, is strongly supported by the increase in intensity of the CD spectrum when the chain is extended to [Nle^{15}]G17(5–17). Further evidence in favor of this hypothesis is that ionization of the glutamyl side chains by addition of base disrupts the CD-observed helical structure, as with α-helical polyglutamyl sequences [208]. The difference CD spectrum of [Nle^{15}]G17(6–17) and [Nle^{15}]G17(5–17) also indicates rapid onset of an α-helix upon the addition of the N-terminal Leu residue, further supporting the likelihood that the N-terminal region of [Nle^{15}]G17(5–17) forms an α-helix segment [208]. Their model, then, consisted of an α-helix in the pentaglutamyl sequence, β-bend or β-turn in the central region, and, consistent with the earlier conformational calculation of Abillion and associates, a C_7 conformation in the biologically essential C-terminal tetrapeptide [208,213].

Following these CD studies, Mammi and coworkers conducted ^1H NMR studies of gastrin peptides in TFE and TFE/H_2O mixtures in order to more clearly define and quantify minigastrin structures, as the presence of numerous aromatic residues, which absorb strongly in the UV region, make defining the backbone structure from CD spectra difficult. Despite solubility problems with the longer gastrin fragments ([Nle^{15}]G17(10–17) and ([Nle^{15}]G17(5–17)) in TFE and in 10% TFE/H_2O the existence of six hydrogen bonds between backbone amide protons and carbonyl groups in the peptides were deduced [209,215]. Six amide protons were determined to be solvent shielded based on low temperature coefficients [214]. Two of these are amide protons of two of the Glu residues (though it is not clear which) in the N-terminal portion of the peptide, while the remaining are those of the residues of the essential C-terminal tetrapeptide sequence (Trp^{14}, Nle^{15} (in their study), Asp^{16}, and Phe^{17}). Based on model proposed by Mammi and associates, they assigned hydrogen bonds between Glu^9-NH and Leu^5-CO and between Glu^{10}-NH and Glu^6-CO, in accordance with the prediction that the N-terminal sequence of minigastrin prior to Gly^{13} forms a α-helical structure in organic solvent. Because they showed the Ala^{11} and Tyr^{12} backbone amide protons are exposed to solvent, they concluded that the α-helix does not extend throughout the peptide, but ends at Glu^{10}. Finally, having considered several possible structures to account for the C-terminal amide protons, they proposed that the C-terminal tetrapeptide sequence forms a 3_{10}-helix, with hydrogen bonds between Trp^{14}-NH and Ala^{11}-CO, Nle^{15}-NH and Tyr^{12}-CO, Asp^{16}-NH and Gly^{13}-CO, and

Phe^{17}-NH and Trp^{14}-CO. The researchers noted that, while the short two-turn α-helix at the N-terminus would likely be unstable, the existence of a flexible region immediately following allows the possibility of the two helices at opposite ends of the peptide interacting and stabilizing each other [210].

Recently, NMR and MD studies have suggested a more complex structure for G17. Through examination of the backbone dihedral angles and alpha carbon intramolecular distances of [Nle^{15}]G17 solvated in a box of water/decane, Stone and coworkers a 3_{10}-helix involving residues Leu^{5}-Glu^{9} and an α-helix terminating in a type I β-turn from Ala^{11} to Asp^{16}, with the C-terminal tetrapeptide folding into a type IV β-turn conformation [211,212]. These investigators suggest that this type I β-turn structure seen in Gly^{13}-Asp^{16} may be indeed be the structure required for binding CCK_2-R.

Understanding the relevance of the folded conformation of gastrin to receptor binding and activation is, of course, of great importance, and, thus, the biological activity of the various C-terminal gastrin fragments and fragments have been studied extensively. As shown previously, the conformation of G17 and its C-terminal analogs in phospholipids and SDS micelles is similar to that in TFE, suggesting that the TFE conformation is close to that of these peptides upon entering the membrane environment [208,213]. Elongation of the chain from the N-terminus of tetragastrin not only changes the conformation, but also the biological potency of the peptide. Extending the chain from [Nle^{15}]G17(11–17) to [Nle^{15}]G17(5–17) increases the biological potency from 20% to 90% of the original activity of G17 [217–218]. This clearly implies that the N-terminal sequence is important in effecting the full biological activity of the peptide, most likely due to the conformational change imparted to the C-terminal tetrapeptide [208]. Additionally, because no ordered structure of the peptide is seen in aqueous solution, if structure is important to the biological activity of G17 and analogs, it should be the structure formed in presence of a lipophilic environment. In continuing ECD and NMR studies, Mammi and associates found that replacing the central Gly residue of [Nle^{15}]G17(5–17) with Ala which is more prone to helix-forming had the effect of extending the α-helix throughout the peptide and changing the environment of the aromatic chromophores of the C-terminus [219]. The analog retains only 10% of the potency of [Nle^{15}]G17(5–17), about the same activity as tetragastrin [220], and has greatly reduced affinity for parietal and nonparietal cells. Replacing the pentaglutamyl sequence with a pentaaspartyl sequence prevents any formation of an α-helix and also significantly reduces potency [221]. These results point to the importance of the suspected “U-shaped” hairpin fold, with the N-terminally-located α-helix stabilizing the active conformation of the C-terminal end, in ensuring the full activity seen in minigastrin and in G17.

5. Structural features required for G17 binding and activation of CCK_2-R

The dependence on the C-terminal sequence, Trp-Met-Asp-Phe-NH_2, for the biological activity of G17 was discovered shortly after the isolation and structural characterization of gastrin [205]. More detailed studies of G17 revealed the importance of individual residues in addition to that of Gly^{13} and terminal configurations described above. J. S. Morley synthesized almost 500 tetrapeptide analogs of gastrin and tested their ability to stimulate acid secretion in rats and dogs [222]. Acylation of the N-terminus did not affect activity negatively, but modification of the C-terminal amide group decreased acid secretion. Meanwhile, replacing the Trp^{14}, Met^{15}, or Phe^{17} residues in some cases resulted in analogs that were still biologically active, but replacing the Asp^{16} residue always gave a loss of activity. It was concluded that the Trp-Met-Asp residues are essential for receptor binding and that the Phe residue is needed for activation.

Later studies expanded on these findings, using radioligand binding assays in addition to functional assays. Blocking the N-terminus of the tetrapeptide with t-Boc, Aloc, or acylation increased binding affinity and biological activity in one study [223]. Substitution of Trp^{14} with Ala caused a 1000-fold decrease in binding affinity, as well as a 36% loss of acid secretion [224–225], and removing of the aromatic ring or formylating the indolyl nitrogen also severely decreased binding and biological activity [226–227]. Substitution of Met^{15} with Ala causes a 100-fold loss in affinity and a 32% loss of acid secretion [224–225], but as aforementioned, it can be replaced with Leu or Nle with no loss of binding or biological activity. Substitution of Asp^{16} with Ala completely destroyed the peptide's ability to bind and activate CCK_2-R, confirming Morley's earlier work [224–225]. Finally, substitution of Phe^{17} with Ala causes a 10^4-10^5-fold decrease in affinity [169,224].

Some groups have also suggested that the binding of metal ions plays an important role in enabling/disabling G17 and G17-Gly activity. Baldwin and colleagues showed that Fe^{3+} ions bind to both Glu^7 and Glu^8-Glu^9 of the pentaglutamyl sequence. An Ala substitution at position 7 lead to the inability of G17-Gly to stimulate proliferation or migration in IMGE-5 cells in vitro, and the iron chelator desferrioxamine blocks the activity of G17-Gly [228]. Another group showed that C-terminal gastrin fragments bind to calcium ions in TFE, and that these ions can affect the conformation of the peptide [229].

6. Colon cancer and the putative high affinity non-CCK G17-Gly receptor

While it has been established that gastrin is a factor in the development and growth of gastric cancers, there is less consensus about the effect of the peptide on colorectal carcinomas. Exogenous gastrin appears to promote the in vitro growth of some human and rat colorectal cancers, as well as the growth of transplanted mouse and rat tumors in nude mice [115,230–241, reviewed in 48]. Additionally, exogenous gastrin promotes the growth of some colorectal cancer cell lines, and this growth can be inhibited by gastrin antagonists [242]. Moreover, hypergastrinemia has been considered a risk factor for colorectal carcinoma [243–245]. However, in recent years, investigators have failed to demonstrate that gastrin plays a role in promoting colorectal carcinoma growth. Removal of the gastric fundus, inducing hypergastrinemia, did not result in greater tumor incidence, nor did treatment of transgenic mice, overexpressing gastrin, with the carcinogen azoxymethane produce increased incidence of tumors [246–248]. Other studies failed to show that hypergastrinemia stimulated the growth of the colonic mucosa [249–251]. Finally, the use of the gastrin receptor antagonist CR2945 to block CCK_2-R over a period of 52 weeks did not reduce tumor numbers in the colonic mucosa [252–253]. Overall, the studies seem to suggest that, while hypergastrinemic antral mucosa may promote gastric carcinomas, if colorectal carcinomas are stimulated by gastrin or gastrin processing intermediates these would have to be local autocrine or paracrine products [251,254–255].

Some colorectal cancers were initially thought to synthesize gastrin and to express gastrin receptors [255]. However, receptor autoradiography indicated that CCK_2-R was expressed in no more than 4% of colorectal carcinomas, while PCR showed CCK_2-R mRNA to be present in only 38% of colorectal carcinomas [256–257]. Equally important was the finding that while gastrin gene product is expressed in the human adult colon [6,235,258–262], progastrin is typically not processed to the mature, amidated peptide in colorectal cancers or in the normal colon [6,260–262]. These revelations, combined with the likelihood that gastrin does not act as an endocrine growth factor through hypersecretion of gastrin elsewhere in the GI tract, strongly suggest that gastrin does not stimulate the colonic mucosa or colorectal cancers either locally or in circulation and that CCK_2-R largely does not mediate these effects [reviewed in 124,255]. Instead, because of the incomplete processing of the gastrin gene product, progastrin and G17-Gly processing intermediates persist in the human adult colon. Resection of colon

cancer in one study reduced circulating levels of gastrin precursors, while leaving the levels of circulating mature gastrins unaffected [263], reflecting the production of precursors but not mature gastrin peptides in the colon. Precursors are found in high concentrations in colorectal tumors and in the blood of colorectal cancer patients [260–262,264, reviewed in 265].

Speculation naturally followed that the incompletely processed peptides might somehow play a role in the stimulation of tumor growth. In fact, gastrin precursors have been shown to stimulate proliferation of colonic mucosa *in vitro* and *in vivo*. Gastrin-deficient mice treated with G17-Gly or progastrin had an increase in proliferation of colonic epithelial cells where as mature gastrin had no effect [266–267]. Treatment with G17-Gly also increased aberrant crypt foci and the sensitivity of cells to azoxymethane [268]. Transgenic mice overexpressing progastrin or G17-Gly had increased epithelial cell proliferation and colonic cell hyperplasia, and those mice overexpressing progastrin treated further with a carcinogen had increased predisposition to neoplasia or adenocarcinoma [248,266,269–270]. Additionally, some groups have shown that gastrin precursors stimulate proliferation of cells that do not express CCK receptors [271–275]. However, there is still some controversy regarding the effect of precursors. Some groups have not found evidence of precursors as growth factors [276], while another group suggests that the precursors merely potentiate the effect of mature gastrin [277–279].

The ability of gastrin precursors to bind and stimulate proliferation of normal and cancerous colonic cells in the absence of CCK_2-R suggests the presence of a putative receptor or receptors that mediate these effects. In fact, high (nanomolar) affinity binding sites have been demonstrated for G17 and G17-Gly on primary and cultured tissues [275,280–281]. HT-29 cancer cells were shown to possess a nanomolar affinity receptor in studies using radioiodinated G17(2–17)-Gly and G17, respectively [274,279]. Additionally, saturation binding studies with 67 primary colon cancer specimens showed 57% to have a site to which gastrin binds with high affinity [281]. However, some studies have instead shown a low (micromolar) affinity binding site; Yang and coworkers revealed the presence of a micromolar affinity site for G17 and G17-Gly on DLD-1 human colon cancer cells [282] and Imdahl et al., which showed a micromolar affinity site on primary colorectal cancer cell lines [283].

The effect of G17-Gly binding on the proliferation of colon cancer cells has been a central topic of investigation of our own laboratory in recent years. Following the discovery of micromolar sites on DLD-1 cells, research was conducted to further characterize the G17-Gly binding receptor using optimal conditions *in vitro* [284]. G17 and G17-Gly were modified by replacing Met^{15} with a leucyl residue to prevent oxidation. It was found that [^{3}H]G17-Gly bound both intact DLD-1 cells and DLD-1 cell membranes, in a saturable and displaceable manner. Both [Leu^{15}]G17 and [Leu^{15}]G17-Gly were able to displace the tritiated G17-Gly dose-dependently. Non-linear regression analysis showed that, in addition to a low affinity (micromolar) binding site for each peptide, both peptides bound at high affinity, with [Leu^{15}]G17-Gly binding with subnanomolar affinity and [Leu^{15}]G17 with nanomolar affinity on DLD-1 membranes. A subsequent experiment using intact DLD-1 cells and a smaller concentration of the higher specific activity peptide [^{125}I-Tyr^{12}, Leu^{15}]G17-Gly to better resolve the two binding sites also showed that [Leu^{15}]G17-Gly displaced the radiopeptide from two binding sites with nanomolar and micromolar affinity. Finally, [^{3}H]CCK-8 that was shown to bind CHO-K1 cells expressing CCK_2-R failed to bind the DLD-1 membranes, and CCK-8 failed to displace [^{125}I-Tyr^{12}, Leu^{15}]G17-Gly from the intact DLD-1 cells. The observations of the failure of CCK-8 to bind DLD-1 membrane or cells as well as the relative similarity of the binding affinities of [Leu^{15}]G17-Gly and [Leu^{15}]G17 clearly indicate the presence of not just non-CCK receptors on the surface of these cells, but possibly a heterogeneous population of such receptors. More curiously, DLD-1 cell proliferation studies with these peptides showed that both stimulated dose-dependent, *biphasic* growth of cell populations, with cell number

reaching a maximum at 10^{-9} M for [Leu^{15}]G17-Gly and at 10^{-8} M for [Leu^{15}]G17, while cells treated with micromolar concentrations showed no significant increase in number over controls. This suggests that at low concentrations of these ligands the observed high affinity sites mediate the proliferatory effect, but when ligand concentrations are increased, the low affinity site is bound and mediates an effect countering that of the high affinity site.

Another study examined the importance of the N- and C-terminal residues of [Leu^{15}]G17-Gly and [Leu^{15}]G17 in the promotion of activity in DLD-1 cells, and extended the investigation to include the HT-29 colon cancer cell line [285]. [^{3}H]G17-Gly bound to HT-29 cell membranes, and [Leu^{15}]G17-Gly displaced it in a dose dependent manner. However, only one low (micromolar) affinity site was seen with non-linear regression analysis, in disagreement with previous studies which showed the presence of a nanomolar site for G17-Gly on intact HT-29 cells. In experiments with intact HT-29 cells and [^{125}I-Tyr^{12}, Leu^{15}]G17-Gly as the radioligand, [Leu^{15}]G17-Gly was seen to bind to two sites with nanomolar and micromolar affinity [285]. The discrepancy between the two experiments was attributed to the relatively low population of high affinity sites compared to low affinity sites, and the inability to resolve them using the lower specific-activity tritiated peptide. A further cell proliferation experiment, however, again revealed increased cell numbers at nanomolar concentrations of [Leu^{15}]G17-Gly and a biphasic growth effect at higher concentrations, as with the DLD-1 proliferation study. Once again, CCK-8 failed to displace radioligand binding, as did the CCK_2-R antagonist L365-260.

Several other important observations were made. [Leu^{15}]G17(2–17)-Gly, an analog created by omitting the N-terminal pyroglutamatyl residue, was able to displace [^{3}H]G17-Gly in competition experiments with DLD-1 membranes. The analog bound to two sites, but at about an order of magnitude lower affinity than that of [Leu^{15}]G17-Gly to both. Conversely, first omission the entire N-terminal pentapeptide preceding the central pentaglutamyl sequence ([Leu^{15}]G17(6–17)-Gly), then the pentaglutamyl sequence as well ([Leu^{15}]G17(11–17)-Gly) resulted in binding only one site at micromolar and supermicromolar affinity, respectively. Lastly, a cell proliferation experiment using HT-29 cells showed that the analog G17(1–12), created by omitting only the C-terminal sequence 13 to 17 of G17 following the pentaglutamyl sequence, stimulated an increase in cell number with a EC_{50} at nanomolar concentration. However, unlike the experiments with [Leu^{15}]G17-Gly, no decrease in cell number was seen at higher concentrations. Combined with the previous proliferation data, this result supports the hypothesis that the high affinity receptor for G17 and G17-Gly mediates the proliferatory effect of these peptides. In all, the binding and proliferation results obtained further suggest that N-terminal residues 1–5 are essential for binding the high affinity site, while the C-terminal 13–17 residues may be important for binding the low affinity site.

Further study of the N-terminal region of G17 in our laboratory has led to some surprising observations. G17(1–12) was seen to stimulate the proliferation of DLD-1 cells in a nonbiphasic manner, similar to that of the HT-29 cells treated with G17(1–12) previously [286]. However, instead of binding selectively to one site, G17(1–12) continued to bind to both high and low affinity sites, with affinities similar to those of the full sequence G17-Gly, suggesting that the peptide activates the high affinity, growth promoting site, while acting as an antagonist at the low affinity site [287]. However, the C-terminally truncated-peptide is sufficient to bind both sites, even without the C-terminal tetrapeptide so vital to activity at the CCK_2 receptor. Secondly, truncating G17(1–12) to G17(1–6)-NH_2 produced a peptide capable of binding a single site with micromolar affinity and the ability to stimulate proliferation of DLD-1 cells [287]. Furthermore, amidated and nonamidated N-terminal peptides as short as G17(1–4) were able to bind a single site on DLD-1 cells albeit with very low affinity (~10^{-5} M). However, these shorter peptides were not able to stimulate proliferation of cells [286].

Structural studies using ECD and VCD spectroscopy as well as MD simulations in a variety of aqueous and membrane-mimicking solvents revealed the presence of a β-turn structure from $pGlu^1$-Trp^4 in these N-terminal peptides of increasing frequency with peptide length [288]. As peptides shorter than G17(1–4) peptides were not seen to bind to a receptor on DLD-1 cells, the 1–4 sequence appears to be important for binding. Additionally, higher affinity for the binding site on DLD-1 cells as well as stimulation of proliferation of DLD-1 cells by G17(1–6)-NH_2 and G17(1–12) may be attributed to the C-terminal amidation and longer sequence, respectively [286–287].

Recent studies have provided some insight into the signaling pathways activated by the putative G17-Gly/G17 receptor leading to cell proliferation. Ogunwobi and Beales showed that MAPK and NF-κB inhibitors significantly reduced the proliferatory effect of G17-Gly administered to HT-29 cells [289]. The activation of ERK in particular supported an earlier report of ERK signaling by G17-Gly in gastric epithelial cells [290]. CCK_2-R activation also triggers the MAPK, ERK, and NF-κB pathways leading to cell proliferation, among other functions. Unlike CCK_2-R, however, using the COX-2 inhibitor celecoxib it was shown that the G17-Gly/G17 pathway is COX-independent. An anti-apoptotic effect of G17-Gly dependent on these pathways was also shown using celecoxib, and a further study revealed that the PI3-K/Akt, JNK and JAK/STAT pathways to be involved as well [291].

Abbreviations

Ach	acetylcholine
CCK	cholecystokinin
COX-2	cyclooxygenase-2
[^{3}H]CCK8	[^{3}H]cholecystokinin-8
DG	1,2-diacylglycerol
DMSO	dimethyl sulfoxide
ECD	electronic circular dichroism
ECL	enterochromaffin-like
EGF	epidermal growth factor
ERK	extracellular signal-related kinase
G17	gastrin-17
G17-Gly	gastrin-17-Gly
GRP	gastrin releasing peptide
HB-EGF	heparin binding epidermal growth factor
IP	inositol phosphate
IP_3	inositol 1,4,5-trisphosphate
JNK	c-jun NH_2-terminal kinase
MAPK	mitogen activated protein kinase
MD	molecular dynamics
NMR	nuclear magnetic resonance
PAM	peptidyl-glycine α-amidating monooxygenase
PI3-K	phosphatidylinositol 3-kinase

PIP_2	phosphatidyl inositol 4,5-bisphosphate
PKC	protein kinase C
PLC-β	phospholipase C-β
SDS	sodium dodecyl sulfate
TFE	2,2,2-trifluoroethanol
TGF-α	transforming growth factor-α
VCD	vibrational circular dichroism
VIP	vasoactive intestinal peptide

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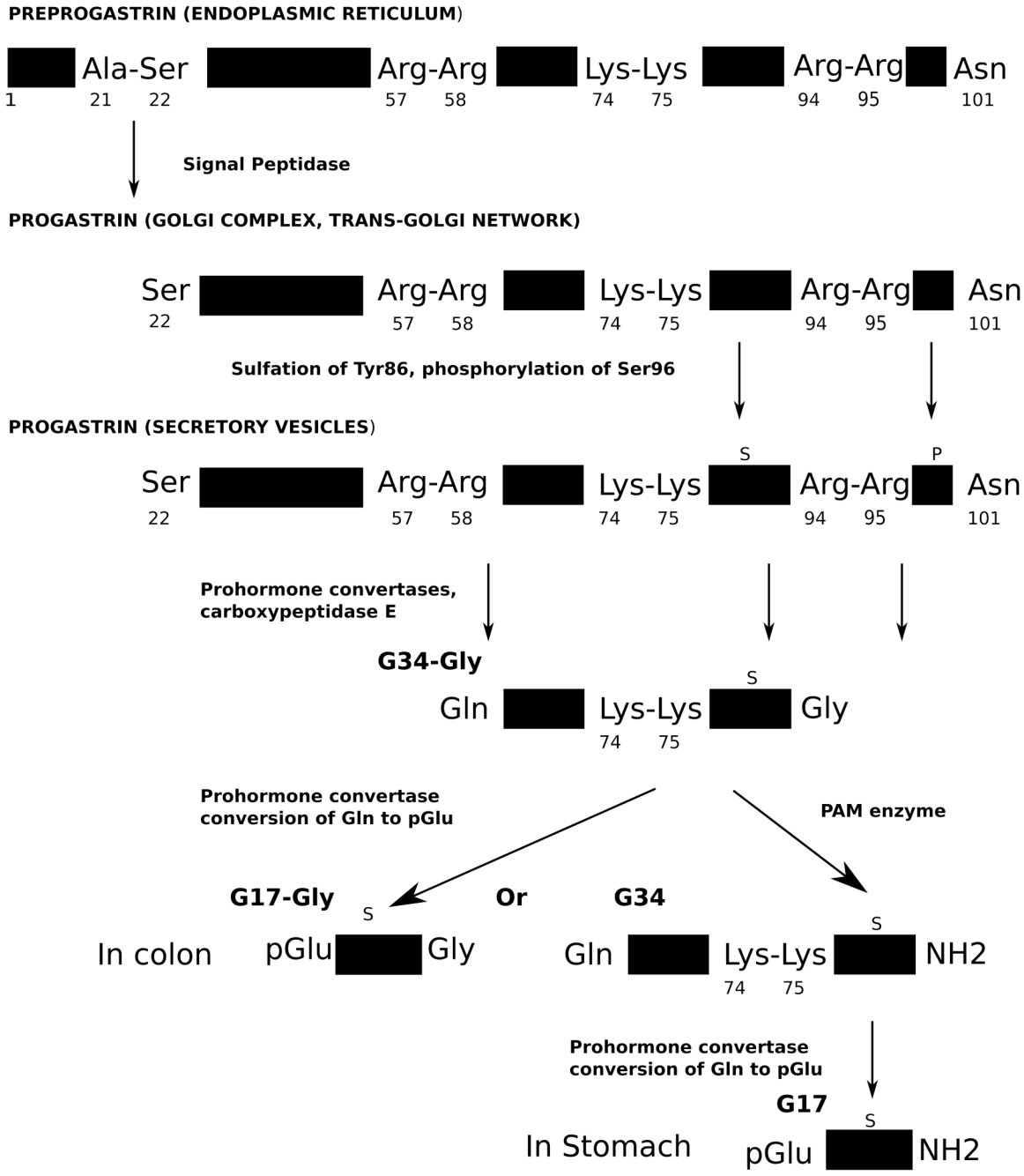

Figure 1.
Processing of preprogastrin in G cells.

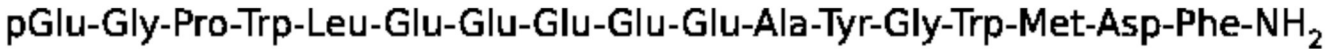

Figure 2.
Primary structure of human G17.