Characterization of gastrin–cholecystokinin
2 receptor interaction in relation to c-fos
induction

Martine J Hollestelle, Peter Timmerman1,2, Rob H Meloen1,3 and
Jo W M Höppener

Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, Lundlaan 6, 3584EA Utrecht, The Netherlands
1Pepscan Therapeutics BV, Runderweg 4-6, 8219PK Lelystad, The Netherlands
2Van’t Hoff Institute for Molecular Sciences, Nieuwe Achtergracht 129, Faculty of Science, University of Amsterdam, 1018WV
Amsterdam, The Netherlands
3Academic Biomedical Centre, University of Utrecht, Yalelaan 1, 3584CL Utrecht, The Netherlands

(Correspondence should be addressed to M J Hollestelle, Department of Clinical Chemistry and Haematology, University Medical
Center Utrecht, G03.550, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands; Email: m.j.hollestelle@umcutrecht.nl)

Abstract

The interaction of gastrin with the cholecystokinin 2 (CCK2)/gastrin receptor has been studied
extensively in relation to gastric acid secretion. However, not much is known about the contribution
of individual amino acids of gastrin interacting with the CCK2 receptor, when gastrin is acting as a
tumor growth factor. The purpose of the present study was to determine the significance of each
individual amino acid residue of human gastrin-17 with respect to CCK2 receptor-mediated cell
proliferation. Activation of this receptor was assessed using an in vitro bioassay based on gastrin-
induced expression of a c-fos-luciferase reporter, transfected in AR42JB13 and Colo 320 cells, a
rat pancreatic and human colorectal cell line respectively. Gastrin-17 dose dependently increased
c-fos induction in both cancer cell lines. L365,260, a known CCK2 receptor antagonist, completely
blocked the gastrin signal, demonstrating the specificity of this assay. We demonstrated for the
first time that four carboxy-terminal amino acids of gastrin-17 are essential for activation of the
CCK2 receptor with respect to c-fos induction. Also other residues of gastrin-17, notably glycine-2
for the rat CCK2 receptor and glutamic acid 8–10 and tyrosine-12 for the human receptor, were
found to be important, although to a lesser extent. Alanine-substitution variants of each of the four
carboxy-terminal amino acids of gastrin-17 showed strongly reduced receptor activation but did
not act as competitive inhibitors of gastrin-17. Identification of the essential role of the carboxy-
terminal tetrapeptide of gastrin-17 in CCK2 receptor-mediated c-fos induction indicates that
gastrin inhibitory therapeutic strategies should mainly be targeted toward this region of gastrin.

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Introduction

Gastrin is a polypeptide hormone known to regulate
gastric acid secretion directly by acting on parietal
cells of the stomach and also indirectly by enhancing
histamine release from enterochromaffin-like (ECL)
cells (Chiba et al. 1988, Edkins 1906, Waldum et al.
1991). Gastrin-17 is one of the biologically active
forms of gastrin, which is most abundantly present in
the antral mucosa (Hilsted & Rehfeld 1987). Other
bioactive forms of gastrin are gastrin-34 and Gly-
extended gastrin (Hilsted & Rehfeld 1987, Seva et al.
1994). In addition to gastric acid secretion, gastrin
plays a role in cell proliferation by stimulating the
expression of early response genes and other growth-
related genes. For example, gastrin has been shown to
upregulate c-fos expression in ECL cells, fibroblasts,
and pancreatic tumor cell line AR42J (Kinoshita et al.
1998, Stepan et al. 1999). On the other hand, c-fos
expression is not enhanced in parietal cells of the
stomach, which secrete acid upon gastrin stimulation
(Kinoshita et al. 1998). These effects of gastrin are
mediated primarily through the cholecystokinin-2
(CCK2) receptor, also named CCKB or gastrin receptor. The CCK2 receptor is a seven transmembrane G-protein-coupled receptor, originally cloned from canine parietal cells (Kopin et al. 1992).

A number of reports demonstrated the important proliferative role of gastrin in a variety of gastrointestinal and non-gastrointestinal tumor types (Smith et al. 1995, 1996, Watson et al. 1996, Thorburn et al. 1998, Haigh et al. 2003). Furthermore, gastrin neutralization is studied in different clinical trials as a therapy for several types of tumors by preventing gastrin to bind to the CCK2 receptor. So far, clinical studies have been performed in pancreatic, colorectal, and gastric cancer patients (Smith et al. 2000, Watson & Gilliam 2001, Brett et al. 2002, Gilliam et al. 2004). Initial results of active gastrin vaccination in pancreatic cancer patients demonstrated prolonged survival for antibody responders compared with non-responders, providing evidence for the fact that gastrin plays an important role in pancreatic tumor development and progression (Brett et al. 2002). The pharmacological mode of action of gastrin in tumor development, however, is still unclear.

Concerning gastric acid secretion, several studies have shown that the carboxy-terminal tetrapeptide of gastrin contains all structural elements required for this physiological function (Tracy & Gregory 1964, Morley et al. 1965). A number of studies have focused on the importance of individual amino acid residues of gastrin-17 using acid secretion as read out. These studies revealed that the Trp14 (Morley 1968, Magous et al. 1982) and the carboxy-terminal Phe-amide residue are essential for gastrin-induced acid secretion mediated by the CCK2 receptor (Morley 1968). Removal of the carboxy-terminal Phe generates a gastrin derivative with antagonistic properties (Martinez & Bali 1984, Yasui et al. 1990). However, a more recent study concludes that the use of des-Phe analogs of gastrin is not a viable strategy for the development of antagonists for the human CCK2 receptor due to decreased receptor affinity (Ahmed et al. 2001). Met at position 15 could be successfully replaced by Leu or Norleucine without loss of activity (Morley 1968, Wunsch et al. 1982). These results suggest that Met15 functions as a spacer or contributes to hydrophobic interactions with the CCK2 receptor. In contrast, even the smallest modifications of the Asp residue at position 16 result in a dramatic loss of activity, suggesting that this residue is particularly important for CCK2 receptor binding and gastric acid secretion (Morley 1968).

In the present study, an in vitro gastrin-specific bioassay was employed to investigate the significance of all individual residues of gastrin-17 for gastrin-induced cell proliferation. To this end, gastrin-17-induced expression of a c-fos-luciferase reporter gene (Stepan et al. 1999) was used as a read out in the rat exocrine pancreatic tumor cell line AR42JB13 (Mashima et al. 1996). The individual residues of gastrin-17 were studied by the substitution of each amino acid with an Ala, except the Ala at position 11, which was substituted for a Gly residue. Furthermore, c-fos stimulatory activity of gastrin-17 was compared with that of other physiologically relevant progastrin processing intermediates, such as gastrin-34 and gastrin-17 extended with a Gly residue. In addition, pentagastrin (the carboxy-terminal pentapeptide of gastrin used in clinical practice (Jepson et al. 1968)), gastrin-17 without carboxy-terminal amidation, and gastrin-17 extended with a Cys residue at the carboxy-terminus were studied in the same bioassay. In this way, important information was obtained about which the residues of gastrin-17 are important for cell proliferation and on which part of the gastrin-17 molecule inhibitory therapy might be focused. In order to validate the relevance of our findings in the rat cancer cell line for such potential clinical application in humans, we also tested the gastrin-17 (Ala-substitution variants) in Colo320 cells, a human colon cancer cell line expressing the CCK2 receptor (Yu et al. 2004).

Materials and methods

Peptides

The following human (derived) peptides were used in the in vitro gastrin bioassay: gastrin-17 (pEGPW-LEEEEAYGWMD, where pE means pyroglutamic acid as in the native peptide), gastrin-34 (pELGPQPGPHLVADPSKKQGPWLEEEEAYGW-MDF), pentagastrin (GWMD), gastrin without carboxy-terminal amidation, Gly-extended gastrin-17, Cys-extended gastrin-17, and Cys-extended gonadotropin-releasing hormone (GnRH: pEHWSYGLRPSC as negative control). Furthermore, the individual residues of gastrin-17 were substituted with an Ala, except the Ala at position 11, which was substituted for a Gly. All peptides were synthesized by Pepscan Therapeutics BV (Lelystad, The Netherlands). All peptides were amidated at the carboxy-terminus, except when mentioned otherwise.

Cell culture, transient transfection, and c-fos-luciferase assay

The rat exocrine pancreatic tumor cell line AR42JB13 (Mashima et al. 1996) was kindly provided by Dr Marlène Dufresne (Institute Louis Bugnard, Utrecht). For transfection, Colo320 cells were cultured in 10% fetal bovine serum (FBS)-fact supplied RPMI-1640 medium. Cells were transiently transfected with 100 ng of the c-fos-luciferase reporter gene (Stepan et al. 1999) and 100 ng of the expression vector for the CCK2 receptor (Haigh et al. 1999) using Lipofectamine (Invitrogen).
RNA isolation and RT-PCR

Total RNA was isolated from AR42JB13 cells by acid guanidinium thiocyanate–phenol–chloroform extraction as previously described (Chomczynski & Sacchi 1987). RNA was quantified by measuring absorption at 260 nm. One microgram total RNA was reverse transcribed using superscript II RT RNase H— (Invitrogen). To demonstrate CCK2 receptor mRNA, a PCR was performed on the cDNA using specific primers (forward: 5'-GACCCACTGCAAGCAGCAGTA-3', reverse: 5'-CAGTAGCACGGACCAGGTTC-3') located in the exons 2 and 3 of the rat CCK2 receptor gene (Wank et al. 1992). This resulted in a single product of 194 bp, representing the CCK2 receptor mRNA.

Statistical analysis

Statistical significance was calculated using the one-sample t-test, unless mentioned otherwise.

Results

Gastrin-specific in vitro bioassay

To check whether the CCK2 receptor gene is expressed in the cell line AR42JB13, used in our experiments, an RT-PCR to demonstrate the presence of CCK2 receptor mRNA is performed (Fig. 1A). After stimulation with 10 nM gastrin-17, a significant and reproducible induction of a c-fos-luciferase reporter was observed (Fig. 1B). Furthermore, AR42JB13 cells were stimulated with 10 nM gastrin-17 after pretreating the cells for 30 min with L365,260, a highly selective CCK2 receptor antagonist (Lotti & Chang 1989). This resulted in a dose-dependent inhibition of the c-fos-luciferase activity, demonstrating that the gastrin-17-induced luciferase activity in our assay is mediated by the CCK2 receptor (Fig. 1B). Also in human Colo 320 cells, a significantly increased c-fos-luciferase signal was detected after stimulation with 10 nM gastrin-17 and complete blocking of the response was achieved with 1 µM L365,260 (Fig. 2A). The effect of gastrin-17 stimulation of Colo 320 cells could be increased by stable transfection with the human wild-type CCK2 receptor (= Colo 320 WT cells) at gastrin-17 concentrations of 1 nM or higher (Fig. 2B).

Effect of various gastrin forms on c-fos promoter induction in AR42JB13 cells

The degree of c-fos induction by gastrin-17 was comparable with that of gastrin-34 (90 ± 4.3% compared with gastrin-17), one of the other biologically active forms of gastrin (Fig. 3). Pentagastrin (G5), used as gastrin analog in clinical practice (Jepson et al. 1968), consists only of the five carboxy-terminal amino acids of gastrin-17 and gastrin-34. G5 was found to induce moderate, but highly reproducible, activation of the c-fos-luciferase gene (78 ± 7.5% compared with gastrin-17), demonstrating the importance of the five carboxy-terminal amino acids. Interestingly, when gastrin-17 was extended at the carboxy-terminus with a Gly residue, which yields a naturally occurring processing intermediate of progastrin, or with a Cys residue, the c-fos activation was almost completely abolished (P < 0.001 and P = 0.004 respectively). This observation that gastrin activity was almost completely

Toulouse, France) with the permission of Dr Itaru Kojima (Gunma University, Maebashi, Japan). The parental cell line AR42J was known to respond to gastrin-17 administration with induction of the c-fos promoter (Todisco et al. 1997, Stepan et al. 1999, Thommesen et al. 2001). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) in 5% CO₂. Colo 320 WT and Colo 320 cells, a human colon cancer cell line with or without stably transfected wild-type human CCK2 receptor respectively were cultured in an RPMI medium supplemented with 10% FBS in 5% CO₂ (Yu et al. 2004). For the in vitro gastrin bioassay, the cells were seeded in 48- or 96-well plates and were transfected with lipofectamine reagent (Medema et al. 2001). The cells were kept with serum-free medium for 24 h and then incubated for 5 h with or without various concentrations of human gastrin-17 or other peptides. In further studies, the cells were first preincubated for 30 min with the CCK2 receptor antagonist, L365,260 (Lotti & Chang 1989; a gift from Dr L Iversen, Panos Therapeutics Ltd, England) prior to the addition of gastrin-17. After this 5-h stimulation, the cells were washed (twice) and lysed in Promega lysis buffer. Luciferase and Renilla measurements were performed on a luminometer (Centro LB 960, Berthold Technologies, Vilvoorde, Belgium) using the Dual-Luciferase Reporter Assay System (Promega). Renilla values were used to correct the luciferase values for cell number and transfection efficiency.

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lost by extending gastrin with an additional amino acid at the carboxy-terminus confirms the importance of the carboxy-terminal part of gastrin. Furthermore, substitution of the carboxy-terminal amide end of gastrin-17 for a free carboxy-terminus (G17-a) completely inactivates the gastrin molecule ($P=0.002$).

**Effect of sequential alanine substitutions on gastrin-17-induced c-fos gene expression in AR42JB13 and Colo 320 cells**

In this Ala-substitution study, complete inactivation (when compared with gastrin-17) was seen at 50 or 10 nM in AR42JB13 and Colo 320 cells respectively, when one of the four carboxy-terminal amino acids was substituted ($P \leq 0.001$). The residual/background activity of these four gastrin variants was comparable with that of GnRH, an arbitrary peptide used as negative control (Fig. 4). This observation correlates very well with the finding that pentagastrin induces moderate c-fos gene expression in AR42JB13 cells (78 ± 7.5% compared with gastrin-17, $P=0.064$), demonstrating the importance of especially the last four amino acids of gastrin-17. Interestingly, in the Colo 320 cells, the Met15/Ala-substitution variant was significantly more active than the Trp14/Ala or Phe17/Ala variant, indicating that the amino acid at position 15 is less critical when compared with these other carboxy-terminal amino acids with respect to gastrin-17-mediated CCK2 receptor activation (Fig. 4B, $P=0.035$ and 0.045 respectively using an independent sample $t$-test; $P=0.058$ for Met15/Ala versus Asp16/Ala). Furthermore, a decrease in c-fos gene expression of 32% when compared with gastrin-17 was observed for the Gly2/Ala-substitution variant in AR42JB13 cells (Fig. 4A). The same phenomenon was not seen for the Colo 320 cells, which indicates therefore that Gly2 is only important for binding to the rat CCK2 receptor and not to the human CCK2 receptor. Substitution of the Glu8–10 residues and Tyr12 caused a decrease in c-fos gene expression in the Colo 320 cells, demonstrating that also these amino acids play a role in activation of the human CCK2 receptor (Fig. 4B). Furthermore, when Pro3 is substituted for Ala, a slight but significant increase in c-fos gene expression was seen in Colo 320 cells, but not in AR42JB13 cells.

To determine whether the four carboxy-terminal Ala mutants of gastrin are also inactive at higher concentrations, these mutants were investigated further in AR42JB13 cells. This experiment clearly demonstrated that all four carboxy-terminal Ala mutants of gastrin-17 can induce c-fos gene expression at concentrations of 1 and 10 µM (to the same extent as gastrin-17), while at

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**Figure 1** Presence of (A) CCK2 receptor mRNA and (B) activity of the CCK2 receptor in AR42JB13 cells. (A) CCK2 receptor mRNA detection in AR42JB13 cells by RT-PCR. A PCR product of the expected size (194 bp) indicates expression of the CCK2 receptor gene. (B) Effect of the CCK2 receptor-specific antagonist, L365,260 on gastrin-17 (G17)-stimulated c-fos-luciferase expression in AR42JB13 cells. Cells were cotransfected with plasmids c-fos-Luc and TK-Renilla and treated with 10 nM G17 alone or in combination with 1, 0.1, or 0.01 µM L365,260. Data are expressed as multiple-fold induction of the Luciferase/Renilla ratio when compared with cells stimulated with G17 alone (set at 1) and are mean ± S.E.M. *$P=0.025$, **$P<0.001$ compared with gastrin-17 alone (control = no gastrin added).
lower concentrations almost no induction was observed (Fig. 5). Furthermore, it can be seen from this experiment that Trp14 and Phe17 are the most critical residues, whereas the Met15/Ala variant is the most active of these four gastrin analogs, as was already shown in the Colo 320 cells. Finally, no inhibitory effect was observed when competition experiments were performed in AR42JB13 cells, using 10 nM gastrin-17 in combination with a tenfold higher concentration of the four carboxy-terminal Ala-substitution variants (Fig. 6).

Discussion
Previous studies have demonstrated that the carboxy-terminal tetrapeptide of gastrin-17 (tetragastrin, W-M-D-F-NH₂) is the minimal sequence required for full gastric acid secretory activity in animal models (Tracy & Gregory 1964, Morley et al. 1965). In addition, tetragastrin was shown to give reproducible gastric acid secretion in human patients (Hart & Madsen 1972). We report, for the first time, the contribution of each individual amino acid of gastrin-17 for induction of c-fos gene expression, previously shown to be involved in proliferative actions of gastrin (Kinoshita et al. 1998, Stepan et al. 1999). Our study demonstrates that the four carboxy-terminal amino acids of gastrin-17 are essential for induction of c-fos gene expression. A complete inactivation of the protein in AR42JB13 cells, at a concentration of 50 nM, was observed when...
one of these last four amino acids of gastrin-17 was substituted for an Ala. The same effect was also seen in Colo 320 cells at a concentration of 10 nM (Fig. 4). In agreement with these results, pentagastrin, which consists of the five carboxy-terminal amino acids of gastrin-17, is still capable of inducing \textit{c-fos} gene expression (with 78% efficacy when compared with gastrin-17, Fig. 3), confirming the importance of the carboxy-terminal part of gastrin-17.

Although gastrin-17 has been studied intensively, almost nothing is known about the importance of the first 12 amino acids of gastrin-17 for interaction with the CCK2 receptor. Here we show for the first time the significance of the Gly\textsubscript{2} residue for activation of the rat CCK2 receptor (Fig. 4A) and of Glu at positions 8–10 and Tyr\textsubscript{12} for activation of the human CCK2 receptor (Fig. 4B).

From an \textit{in vivo} study in dogs, by measuring acid secretion, Morley \textit{et al.} concluded that Asp\textsubscript{16} is the most important residue of gastrin-17, because all amino acid changes at this position lead to virtually inactive compounds (Morley \textit{et al.} 1965). In our study, substitution of Asp\textsubscript{16} for Ala also showed complete inactivation of \textit{c-fos}-induced luciferase activity in

![Figure 4](https://example.com/figure4.png)

**Figure 4** Effect of sequential alanine substitutions on gastrin-17-induced \textit{c-fos} promoter activation. (A) AR42JB13 cells and (B) Colo 320 cells were cotransfected with plasmids \textit{c-fos}-Luc and TK-Renilla and treated with (A) 50 nM or (B) 10 nM G17, sequential alanine-substitution variants of G17 or gonadotropin-releasing hormone (GnRH, negative control peptide) respectively. Data are expressed as multiple-fold induction of the Luciferase/Renilla ratio over G17-stimulated cells (set at 1) and are mean $\pm$ S.E.M. ($n=4$ for AR42JB13 cells and $n=3$ (except GnRH $n=2$) for Colo 320 cells). $^*P<0.05$, $^{**}P \leq 0.008$ compared with gastrin-17 without any substitution.
AR42JB13 cells and almost complete loss of activity in Colo 320 cells, indicating that for cell proliferation Met15 is essential (Fig. 4). Similarly, the Phe17/Ala-substitution variant was shown to be still active on gastric acid secretion, although to a lesser extent when compared with the native peptide (Morley 1968), whereas in our study no activity of the Phe17/Ala-substitution variant with respect to c-fos induction was observed using a concentration of 50 nM (in AR42JB13 cells, Fig. 4A) or 10 nM (in Colo 320 cells, Fig. 4B). The apparent discrepancies between our and previous results can be due to different experimental approaches. Our study focuses on c-fos activation, while previous studies on gastric acid secretion. Furthermore, the effective concentrations of the peptides that are used cannot be compared properly between these different studies. When the concentration of the four carboxy-terminal Ala mutants of gastrin-17 was increased to 1 µM or more, c-fos-induced luciferase activity was observed, and almost comparable with that of gastrin-17 (Fig. 5). This result indicates that these Ala substitutions probably decrease the affinity of gastrin-17 for the CCK2 receptor to a large extent, but that these gastrin variants are not completely inactive. The finding that even a tenfold excess of these four carboxy-terminal Ala mutants of gastrin-17 did not have an inhibitory effect on gastrin-17-induced c-fos activation (Fig. 6) is in agreement with the notion that these mutants indeed have a much lower affinity for the CCK2 receptor. This indicates also that such molecules are unlikely to be successful as competitive inhibitors of gastrin in a therapeutic setting.

The functional importance of the carboxy-terminus of gastrin-17 for cell proliferation was further demonstrated by the (almost) complete loss of activation of the rat CCK2 receptor, when gastrin-17 was extended with a Gly or Cys next to the carboxy-terminal Phe (Fig. 3). Previously, it was also demonstrated that gastrin-17 is able to upregulate c-fos gene expression in AR42J cells, whereas Gly-extended gastrin had no effect (Todisco et al. 1995). In addition, amidation of the carboxy-terminal Phe was shown to be an absolute requirement for biological activity, i.e., stimulation of gastric acid secretion (Sugano et al. 1985, Matsumoto et al. 1987, Hilsted 1991). Our results show that removal of the carboxy-terminal amide also completely inactivates gastrin-17-induced c-fos expression (Fig. 3) in agreement with the notion that carboxy-terminal amidation is essential for biological activity of many polypeptide hormones (Merkler 1994).
In conclusion, our results show that the four carboxy-terminal amino acids of gastrin-17 are essential for its stimulatory effect on c-fos gene expression, regulating cellular proliferation. Ala-substitution variants of each of these four carboxy-terminal amino acids showed strongly reduced CCK2 receptor activation but could not act as competitive inhibitors of native gastrin. A few other amino acids of gastrin-17 showed minor effects on CCK2 receptor-mediated c-fos induction. Overall, this knowledge can be used in the development of novel strategies for therapeutic inhibition of gastrin’s proliferative actions, notably involved in gastrointestinal cancer.

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