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Importance of N- and C-terminal regions of gastrin-Gly for preferential binding to high and low affinity gastrin-Gly receptors

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Abstract

G17-Gly has been shown to stimulate the growth of DLD-1 human colon cancer cells in a biphasic manner via high and low affinity receptors. In the current study, the existence of heterogeneous receptor populations for G17-Gly on the HT-29 human colon cancer cell line was investigated. The effect of either N- or C-terminal peptide truncation on receptor binding and cell growth stimulation was also explored. [Leu^{15}]G17-Gly bound to both high (nM) and low (μM) affinity sites on HT-29 cells. The peptide stimulated cell growth in a dose-dependent and biphasic manner with maximal stimulation at 10^{-9} M peptide concentration, suggesting that, as in the case of DLD-1 cells, it is the high affinity receptor which is responsible for the growth-promoting effects. In contrast, G17(1–12) stimulated the growth of HT-29 cells in a sigmoidal fashion with an EC_{50} of 4.6×10^{-9} M. Sequential N-terminal truncation of [Leu^{15}]G17-Gly results in decreased binding to the high affinity G17-Gly receptor on DLD-1 cells. [Leu^{15}]G17(11–17)Gly bound to the low affinity G17-Gly receptor with an affinity similar to that of the full sequence peptide but was unable to displace the radioligand from high affinity sites. G17(1–6)-NH_2 was unable to displace [^{3}H]G17-Gly from either site. These results suggest that the important residues for binding to the low affinity receptor are in the C-terminal region of the peptide while those required for interaction with the high affinity receptor lie further towards the N-terminus.

Keywords: Glycine-extended gastrin; Colon cancer; Biphasic growth response; High affinity receptor; Low affinity receptor; Gastrin fragment

1. Introduction

G17-Gly (Fig. 1) is not converted to G17 but is rather one of the end products of progastrin processing and is present in the circulation at concentrations similar to that of G17 [20,4]. G17-Gly stimulates the growth of colon cancer cells in vitro and in vivo [9–11,6]. In addition, colon cancer cells secrete gastrin processing intermediates but not the mature, amidated peptide [14,19]. A receptor for G17-Gly has yet to be cloned and considerable controversy surrounds the identity and properties of such a receptor [21,7,16,12,18]. Recently, studies here showed that the DLD-1 colon cancer cell line possesses both high (nM) and low (μM) affinity receptors for G17-Gly [1]. The peptide stimulates the growth of these cells in a dose-dependent and biphasic manner with maximal stimulation at 10^{-9} M concentration. These results suggest that the high affinity receptor is responsible for the growth stimulatory effects while activation of the low affinity site may account for the decreased growth stimulation at higher peptide concentrations. In the present study, the occurrence of heterogeneous receptor populations for G17-Gly on the HT-29 human colon cancer cell line and the importance of N- and C-terminal regions of the peptide for differential re-

Abbreviations: BSA, bovine serum albumin; CCK-8, cholecystokinin-8; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ESI-MS, electrospray ionization mass spectrometry; FCS, fetal calf serum; Fmoc, 9-fluorenylmethyloxycarbonyl; G17, gastrin; G17-Gly, *N*-carboxymethyl gastrin; [^{3}H]G17-Gly, [3′,5′-3H-Tyr12,Leu15]G17-Gly; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; TFA, trifluoroacetic acid; Tris–HCl, tris hydroxymethyl aminomethane hydrochloride

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[Leu^{15}]G17	pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-NH_2
[Leu^{15}]G17-Gly	pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-Gly-OH
G(1-6)NH_2	pGlu-Gly-Pro-Trp-Leu-Glu-NH_2
G(1-12)	pGlu-Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-OH
[Leu^{15}]G(2-17)-Gly	Gly-Pro-Trp-Leu-Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-Gly-OH
[Leu^{15}]G(6-17)-Gly	Glu-Glu-Glu-Glu-Glu-Ala-Tyr-Gly-Trp-Leu-Asp-Phe-Gly-OH
[Leu^{15}]G(11-17)-Gly	Ala-Tyr-Gly-Trp-Leu-Asp-Phe-Gly-OH

Fig. 1. Sequence alignment of synthetic gastrin analogs.

ceptor binding and growth stimulation of HT-29 and DLD-1 cells were investigated.

2. Materials and methods

2.1. Cells and reagents

HT-29 and DLD-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). HT-29 cells were cultured in McCoy 5A medium containing 7.5% $NaHCO_3$ and 10% FCS while DLD-1 cells were cultured in RPMI 1640 medium containing 10% FCS. Cells were grown at 37 °C in an atmosphere of 5% CO_2 and maintained at sub-confluent levels. *N*-α-Fmoc amino acids were purchased from Advanced ChemTech (Louisville, KY, USA). Fmoc-Gly-Wang resin, Fmoc-Tyr-Wang resin, Fmoc-amide resin with Knorr linker, and peptide synthesis grade DMSO, DMF, THF, HBTU, HoBT, DIEA, and piperidine were purchased from Applied Biosystems/Perkin-Elmer (Foster City, CA, USA). Diethylether, $MgCl_2$, $NaCl_2$, NH_4OH, Tris–HCl, and TFA were from Fisher Scientific (Rockford, IL, USA). EDT, thioanisole, HEPES, EGTA, $NaHCO_3$, and Trypan Blue were from Sigma–Aldrich (Milwaukee, WI, USA).

2.2. Peptide synthesis

Peptides (Fig. 1) were synthesized using Fmoc chemistry at a 0.025 mM scale on an ABI 432A peptide synthesizer (Applied Biosystems/Perkin-Elmer) as reported previously [1,2]. Peptides were cleaved from the resin using TFA/thioanisole/EDT/H_20 (36:2:1:1, v/v/v/v) for 15 min at 0 °C followed by 105 min at room temperature.

2.3. Peptide purification and characterization

Peptides were analyzed and purified on a dual pump Gilson HPLC apparatus. Solvent A was 50 mM NH_4HCO_3 and solvent B was 60% CH_3CN in 50 mM NH_4HCO_3. Analytical HPLC was carried out on a Phenomenex 00G-4252-Y0 column (spherical C_{18}, 5 μm particle size, 3 mm × 250 mm) at a flow rate of 0.5 ml/min. For preparative HPLC, a Phenomenex semi-preparative 00G-4252-NO column (spherical C_{18}, 5 μm particle size, 10 mm × 250 mm) column at a flow rate of 4 ml/min was used. Peptides were obtained at >95% purity. Molecular weights of peptides were verified using ESI-MS with a Perkin-Elmer quadrupole mass spectrometer.

2.4. Production of radiolabeled [Leu^{15}]G17-Gly

Custom radioiodination of [Leu^{15}]G17-Gly using Chloramine T and subsequent HPLC purification was carried out by Peninsula Laboratories. The specific activity of the ^{125}I-peptide was 1300 Ci/mmol.

[3′,5′-I-Tyr^{12}, Leu^{15}]G17-Gly was synthesized at a 0.025 mM scale on an ABI 432A peptide synthesizer using *N*-α-Fmoc amino acids and tritiated using catalytic hydrogenation [17] as previously published [1]. The specific activity of the tritiated peptide was 25 Ci/mmol.

2.5. Radioligand binding

Binding of [^{3}H]G17-Gly (50 nM) to HT-29 cell membranes in competition with [Leu^{15}]G17-Gly and to DLD-1 cell membranes in competition with [Leu^{15}]G17(2–17)Gly, [Leu^{15}]G17(6–17)Gly, [Leu^{15}]G17(11–17)Gly, to G17(1–12) was assessed by previously described methods [1]. Binding of [^{125}I-Tyr^{12}, Leu^{15}]G17-Gly (50 pM), and HT-29 whole cells in competition with [Leu^{15}]G17-Gly was also determined. Cells (2×10^6 cells/well) were incubated in 100 mM Tris (pH 7.4) for 60 min at 27 °C with 50 pM [^{125}I-Tyr^{12}, Leu^{15}]G17-Gly and concentrations of [Leu^{15}]G17-Gly from 10^{-12} to 10^{-4} M. Binding of ^{125}I-labeled peptide in the presence of CCK-8 and the CCK_2 receptor antagonist L-365, 260 at a concentration of 10^{-4} M was also assessed in triplicate. The final reaction volume was 250 μl. Non-specific binding was determined in the presence 10^{-4} M unlabeled [Leu^{15}]G17-Gly. The binding was terminated by rapid filtration through Whatman GF/C glass fiber filters, that had been presoaked at 4 °C in incubation buffer containing 0.1% BSA, using a Brandell cell harvester (Biomedical R&D Laboratories Inc., Gaithersburg, MD, USA). The filters were then washed 3 times with ice-cold 50 mM Tris–HCl buffer, pH 7.4,