Gastrin regulates the TFF2 promoter through gastrin-responsive cis-acting elements and multiple signaling pathways

Shuiping Tu,* Alfred L. Chi,* SeonHee Lim, Guanglin Cui, Zina Dubeykovskaya, Wandong Ai, John V. Fleming, Shigeo Takaishi, and Timothy C. Wang

Division of Digestive and Liver Diseases, Department of Medicine, College of Physicians and Surgeons, Columbia University, New York, New York

Submitted 28 July 2006; accepted in final form 16 February 2007

Tu SP, Chi AL, Lim SH, Cui G, Dubeykovskaya Z, Ai W, Fleming JV, Takaishi S, Wang TC. Gastrin regulates the TFF2 promoter through gastrin-responsive cis-acting elements and multiple signaling pathways. Am J Physiol Gastrointest Liver Physiol 292: G1726–G1737, 2007. First published March 1, 2007; doi:10.1152/ajpgi.00348.2006.—Trefoil family factor 2 (TFF2) is expressed in gastrointestinal epithelial cells where it serves to maintain mucosal integrity and promote epithelial repair. The peptide hormone, gastrin, stimulates acid secretion but also induces proliferation of the acid-secreting mucosa. Because the relationship between these peptides of overlapping function is not understood, we chose to investigate the regulatory effect of gastrin on TFF2 expression. The expression of mRNA and protein of TFF2 was determined by RT-PCR and immunohistochemical staining, respectively. A series of truncated and mutant murine TFF2 promoter constructs was generated. Promoter activity was assessed using dual luciferase reporter assays. Gastrin-responsive DNA-binding sites in the TFF2 promoter were evaluated by electrophoretic mobility shift assay. Gastrin significantly increased the level of endogenous mRNA of TFF2 in the gastrin receptor-expressing AGS-E gastric cancer cell line in a time- and dose-dependent manner. TFF2 protein expression in the gastric fundus was elevated in hypergastrinemic (INS-GAS) transgenic mice and reduced in gastrin-deficient mice. Gastrin treatment increased TFF2 promoter activity through cis-acting regions, containing CCAATA- and GC-rich enhancers. Pretreatment with Y-F476, a gastrin/CCKB receptor antagonist, abolished gastrin-dependent promoter activity. Inhibitors of protein kinase C (PKC), mitogen/extracellular signal-regulated kinase (MEK1), and phosphatidylinositol 3-kinase (PI 3-kinase) reduced gastrin-dependent TFF2 promoter activity, whereas an epithelial growth factor receptor (EGFR) inhibitor had no effect. We found that gastrin regulates TFF2 transcription through a GC-rich DNA-binding site and a PKC-, MEK1- and PI 3-kinase-dependent but EGFR-independent pathway. Regulation of TFF2 by gastrin may play a role in the maintenance and repair of the gastrointestinal mucosa.


gastrin; trefoil family factor 2; gastrin-responsive element; gene

THE TREFOIL FAMILY FACTOR (TFF) comprises a group of three small proteins bearing one or more trefoil motifs, triple loop structures maintained by disulfide bridges between highly conserved cysteine residues (36, 38, 40). Under normal conditions, TFF1, TFF2, and TFF3 are differentially expressed in surface (foveolar) mucus cells of the stomach, mucous neck cells of the stomach, and goblet cells of the intestinal epithelium in rodent, respectively (36). In humans, TFF2 is expressed in Brunner’s glands in the duodenum, the basal portion of the antral/pyloric glands, and in the midzone of the gastric/body mucosa, where it is localized to mucous neck cells and rarely to a few deeper cells (15, 29). TFFs constitute an established class of regulatory peptides involved in mucosal protection and repair of the gastrointestinal tract (15, 29, 36, 38, 40). TFF2, also known as spasmolytic polypeptide, was the first member of the family to be identified. In addition to roles in gastric cytoprotection and repair (11, 15, 29, 39), TFF2 is also thought to participate in the mucosal immune response (9) and is regulated by both proinflammatory (27) and anti-inflammatory cytokine (5). Treatment with exogenous TFF peptides alleviates gastric damage (3, 30) and colon inflammation through upregulation of repairing mechanisms and modulation of the inflammatory response (13, 35).

Gastrin is a peptide hormone produced primarily by G cells, endocrine cells located in the gastric antrum that regulate acid secretion. However, gastrin also acts as a growth factor for normal gastric oxyntic mucosa, stimulating the proliferation and migration of acid-secreting cells (parietal cells) and histamine-producing cells (ECL cells; see Ref. 12). A number of studies have indicated that, in the setting of gastric ulceration or injury, gastrin can promote ulcer healing and repair (26, 37), and overexpression of gastrin in transgenic animals leads to mucosal hyperplasia (20). The physiological actions of gastrin are mediated by the gastrin-CCKB subtype receptors (CCKBR; see Ref. 12), which are expressed normally on ECL cells, parietal cells, and mucous neck cells (15). CCKBR has also been implicated in gastric mucosal wound healing, with increased CCKBR expression noted in epithelial cells at the regenerative mucosal ulcer margin (32). To date, a number of downstream target genes of gastrin have been reported, including matrix metalloproteinase 9 (44), plasminogen activator inhibitor 2 (41), tenascin-c, S100A6, myosin light chain kinase (25) and cyclooxygenase-2 (20), and TFF1 (23).

Hypergastrinemic (INS-GAS) transgenic mice, with or without Helicobacter infection, show increased mucosal proliferation and TFF2-expressing cell types that precedes the development of gastric cancer (42). In the rat stomach, the TFF2-expressing lineage, designated spasmolytic polypeptide-expressing metaplasia (SPEM), is typically found at the base of fundic glands, and the lineage is often positive for proliferating cell nuclear antigen (45). In Helicobacter-infected wild-type C57BL/6 and INS-GAS transgenic mice, the TFF2-expressing SPEM lineage is the precursor for invasive cancer (28, 42).

*S. Tu and A. L. Chi contributed equally in this study.

Address for reprint requests and other correspondence: T. C. Wang, Division of Digestive and Liver Diseases, Dept. of Medicine, College of Physicians and Surgeons, Columbia Univ., 1130 St. Nicholas Ave., Rm. 925, 9th Fl., New York, NY 10032 (e-mail: tcw21@columbia.edu).

G1726 0193-1857/07 $8.00 Copyright © 2007 the American Physiological Society http://www.ajpgi.org
One study has shown that *H. pylori* infection leads to a reduction in luminal TFF2 in the human stomach (18). However, other studies have suggested that *H. pylori* infection results in significantly increased expression of TFF2 in chronic fundic gastritis, particularly in association with dysplasia (14, 33). In one study, the SPEM lineage was present in 68% of fundic biopsies from patients with *H. pylori*-associated gastritis but was absent in biopsies of fundic mucosa from patients without *H. pylori* infection. The SPEM lineage was found in 91% of gastric cancer, typically located in mucosa adjacent to the carcinoma or areas of dysplasia (33). In another study, SPEM was noted within cancer cells in 62% of early gastric cancer, within dysplastic cells in 76% of resections where dysplasia was present, and in 82% of the biopsies obtained before the diagnosis of gastric cancer, compared with only 37% in the gastritis cohort (14). Because serum gastrin levels are usually elevated in the stomachs of *H. pylori*-infected patients and mice, this suggests a possible association between gastrin stimulation, the TFF2-expressing SPEM lineage, and gastric cancer.

Thus gastrin and TFF2 appear to share overlapping roles in gastric repair and neoplasmia. Although the mechanism of action of trefoil factors remains undefined, the observation that *H. pylori*-induced hypergastrinemia is often associated with increased TFF2 expression in gastric tissues raises the possibility that gastrin may regulate TFF2 expression. Previous studies by our group and others have investigated the TFF2 promoter and shown it to be a complex promoter/enhancer responsive to several growth factors and basal transcription factors such as GATA-6 and hepatocyte nuclear factor responsive to several growth factors and basal transcription.

**MATERIALS AND METHODS**

**Cell culture and animals.** The human cell line, AGS-E (ATCC, Manassas, VA), which stably expresses CCKBR, was used in this study (23). AGS-E cells were maintained under selection by supplementing medium with puromycin (2 μg/ml) every 4 wk. Cells were cultured in DMEM medium supplemented with 10% FBS and 100 IU/ml penicillin/streptomycin in a humidified incubator at 37°C under 5% CO2-95% O2 conditions. The transgenic animals used in this study (23). AGS-E cells were maintained under selection by supplementing medium with puromycin (2 μg/ml) every 4 wk. Cells were cultured in DMEM medium supplemented with 10% FBS and 100 IU/ml penicillin/streptomycin in a humidified incubator at 37°C under 5% CO2-95% O2 conditions. The transgenic animals used in this study.

**Cell transfection and luciferase assays.** AGS-E cells (1.0 × 10^5 cells/well) were seeded in 12-well plates 24 h before transfection. Cells were transfected with either 1.5 μg TFF2-luciferase construct in pancreatic β-cells and are hypergastrinemic (42). The background strains for GAS-KO and INS-GAS mice are C57BL/6 and FVB/N, respectively.

**Cell transfection and luciferase assays.** AGS-E cells (1.0 × 10^5 cells/well) were seeded in 12-well plates 24 h before transfection. Cells were transfected with either 1.5 μg TFF2-luciferase construct plasmid or 1.5 μg empty reporter vector DNA using 4 μl Superfectin (Qiagen, Valencia, CA) for 3 h. To control for background luciferase activity, 0.05 μg/well of a Renilla luciferase reporter vector DNA, driven by a minimally active thymidine kinase promoter (pRL-TK; Promega), was cotransfected with all promoter constructs, and the ratio of firefly to Renilla luciferase activity was calculated. The total amount of DNA transfected was kept constant by addition of appropriate amounts of the pCMV vector. The cells were cultured in full medium for 24 h before luciferase measurements. Luciferase activity was determined using the dual-luciferase assay system (Promega) and a Monolight 3010 luminometer (Pharmingen). CCKBR antagonist YF-476 was a gift from Dr. Keiji Miyata and Dr. Hidenobu Yuki (Yamanouchi Pharmaceutical, Tsukuba, Japan). Immunohistochemical staining. Gastric expression of TFF2 was examined by immunohistochemical staining in 2-mo-old INS-GAS, GAS-KO, and control mice. Following death, midline strips removed from the lesser curvature of the stomach were fixed in 10% neutral buffered formalin overnight, processed routinely, and embedded in paraffin. Sections were cut at 5 μm followed by antigen retrieval, achieved by boiling sections for 15 min in 0.01 M citrate buffer, pH 6.0. The rabbit polyclonal TFF2 antibody was raised to the COOH-terminal 16 amino acids (EVPWCFFPSVQDCHY) from mouse TFF2 but which are highly conserved (e.g., 15 of 16 residues) in the human COOH-terminal peptide. We have tested this antibody in immunohistochemical stains of human tissue and in Western blots against recombinant human TFF2. The antibody detects quite cleanly the 14-kDa human TFF2 peptide (unpublished data). Tissue sections were incubated with the TFF2 antibody at a dilution of 1:200 at 4°C overnight in a humidified chamber. Indirect labeling was performed with species-appropriate biotinylated secondary antibodies followed by avidin-biotin complex (ABC)-peroxidase (ABC kit; Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol. 3-Amino-9-ethylcarbazole (Vector Laboratories) was used as chromogen, and slides were counterstained with Mayer’s hematoxylin. Stained sections were examined by light microscopy (CX31; Olympus Optical, Cebu, Philippines). Double immunohistochemistry for the CCKBR and TFF2 was performed as described previously (23). Tissue sections were incubated with the anti-human gastrin/CCKBR antibody at 4°C overnight and visualized with fluorescence isothiocyanate-conjugated affiniPure goat anti-serum against rabbit IgG (1:200; Jackson ImmunoResearch, West Grove, PA). Staining for TFF2 was visualized with a rhodamine-conjugated mouse antisera against rabbit IgG (1:200; Jackson ImmunoResearch), and double-stained sections were examined with a fluorescence microscope (Olympus BX 51; Olympus Optical, Melville, MD).

**Quantitative and semiquantitative PCR.** Total RNA was extracted from AGS-E cells and whole murine stomach tissues by TRIZOL (Invitrogen, Carlsbad, CA). RT was performed using SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufactures’ protocol. Semiquantitative PCR reactions were performed with a GeneAmp PCR System 9700 (Applied Biosystems) using PCR Core Kit. The quantity of cDNAs generated in PCR reactions were standardized against glyceraldehyde 3-phosphate dehydrogenase amplification. The sense and the antisense mouse and human TFF2 primers were designed to cross exon-intron boundaries to avoid amplification from contaminating DNA. The sequences of human TFF2 primers were as follows: forward, 5'-atgggagggagagccgcca-3' and reverse, 5'-tatgattgagttccagc-3'. The sequences of mouse TFF2 primer were as follows: forward, 5'-GCAGTGCTGTGATCTTGATGC-3' and reverse, 5'-TCAGGTTAGAAACGACAGTT-3'. The PCR products were analyzed by agarose gel electrophoresis. Quantitative real-time PCR was performed with a three-step method using the Bio-Rad iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Each reaction was carried out in a 10-μl mixture consisting of QuantiTect SYBR Green PCR Master (Qiagen). All primer pairs were optimized to amplify only a single product. Efficient amplification under the reaction conditions. The PCR conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

**Western blot analysis.** Cells were lysed in buffer containing 50 mM Tris·HCl, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, and 5 mM EGTA, 50 mM sodium fluoride, 60 mM β-glycerophosphate, 0.5 mM sodium vanadate, 0.1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, and 10 μM leupeptin. Protein extracts of whole gastric fundus from 2-mo INS-GAS, GAS-KO, and control mice were prepared in lysis buffer. Protein samples were electrophoresed on 4–20% denaturing SDS gels (Invitrogen), and transferred to an
Immobilon-P membrane (Millipore, Bedford, MA). Blots were fixed 0.2% glutaraldehyde for 25 min, incubated with rabbit polyclonal TFF2 antibody (6 μg/ml) overnight, and then incubated at room temperature for 1 h with a peroxidase-conjugated goat anti-rabbit secondary antibody (Amersham, Piscataway, NJ). Immunobands were visualized by enhanced chemiluminescence (Amersham).

Reporter constructs and PCR mutagenesis. A 2.5-kb fragment of the TFF2 promoter was obtained through PCR amplification from mouse genomic DNA (Promega) using synthetic primers (Invitrogen) based on published sequences. Part of sequences of mouse promoter were shown in Fig. 3A. The forward primer was located at 2583 nucleotides upstream of the published TFF2 transcriptional start site, and the reverse primer corresponded to nucleotides +20 to +34. The promoter fragment was cloned directly into pBluescript (Invitrogen) and subsequently subcloned into the Hind III-XhoI site of the luciferase reporter vector pGL2-basic to generate the TFF2-2583 plasmid. This construct was used as a reporter template to generate the other truncated promoter constructs, namely TFF2-1842, TFF2-965, TFF2-324, TFF2-305, TFF2-222, TFF2-79, and TFF2-46. To verify gastrin-responsive elements contained in the TFF2-305 (P-305) promoter construct, several additional constructs containing point mutations were generated derived from the P-305 construct using the Site-Directed Mutagenesis kit (Stratagene). All mutants were confirmed by sequencing.

Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA) were performed as described previously (6) using nuclear extracts from AGS-E cells. Double-stranded oligonucleotides (5 pmol) were radiolabeled with [α-32P]dCTP with Klenow fragment of DNA polymerase. Probes (10 fmol) were incubated with nuclear extracts (6 μg). DNA-protein complexes were electrophoresed through 6% nondenaturing polyacrylamide gels, and the dried gels were exposed to phosphor storage screens. Images were revealed with a PhosphorImager (GE Healthcare Bio, Piscataway, NJ). The probes used were designed, and the sequences are shown in Table 1.

Statistical analyses. Data are presented throughout as means ± SE. Statistically significant differences (P < 0.05) were decided by one-way ANOVA and unpaired t-test.

RESULTS

Gastrin upregulates TFF2 gene expression in vitro and in vivo. We first investigated by RT-PCR the effect of gastrin on endogenous TFF2 mRNA expression in AGS-E cells that stably express CCKAR. The level of TFF2 mRNA was markedly increased in AGS-E cells in response to 10^{-9} to 10^{-7} M (1–100 nM) gastrin for 24 h (Fig. 1A). Real-time PCR confirmed that gastrin stimulated expression of TFF2 gene expression in a time- and dose-dependent manner (P < 0.05; Fig. 1B). Consistent with previous reports (6, 8), we failed to detect by Western blot TFF2 protein expression in human gastric cancer cell lines.

We next asked whether gastrin overexpression or deficiency influences the in vivo expression of TFF2 in the gastric mucosa. Because older INS-GAS and GAS-KO mice exhibit increased number of TFF2-expressing cells (22, 42), we examined younger (2-mo-old) INS-GAS and GAS-KO mice. Real-time PCR revealed that TFF2 mRNA expression was downregulated significantly in GAS-KO mice and upregulated significantly in INS-GAS mice compared with strain- and age-matched control animals (P < 0.05, Fig. 2A). Western blot indicated a similar downregulation in TFF2 protein levels in gastrin-deficient mice and upregulation in hypergastrinemic mice compared with strain- and age-matched control animals (Fig. 2B). Densitometric analysis of TFF2-specific bands noted on Western blots from cohorts of six mice per group showed a 2.3-fold decrease in the abundance of TFF2 in GAS-KO mice and a 1.89-fold increase in the abundance of TFF2 in INS-GAS mice compared with control mice (P < 0.05). In addition, immunohistochemical staining showed that TFF2 protein expression was reduced in the gastric fundus of GAS-KO mice compared with the C57BL/6 controls, whereas TFF2 protein expression was increased in INS-GAS mice (Fig. 2C). Expression of TFF2 protein was localized primarily to gastric mucous neck cells (Fig. 2C), whereas expression of the CCKAR was found in both neck cells and the deeper regions of the gastric glands (Fig. 2D). Nevertheless, some colocalization of TFF2 and CCKAR was observed in occasional glandular cells (Fig. 2E), raising the possibility that gastrin might directly stimulate TFF2 expression. These results strongly suggested that gastrin regulates the expression of TFF2 in vitro and in vivo.

Table 1. Sequences and location of TFF2 probes used in EMSA

<table>
<thead>
<tr>
<th>Probes</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT1</td>
<td>-46·GCAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M1</td>
<td>-46·TACGCTCAGCTAAGAACAAATATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M2</td>
<td>-46·GCAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M3</td>
<td>-46·GGAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M4</td>
<td>-46·GGAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M5</td>
<td>-46·GGAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M6</td>
<td>-46·GGAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M7</td>
<td>-46·GGAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
<tr>
<td>M8</td>
<td>-46·GGAACAAACAGGAGCCATTTTTATGCAGCTTGCTGGGACCTGGGACCTGGGCC·1</td>
</tr>
</tbody>
</table>
| WT2      | -305·CTATCCCTTTCTCGTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCTGGGACCT
Gastrin regulation of the TFF2 promoter is dependent on the CCK$_B$R. To determine whether gastrin-dependent upregulation of TFF2 gene expression could be attributed to stimulated transcription, a series of murine TFF2 promoter reporter constructs were generated containing the downstream 3'-untranslated sequence (+34) and various lengths of upstream mouse TFF2 5'-flanking sequences (Fig. 3, A and B). Basal promoter activity of the TFF2-2583 construct in AGS-E cells was >14-fold higher than that of the empty vector pGL-2. With deletion to −965 nucleotides (in the TFF2-965 construct), promoter activity was reduced significantly ($P < 0.01$), and, with further deletions, promoter activity continued to decline gradually ($P < 0.05$; Fig. 3B). The minimal basal promoter could be localized to the region between −46 and +34 bp (Fig. 3B).

Next, we determined the effect of gastrin on TFF2 promoter activity in AGS-E cells. Gastrin stimulation increased significantly the activity of all TFF2 promoter-reporter gene constructs ($P < 0.01$; Fig. 3C). The promoter activity of the TFF2-2583 construct showed minimal responses to lower concentrations of gastrin ($10^{-11}$ to $10^{-9}$ M) but showed a 9- to 10-fold increase in response to higher concentrations of gastrin ($10^{-8}$ to $10^{-7}$ M; $P < 0.01$; Fig. 4A). The promoter activity of the TFF2-2583 construct began to increase at 4 h after gastrin stimulation and peaked at 12 h ($P < 0.01$; Fig. 4B). The results showed that gastrin activates TFF2 transcription in a time- and dose-dependent manner.

To confirm that gastrin activation of TFF2 transcription is dependent on the CCK$_B$R, AGS-E cells were pretreated with CCK$_B$R antagonist, YF-476 (23), before gastrin stimulation. These results showed the YF-476 pretreatment significantly abolished gastrin-dependent TFF2 promoter activity in a dose-dependent manner ($P < 0.05$; Fig. 4C). The response of all TFF2 promoter constructs to $10^{-7}$ M to $10^{-8}$ M of gastrin was completely inhibited in the presence of YF-476 at $10^{-7}$ M ($P < 0.01$; Figs. 3C and 4A). Thus gastrin-dependent TFF2 promoter activity is specifically dependent on signaling through the CCK$_B$R.

We further sought to identify gastrin-responsive cis-acting elements in the TFF2 promoter. AGS-E cells were transfected with a series of truncated TFF2 promoter constructs and treated with and without gastrin stimulation before luciferase assays. These results demonstrated that gastrin treatment led to significant induction of all TFF2 promoter constructs (Fig. 3C). A significant reduction in stimulation occurred upon deleting from −305 to −222 and from −46 to pGL2, suggesting that gastrin-responsive elements may be located in both the −305 to −222 bp and the −46 to +34 bp regions, respectively (Fig. 3C).

The CCAATA enhancer element located at −35 to −25 bp of the TFF2 promoter is a gastrin-responsive site. To further define the gastrin-responsive elements in the TFF2 promoter, we performed EMSA using AGS-E extracts and wild-type and mutant oligonucleotides derived from the sequences between −46 to −1 bp and −305 to −222 bp upstream of the TFF2 transcriptional start site. These EMSA experiments were performed to directly detect DNA-protein complex formation using 32P-labeled wild-type (WT) 1 to WT3 and mutant (M) 1
to M4 probes (Table 1). DNA-protein complexes could be detected using WT1 probe (−46 to −1), M1 to M3 probes (Fig. 5A), and WT2 (−305 to −264) and WT3 (−263 to −222) probes (Fig. 5B). Only the M4 (−46 to −1) probe with mutant sequences located at −34 to −25 bp significantly attenuated the formation of DNA-protein complexes (Fig. 5A). These results suggested that the sequences between −34 and −25 bp (M4), which contains the sequence 5′-GAGC-CAATAA-3′, were responsible for the most prominent DNA-protein complexes observed in EMSA.

To further map the specific binding elements in this region, a series of additional oligonucleotides was generated for EMSA with 3 bp cluster mutations replacing the −34 to −25 bp region, and designated M5, M6, M7, and M8 (Table 1). EMSA results showed that M6 and M7 failed to form DNA-protein complexes, suggesting that the nucleotides contained in this region (−32 CCAATA −27) were directly responsible for DNA complex formation. EMSA probes M5 and M8, which contained mutations outside of the core CCAATA sequence, were still able to form DNA-protein complexes. These results demonstrate that the −32 to −27 bp sequence (−CCAATA−) is largely responsible for the DNA-protein complex formation. A GC-rich element (CCCTGTGG) is also required for gastrin-dependent TFF2 promoter activity. We next investigated the gastrin-responsive cis-acting elements in the −305 to −222 bp region of the TFF2 promoter using wild-type and mutant oligonucleotides in EMSA studies. The mutant oligonucleotides contained 10-bp mutations in the original −305 to −264
bp (WT2) and −263 to −222 bp (WT3) wild-type oligonucleotides and were designated M9A, M9B, M9C, and M9D, and M10A, M10B, M10C, and M10D, respectively (Table 1). EMSA studies employing these mutant oligos as competitors showed that the DNA-protein complexes formed with the WT2 probe were almost completely abolished by competition with unlabeled M9A, M9B, and M9D but not the M9C mutant oligo (Fig. 6A). Similarly, when used as competitors in EMSA, the M10A, M10B, M10C, and M10D oligos all reduced binding and complex formation with the WT3 wild-type probe (Fig. 6B). Thus the finding that the M9C mutant oligo failed to reduce the binding to the WT2 probe suggests that the sequences mutated in this oligo, ACCCTGTGGGTG between −285 to −274 bp regions, were critical for factor interaction with the TFF2 promoter.

To determine the functional contribution of the −285 to −274 bp region to gastrin responsiveness of the promoter, we generated a series of TFF2 promoter constructs in which point mutations in the −285 to −274 region were introduced on the backbone of TFF2-305 luciferase constructs. These constructs, which were designated TFF2-305-9CM1, -9CM2, -9CM3, and -9CM4 (Fig. 6C), were then tested for gastrin responsiveness. These studies revealed that the mutant promoter constructs TFF2-305-9CM1, -9CM2, and -9CM3, showed significantly decreased responses to gastrin (P < 0.05), whereas the activity of mutant TFF2-9CM4 promoter showed a normal gastrin response (Fig. 6D). These findings indicate that the sequence 5′-CCCTGTGGGTTG-3′ between −284 to −277 bp regions was required for full gastrin-dependent activation of the TFF2 promoter.

Gastrin activates the TFF2 promoter through a protein kinase C-, mitogen/extracellular signal-regulated kinase 1-, and phosphatidylinositol 3-kinase-dependent pathway. Last, we used specific inhibitors to investigate the signal transduction pathways involved in gastrin-dependent regulation of TFF2 promoter activity. The protein kinase C (PKC)-
specific inhibitor staurosporine (23) and the mitogen/extracellular signal-regulated kinase (MEK) 1 inhibitor PD-098059 (23) significantly reduced gastrin-dependent activity of the TFF2-305 promoter construct ($P < 0.05$; Fig. 7A). Similarly, the phosphatidylinositol 3-kinase (PI 3-kinas) inhibitor LY-294002 (23) almost completely reversed the effect of gastrin on the induction of TFF2 promoter activity ($P < 0.01$; Fig. 7A). Furthermore, the inhibitors staurosporine, PD-098059, and LY-294002 also suppressed the effects of gastrin on endogenous TFF2 mRNA expression in AGS-E cells (Fig. 7A). Intriguingly, these specific inhibitors had no effect on gastrin-dependent activity of the TFF2-46 promoter construct, suggest-
ing that the upregulation by gastrin of this basal promoter may be mediated through other signaling pathways. Overall, these results indicate that gastrin-dependent regulation of the CCCTGTGG cis-acting element is largely dependent on the PKC, MEK1, and PI 3-kinase pathways.

We also investigated the effects of the epithelial growth factor (EGF) receptor (R)-specific inhibitor tyrphostin, AG-1478, on TFF2 promoter activity. Both EGF and gastrin stimulated the TFF2 promoter and upregulated the level of endogenous TFF2 mRNA (P < 0.01; Fig. 7, B and C). However, AG-1478 significantly reduced EGF-stimulated TFF2 gene expression and transcription (P < 0.01) but did not inhibit gastrin-stimulated TFF2 promoter activity and TFF2 gene expression in AGS-E cells (P > 0.05; Fig. 7, B and C). Thus gastrin-dependent activation of TFF2 transcription appears to be independent of EGFR signaling.

DISCUSSION

In this study, we found that gastrin regulates TFF2 gene expression both in vitro and in vivo. Expression of the TFF2 gene was reduced in the gastric fundus of gastrin-deficient mice and increased in the proximal stomach of our hypergastrinemic INS-GAS mice. Thus expression of the TFF2 gene appears to be highly responsive to the normal physiological range of circulating amidated gastrin. Gastrin treatment increased endogenous TFF2 mRNA expression in gastric cancer cells in a dose- and time-dependent manner. Using TFF2 promoter-reporter gene constructs, we showed that gastrin induced TFF2 transcription through two discrete gastrin-responsive promoter elements and through a PKC-, MEK1-, and PI 3-kinase-dependent but EGFR-independent pathway.

The TFF2 promoter contains a complex enhancer region responsive to a number of agents, including estrogens and phorbol esters (7, 21). In addition, basal transcription factors like GATA-6 and HNF-3 have been demonstrated to activate both the TFF1 and TFF2 promoters (1, 4). Studies from our group have shown that TFF2 is an immediate early gene capable of regulating its own expression through activation of the TFF2-promoter, and we have reported on a cis-acting element, designated spasmolytic polypeptide responsive element, located between −191 and −174 upstream of the TFF2 transcriptional start site (8). In the current study, we found that gastrin stimulated the transcription of TFF2 through both the proximal CCAATA elements located at −32 to −27 bp and the more distal CCCTGTGG element located at −284 to −277 bp upstream of the TFF2 transcription start site. Many studies have shown that the CCAAT sequences are consensus regulatory sites of a number of promoters and bind CCAAT/enhancer binding proteins consensus-binding site. Mutation of this consensus site reduced the basal promoter activity by >50% in MCF-7 cells but had no effect on basal promoter activity in human gastric cancer cells (6). In the current study, we found that gastrin stimulated the transcription of TFF2 though both the proximal CCAATA elements located at −32 to −27 bp and the more distal CCCTGTGG element located at −284 to −277 bp upstream of the TFF2 transcription start site. Many studies have shown that the CCAAT sequences are consensus regulatory sites of a number of promoters and bind CCAAT/enhancer binding proteins, a family of structurally related transcription factors critical for normal tissue development, proliferation, and differentiation (19). For the GC-rich element containing

---

Fig. 5. Electrophoretic mobility shift assays (EMSA) studies demonstrate factor binding to the downstream gastrin-responsive TFF2 promoter element −46 to −1 bp. A: nuclear proteins bind to the −46 to −1 bp region of the TFF2 promoter. EMSA was performed using nuclear proteins from gastrin-stimulated AGS-E cells, and WT (WT1) and mutant double-stranded oligonucleotide probes M1 to M4 were labeled with [32P]dCTP. B: EMSAs were performed using nuclear extract from gastrin-stimulated AGS-E cells and the end-labeled (α-32P) M5-M8, WT2, and WT3 double-stranded oligos as probes. The specific DNA-protein complexes are indicated by arrows C1 and C2. Treatment with (+) or without (−) gastrin is noted. P0, P1, P2, P3, P4, P5, P6, P7, P8, P9, and P10 indicate without nuclear protein loading.
the CCCTGTGG sequence, we did not identify any homology to other transcriptional factors known by computer-based homology search, suggesting a novel gastrin-responsive cis-acting element. Mutation of both the CCAAT and CCCTGTGG sequences abolished gastrin-dependent TFF2 promoter activity, indicating that both elements are required for gastrin induction of TFF2 transcription. EMSA studies indicated binding by specific factor complexes, but further investigations will be required to define the precise nuclear factors involved in TFF2 transcriptional control through the CCAAT element.

Gastrin activation of TFF2 transcription is dependent on its receptor, the CCKbR. In this study, the CCKbR antagonist YF-476 pretreatment significantly abolished gastrin-dependent TFF2 promoter activity in a dose-dependent manner. YF-476 is an extremely potent selective human CCKbR antagonist with a reported inhibitory constant value of 0.068–0.19 nM in different tissues. The response of all TFF2 promoter constructs to $10^{-7}$–$10^{-8}$ M of gastrin was completely inhibited in the presence of YF-476 at $10^{-7}$ M. Notably, YF-476 at $10^{-11}$ M inhibited 50% of the transactivating effects of $10^{-7}$ M gastrin on the TFF2 promoter in AGS-E cells. The greater apparent affinity of YF-476 for the CCKbR in this study, compared with previous studies, is not currently understood.

Gastrin-dependent TFF2 promoter activity was inhibited significantly by the MEK1 inhibitor (PD-98059) and the PKC inhibitor (staurosporine), suggesting activation of the CCKbR of a PKC- and MEK1-dependent pathway. The CCKbR is a Gq-linked seven-transmembrane receptor, and gastrin has been shown to regulate numerous genes (e.g., TFF1, histidine decarboxylase and heparin-binding EGF) through a PKC-dependent HDC- and MEK1-dependent pathway (16, 17, 23, 46). Studies with recombinant CCKbR expressed in eukaryotic cells have indicated that the receptor showed near-maximal

---

**Fig. 6.** EMSA studies demonstrate factor binding to the upstream gastrin-responsive TFF2 promoter element −305 to −222 bp. A: nuclear proteins bind to the −305 to −222 bp region of the TFF2 gene. EMSA was performed using nuclear proteins from AGS-E cells with the 32P-labeled WT2 probe and unlabeled mutant probes M9A–D as competitors. The specific DNA-protein complexes are indicated by arrow C1. B: EMSA was performed using nuclear extracts from AGS-E cells with the 32P-labeled WT3 probes and unlabeled mutant probes M10A–D as competitors. The specific DNA-protein complexes are indicated as arrows C1 and C2. C: schematic outline of WT and mutant TFF2-305 plasmids. The mutated sequences of the TFF2-305 mutant plasmids are designated by italic bold letters and underlines. D: effect of gastrin on the activity of WT and mutant TFF2-305 promoters. AGS-E cells were cotransfected with the indicated TFF2 promoter plasmids or empty pGL2 vector and Renilla luciferase vector for 3 h before $10^{-7}$ M gastrin treatment. The luciferase activities were determined by the dual-luciferase assay system 48 h after transfection. Data represent the mean luciferase activity ± SE and are degree of induction compared with the empty vector in 3 independent experiments. *P < 0.05 vs. TFF2-305 construct.
binding with gastrin concentrations between $10^{-9}$ and $10^{-10}$ M. In the original cloning of the CCK_{2R}, the IC{50} for gastrin was 0.26 nM (43); in the generation of CCK_{2R}-expressing AGS cells, the IC{50} for gastrin binding to AGS/CCK_{2R} cells was 0.6 nM (17). With respect to transcriptional studies, CCK_{2R}-expressing AGS cells have shown near-maximal transcriptional responses with gastrin concentrations ranging from $10^{-9}$ to $10^{-8}$ M or up to one log greater gastrin concentrations. Our previous publications have demonstrated that 5 $\times$ $10^{-9}$ M gastrin can induce 50% maximal promoter upregulation of the rat HDC promoter (17), the human HDC promoter (46), the mouse chromogranin A promoter (16), and the murine TFF1 promoter (23) when transfected in AGS cells. The reason for this slight discrepancy in curves for binding vs. transactivation is not clear but might relate to the need for a certain threshold occupancy level for signaling. In some cases, signaling from the CCK_{2R} to the MEK/ERK pathway is direct, while in other cases it is indirect, involving transactivation of the EGFR by EGFR ligands. For example, previous studies have demonstrated that gastrin regulates the HB-EGF promoter via an EGFR-dependent pathway (34). Nevertheless, in the current study, EGFR inhibitors did not attenuate gastrin-dependent TFF2 promoter activity, indicating that gastrin regulation of TFF2 expression was independent of the EGFR pathway.

TFF2 is upregulated in diverse pathological conditions of the gastrointestinal tract in human and mouse (38), such as gastric ulceration, duodenal ulceration, and Crohn’s disease of the small intestine (36, 38, 40). It is in fact one of the earliest genes upregulated after injury to the gastrointestinal tract, preceding the activation of other trefoil genes and growth factors (31, 43). In experimentally induced gastric ulceration in rats, the increase in TFF2 mRNA expression occurs within minutes (43). Although the CCK_{2R} is not so promptly induced, a similar pattern of altered expression of the CCK_{2R} has been observed during the later stages of ulcer healing (32). During wound healing, CCK_{2R} are specifically expressed and localized to the regenerative mucosal ulcer margin and correlated with proliferative activity in these tissues. Thus it seems likely that sustained elevations in TFF2 gene expression in the setting of
wound healing or regeneration may be sustained in part through gastrin stimulation.

Gastrin is not only a trophic factor for normal gastric epithelial cells but also has been postulated to play a role in a number of malignancies (12). Expression of CCKbR has also been observed in mucous neck cells in the fundic mucosa of mouse, and, in H. pylori-infected patients, an increase in the number of TFF2-expressing mucosa neck cells has been observed in the mouse and human stomach (14, 42). Helicobacter-infected mice show increases in serum gastrin levels before the emergence of preneoplastic and neoplastic TFF2-expressing gastric metaplasia and dysplasia (42). Both gastrin and TFF2 have been linked to cellular migration, and TFF2 has been shown to be expressed by some human gastric cancers where it is associated with a significantly shorter disease-free survival (10). Thus induction by gastrin of TFF2 expression could potentially contribute to cancer cell migration, invasion, and spread. Further studies will be required to determine the importance of the gastrin-TFF2 link in neoplastic disease.

ACKNOWLEDGMENTS

We thank Dr. S. Lei for technical assistance in EMSA experiments.

GRANTS

This work was supported by a National Institute of Diabetes and Digestive and Kidney Diseases Grant R01.DK-58889-01 to T. C. Wang.

REFERENCES


