Stimulation of gastrin-CCKB receptor promotes migration of gastric AGS cells via multiple paracrine pathways

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Noble, Peter J. M., Geraint Wilde, Michael R. H. White, Steven R. Pennington, Graham J. Dockray, and Andrea Varro. Stimulation of gastrin-CCKB receptor promotes migration of gastric AGS cells via multiple paracrine pathways. Am J Physiol Gastrointest Liver Physiol 284: G75–G84, 2003; 10.1152/ajpgi.00300.2002.—Responses to G protein-coupled receptor stimulation may be mediated by paracrine factors. We have developed a coculture system to study paracrine regulation of migration of gastric epithelial (AGS) cells after stimulation of gastrin-CCKB receptors. In cells expressing this receptor, G-17 stimulated migration by activation of protein kinase C. However, G-17 also stimulated the migration of cells expressing green fluorescent protein, but not the receptor, when they were cocultured with receptor-expressing cells consistent with activation of paracrine signals. The use of various pharmacological inhibitors indicated that gastrin stimulated migration via activation of the EGF receptor (EGFR), the erbB-2 receptor tyrosine kinase, and the MAP kinase pathway. However, gastrin also released fibroblast growth factor (FGF)-1, and migration was inhibited by the FGF receptor tyrosine kinase inhibitor SU-5402. Flow cytometry indicated that in both cell types, gastrin increased MAP kinase via activation of EGFR but not FGF-R1 or erbB-2. We conclude that gastrin-CCKB receptors stimulate epithelial cell migration partly via paracrine mechanisms; transactivation of EGFR is only one component of the paracrine pathway.

epidermal growth factor; epithelial cell migration; G protein-coupled receptor; fibroblast growth factor

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 Typically, cells were mixed, but in some experiments, the two cell types were segregated into different parts of the well by applying them to either side of a divider that was subsequently removed. Studies of migration were made using scratch wound assays executed by making a linear denuded region using a pipette tip. Cells were washed four times with serum-free medium and human G-17 (Bachem, St Helens, Merseyside, UK) applied either alone or with one of the following: antibody to EGF-R (Oncogene Research Products, Boston, MA) or the metalloproteinase inhibitor GM-6001 or the inhibitors of intercellular signaling AG-1478, AG-825, SU-5402, Ro-32,0432 (CN Biosciences, Nottingham, UK) or PD-98059 (Promega, Southampton, UK). The number of cells crossing a margin of 500 μm into an acellular area was counted. Alternatively, cells were cultured on glass coverslips in 30-mm Petri dishes that were then mounted on the heated stage of a Zeiss Axiovert 100 microscope in a humidified chamber, and images were captured at 5-min intervals with a Hamamatsu 480–800 CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan) and Kinetic Imaging AQM-2000 software (Kinetic Imaging, Liverpool, UK). Time-lapse image series were analyzed using Scion Image software (Scion, Frederick, MD), which is based on National Institutes of Health Image. Cell tracking and frame-by-frame recording of movements were used to calculate cell speed over 30-min periods.

Flow cytometry. Phospho-MAP kinase was detected by flow cytometry (7). Briefly, cells were suspended in 2% EDTA and fixed in 2% paraformaldehyde at 37°C before permeabilization in 90% methanol at 4°C. Cells were then incubated with antibodies to phospho-MAP kinase or total MAP kinase (New England BioLabs, Beverly, MA) followed by AlexaFluor-647-conjugated secondary antibodies (Molecular Probes, Leiden, Netherlands). Cells were passed through a flow cytometer (FACS Vantage SE; Becton Dickinson) and results analyzed with Cell Quest software (Becton Dickinson). AlexaFluor-647 fluorescence excited by the 633-nm laser was analyzed, gating separately for AGS-GFr and AGS-GFP cells on the basis of GFP fluorescence excited by the 488-nm laser.

Identification of putative mediators by proteomic methods. Cells were cultured in 75-ml flasks to 70% confluence in full medium, then washed three times in serum-free medium and cultured in the latter for 24 h either with or without G-17 (1 nM). Medium was concentrated on Sep-Pak C18 cartridges according to the manufacturer’s instructions and eluted with acetonitrile (50% vol/vol in water) containing 0.02 M sodium phosphate pH 7.4 and lyophilized. Aliquots of samples were reconstituted in isoelectric focusing sample buffer (7 M urea, 25% acetonitrile, 2% 3-2 mercaptopropanol, 0.5% Triton X-100, 0.1 M Tris-HCl pH 8.5, 0.2% sodium dodecyl sulfate). Samples were applied to 1-D gels (12% acrylamide), run using standard slab gel electrophoresis conditions, and stained with silver nitrate. The gel was scanned at 700 nm and analyzed using PDQuest software (Bio-Rad).
2 M thiourea, 4% wt/vol 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 40 mM Tris, 1% wt/vol DTT). Samples containing equal quantities of protein were diluted to 240 μl with rehydration buffer (9 M urea, 2% wt/vol CHAPS, bromophenol blue, 2% IPG buffer (Amersham BioSciences Pharmacia, Amersham, UK), 0.28% DTT) and incubated overnight with nonlinear IPG strips (pH 3–10NL, Amersham Biosciences) in a reswelling chamber. Two-dimensional polyacrylamide gel electrophoresis (2-DE) was undertaken as described (16). Isoelectric focusing was on a Multiphor II system (Amersham BioSciences), with an initial linear gradient of 0–500 V over 1 min, then 500–3,500 V linearly over 1 h and 30 min, followed by 3,500 V for 5 h and 40 min. The IPG strips were then incubated with SDS equilibration buffer (50 mM Tris, 6 M urea, 30% vol/vol glycerol, 2% wt/vol SDS, bromophenol blue, containing 1% wt/vol DTT) for 15 min followed by incubation in the same buffer containing 2.5% (wt/vol) iodoacetamide for a further 15 min. The strips were applied to the surface of 12% SDS-PAGE gels and sealed with agarose (0.5% wt/vol agarose in electrophoresis buffer containing a trace of bromophenol blue). Electrophoresis was carried out using an Amersham BioSciences Dalt II apparatus at 25°C, with initial separation at a constant 5 W/gel for 30 min followed by 20 W/gel until the dye front had migrated ~18 cm (typically 3–3.5 h). Gels were then transferred to polypropylene containers and immersed in fixative (40% methanol and 7% acetic acid in water), before staining with colloidal Coomassie blue (25), for 3 h (4:1 mixture of 0.1% wt/vol Coomassie brilliant blue-G250 dye, 2% orthophosphoric acid, and 10% wt/vol ammonium sulphate, and methanol). Gels were destained in 10% vol/vol acetic acid, 25% vol/vol methanol, and subsequently stored in 25% methanol at 4°C. Protein spots were isolated from Coomassie-stained 2-DE gels, sliced into 1-mm² pieces washed in 50% acetonitrile/25 mM ammonium bicarbonate, pH 7.8, and dried in a SpeedVac. The dried gel

![Image of migration assay](https://example.com/migration-assay.png)

**Fig. 2.** Gastrin stimulated migration of AGS-GFP cells in coculture with AGS-Ga cells. A and B: phase (top) and fluorescence (bottom) show scratch wound assays of cocultured cells treated with G-17 (1 nM). The denuded area is indicated by horizontal lines and is acellular at the start (A; 0 h) and filled with cells after 8 h treatment with gastrin. Arrows indicate the position of AGS-green fluorescence protein GFP cells (scale bars, 50 μm). C: quantitative data of numbers of AGS-Ga and AGS-GFP cells migrating in response to G-17 (1 nM, 8 h); means ± SE, n = 8. D: speed of AGS-Ga and AGS-GFP cells after 5 h stimulation by G-17 (1 nM). Values are means ± SE; n = 10; *P < 0.05.
spots were rehydrated with 4–10 μl digestion buffer [10 μg/ml modified sequencing grade trypsin (Promega) in 25 mM NH₄HCO₃] and incubated overnight at 37°C. The resulting peptides were extracted in 4 μl water followed by 7 μl 30% acetonitrile/0.1% trifluoroacetic acid (TFA), centrifuged, and the supernatants were mixed 1:1 with matrix (10 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/50% ethanol/0.001% TFA) containing adrenocorticotropic hormone (50 fmol/μl), and 1 μl of the mixture was spotted onto a 96-position target (8). Peptide mass fingerprints were obtained semiautomatically on a MALDI mass spectrometer (Micromass, Manchester, UK), and resultant mass lists were searched against a nonredundant protein database (Swiss-Prot/Trembl) using ProteinLynx 3.4 (Micromass) and Mascot (Matrix Sciences).

**Statistics.** Results are expressed as means ± SE, and comparisons made by t-test were considered significant at P < 0.05.

**RESULTS**

*Gastrin increases cell migration.* The effect of G-17 on cell migration was first examined in scratch-wound assays. Confluent monolayers of the parental or the empty vector transfected cells cultured on plastic showed little or no migration into an acellular zone over 24 h and did not respond to G-17. In contrast, AGS-GR cells stimulated by gastrin exhibited little movement up to 6 h, but thereafter, there was rapid migration of individual cells that resulted in complete

Fig. 3. Gastrin-stimulated migration of AGS-GFP cells was dependent on EGF-R, erbB-2, and MEK. A: MEK inhibitor PD-98059 (20 μM) significantly reduced AGS-GFP cell migration in response to G-17 (1 nM, 8 h). B: inhibitors of EGF-R, AG-1478 (3 μM), and erbB-2, AG-825 (5 μM), both significantly reduced AGS-GFP cell migration. Values are means ± SE; n = 3; *P < 0.05.

Fig. 4. AGS-GFP cell migration in response to activation of EGF-R. A: immunoneutralisation of EGF-R (10 μg/ml of antibody to EGF-R) inhibited the response to G-17 (1 nM, 8 h). B: transforming growth factor (TGF-α; 100 ng/ml) stimulated migration of AGS-GR cells, although responses were less than those to G-17 (1 nM) at 8 h. C: effect of TGF-α was inhibited by AG-1478 (3 μM) but not AG-825 (5 μM). Values are means ± SE; n = 3–5; *P < 0.05.
filling of the acellular zone by ~8 h (Fig. 1, A–C). The responses were concentration dependent over the range of 30 pM to 3 nM (Fig. 1D). Similar responses were stimulated by PMA (100 nM) in both the parental cell line (not shown) and AGS-GR cells (Fig. 1E).

The response to gastrin-CCKB receptor stimulation was not secondary to proliferation, because G-17 inhibits proliferation in these cells (34). There was, however, a requirement for transcription, because cell migration in response to gastrin was inhibited by actinomycin D (G-17; 39.5 ± 8.0 cells entering an acellular zone of side length 500 µm; G-17 and 2.0 µg/ml actinomycin D: 14.3 ± 5.5; n = 3, P < 0.05). Time-lapse videomicroscopy revealed cell movements within confluent monolayers in the control cultures and an approximately twofold increase in the speed of these movements in response to gastrin. The peak increase in speed occurred at ~5 h, i.e., before the maximal migration of cells into the acellular zone.

The migration of AGS-GR cells in response to gastrin-CCKB receptor stimulation was inhibited by the receptor antagonist L-740,093 (100 nM, not shown). The response to G-17 was partially blocked by the PKC inhibitor Ro-32,0432 (1 µM); the same concentration of Ro-32,0432 fully inhibited the effect of PMA (Fig. 1E). In addition, migration in response to G-17 was significantly reduced by the EGF-R tyrosine kinase inhibitor AG-1478 (3 µM) and by the MAP ERK kinase (MEK) inhibitor PD-98059 (20 µM; Fig. 1F), indicating that activation of EGF-R and MEK contributed to the migratory response to gastrin.

Gastrin-stimulated migration is partly mediated by paracrine mechanisms. Because gastrin stimulates EGF-R ligand production in vivo (32, 36), we asked whether paracrine actions of EGF-R ligands might account for gastrin-stimulated migration of AGS-GR cells. We, therefore, examined migration of AGS cells stably transfected with GFP (and not expressing the CCKB receptor) when cocultured with AGS-GR cells. We, therefore, examined migration of AGS cells stably transfected with GFP (and not expressing the CCKB receptor) when cocultured with AGS-GR cells (Fig. 1A–C) and exhibited a significant increase in speed, although this was lower than AGS-GR cells (Fig. 1D). The migration of AGS-GFP cells in response to G-17 was not observed when they were cultured alone, which is compatible with the idea that gastrin increases cell migration by release of a motogen.

In a coculture model in which AGS-GR and AGS-GFP cells were segregated into spatially distinct regions within the wells, gastrin increased the numbers of AGS-GR cells migrating into a denuded zone (5.05 ± 1.5-fold increase over unstimulated controls; P < 0.05) but had no effect on the numbers of migrating AGS-GFP cells (1.05 ± 0.27-fold relative to control). The results therefore suggest that relatively close proximity of AGS-GR and AGS-GFP cells is required for stimulation of the latter by gastrin.

We then examined the relative responses of AGS-GR and AGS-GFP cells to inhibition of EGF-R and MEK. There was a profound inhibition of AGS-GFP migration in response to both PD-98059 (20 µM) and AG-1478 (3 µM; Fig. 3, A and B). For comparison, we also examined the effect of an inhibitor of erbB-2 receptor tyrosine kinase, AG-825 (5 µM), and an inhibitor of platelet-derived growth factor receptor tyrosine kinase, AG-1295 (5 µM). The effect of G-17 on migration of AGS-GFP cells was fully inhibited by AG-825 and was significantly reduced in AGS-GR cells (Fig. 3B). In contrast, AG-1295 had no effect (not shown).

Consistent with the idea of paracrine release of an EGF-R ligand, we found that in cocultures of AGS-GR and AGS-GFP cells, addition of an antibody to EGF-R (10 µg/ml) inhibited the migration of AGS-GFP cells but had only a modest effect on cocultured AGS-GR cell migration (Fig. 4A). Moreover, addition of TGF-α (100 ng/ml) stimulated migration; although, when comparisons were made after 8 h, the response was less than that to G-17 (Fig. 4B). The effect of TGF-α was inhibited by AG-1478 but not by AG-825 (Fig. 4C).

Metalloproteinases mediate gastrin-stimulated cell migration. The liberation of membrane-bound precursors of EGF-R ligands from the cell surface can be prevented by inhibitors of metalloproteinases (13), and so we asked whether the same inhibitors prevented gastrin-stimulated cell migration. The metalloproteinase inhibitor GM-6001 (25 µM) significantly inhibited gastrin-stimulated migration of AGS-GFP cells in cocultures with AGS-GR cells (Fig. 5).

Identification of FGF-1 as a gastrin-regulated stimulant of migration. To identify potential paracrine factors that might mediate the action gastrin, we concentrated from gastrin-treated AGS-GR cells and separated the material by 2-DE (Fig. 6). Differentially expressed spots were taken for MALDI-TOF analysis of tryptic peptides. Two adjacent spots identified in this way corresponded to FGF-1. The relevant spectra (Fig. 6, Ba and Bb) corresponded to seven and six tryptic peptides of FGF-1, respectively, and gave a 28% coverage of the entire sequence in each case. This assignment was also confirmed in a second independent experiment.

Because gastrin increased FGF-1 in the medium, we examined the effect of FGF on migration. After 8 h,
GPCR STIMULATION OF EPITHELIAL CELL MIGRATION

A

Control

G-17

B

a

b

C

AGS-GFP

AGS-GFP

Cells

G-17

SU-5402

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there was stimulation by FGF, although responses were smaller than those to gastrin (not shown). The FGF-receptor 1 (FGF-R1) inhibitor SU-5402 (25 \( \mu \)M) virtually abolished the response of AGS-GFP cells to gastrin and significantly reduced gastrin-stimulated cell migration in AGS-GR cells (Fig. 6).

**Activation of the MAP kinase pathway in cocultured cells.** The experiments described above, using inhibitors of various receptors and transduction pathways, leave open the issue of whether these agents are acting on AGS-GR cells to regulate production of a paracrine mediator or on AGS-GFP cells to block their responses.

To determine directly whether there was activation of the MAP kinase pathway in both AGS-GR and AGS-GFP cells, we used flow cytometry to separately estimate phosphorylated p42/44 MAPK in the two cell types when they were cocultured. In response to G-17, there was significantly increased p42/44 MAPK phosphorylation in both AGS-GR and AGS-GFP cells as previously described (34). Activation was de-
detectable at 15 min, and the maximum was at 30 min; the time course was similar in the two cell populations. Parallel studies using antibodies to total p42/44 MAP kinase revealed no change in response to G-17 (Fig. 7A). As expected, TGF-α stimulated phosphorylation of p42/44 MAP kinase, and there was no difference between AGS-GFP cells and AGS-GR cells (not shown).

The increase in p42/44 MAP kinase phosphorylation in response to gastrin in both cells was inhibited by PD-98059 (20 μM; Fig. 7B). Interestingly, the increase in phosphorylated p42/44 MAP kinase in AGS-GFP cells was completely inhibited by AG-1478 (3 μM), whereas that in AGS-GR cells was only partially reversed (Figs. 7C and 8). Because erbB-2 and FGF-1 were implicated in the paracrine migratory response to G-17, we then examined the effect of inhibitors of these tyrosine kinases (AG-825 and SU-5402, respectively) on phosphorylation of p42/44 MAP kinase. In both AGS-GF and AGS-GFP cells, AG-825 (5 μM) and SU-5402 (25 μM) had no effect on MAP kinase phosphorylation (Fig. 8).

**DISCUSSION**

The results presented here show that activation of a GPCR, the gastrin-CCKB (or CCK2) receptor, stimulates cell migration through mechanisms involving PKC and stimulation of EGF-R and the MAP kinase pathway. With the use of cocultures of cells that expressed GFP but not the gastrin-CCKB receptor, we showed that gastrin-stimulated migration was partly mediated through local paracrine signals sensitive to inhibition of metalloproteinase activity, EGF-R, erbB-2, and the MAP kinase pathway. Moreover, with the use of a proteomic approach, we identified FGF-1 as a gastrin-stimulated motogen and showed that FGF-R1 inhibition reduces migration, although this seems not to be mediated by the MAP kinase pathway. The data indicate GPCR activation induces a program of cell migration, both directly and indirectly via multiple paracrine pathways.

Signaling through GPCRs is recognized to stimulate EGF-R phosphorylation (9, 22, 10, 21), which, in turn, leads to activation of the MAP kinase cascade. Multiple mechanisms may mediate these events. In particular, both intracellular activation of EGF-R and extracellular pathways mediated by release of EGF-R ligands have been identified (21, 28). Stimulation of GPCRs coupled to Gαq/11 activates PKC and increases intracellular calcium, both of which lead to proteolysis of the membrane-bound precursor forms of HG-EGF or TGF-α (11, 15). The latter event is thought to be mediated by members of the ADAM (a disintegrin and a metalloproteinase) family and can be suppressed by metalloproteinase inhibitors (13). The present data indicate that GPCR-stimulated shedding of EGF-R ligands contributes to increased migration in gastric epithelial cells. However, several lines of evidence indicate that this is not the only mechanism regulating migration. Thus the effect of TGF-α on migration was relatively modest over periods up to 8 h when responses to gastrin were maximal. Second, the effect of gastrin was reduced by the erbB-2 tyrosine kinase inhibitor AG-825, which had no effect on TGF-α-induced migration, or induction of MAP kinase activity. Third, we identified FGF-1 as a gastrin release motogen and showed that inhibition of FGF-R1 blocked gastrin-stimulated migration but had no effect on the phosphorylation of MAP kinase. Together then, the data support the idea that although GPCR stimulation of epithelial cell migration may be partly mediated via paracrine activation of EGF-R, this cannot fully account for migratory responses, and other mediators are likely to be involved.

To identify paracrine activation of the MAP kinase pathway, we applied flow cytometry with immunodetection of activated MAP kinase (7) to cocultured cells distinguished by GFP expression. A similar approach has recently been applied to other cell types (7, 27). The approach is potentially valuable in separately dissecting signaling cascades in mixed populations of cells interacting through paracrine messengers. In the present study, we were able to show that although...
inhibitors of three different receptor tyrosine kinase reduced paracrine-stimulated migration (i.e., EGF-R, erbB-2, FGF-R1), only EGF-R was linked to MAP kinase activation. Previous studies (34) using the present cellular system have shown that gastrin stimulation of HB-EGF shedding accounts for a proliferative response in AGS-GFP cells. We have also identified a paracrine mechanism regulating expression of a promoter-luciferase reporter for plasminogen activator inhibitor type 2 (PAI-2) in AGS cells cocultured with AGS-GFP cells (33). The threshold concentration of gastrin for paracrine stimulation of migration (100–300 pM) was similar to that for proliferation (34). However, it seems likely that there are differences in the mechanisms involved in gastrin-regulated migration, proliferation, and gene expression. Thus the proliferative response could be accounted for by HB-EGF stimulation of EGF-R, whereas paracrine regulation of PAI-2 expression did not involve EGF-R. Moreover although activation of EGF and FGF receptors influences migration in both AGS-GFP and AGS-GFP cells, the proliferative response is seen only in the latter (34). Analysis of the mechanisms would therefore seem to be complicated by interactions between different paracrine mediators and between paracrine-regulated events and events determined as a direct consequence of gastrin-CCKB receptor activation.

Cell migration is important in normal development and in the maintenance of gastric epithelial integrity during wound healing. Dysfunctional migration is a feature of tumor invasion in cancer. The present data suggest stimulation of the gastrin-CCKB receptor might regulate migration both by direct intracellular effects and indirectly by paracrine mediators. There are several ways these observations might relate to phenomena in vivo. First, the gastrin-CCKB receptor is normally expressed by parietal and enterochromaffin-like cells. There is parietal cell migration from the proliferating zone of the gastric glands in the isthmus toward the base of the gland, and studies in transgenic mice suggest gastrin stimulates the migration of these cells (20). The paracrine action of gastrin-stimulated motogens could provide a mechanism for coordinated migration of different cell populations in response to gastrin, although some cell types might not express the gastrin-CCKB receptor. Second, recent work suggests that, in vivo, there is increased expression of the gastrin-CCKB receptor by surface epithelial cells in response to wounding (30). Together with the present data, this observation suggests a functional role for the gastrin receptor in enhancing wound healing by migration of adjacent undamaged epithelial cells (which do not express the receptor). Third, there is evidence from transgenic mice that increased gastrin gene expression, particularly in the presence of Helicobacter felis infection, may stimulate a progression to invasive gastric cancer (36). Interestingly, it has been reported (24) that the gastrin-CCKB receptor might be expressed by proliferating cells in hypergastrinemic mice, raising the possibility that, in some circumstances, gastrin might act directly to regulate migration of proliferating cells. Finally, it is worth noting that other products of the gastrin gene might also regulate epithelial cell migration. In particular, the COOH-terminal Gly-extended gastrins, which have very low affinity for gastrin-CCKB receptors, stimulate migration of a non-transformed gastric epithelial cell line, whereas stimulation of gastrin-CCKB receptors was without effect (18). The cells used in the present study do not respond to Gly gastrins (unpublished observations). However, there is evidence that, in vivo, there might be interactions between amidated and Gly gastrins for stimulation of acid secretion (6), and possible interactions for cell migration cannot be excluded.

There is a growing body of evidence that stimulation of the gastrin-CCKB receptor in vivo is associated with production of multiple paracrine mediators. These include histamine, which controls acid secretion (2), somatostatin (38), growth factors of the EGF family (23, 34, 36), members of the Reg family (14, 17), and members of the FGF family (3). These paracrine mediators allow control of acid secretion and cell growth. The present data indicate that paracrine control mechanisms might also extend to control of epithelial cell migration. The normal organization of gastric epithelial cells into glands also requires that, during migration, cells maintain appropriate cell-cell and cell-matrix interactions. Pagliocca et al. (26) have recently shown that gastrin also regulates the assembly of AGS-GFP cells into tubularlike cellular arrays when the cells are cultured on extracellular matrix. Together with the present data, it would appear that stimulation of migration depends on a set of complex interactions that include the production of several paracrine mediators.

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