High and low affinity receptors mediate growth effects of gastrin and gastrin-Gly on DLD-1 human colonic carcinoma cells

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Abstract Gastrin (G17) and *N*-carboxymethylgastrin (G17-Gly) have been shown to stimulate the growth of colon cancer cells both in vivo and in vitro. The identity of the receptor mediating these effects is controversial. A recent study demonstrated the presence of a low affinity binding site for G17 and G17-Gly on the DLD-1 human colon cancer cell line. The goal of the current study was to further investigate the role of this receptor in mediating the growth-promoting effects of gastrin peptides. Binding of [Leu^{15}]G17 and [Leu^{15}]G17-Gly to DLD-1 cell membranes in competition with [^{3}H]G17-Gly was examined. Binding of [^{3}H]cholecystokinin-8 (CCK8) to DLD-1 cell membranes was also assessed. Whole cell binding experiments were carried out using [^{125}I-Tyr^{12},Leu^{15}]G17-Gly. In addition, the ability of [Leu^{15}]G17 and [Leu^{15}]G17-Gly to stimulate cell growth, as determined by cell counting, was tested. [Leu^{15}]G17 and [Leu^{15}]G17-Gly competed with [^{3}H]G17-Gly at both a high and a low affinity site on DLD-1 membranes. The IC_{50} values for [Leu^{15}]G17 were 6.0×10^{-8} M and 6.9×10^{-6} M while those for [Leu^{15}]G17-Gly were 3.2×10^{-9} M and 4.9×10^{-6} M. [^{3}H]CCK8 did not bind to either site. [Leu^{15}]G17-Gly also competed with [^{125}I-Tyr^{12},Leu^{15}]G17-Gly at both a high and a low affinity site on DLD-1 cells with similar affinities as observed with membranes. [Leu^{15}]G17 and [Leu^{15}]G17-Gly significantly stimulated the growth of DLD-1 cells in a dose-dependent and biphasic manner. The binding profiles of the peptides tested suggest that these sites are different from previously identified wild-type and mutant CCK_1 or CCK_2 receptors.

Key words: Gastrin; Glycine-extended gastrin; Colon cancer; Cell growth; Receptor binding

1. Introduction

Receptors belonging to the cholecystokinin (CCK) family are known to mediate the biological effects of gastrin and cholecystokinin. To date, two such receptors (CCK_1 and CCK_2) have been identified and cloned [1,2]. The receptors are commonly distinguished by their selectivity for gastrin 17 (G17) and CCK8. CCK8 binds both the CCK_1 and CCK_2 receptors with high affinity while gastrin is selective for the CCK_2 receptor. In addition to binding G17 and CCK8, the CCK_2 receptor also interacts with gastrin fragments and processing intermediates as well as a variety of non-peptide antagonists. The human CCK_2 receptor has been pharmacologically characterized and shown to have the following rank order of affinities for its ligands: CCK8 > G17 > pentagastrin > L-365,260 > CCK4 > *N*-carboxymethylgastrin (G17-Gly) [3,4]. The highest affinity ligands bind with sub-nanomolar affinity while the lowest (G17-Gly) binds with micromolar affinity. In addition to the wild-type receptor, mutant CCK_2 receptors which are the result of alternative splicing or point mutations have also been discovered [5–8]. These receptors bind CCK_2 receptor ligands with similar affinities and the same rank order as the wild-type receptor. The role and distribution of these variant receptors have yet to be clarified.

The observation that G17 is a potent growth factor for gastrointestinal mucosa has led to investigations of a role for this hormone in the growth of gastrointestinal cancers. Studies have indicated a possible role in the growth of gastric carcinomas [9–11] but the effect on colon cancer is more controversial. Early studies demonstrated a mitogenic effect of G17 on both normal and cancerous colonic epithelia [12–14]. The normal adult colon, however, does not express CCK_2 receptors [15]. This is also the case for the majority of colon cancer specimens [16–18]. In addition, colon cancer patients do not show increased serum levels of G17 [19–21] and there appears to be no correlation between a hypergastrinemic state and disease [22,23].

It has been shown that colon cancer cells secrete intermediates of progastrin processing but not the mature, amidated peptide [24,25]. G17 and G17-Gly are separately derived from G34-Gly in normal gastric G cells and are found at comparable concentrations in the circulation [26,27]. Numerous studies have shown that G17-Gly stimulates the growth of colon cancer cells in vitro and in vivo [28–31]. The results of these studies suggest that the effect of G17-Gly is mediated by a receptor different from the wild-type CCK_1 or CCK_2 receptors. However, reports differ as to the affinity and selectivity of such a receptor. A recent study demonstrated the presence of a micromolar affinity receptor for G17-Gly on the DLD-1

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Abbreviations: CCK8, cholecystokinin-8; [^{3}H]CCK8, [^{3}H]propionyl-CCK8; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; G17, gastrin 17; G17-Gly, *N*-carboxymethylgastrin; [^{3}H]G17-Gly, [3′,5′-^{3}H-Tyr^{12},Leu^{15}]G17-Gly; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid

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human colon cancer cell line [32]. The current study was undertaken to further characterize the receptor for G17-Gly on DLD-1 cells using optimized conditions.

2. Materials and methods

2.1. Cells and reagents

DLD-1 and CHO-K1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). DLD-1 cells were cultured in RPMI 1640 containing 10% fetal calf serum while CHO-K1 cells were grown in F12-K medium containing 10% fetal calf serum. Cells were grown at 37°C in an atmosphere of 5% CO_2 and maintained at sub-confluent levels. *N*-α-9-Fluorenylmethyloxycarbonyl (Fmoc) amino acids and *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), piperidine, and *N*,*N*-diisopropylethylamine (DIEA) for the ACT 90 peptide synthesizer were purchased from Advanced ChemTech (Louisville, KY, USA). Rink Amide Am resin and Fmoc-Gly-Wang resin were purchased from NovaBiochem (San Diego, CA, USA). Fmoc-amide resin with Knorr linker and peptide synthesis grade dimethylsulfoxide, *N*,*N*-dimethylformamide (DMF), tetrahydrofuran, HBTU, HOBt, DIEA and piperidine for the ABI 432A peptide synthesizer were purchased from Applied Biosystems/Perkin-Elmer (Foster City, CA, USA). Diethylether, NH_4OH, $MgCl_2$, Tris–HCl, trifluoroacetic acid (TFA), DIEA, and peptide synthesis grade dichloromethane (DCM) and DMF for the ACT90 peptide synthesizer were from Fisher Scientific (Rockford, IL, USA). Ethanedithiol, thioanisole, HEPES, EGTA, and trypan blue were from Sigma Aldrich (Milwaukee, WI, USA). [^{3}H]Propionyl-CCK8 ([^{3}H]CCK8; specific activity of 60 Ci/mmol) was purchased from Amersham Life Sciences (Elk Grove, IL, USA).

2.2. Peptide synthesis

Peptides were synthesized using Fmoc chemistry at either a 0.025 mM scale on an ABI 432A peptide synthesizer (Applied Biosystems/Perkin-Elmer) as reported previously [33] or at a 0.5 mM scale on an ACT 90 peptide synthesizer (Advanced ChemTech). With the ACT 90 peptide synthesizer, G17 and CCK8 were synthesized on Rink Amide Am resin while G17-Gly was synthesized on Gly-Wang resin. The reactive side chains of tyrosine, aspartic acid, and glutamic acid were *t*-but protected. Fmoc removal was accomplished using 20% piperidine in DMF and monitored at 290 nm. Couplings were carried out with a four-fold excess of amino acids using in situ activation with HBTU in 30% DCM/DMF. Coupling times were 40 min and completion was monitored using the Kaiser test [34]. Deprotection times were 20 min up to residue 10 and 35 min for the remainder of the synthesis. Peptides were cleaved from the resin using TFA/thioanisole/EDT/H_2O (36:2:1:1, 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 high performance liquid chromatograph (HPLC). 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 either a Vydac 218TP54 column (C_{18}, 5 μm particle size, 4.6 mm×250 mm) or a Phenomenex 00G-4252-Y0 column (spherical C_{18}, 5 μm particle size, 3 mm×250 mm) at flow rates of 1 ml/min or 0.5 ml/min, respectively. For preparative HPLC, either a preparative Vydac 218TP1022 column (C_{18}, 5 μm particle size, 22 mm×250 mm) with a flow rate of 9 ml/min or a Phenomenex semi-preparative 00G-4252-NO column (spherical C_{18}, 5 μm particle size, 10 mm×250 mm) column with a flow rate of 4 ml/min was used. Peptides were obtained at >95% purity. Molecular weights of peptides were verified using electrospray ionization mass spectrometry on a Perkin-Elmer quadrupole mass spectrometer.

2.4. Radiolabeled peptides

Custom iodination of [Leu^{15}]G17-Gly and subsequent HPLC purification was carried out by Peninsula Laboratories using the chloramine T method (Peninsula Laboratories, San Carlos, CA, USA). The specific activity of the ^{125}I-peptide was 1582 Ci/mmol. For the tritiated peptide, [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. Tritiation of the precursor peptide was carried out using catalytic dehalogenation as previously published [35]. The specific activity of the resulting labeled peptide was 25 Ci/mmol.

2.5. Radioligand binding

Experiments using [3′,5′-^{3}H-Tyr^{12},Leu^{15}]G17-Gly ([^{3}H]G17-Gly) were performed with membrane fractions prepared from low passage cells (29–33). Membrane preparations (240 μg/ml protein concentration) were incubated in 10 mM HEPES containing 1 mM EGTA, 130 mM NaCl, and 5 mM $MgCl_2$ for 60 min at 27°C with 50 nM [^{3}H]G17-Gly and concentrations of [Leu^{15}]G17-Gly or [Leu^{15}]G17 from 10^{-12} M to 10^{-4} M. The low specific activity of the tritiated peptide did not allow discrimination of both sites at lower radioligand concentration. Therefore, binding of [Leu^{15}]G17-Gly, to receptors on whole cells, was also assessed in competition with a low concentration of [^{125}I-Tyr^{12},Leu^{15}]G17-Gly. DLD-1 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} M to 10^{-4} M and displacement of [^{125}I-Tyr^{12},Leu^{15}]G17-Gly from whole cells by CCK8 was also tested. The final reaction volume for all experiments was 250 μl. Non-specific binding was determined in the presence of 10^{-4} M [Leu^{15}]G17-Gly. The reaction 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% bovine serum albumin. Filtration was carried out on a Brandell cell harvester (Biomedical R&D Laboratories, Gaithersburg, MD, USA). Filters were washed three times with ice-cold 50 mM Tris–HCl buffer, pH 7.4. Filters were air-dried and then soaked in Ultima Gold MV scintillation cocktail and the bound radioactivity was measured with a 1209 Rackbeta liquid scintillation counter (Wallac, Gaithersburg, MD, USA). Binding of [^{3}H]CCK8 to DLD-1 cell membranes and CHO-K1 cell membranes expressing human CCK_2 receptors was also tested at concentrations of 0.1 and 50 nM as previously reported [33].

2.6. Cell growth studies

Before beginning growth experiments, the stability of the test peptides in the presence of cells was determined using HPLC. This was done by incubating 20 000 cells/well in 24-well plates with serum free medium containing 10^{-5} M [Leu^{15}]G17-Gly or 10^{-5} M [Leu^{15}]G17. Samples of media were removed at 24-h intervals for HPLC analysis. The presence of mycoplasma in cells was examined using the Mycoplasma Detection Kit from American Type Culture Collection. Growth studies were performed using either 48- or 96-well plates. Low passage cells (29–33) were harvested at 70–90% confluence and used for growth studies. Cells were seeded in 48-well plates at a density of 7000 cells/well or in 96-well plates at a density of 3000 cells/well in serum-containing medium and allowed to attach for 48 h. The medium was removed and replaced with serum-free medium. Various concentrations of test peptide were added to the inner 24 wells of 48-well plates or inner 60 wells of 96-well plates. Cells were allowed to grow for an additional 72 h and the medium was removed. Cells were detached using 0.05% trypsin and 0.02% EDTA and counted with the aid of a hemocytometer under an inverted light microscope.

2.7. Statistical analysis

All data were analyzed using Graphpad Prizm software (Graphpad Software, San Diego, CA, USA). Comparison of fit for one- and two-site binding was determined using the *F*-test. Statistical significance of growth data was assessed using the one-way analysis of variance.

3. Results

3.1. Binding of G17 and G17-Gly to DLD-1 cells and membranes

Both [^{125}I-Tyr^{12},Leu^{15}]G17-Gly and [^{3}H]G17-Gly bound in a saturable and displaceable manner to DLD-1 cells and membrane preparations, respectively. Total binding represented less than 1% of the total added radioligand in all experiments and specific binding was 60–70% of total binding. Both [Leu^{15}]G17-Gly and [Leu^{15}]G17 were able to displace the [^{3}H]G17-Gly from DLD-1 membranes in a dose-dependent manner (Fig. 1). Non-linear regression analysis of the

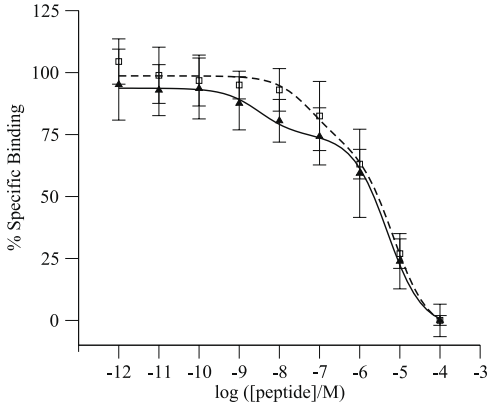

Fig. 1. Binding of [Leu^{15}]G17 (□) and [Leu^{15}]G17-Gly (▲) to DLD-1 membranes. [Leu^{15}]G17 results are the average of two experiments with four replicates each. [Leu^{15}]G17-Gly results are the average of five experiments with three to six replicates for each concentration. Results are presented as mean ± S.D. $P < 0.0001$ for two-site binding fit.

results fit the data for binding of both peptides to a two-site model with a high level of significance ($P < 0.0001$). The IC_{50} values for binding to the two sites were 3.2×10^{-9} M and 4.9×10^{-6} M for [Leu^{15}]G17-Gly and 6.0×10^{-8} M and 6.9×10^{-6} M for [Leu^{15}]G17. The fractional abundance of the two sites was in good accord with high affinity binding comprising 20 ± 5% and 26 ± 12% of the total bound radioactivity for [Leu^{15}]G17-Gly and [Leu^{15}]G17, respectively. Competition of [Leu^{15}]G17-Gly with [^{125}I-Tyr^{12},Leu^{15}]G17-Gly for receptors on whole cells also showed two sites ($P < 0.01$) with IC_{50} values of 0.83×10^{-9} M and 8.2×10^{-6} M. High affinity binding comprised 18 ± 5% of the total bound radioactivity. [^{3}H]CCK8 was unable to bind to DLD-1 cell membranes at concentrations of 0.1 nM and 50 nM and CCK8 was unable to displace [^{125}I-Tyr^{12},Leu^{15}]G17-Gly from DLD-1 cells at concentrations as high as 10^{-4} M. By contrast, [^{3}H]CCK8 bound in a saturable and displaceable manner to CHO-K1 cells expressing human CCK_2 receptors (data not shown).

3.2. Mitogenic effects of G17 and G17-Gly on DLD-1 cells

Both [Leu^{15}]G17-Gly and [Leu^{15}]G17 were shown to be stable in the presence of DLD-1 cells for a period of 72 h at 37°C with no appreciable decrease in HPLC peak size (data not shown). [Leu^{15}]G17-Gly and [Leu^{15}]G17 stimulated cell growth in a dose-dependent and biphasic manner with maximal stimulation at 10^{-9} M and 10^{-8} M, respectively (Fig. 2).

4. Discussion

Our results show that a heterogeneous population of receptors for both G17 and G17-Gly is present on DLD-1 cells. The presence of these sites in similar proportions on both whole cells and membrane preparations suggests that their numbers are not regulated by the presence of guanine nucleotides as is the case for high and low affinity states of G protein-coupled receptors. The inability of [^{3}H]CCK8 to bind to either site suggests that these are different from both wild-type and mutant CCK_1 and CCK_2 receptors identified to date. The higher affinity of G17-Gly than of G17 at both sites also supports this conclusion. The ability of the G17 and G17-Gly to stimulate growth of DLD-1 cells at low concentrations ($< 10^{-8}$ M) suggests that it is the high affinity site that mediates the mitogenic effects of these peptides.

The identity of the receptor which mediates the trophic effects of G17 and G17-Gly on both normal and cancerous colonic cells is controversial. Some groups have demonstrated the presence of nanomolar affinity sites on various primary and cultured tissues [36–38] while others have detected sites with micromolar affinity [32,39]. The selectivity of these sites for G17 and G17-Gly is also in dispute. Stepan et al. showed the presence of a nanomolar affinity receptor on HT-29 cells by saturation studies using ^{125}I-G(2–17)-Gly up 0.5 nM [36]. Mauss et al. demonstrated the presence of a similar site on HT-29 cells using ^{125}I-G17 [37]. In addition, saturation experiments with 0.01–0.5 nM ^{125}I-G17 showed the presence of high affinity sites for gastrin on 57% of 67 primary colon cancer specimens [38]. In contrast, Yang et al. found a micromolar affinity site for both G17 and G17-Gly on DLD-1 cells by competition experiments with ^{125}I-G17-Gly and G17-Gly [32]. Imdahl et al. showed the presence of a similar, micromolar affinity gastrin binding site on 36% of 102 primary colorectal cells by competition experiments with ^{125}I-G17 and G17 [39].

Assuming that both binding sites exist, it is not surprising that the various studies above could detect only a single site. The groups reporting only nanomolar binding sites for G17 and G17-Gly relied on saturation experiments with low concentrations of ^{125}I-labeled peptides. The conditions used in each of these studies would be insufficient to detect the presence of micromolar sites since the K_d of such a site would be considerably higher than the highest radioligand concentration used. Thus, a normal saturation assay using ^{125}I-labeled peptides would be unable to detect the presence of a heterogeneous population of receptors with nanomolar and micromolar affinities since the radioligand would ultimately saturate only the high affinity sites. By contrast, the reports in which only micromolar affinity sites were detected were based on competition experiments with a small range (10^{-9}–10^{-4} M) and number (5) of unlabeled ligand concentrations. Our early

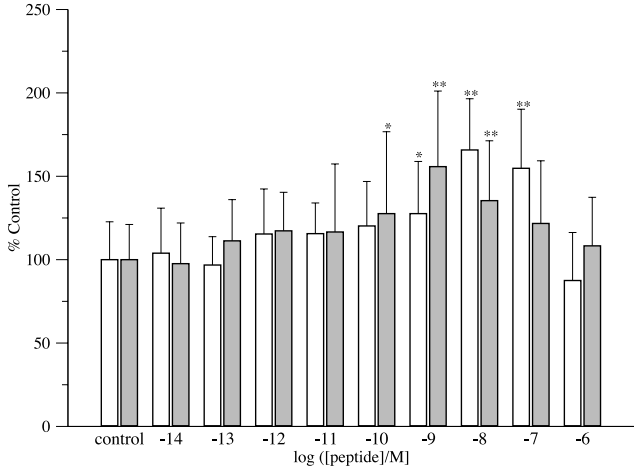

Fig. 2. Stimulation of growth of DLD-1 cells by [Leu^{15}]G17 (white bars) and [Leu^{15}]G17-Gly (gray bars). Results are mean ± S.D. and represent six experiments with four to five replicates each for [Leu^{15}]G17-Gly and four experiments with four to five replicates each for [Leu^{15}]G17. *$P < 0.05$, **$P < 0.01$.

radioligand binding experiments with both the DLD-1 and HT-29 cell lines demonstrated the presence of a single, micromolar affinity site similar to that shown by Yang et al. [32]. These experiments used [^{3}H]G17-Gly with the same number and range of ligand concentrations used by Yang et al. Such conditions would not be adequate to detect the presence of a high affinity site since cold ligand concentrations spanning at least two orders of magnitude on either side of the IC_{50} should be used. Further optimization of conditions, including the use of a larger number and range of cold ligand concentrations from 10^{-12} M to 10^{-4} M G17 or G17-Gly, allowed us to detect a second, high affinity site on DLD-1 cells. Further experiments are currently being performed using HT-29 cells.

Studies by Ishizuka et al. on the mitogenic effects of neurotensin on pancreatic cancer cells demonstrated a similar biphasic growth response to that observed here [40]. The group showed dose-dependent phospholipase C activation in response to neurotensin. In addition, they also observed cAMP activation but only at high peptide concentrations. They proposed that both high and low affinity receptors mediate the biphasic growth response. Growth experiments carried out by Stepan et al. [36] and Mauss et al. [37] with HT-29 cells showed a similar biphasic growth response to G17 and G17-Gly to that shown here with DLD-1 cells. It is possible that, in colon cancer cells, high affinity receptors mediate a G17- or G17-Gly-induced stimulation of growth and activation of low affinity receptors results in decreased proliferation. Further investigation of the secondary messenger responses in these cells is required.

The results presented here may help to clarify some of the current controversy surrounding the binding affinity of the so-called glycine-extended gastrin receptor. These results together with the discovery of multiple affinity states for other receptors including the CCK_1 and CCK_2 receptors [41,42] emphasize the importance of using appropriate radioligands and conditions that allow detection of high and low affinity receptors at both ends of the affinity spectrum. Further studies using other colon cancer cell lines and primary tissue cultures are warranted to investigate the possibility of heterogeneous receptor populations for G17 and G17-Gly.

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