Binding of progastrin fragments to the 78 kDa gastrin-binding protein

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Abstract The non-selective gastrin/cholecystokinin receptor antagonists proglumide and benzotript inhibit colon carcinoma cell proliferation by binding to the 78 kDa gastrin-binding protein (GBP) (Baldwin, Proc. Natl. Acad. Sci. USA, 91 (1994) 7593–7597). However, although most colon carcinoma cell lines synthesize progastrin, production of mature amidated gastrin$_{17}$ has not been observed. In order to define the structural requirements for the binding of gastrin to the GBP the affinities of various fragments of amidated amd C-terminally extended gastrin$_{17}$ for the GBP have been measured. The results indicate that the GBP recognizes both N- and C-termini of gastrin$_{17}$. Moreover since C-terminal amidation is not a prerequisite for binding of gastrin to the GBP, the GBP is a potential target for the autocrine effects of progastrin.

Key words: Autocrine loop; Gastrin; Gastrin receptor; Colorectal carcinoma

1. Introduction

Although evidence is accumulating that colorectal carcinomas utilize progastrin as an autocrine growth factor, there has been some dispute over the receptor involved [1]. Based on reports of high affinity gastrin receptors on the mouse colon carcinoma cell line MC26 [2] and on over 50% of primary human colon cancer specimens [3], it has often been assumed that the autocrine loop utilizes a gastrin/cholecystokinin (CCK)-B receptor. However the majority of colon carcinoma cell lines express only gastrin/CCK-C receptors, which have a low affinity for gastrin [4–6]. In addition the observation that the CCK-A receptor-selective antagonist, L364,718, and the gastrin/CCK-B selective antagonist, L365,260, had no effect on colon carcinoma cell proliferation at concentrations sufficient to saturate their respective receptors suggested that neither the A nor the B receptor participated in the autocrine loop [7]. The gastrin/CCK-C receptor, which is the target for the inhibitory effects of gastrin/CCK receptor antagonists on colon carcinoma cell proliferation [5], is identical to a 78 kDa gastrin-binding protein (GBP) [8]. The GBP, which has been purified from porcine gastric mucosal membranes [9,10], is a member of the family of fatty acid oxidation enzymes possessing enoyl CoA hydratase and 3-hydroxyacyl CoA dehydrogenase activities [11].

Since colon carcinoma cell lines synthesize progastrin, but fail to process the prohormone to mature C-terminally amidated gastrin$_{17}$ as shown in Fig. 1 [13–15], a candidate receptor for autocrine gastrin should not require an amidated C-terminus for binding. A cell-surface receptor binding glycine-extended gastrin$_{2-17}$ and capable of stimulating proliferation of the rat pancreatic carcinoma cell line AR4-2J has recently been reported [16]. Both binding and proliferation were uneffected by gastrin$_{17}$ or by the gastrin/CCK-B receptor antagonists, L365,260 and PD134,308. The affinity of the non-selective gastrin/CCK receptor antagonists proglumide and benzotript for the new receptor, and its structural relationship to other members of the gastrin/CCK receptor family, has not been reported yet.

Since the GBP is the target for the anti-proliferative effects of proglumide and benzotript on colon carcinoma cell lines [8], the structural requirements for binding of gastrin to the GBP were investigated, and are reported in the present paper. In particular the observation that the affinity of the GBP for glycine-extended gastrin$_{17}$ is very similar to the affinity for gastrin$_{17}$ implies that the GBP is a potential target for the autocrine effects of progastrin.

2. Experimental

2.1. Materials

Gastrin$_{17}$ was from Research Plus, Bayonne, NJ. The purity and composition of gastrin-related peptides was assessed by the manufacturer (Chiron Mimotopes, Clayton, Australia) by reversed phase high pressure liquid chromatography and by ion spray mass spectrometry, respectively. Pepstatin, benzamidine, hexamethylphosphoramide and aprotinin were from Sigma, St.Louis, MO. Na^{125}I was from NEN, North Ryde, Australia.

2.2. Gastrin cross-linking assay

The 78 kDa GBP was partially purified from detergent extracts of porcine gastric mucosal membranes by lectin and ion-exchange chromatography [9,10]. The following protease inhibitors were included in all buffers to prevent proteolysis: pepstatin 1 μM, benzamidine 1 mM, hexamethylphosphoramide 0.1% (w/v), aprotinin 500 units/ml. Crosslinking of [^{125}I]Nle^{15}-gastrin$_{2-17}$ to the GBP with disuccinimidylsuberate was measured as described previously [9] in the presence of increasing concentrations of gastrin analogues. The products of the cross-linking reaction were separated by polyacrylamide gel electrophoresis, and radioactivity associated with the 78 kDa GBP was detected and quantitated with a phosphorimager (Molecular Dynamics, Sunnyvale, CA). Initial estimates of IC$_{50}$ values, and of the levels of [^{125}I]gastrin$_{2-17}$ bound in the absence of competitor, were obtained by fitting the data with the program EBDA, and were refined with the program LIGAND as described previously [4].

3. Results

3.1. Amidation of gastrin$_{17}$ is not required for GBP binding

The effect of extending the C-terminus of gastrin$_{17}$ on binding to the GBP was investigated by comparing the potencies of glycine-extended gastrin$_{17}$ (Fig. 1) and gastrin$_{17}$ as inhibitors of cross-linking of [^{125}I]Nle^{15}-gastrin$_{2-17}$ to the GBP. The IC$_{50}$

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Abbreviations: CCK, cholecystokinin; GBP, gastrin-binding protein; IC$_{50}$, concentration required for 50% inhibition of cross-linking.

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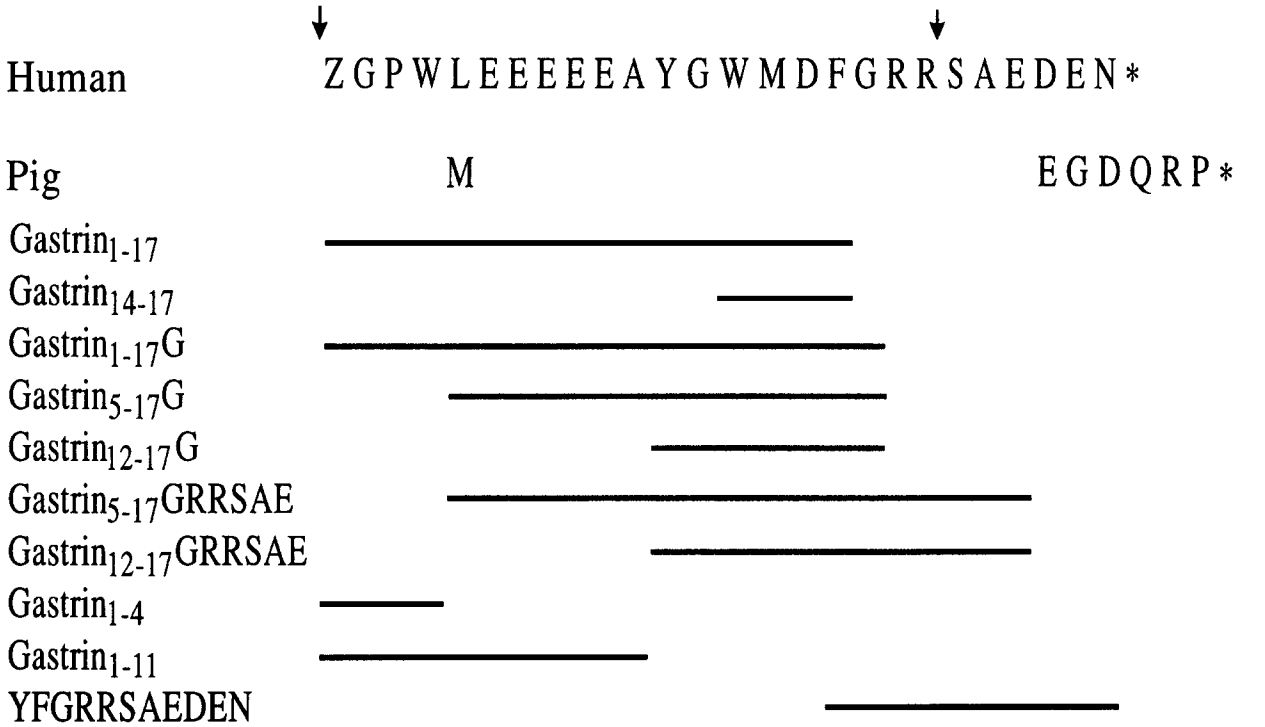

Fig. 1. Processing and sequences of gastrin-related peptides. The C-terminal sequence of human progastrin is shown in the one letter code. The corresponding region of porcine progastrin is only shown where it differs from human progastrin. Processing begins by cleavage by a dibasic-specific endopeptidase at the sites marked by arrowheads [12]. Removal of the basic amino acids by carboxypeptidase E yields glycine-extended gastrin$_{17}$ (gastrin$_{1-17}$G), which is transamidated by peptidyl-glycine α-amidating monooxygenase to form mature amidated gastrin$_{1-17}$. The indicated peptides (numbered from the N-terminus of gastrin$_{1-17}$) were synthesized according to the human sequence, with the N-terminal glutamic acid of gastrin$_{1-4}$, gastrin$_{1-11}$, gastrin$_{1-17}$ and gastrin$_{1-17}$G cyclized to pyroglutamate. The C-termini of gastrin$_{1-17}$ and gastrin$_{14-17}$ were amidated.

value for glycine-extended gastrin$_{17}$ was slightly lower than the IC_{50} value for gastrin$_{17}$ (Fig. 2, Table 1). Addition of a further 5 residues to the C-terminus of glycine-extended gastrin$_{5-17}$ did not alter the affinity of the resultant peptide for the GBP, while addition of 5 residues to the C-terminus of glycine-extended gastrin$_{12-17}$ actually resulted in a 25-fold reduction in affinity.

3.2. *Gastrin$_{17}$ binding determinants*

The regions of gastrin$_{17}$ contributing to GBP binding were investigated by testing the effect of N- and C-terminal deletion on the potency of glycine-extended gastrin$_{17}$ as an inhibitor of cross-linking of [^{125}I]Nle^{15}-gastrin$_{2-17}$ to the GBP. Removal of 4 or 11 residues from the N-terminus of glycine-extended gastrin$_{17}$ resulted in 140- and 160-fold increases, respectively, in the IC_{50} value for inhibition of cross-linking of [^{125}I]Nle^{15}-gastrin$_{2,17}$ to the 78 kDa GBP (Fig. 2, Table 1). Similarly removal of 7 residues from the C-terminus of glycine-extended gastrin$_{17}$ resulted in a 110-fold reduction in affinity. Removal of a further 7 residues from the C-terminus of glycine-extended gastrin$_{17}$ resulted in almost complete loss of binding affinity.

4. Discussion

4.1. *Comparison of gastrin receptor binding sites*

Comparison of the affinities of gastrin analogues for the 78 kDa GBP revealed that both ends of gastrin$_{17}$ contribute to binding. Thus removal of 4 residues (pyroglutamylGPW) from the N-terminus of glycine-extended gastrin$_{17}$ resulted in a 140-fold decrease in affinity, while removal of 7 residues (YGWMDFG) from the C-terminus of glycine-extended gastrin$_{17}$ resulted in a 110-fold decrease in affinity (Table 1). In reciprocal experiments the binding of the deleted peptides to the GBP was also measured. As expected gastrin$_{14-17}$ bound weakly to the GBP, but binding of the N-terminal tetrapeptide pyroglutamylGPW was barely detectable. Since benzotript, the best available antagonist for the GBP [8], is an acylated tryptophan derivative (*N*-4-chlorobenzoyl-L-tryptophan), it seems likely that the tryptophan residue of either the N-terminal or C-terminal tetrapeptide, or both, makes a significant contribution to binding.

The role of the five glutamic acid residues of gastrin$_{17}$ in GBP binding is not clear at present. The similar affinities of glycine-extended gastrin$_{5-17}$ and glycine-extended gastrin$_{12-17}$ for the GBP (Table 1) suggest that the polyglutamate sequence makes no contribution. However, deletion of the five glutamate residues from gastrin$_{1-11}$ to yield gastrin$_{1-4}$ resulted in a 60-fold decrease in affinity.

In contrast to the present results with the GBP, two lines of evidence demonstrate that the gastrin/CCK-B receptor does not recognize the N-terminus of gastrin$_{17}$. Firstly no binding of the N-terminal tridecapeptide to the gastrin/CCK-B receptor on canine parietal cells was observed even at concentrations as high as 100 μM [17]. Secondly removal of 4 residues from the N-terminus of gastrin$_{17}$ had no effect on binding to rabbit parietal cells [18]. The observation that removal of a further 5 residues, including 4 of the 5 glutamic acid residues, reduced binding to rabbit parietal cells by 100-fold additionally implied

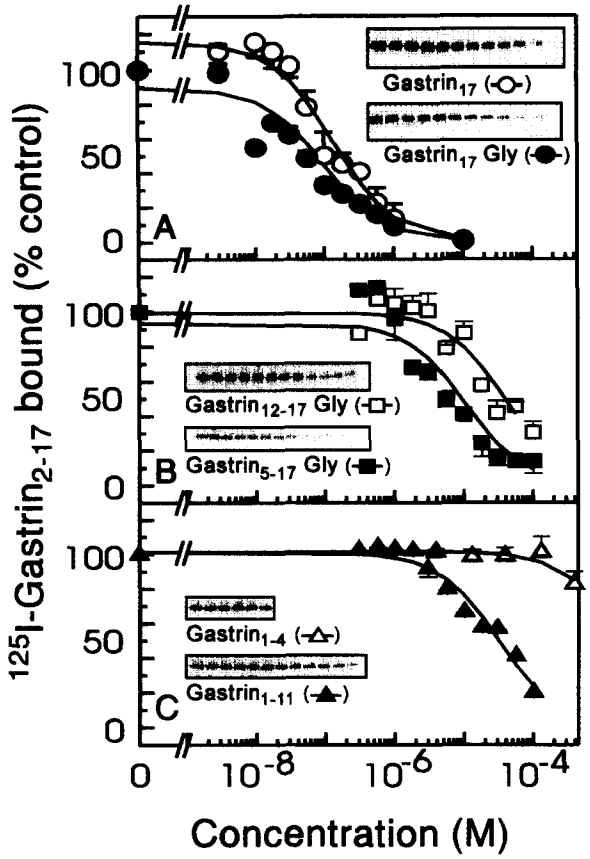

Fig. 2. Binding of gastrin-related peptides to the 78 kDa GBP. Cross-linking of [^{125}I]Nle^{15}-gastrin$_{2-17}$ to the 78 kDa GBP was measured as described in section 2 in the presence of increasing concentrations of gastrin-related peptides. Duplicate samples were subjected to electrophoresis on NaDodSO$_4$-polyacrylamide gels and the radioactivity associated with the 78 kDa GBP was quantitated by phosphorimager scanning (insets), and expressed as a percentage of the value obtained in the absence of competitor. The following values for IC_{50} and for the predicted ordinate intercept were obtained by computer fitting as described previously [4], and used to construct the indicated lines of best fit: (A) gastrin$_{1-17}$ (138 nM, 115.5%) or glycine-extended gastrin$_{1-17}$ (98 nM, 90.0%); (B) glycine-extended gastrin$_{5-17}$ (11 μM, 93.8%) or glycine-extended gastrin$_{12-17}$ (40 μM, 99.6%); or (C) gastrin$_{1-4}$ (1.96 mM, 101.7%) or gastrin$_{1-11}$ (31 μM, 101.3%). These values were averaged with the results of two similar experiments to obtain the mean values presented in Table 1.

that the pentaglutamic acid sequence contributed to gastrin/ CCK-B receptor binding [18]. The gastrin binding sites of the GBP and the gastrin/CCK-B receptor are thus clearly distinct.

4.2. Role of progastrin in colorectal carcinoma growth

There is now abundant evidence that colorectal carcinomas produce progastrin, but fail to process it into mature amidated gastrin. Gastrin mRNA has been demonstrated in both normal and neoplastic colorectal mucosa by Northern blotting [15], and in colon carcinoma cell lines by PCR [15,19,20]. The levels of gastrin mRNA detected by quantitative PCR were always less than 1 molecule/cell [20]. At the peptide level gastrin immunoreactivity was detected in 21% of colonic tumours [21]. More recently both progastrin and gastrin were detected by immunohistochemistry in more than 80% of the tumour cells in 20/23 colorectal adenocarcinomas, whereas in normal colonic mucosa only occasional crypt cells stained simultaneously for progastrin and gastrin [22]. An increase in progastrin production in colorectal tumour tissue compared to normal mucosa has also been demonstrated in tissue extracts [14, 15]. In contrast to the immunohistochemical results levels of mature gastrin were low, and did not differ between normal and tumour tissue [13–15]. Although an early report indicated that all 5 colonic carcinoma cell lines tested secreted gastrin immunoreactivity [23], more recent work revealed that all (5/5) colonic carcinoma cell lines tested produced progastrin, but that mature amidated gastrin was not present [15].

Any gastrin receptor participating in an autocrine loop in colon carcinoma cells must therefore be capable of recognizing C-terminally extended forms of gastrin. One possible candidate is the receptor for glycine-extended gastrin$_{2-17}$ recently described by Seva and coworkers [16] on the surface of the rat pancreatic carcinoma cell line AR4–2J. The new receptor binds glycine-extended gastrin$_{2-17}$ with high affinity (K_d = 0.45 nM), but does not recognize gastrin$_{17}$, CCK$_8$ or the gastrin/CCK-B receptor-selective antagonists L365,260 and PD134308. The 78 kDa GBP also fits the requirements for the autocrine receptor, since it binds glycine-extended forms of gastrin with similar affinity to mature amidated gastrins (Table 1). Although the affinity of the GBP for gastrin$_{17}$ is low (230 nM), the intracellular concentration of progastrin may be considerably higher than has been supposed previously, as discussed in a later section. Further C-terminal extension of glycine-extended gastrin$_{5-17}$ did not change its affinity for the GBP, while extension of glycine-extended gastrin$_{12-17}$ resulted in a 25-fold reduction in affinity (Table 1). In contrast to the GBP the gastrin/CCK-B receptor on canine parietal cells has much lower affinity for

Table 1
Affinities of gastrin-related peptides for gastrin receptors

Peptide	Purity (%)	IC_{50} (μM)	
		78 kDa Gastrin-binding protein	Gastrin/ CCK-B receptor
Gastrin$_{1-17}$	82	0.23 ± 0.15	5×10^{-4}
Gastrin$_{1-17}$G	78	0.19 ± 0.10	ND
Gastrin$_{5-17}$G	93	26 ± 15	ND
Gastrin$_{5-17}$GRRSAE	87	20 ± 11	ND
Gastrin$_{12-17}$G	81	31 ± 17	14 ± 71
Gastrin$_{12-17}$GRR	ND	ND	44 ± 21
Gastrin$_{12-17}$GRRSAE	91	760 ± 550	ND
Gastrin$_{14-17}$	95	370 ± 280	0.1
Gastrin$_{1-11}$	97	21 ± 9	ND
Gastrin$_{1-4}$	98	> 1000	ND
YFGRRSAEDEN	62	105 ± 21	ND

IC_{50} values (mean ± S.D. of at least 3 separate determinations, one of which is shown in Fig. 2) for the inhibition of cross-linking of [^{125}I]Nle^{15}-gastrin$_{2-17}$ to the 78 kDa gastrin-binding protein by the indicated peptides were determined as described in the legend to Fig. 2. IC_{50} values for gastrin$_{12-17}$GRRSAE, gastrin$_{1-4}$ and YFGRRSAEDEN should be regarded as estimates only because the limiting amounts of peptide available precluded assay at concentrations greater than 100, 400 and 100 μM, respectively. IC_{50} values for the gastrin/CCK-B receptor on canine parietal cells, and for the binding of gastrin$_{1-17}$ and gastrin$_{14-17}$ to the GBP, are taken from [17] and [8], respectively. The amino acid sequences of C-terminal extensions are shown in the one letter code; peptide sequences are shown in full in Fig. 1.

glycine-extended gastrin$_{12-17}$ than for mature amidated gastrins [17] (Table 1). Thus the affinities of the 78 kDa GBP and the gastrin/CCK-B receptor for glycine-extended gastrin$_{12-17}$ are similar (31 μM and 14 μM respectively), despite a 400-fold difference in their affinities for gastrin$_{17}$ (0.5 nM and 0.2 μM, respectively). A critical experiment in deciding which receptor is involved in the autocrine loop will be comparison of the affinities of all known gastrin/CCK receptors for full-length progastrin. However, the length of the prohormone (104 amino acids) has precluded organic synthesis, and will necessitate progastrin expression in, and purification from, bacterial or baculovirus systems.

Interestingly the intracellular concentration of progastrin in colon carcinoma cells reaches μM levels. Values for extracts from 5 different cell lines ranged from 17 to 54 fmol/10^6 cells [15]. Assuming a cell diameter of 20 μm, and an even distribution of progastrin throughout the cell, the internal concentrations of progastrin are in the range 4 to 13 μM. If the intracellular population of the 78 kDa GBP is accessible to progastrin, and if the binding affinity for progastrin is similar to glycine-extended gastrin$_{17}$, then the percent saturation will be between 95% and 98%. Although the gastrin/CCK-B receptor would also be saturated by the levels of progastrin present within colon carcinoma cell lines, receptor expression in this case is limited to the cell surface. In conclusion previous data on agonist and antagonist affinities [8], and the present report that the GBP binds glycine-extended gastrin$_{17}$ with an affinity similar to gastrin$_{17}$, are consistent with the conclusion that the GBP is the target for the autocrine effects of progastrin in colonic carcinoma.

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