Gastrin regulates the heparin-binding epidermal-like growth factor promoter via a PKC/EGFR-dependent mechanism

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Gastrin is a peptide hormone that is important as both an acid secretagogue (2) and as a trophic factor for the gastrointestinal mucosa (4, 9, 10). Infusion of gastrin into rats has been associated with increased gastric proliferation and increased mucosal thickness (4), and studies in transgenic mice have shown that overexpression of amidated gastrin results in parietal cell atrophy, foveolar hyperplasia, increased gastric mucosal proliferation, and eventually invasive gastric cancer (25).

Heparin-binding epidermal-like growth factor (HB-EGF) is the major EGF receptor (EGFR, HER) ligand expressed in the gastric mucosa (16) and has been shown to activate both HER1 and HER4 as well as HER3 through receptor transactivation (19). Effects include increasing mucosal proliferation, prostaglandin synthesis, and cell migration while inhibiting gastric acid and pepsinogen secretion. EGFRs are expressed by parietal cells, G cells, and chief cells, including cells in the neck region of the gastric gland where the gastric stem cells reside (16).

More specifically, HB-EGF, EGFR (HER1), and the gastrin receptor (CCK-2R) are coexpressed by parietal cells. The precise role of CCK-2R on parietal cells is unclear, but parietal cells have been implicated in directing gastric differentiation (1, 12), as has gastrin (4, 9, 10, 25). Studies have shown that administration of gastrin stimulates the cleavage of pro-HB-EGF into mature HB-EGF and stimulates expression of HB-EGFs at the mRNA and protein levels by Northern blot analysis in both whole rat stomach and rat gastric epithelial cell lines (15, 24). Moreover, transgenic mice that overexpress amidated gastrin and develop invasive gastric cancer demonstrate an upregulation of HB-EGF at the time when foveolar hyperplasia occurs (25). Hence, CCK-2R on parietal cells may regulate HB-EGF expression and cleavage and in so doing regulate gastric mucosal growth and differentiation.

The human HB-EGF promoter has one major transcription initiation site. Several potential binding sites for transcription factors have been identified, but to date most of the studies have been performed in nonepithelial cell lines. MyoD has been shown to regulate HB-EGF expression in skeletal muscle cells through binding to an E-box element located ∼510 from the transcriptional start site (27). Ap-1 has been shown to mediate stretch-induced expression of HB-EGF in bladder smooth muscle cells by binding to an element ∼993 from the transcriptional start site (17). It has also been shown that activation of MAP kinase by oncogenic raf can upregulate the HB-EGF promoter through binding to an Ets-2 site (∼974 to ∼988) in NIH/3T3 cells (13). The regulation of the HB-EGF promoter in gastric epithelial cells has not yet been studied.

To better define the mechanisms by which gastrin regulates the HB-EGF promoter, we performed studies to define the signaling pathways and cis-acting regulatory elements involved in mediating gastrin’s effects on the HB-EGF promoter.

MATERIALS AND METHODS

Construction of the human HB-EGF reporter constructs. A 2.0-kb human HB-EGF clone of 5′-flanking sequence (kind gift of Mu-En Lee, Boston, MA) was cloned into the Psp-2 luciferase reporter construct for use in transient transfection studies. Serial deletion constructs were generated by PCR and subcloned into the XhoI and HindIII sites of the Psp-2 luciferase reporter construct.

Heterologous promoter constructs were generated by PCR amplification with the resulting products being subcloned into the enhancerless promoter-luciferase construct pT81. Mutant promoter constructs were generated through PCR mutagenesis and were subcloned into the enhancerless promoter-luciferase construct pT81.

Tissue culture and transfections. The AGS-E cell line was generated by transfection of AGS cells (ATCC CRL 1739) with the expression vector pEF1a-CCK-2R (6) (kind gift of Ramnik Xavier, University of Massachusetts Memorial Medical Center, Division of Digestive Diseases and Nutrition, 55 Lake Ave. North, Worcester, MA 01655 (E-mail: timothy.wang@umassmed.edu)).
Boston, MA), which expresses full-length human CCK-2 receptor mRNA. Stably transfected cells were initially selected in puromycin (1 mg/ml in complete medium). As a positive control, these cells demonstrated stimulation of the human histidine decarboxylase promoter by gastrin in transient transfection studies (data not shown). The clone exhibiting the greatest gastrin response, AGS-E, was used in subsequent experiments. Cells were grown in DMEM containing 10% fetal bovine calf serum, 100 IU/ml penicillin, and 100 IU/ml streptomycin.

Transient transfection experiments were performed using the calcium phosphate precipitation method (5 Prime → 3 Prime, Boulder, CO). The medium was changed to serum-free conditions (Ultraculture) 12–14 h after transfection. Gastrin or PMA (Sigma, St. Louis, MO) was added the next day to stimulate HB-EGF promoter activity. Inhibitors such as forskalin, wortmannin, H7, PD-98059, CRM 197 (Sigma); matrix metalloproteinase (MMP)-II and AG 1478 (Calbiochem, San Diego, CA); and CRM-197 (kind gift of Gerhard Raab, Boston, MA) were added up to 4 h before stimulation, after dose–response studies determined their optimal concentration. Cells were then harvested, and luciferase assays were performed after 1, 2, 4, 6, 8, 16, 24, or 48 h of stimulation. Luciferase assays were performed in triplicate using luciferin (Promega, Madison, WI) using a Monolight luminometer (Analytical Luminescence Laboratory) as previously described (25). HB-EGF-luciferase activity is expressed as fold increase compared with unstimulated controls. The basal promoter-luciferase construct pT81 was used as an additional control. All experiments were repeated at least four independent transfections. A thymidine kinase-Renilla luciferase expression construct (Promega) was cotransfected in at least one of the four independent transfections to control for transfection efficiency.

Electromobility shift assays. Nuclear extracts were obtained from gastrin-deficient mice (11) receiving an 8-kw infusion of either saline or gastrin (10 μmol·kg −1·h −1), as well as from a strain-matched wild-type 129/Sv × C57BL/6 control mouse and from AGS-E cells as previously described (25). One-hundred milligrams of lysate were then run on a 16% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ). HB-EGF was detected by an enhanced chemiluminescence method using a 1:1,000 primary anti-membrane (Amersham, Piscataway, NJ). HB-EGF was detected by an enhanced chemiluminescence method using a 1:1,000 primary anti-membrane (Amersham, Piscataway, NJ). HB-EGF was detected by an enhanced chemiluminescence method using a 1:1,000 primary anti-membrane (Amersham, Piscataway, NJ). HB-EGF was detected by an enhanced chemiluminescence method using a 1:1,000 primary anti-membrane (Amersham, Piscataway, NJ). HB-EGF was detected by an enhanced chemiluminescence method using a 1:1,000 primary anti-membrane (Amersham, Piscataway, NJ). HB-EGF was detected by an enhanced chemiluminescence method using a 1:1,000 primary anti-membrane. The resulting RNA blots were then hybridized with an [γ-32P]ATP and then incubated with 10 μg of crude nuclear extracts from AGS-E cells in EMSA buffer for 30 min on ice, followed by UV cross-linking for 5 min using a UV transilluminator (TM-36, UVP, San Gabriel, CA). The DNA-protein complexes were then boiled for 10 min with 2× SDS-loading buffer and electrophoresed on a 12% SDS-PAGE gel along with prestained protein markers (Invitrogen). The gel was then dried and exposed to a Fuji phosphoimaging screen overnight, and the screen was scanned using a phosphoimager (Fuji FLA-5000).

RESULTS

Gastrin induces HB-EGF promoter activity in a luciferase reporter assay. Gastrin-induced HB-EGF promoter activity was confirmed through transient transfection studies using a 1.8-kb human HB-EGF promoter-luciferase reporter construct transfected into AGS-E cells. Gastrin stimulation resulted in a four- to fivefold induction of HB-EGF promoter activity, with an initial increase in HB-EGF promoter activity seen at 2 h and a peak effect at 24 h (Fig. 2A). Response to gastrin was also dose dependent, with peak activity seen with the 10−7 M dose (Fig. 2B).

Protein samples were obtained from gastrin-treated AGS-E cells either before or after 24-h stimulation with 10 μg of crude nuclear extracts from AGS-E cells as previously described (25). One-hundred milligrams of lysate were then run on a 16% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ). HB-EGF was detected by an enhanced chemiluminescence method using a 1:1,000 primary anti-HB-EGF M-18 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) dilution and a 1:10,000 secondary dilution.

Nuclear extracts were obtained from gastrin-deficient AGS-E cells, either before or after 24-h stimulation with gastrin (10−7 M) as previously described (25). Briefly, 10 μg of nuclear extract protein were incubated with an [α-32P]-labeled double-strand oligonucleotide probe of interest (Table 1) in a buffer containing 10 mM Tris·HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, 1 μg of poly(dA·dT), and 10% glycerol with a final volume of 20 μl for 20 min at room temperature. The resulting DNA-protein complexes were then run on a 6% nondenaturing polyacrylamide gel containing 0.25% Tris borate/EDTA buffer at a constant current of 15 mA.

For competition experiments, the nuclear extracts were incubated with an excess of competitor double-strand oligonucleotides (Table 1) at room temperature for 10 min before the addition of the radiolabeled probe. For supershift experiments, the nuclear extracts and antibodies (Santa Cruz Biotechnology) were incubated for 10 min at room temperature followed by a 30-min incubation at 4°C before the addition of the radiolabeled probe. Densitometry was performed as above.

UV cross-linking. Wild-type and mutant 2a (Mut 2a) double-strand oligonucleotides were end-labeled with [γ-32P]ATP and then incubated with 10 μg of crude nuclear extracts from AGS-E cells in EMSA buffer for 30 min on ice, followed by UV cross-linking for 5 min using a UV transilluminator (TM-36, UVP, San Gabriel, CA). The DNA-protein complexes were then boiled for 10 min with 2× SDS-loading buffer and electrophoresed on a 12% SDS-PAGE gel along with prestained protein markers (Invitrogen). The gel was then dried and exposed to a Fuji phosphoimaging screen overnight, and the screen was scanned using a phosphoimager (Fuji FLA-5000).

RESULTS

Gastrin stimulates HB-EGF expression in the mouse stomach and in the human gastric cancer cell line AGS-E. To show that gastrin can upregulate HB-EGF in an intact animal, we performed a Western blot analysis of stomachs from gastrin-deficient mice infused with either amidated gastrin (10 μmol·kg −1·h −1) or saline for 8 wk. A strain-specific 129/Sv × C57BL/6 wild-type mouse was used as a control. These studies revealed that the gastrin-deficient mice had a threefold decrease in HB-EGF protein expression and that administration of amidated gastrin for 8 wk resulted in a nearly 10-fold increase in HB-EGF expression (Fig. 1A).

We then made similar observations in a model gastric cancer cell line. Amidated gastrin was administered to the human gastric cancer cell line AGS-E for 24 h, RNA was harvested for Northern blot analysis, and protein was extracted for Western blot analysis. This experiment revealed that gastrin administration results in a marked induction of HB-EGF mRNA expression when controlled for GAPDH expression (Fig. 1B), and a marked increase in HB-EGF protein expression when controlled for β-actin expression (Fig. 1C), that is comparable with the response seen after gastrin administration in mice (Fig. 1A) and in rats (15). This demonstrated that the AGS-E cell line would be a useful model system for studying gastrin’s regulation of the HB-EGF promoter in gastric epithelial cells.

Table 1. Oligonucleotides used for EMSA studies

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<tr>
<th>Oligo</th>
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<tr>
<td>Wt</td>
<td>−69 GCGGCGGCGGCGG −58</td>
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<td>Mut 2a</td>
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<tr>
<td>Mut 2b</td>
<td>−69 CGTTTGGCGGCGG −58</td>
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<tr>
<td>Mut 3a</td>
<td>−69 GCGGCTTGGCGG −58</td>
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<tr>
<td>Mut 3b</td>
<td>−69 GCGGCGGTTGGC −58</td>
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clearly essential region from /H11002/ secondary to impairment of the basal promoter activity. The HB-EGF promoter (19) (Fig. 2C) were stimulated with gastrin (10⁻⁸ M) for 24 h and were then harvested for protein isolation. A Western blot was then performed using 100 μg of protein per lane, and probed with an anti-HB-EGF antibody. Gastric tissue from a strain-specific mice were infused with 8 wk of gastrin (10⁻⁹ M: gastrin-deleted mice) or saline (KO) and then killed, gastric mucosal tissue was isolated, and 100 mg of protein were run on a Western blot probing /H9262/RNA collection. A Northern blot was performed using 10⁻⁹ M: semicon-primed GAPDH probe was used as an internal control. C: lane and probed with a semicon-primed human HB-EGF cDNA probe. A consistent band C1 (Fig. 3A) was highly competitive to nuclear protein binding compared with the wild-type competitor and Mut 2b (P < 0.02). Addition of 25 mM EDTA to chelate zinc resulted in a significant decrease in binding to the probe, suggesting that a zinc-finger transcription factor is binding this response element (Fig. 3A).

Sp1 does not mediate gastrin responsiveness in the HB-EGF promoter. Analysis of the sequence of the gastrin response element revealed the presence of a consensus Sp1 site (-62 -GGGCGG- -57). This, coupled with the fact that the nuclear protein involved appeared to belong to the zinc-finger family of transcription factors, raised the possibility that the gastrin response was mediated by the transcription factor Sp1. The importance of Sp1 mediating gastrin responsiveness was tested by cotransfection of a dominant-negative Sp1 expression construct into AGS cells with the full-length 2.0-kb HB-EGF-luciferase reporter constructs. There was no effect on gastrin responsiveness by the dominant-negative Sp1 expression construct on the full-length HB-EGF promoter or the heterologous promoter construct containing the gastrin response element (Fig. 3B). As a control, we also showed that cotransfection of the dominant-negative Sp1 expression construct could inhibit EGF induction of the gastrin promoter (Fig. 3C), which has previously been shown to be mediated through binding of Sp1 (14).

These findings were supported by EMSA assays using antibodies to Sp1 and Sp4, which showed no significant supershifting of the bands (Fig. 3A).
UV cross-linking suggests that at least two transcription factors bind the gastrin response element. To discern whether one or more transcription factors are involved in the DNA-binding band seen on EMSA, UV cross-linking was performed using the wild-type probe or the Mut 2a probe (see Table 1). As seen in Fig. 3D, following electrophoresis on a denaturing gel, there is distinct binding of probe to protein or protein complexes at 80, 120, and 200 kDa when the wild-type probe is used. Hence, it is likely that the single band observed on the nondenaturing gel necessary for the EMSA study (Fig. 3A) corresponds to the larger 200-kb band on the cross-linked gel (Fig. 3D). In contrast with the Mut 2a probe, the 80-kDa band and, to a lesser extent, the 120-kDa band are weaker compared with the wild-type probe, and the composite 200-kDa band is absent. This would suggest that the 80- and 120-kDa proteins form a complex, at its simplest a heterodimer, and that the mutation in the −69 to −67 region inhibits binding of these proteins to the gastrin response element.

Gastrin stimulates HB-EGF promoter activity through a PKC-dependent signaling pathway. Because gastrin has previously been shown to activate the PKC and PKA pathways in AGS cells expressing the CCK-2 receptor (8) and the phosphoinositid 3-kinase (PI3-K) pathway in AR4–2J cells (22), we performed experiments to establish which signaling pathway mediated gastrin-induced HB-EGF expression. Gastrin-induced stimulation of the 2.0-kb HB-EGF-luciferase construct was inhibited by coadministration of the PKC inhibitor H7 (Fig. 4A) and the MEK inhibitor PD-98059 (Fig. 4B) and by cotransfection with a dominant-negative Erk1 expression construct (Fig. 4C). In contrast, the PI3-K inhibitor wortmannin (Fig. 4D) and the PKA antagonist H-89 (Fig. 4E) did not significantly decrease gastrin-induced HB-EGF promoter activity at all concentrations tested; in fact, both inhibitors could increase gastrin-stimulated HB-EGF promoter activity. In keeping with this, stimulation of AGS-E cells transiently transfected with the 2.0-kb HB-EGF-luciferase construct with the PKC agonist phorbol ester resulted in a fivefold increase in promoter activity, whereas stimulation with the PKA agonist forskolin did not result in increased promoter activity (data not shown).

Stimulation of the HB-EGF promoter by gastrin is partially mediated by activation of the EGF. Studies have shown that G protein-coupled receptor-mediated signaling is often depen-
dent on the activation of the EGFR (23). It has also been reported that the activation of EGFR is dependent on cleavage of pro-HB-EGF by MMP-3 (18). Additionally, it has been shown that gastrin can induce cleavage of pro-HB-EGF via MMP-3, releasing mature HB-EGF that can then activate the EGFR as evidenced by tyrosine phosphorylation (16). To see if cleavage of pro-HB-EGF resulting in EGFR activation was important in gastrin-induced HB-EGF promoter activity, we first assessed whether addition of the MMP-3 inhibitor MMP-II affected gastrin responsiveness. Preincubation with 3 nM MMP-II (Calbiochem) resulted in a 43% decrease in gastrin-induced HB-EGF promoter activity at 24 h (Fig. 5A). These results were confirmed using CRM-197, a mutant diptheria toxin that is specific for inhibiting HB-EGF activity by deplet-
It is likely that the effect of both the MMP-3 inhibitor and CRM-197 is to block activation of EGFR. To show that EGFR activation is important in mediating gastrin-induced HB-EGF expression, the EGFR antagonist AG 1478 (100 nM) was added to AGS-E cells transiently transfected with the 2.0-kb human HB-EGF promoter-luciferase promoter construct and stimulated with gastrin for 24 h. The addition of AG 1478 resulted in a 55% decrease in gastrin-induced HB-EGF promoter activity (Fig. 5C).

DISCUSSION

In this study, we used AGS-E cells as a model system to study how gastrin regulates HB-EGF expression. Gastrin results in increased mRNA and protein expression in AGS-E cells, which mimics the results seen with gastrin infusion on the rat and murine gastric mucosa. This occurs in a time- and dose-dependent manner through the activation of the PKC/Erk-1 signaling pathway. The cis-regulatory elements lie in the region −69 to −58 from the previously mapped transcriptional start site on the HB-EGF promoter (3). EMSA analysis using the region from −69 to −58 as a probe reveals that nuclear protein(s) bind this region, and one likely represents a zinc-finger transcription factor. Although there is a Sp-1 site within the −69 to −58 region of the HB-EGF promoter, cotransfection of dominant-negative Sp1 expression constructs does not affect gastrin responsiveness of the HB-EGF promoter, and EMSA analysis revealed that antibodies to Sp1 and Sp4 failed to supershift the bands, suggesting that this family is not involved in mediating gastrin responsiveness. Finally, we showed that activation of the EGFR through cleavage of pro-HB-EGF is an important component of gastrin-induced HB-EGF expression.

Fig. 3. Zinc-finger transcription factor binds the region between −69 and −58 that is not Sp1. A: EMSA were performed using nuclear extracts from gastrin-stimulated AGS-E cells with the −64 to −53 region of the HB-EGF promoter used as a probe. Lane 1: probe alone without nuclear extract. Lane 2: probe and nuclear extract. Lanes 3–7: a 50-fold excess of cold probe has been added as a competitor probe (sequences as Table 1). Lane 3: with wild type. Lane 4: mutant 2a. Lane 5: mutant 2b. Lane 6: mutant 3a. Lane 7: mutant 3b. Lane 8: has WT probe with preincubation with 25 mM EDTA to chelate zinc. Lane 9: has WT probe with anti-Sp1 antibody. Lane 10: has WT probe with anti-Sp4 antibody.

B: AGS-E cells were transfected with the 2.0-kb human HB-EGF-luciferase reporter construct (gray bars) and stimulated with gastrin (10⁻⁴ M) with or without cotransfection with a dominant-negative Sp1 expression construct (dnSp1).

C: to verify that the dominant-negative Sp1 expression construct was functional, AGS-E cells were transfected with a 1.3-kb human gastrin promoter-luciferase reporter construct and stimulated with EGF (10⁻⁴ M) with or without cotransfection with a dominant-negative Sp1 expression construct (dnSp1).

D: UV cross-linking experiments were performed using either the wild-type −64 to −53 probe or the Mut 2a probe. The 200-kDa band (arrow) is absent in the Mut 2a lane.
Gastrin is a known gastric growth/differentiation factor, as evidenced by the altered gastric mucosal architecture seen in gastrin-overexpressing (25) and gastrin-deficient mice (7, 11) and the development of gastric cancer in the gastrin-overexpressing mice (25). Taken together, these studies suggest that gastrin regulates the HB-EGF promoter.

Fig. 4. Gastrin stimulates HB-EGF expression through a PKC-dependent signaling pathway. Transient transfection studies were performed in AGS-E cells with the 2.0-kb HB-EGF promoter-luciferase reporter construct and then stimulated with gastrin (10^{-7} M) for 24 h in the presence of the H7 (A), PD-98059 (B), a cotransfected dominant-negative Erk1 expression construct (C), wortmanin (D), or H-89 (E). Luciferase activity is expressed as a percentage relative to gastrin-stimulated cells in the absence of any inhibitor and represents the mean of 4 independent experiments. *P < 0.05.

Fig. 5. Gastrin stimulation of the HB-EGF promoter is partially dependent on transactivation of the EGF receptor (EGFR). AGS-E cells were transiently transfected with the 2.0-kb HB-EGF promoter-luciferase reporter construct and then stimulated with gastrin (10^{-7} M) for 24 h with or without pretreatment with various doses of MMP-II (A), 10^{-4} M CRM-197 (B), or various doses of AG 1478 (C). Luciferase activity is expressed as a fold increase relative to unstimulated controls and represents the mean of 4 independent experiments. *P < 0.05.

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the trophic actions of gastrin in the gastric mucosa are mediated through upregulation of HB-EGF.

It has been shown that activation of the CCK-2R on parietal cells results in increased HB-EGF expression and cleavage of pro-HB-EGF resulting in release of mature HB-EGF that can then activate the EGF receptor (15). This suggests that one of the major functions of the CCK-2R in parietal cells may be to mediate the regulation of HB-EGF, which in turn can regulate gastric mucosal growth/differentiation. Thus understanding the regulation of the HB-EGF by gastrin may lead to valuable insight into the regulation of gastric differentiation.

Gastrin has also been shown to upregulate the HDC, chromogranin-A genes, and VMAT-2 through a PKC-dependent pathway (8, 26, 28). Interestingly, it appeared that inhibitors to the PKA and PI3-K signaling pathways can increase gastrin-stimulated HB-EGF promoter activity, suggesting that the PKA/PI3-K signaling pathways may normally inhibit gastrin-stimulated HB-EGF promoter activity.

The cis-regulatory elements in the HB-EGF promoter differ from those seen in the aforementioned gastrin-responsive genes, with the key area on the HB-EGF promoter being located at −69-CGGGGGGGGGCGG−58 from the transcriptional start site. Gastrin regulation of the HDC promoter is mediated by three overlapping gastrin cis-acting response elements, which bind novel and as of yet undefined transcription factors (20, 21). Gastrin induced activation of the chromogranin A promoter mediated by Sp1/Egr1 binding site −88-CGGGGGGGGC−77 and CREB site −71-TGACGTAAA−64 (8). Finally, gastrin has been shown to upregulate the vesicular monoamine transporter-2 (VMAT2) promoter through an AP-2/Sp-1 binding site −61-CCCTCCGCCC−48 (26). Although most of these elements are GC rich, they do not share significant homology with the gastrin response element found on the HB-EGF promoter.

EMSA analysis reveals that nuclear proteins can bind this cis-regulatory element. Competition studies using mutant inhibitors show that the mutant competitor containing a mutation between −66 and −64 on the HB-EGF promoter (Mut 2b) exerted the most competition on nuclear protein binding to the gastrin response element (−69 to −58), whereas the mutant competitor spanning −69 to −67 (Mut 2a) and mutant competitors spanning −63 to −61 (Mut 3a) and −60 to −58 (Mut 3b) exerted significantly less inhibition on binding of the nuclear protein(s) to the element. This suggests that the area of the gastrin response element most important to nuclear binding lies between −69 to −67. Additionally, when these poorly competitive mutations (Mut 2a, 3a, and 3b) are placed upstream of a luciferase reporter construct, they exhibit significantly decreased gastrin responsiveness, supporting these regions as important in mediating gastrin responsiveness.

Given that there is an Sp1 site in this element, that Sp1 expression is upregulated by gastrin in AGS-E cells, and that Sp1 plays a role in gastrin induction of the chromogranin-A promoter, we investigated Sp1’s role in gastrin’s induction of the HB-EGF promoter. EMSA studies revealed no significant supershift with a Sp1 antibody. More importantly, coexpression of a dominant-negative Sp1 construct did not affect gastrin induction of the HB-EGF promoter, whereas it did inhibit EGF induction of the gastrin promoter, a system that has been previously described to be Sp1 dependent (14). SP4 likewise did not appear to be playing a role in gastrin-induced HB-EGF promoter activity by EMSA analysis.

UV cross-linking studies suggest that the band seen on EMSA represents a complex of at least two proteins that runs at 200 kDa, made up of an 80- and a 120-kDa component. At least one of these proteins would appear to be a zinc-finger transcription factor, as the addition of EDTA in concentrations sufficient to chelate zinc results in the loss of the gel shift band. The identities of these two proteins remain to be elucidated; unfortunately, it is difficult to predict the transcription factors that bind GC-rich DNA-binding elements by DNA sequence alone. We are currently attempting to identify the transcription factors involved by yeast one-hybrid screening and mass spectrometry.

Studies have suggested that mitogenic G protein-coupled receptor signaling is dependent on transactivation of EGFR (HER1) (23) and that this transactivation may require cleavage of pro-HB-EGF through a PKC-dependent pathway (18). Pre-incubation with a MMP-3 inhibitor (which would prevent cleavage of pro-HB-EGF), or with mutant dipheria toxin (which depletes membrane-bound pro-HB-EGF), significantly inhibited gastrin induction of the HB-EGF promoter, suggesting that activation of the EGFR does play an important role in gastrin’s regulation of HB-EGF. This was supported when the EGFR antagonist AG 1478 also attenuated induced HB-EGF activity.

Thus it appears that gastrin-stimulated activation of its cognate GPCR results in activation of the PKC signaling pathway. One of the downstream effects of PKC signaling is the activation of MMP-3, allowing for the cleavage of pro-HB-EGF and release of mature HB-EGF. Mature HB-EGF is then free to bind the EGFR, resulting in EGFR activation. EGFR activation then contributes to increased HB-EGF expression (Fig. 6).

In summary, we further defined the mechanisms by which gastrin upregulates HB-EGF expression. We defined novel cis-acting regulatory elements in the HB-EGF promoter and showed that the transcription factor(s) involved differ from those previously described to mediate gastrin-induced signaling, as well as from those previously described to regulate gastrin-stimulated HB-EGF promoter activity, whereas it did inhibit EGF induction of the gastrin promoter, a system that has been previously described to be Sp1 dependent (14). SP4 likewise did not appear to be playing a role in gastrin-induced HB-EGF promoter activity by EMSA analysis.

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Studies have suggested that mitogenic G protein-coupled receptor signaling is dependent on transactivation of EGFR (HER1) (23) and that this transactivation may require cleavage of pro-HB-EGF through a PKC-dependent pathway (18). Pre-incubation with a MMP-3 inhibitor (which would prevent cleavage of pro-HB-EGF), or with mutant dipheria toxin (which depletes membrane-bound pro-HB-EGF), significantly inhibited gastrin induction of the HB-EGF promoter, suggesting that activation of the EGFR does play an important role in gastrin’s regulation of HB-EGF. This was supported when the EGFR antagonist AG 1478 also attenuated induced HB-EGF activity.

Thus it appears that gastrin-stimulated activation of its cognate GPCR results in activation of the PKC signaling pathway. One of the downstream effects of PKC signaling is the activation of MMP-3, allowing for the cleavage of pro-HB-EGF and release of mature HB-EGF. Mature HB-EGF is then free to bind the EGFR, resulting in EGFR activation. EGFR activation then contributes to increased HB-EGF expression (Fig. 6).

In summary, we further defined the mechanisms by which gastrin upregulates HB-EGF expression. We defined novel cis-acting regulatory elements in the HB-EGF promoter and showed that the transcription factor(s) involved differ from those previously described to mediate gastrin-induced signaling, as well as from those previously described to regulate
HB-EGF expression. Transactivation of the EGFR by CCK-2R activation by gastrin plays an important role in the regulation of HB-EGF by gastrin. Given the tight regulation of HB-EGF by gastrin, we speculate that one of the major functions of gastrin-induced activation of CCK-2R in parietal cells is to regulate expression of HB-EGF and in so doing modulate the growth/differentiation of the gastric mucosa.

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