ATTENUATION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR GAMMA (PPARγ) MEDIATES GASTRIN-STIMULATED COLORECTAL CANCER CELL PROLIFERATION

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Peroxisome proliferator activated receptor gamma (PPARγ) has been shown to suppress cell proliferation and tumorigenesis, while the gastrointestinal regulatory peptide gastrin stimulates the growth of neoplastic cells. The present studies were directed to determine whether changes in PPARγ expression might mediate the effects of gastrin on the proliferation of colorectal cancer (CRC).

Initially, using growth assays, we determined that the human CRC cell line DLD-1 expressed both functional PPARγ and gastrin receptors. Amidated gastrin (G-17) attenuated the growth suppressing effects of PPARγ by decreasing PPARγ activity and total protein expression, in part through an increase in the rate of proteasomal degradation. G-17-induced degradation of PPARγ appeared to be mediated through phosphorylation of PPARγ at serine 84 by a process involving the biphasic phosphorylation of ERK1/2 and activation of the epidermal growth factor receptor (EGFR). These results were confirmed through the use of EGFR antagonist AG1478 and MEK1 inhibitor PD98059. Furthermore, mutation of PPARγ at serine 84 reduced the effects of G-17, as evident by inability of G-17 to attenuate PPARγ promoter activity, degrade PPARγ, or inhibit the growth suppressing effects of PPARγ. The results of these studies demonstrate that the trophic properties of gastrin in CRC may be mediated in part by transactivation of the EGFR and phosphorylation of ERK1/2, leading to degradation of PPARγ protein and a decrease in PPARγ activation.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cause of cancer-related death in the United States, accounting for 10% of all deaths due to malignancy. The American Cancer Society estimates that in 2005, approximately 145,000 Americans will be diagnosed with CRC, of whom 56,000 will die (1). CRC generally arises from benign adenomas, which progress into malignant adenocarcinomas (2,3). The development of CRC appears to involve a multi-step process of genetic mutations combined with largely undefined environmental factors whereby normal epithelial cells undergo dysplastic transformation, followed by proliferation and eventual histological progression to neoplasia (2). This transition appears to require multiple genetic alterations, such as mutations in the adenomatous polyposis coli (APC) gene. After the initial mutations have established a neoplastic phenotype, other factors appear to promote proliferation and neoplastic progression (2).

One such potential growth factor is the polypeptide hormone gastrin. In addition to its recognized role in the physiological regulation of acid secretion (4-7), another biological property attributed to gastrin is its trophic effect on the gastrointestinal (GI) mucosa (6-8). Numerous studies have provided evidence that gastrin peptides play an integral role in promoting colorectal tumor growth (3), as well as other malignancies throughout the GI tract (8,9). In a well-
controlled, elegantly designed epidemiologic study of nearly 130,000 individuals, Thorburn et al. found that prolonged hypergastrinemia comprised a risk factor for the development of CRC. This study found a 3.9-fold increased risk of CRC due to elevated circulating gastrin levels, prompting the authors to conclude that 8.9% of all CRCs may be attributed to hypergastrinemia (10). Studies in transgenic mice overexpressing gastrin have demonstrated increased proliferation of the gastric and colonic epithelium after eight months. When these mice were followed for longer periods (~20 months), an increased tendency to develop neoplasia was observed (11).

Several molecular forms of gastrin are synthesized and released into the circulation, with the predominant peptide being α-amidated gastrin-17 (G-17) (9). Progastrin precursor peptides such as glycine-extended gastrin (Gly-G) appear to affect CRC in vivo principally via autocrine pathways, while fully processed gastrin also utilizes endocrine pathways (12-16). Smith et al. reported that ~80% all colorectal adenomas express gastrin/cholecystokinin-2 receptors (CCK-2R) (17), and Ciccotosto et al. found that while approximately 70% of CRC expressed fully processed α-amidated gastrin, 100% produced progastrin precursor peptides (18). These studies all strongly suggest a role for gastrin in GI cell proliferation and carcinogenesis.

Although the trophic effects of gastrin are well recognized, the molecular and intracellular mechanisms by which gastrin modulate cell growth in the GI tract have not been fully elucidated. Previous studies have reported that stimulation of the CCK-2R by gastrin activates various signal transduction pathways implicated in cell proliferation, such as the mitogen activated protein kinases (MAPK), which include ERK, JNK, and p38 kinase (8). One potential downstream target of the MAPKs is peroxisome proliferator activated receptor gamma (PPARγ) (19-24). The possibility of a functional relationship between gastrin and PPARγ has not been previously evaluated.

PPARγ, a member of the nuclear hormone receptor family, functions as a transcription factor that regulates several biological processes, including growth and differentiation (25,26). In addition to its recognized role in adipogenesis (31), PPARγ has been shown to modulate the growth of cells in various organs. This trophic effect is most evident in the colon, where normal human colonic mucosa, colon adenocarcinoma, and cultured CRC cells express levels of PPARγ1 equivalent to that detected in adipocytes (27,28). Activation of PPARγ in cultured colon cells inhibits growth and induces differentiation, reverses the malignant phenotype, and promotes apoptosis (27,29-32). In CRC cells, PPARγ activation results in both an increase in the cyclin dependent kinase inhibitors, p21 and p27 (33), which repress cell cycle progression, leading to a decrease in cell growth and an increase in the differentiation of cancer cells, and upregulation of caspase activity (34,35), resulting in DNA fragmentation and apoptosis. Moreover, a recent study demonstrated that 8% of primary colorectal tumors harbor a functional mutation in one allele of the PPARγ gene, further supporting the role of PPARγ as a tumor suppressor in humans (36).

The present studies were directed to determine whether changes in PPARγ expression might mediate the effects of gastrin on the proliferation of CRC. We have demonstrated that gastrin-stimulated proliferation of CRC cells is associated with a significant concomitant decrease in cellular PPARγ levels. Moreover, gastrin attenuates the inhibition of cell growth induced by PPARγ agonists. Finally, our studies demonstrate that the trophic properties of gastrin may be mediated in part by transactivation of the epidermal growth factor receptor (EGFR) and phosphorylation of ERK1/2, leading to degradation of PPARγ protein and a decrease in PPARγ activation.

**MATERIALS AND METHODS**

**Materials.** DLD-1 human adenocarcinoma cells were obtained from the American Type
Culture Collection (Manassas, VA), SW48 human colon adenocarcinoma cells were kindly donated by Dr. B. Vogelstein (Baltimore, MD), and MC-26 cells, a transplantable mouse CRC cell line, were obtained from Dr. K.K. Tanabe (Boston, MA). The PPARγ monoclonal (E-8, no sc-7273) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and α-amidated gastrin-17 (G-17) and Gly-G were purchased from Bachem (King of Prussia, PA) and Anspep (Parkville, Victoria, Australia), respectively. The phospho-specific PPARγ polyclonal antibody (clone AW504) and the phospho-specific monoclonal antibody to the epidermal growth factor receptor (pTyr1173) were purchased from Upstate Biotechnology (Waltham, MA). The phospho-specific monoclonal antibody to ERK1/2 (Thr202/Tyr204) antibody was purchased from Cell Signaling Technologies (Beverly, MA), and the proteasome inhibitor, MG132 (N-carbobenzoxyl-Leu-Leu-Leucinal), cycloheximide, and thymidine were purchased from Sigma (St. Louis, MO). [3H]-Thymidine was purchased from NEN (Boston, MA). FuGENE 6 and Complete Protease Inhibitor Cocktail Tablets were purchased from Roche Molecular Biochemicals (Indianapolis, IN), and the thiazolidinediones, ciglitazone and rosiglitazone, which stimulate PPARγ activity, were purchased from Cayman Chemicals (Ann Arbor, MI). The pHD(x3)Luc vector was a kind gift from Dr. John Capone of McMaster University (Hamilton, ON). This vector contains three tandem repeats of the peroxisome proliferators response element from the promoter of the rat hydratase-dehydrogenase gene subcloned into the BamHI site of pCPSluc located immediately upstream of the carbamoyl-phosphate synthetase promoter. L-365,260 was generously provided by Dr. L. Iverson (Oxford, UK), pCMV-β-gal was purchased from Life Technologies (Grand Island, NY), and PD98059, SB203580, and lactacystin were purchased from Calbiochem (San Diego, CA). The RNAeasy Mini Kit and the SYBR green quantitative PCR master mix were purchased from Qiagen (Valencia, CA), and pTracerA/Bsd plasmid and ThermoScript™ Reverse Transcriptase were purchased from Invitrogen (Carlsbad, CA). The Dual-Luciferase® Reporter Assay System, the β-Galactosidase Enzyme Assay System, and the CellTiter 96® AQueous One Solution Cell Proliferation (MTT) Assay Kit were purchased from Promega (Madison, WI). The Quikchange XLII Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, California). Kpn I, Xba I, and the Quick Ligase Kit were purchased from New England Biolabs (Ipswich, MA).

Site-directed Mutagenesis and Generation of PPARγ phosphorylation mutants. Site-directed mutagenesis was performed utilizing the Quikchange XLII kit with the forward primer, 5′-GTGGAGCCTGCAGCTCCACCTTATTAT TCTG-3′, and the reverse primer, 3′-CACCTCGGACGTGAAGTTGAATAATA AGAC-5′, and the pcDNA3-Flag-wtPPARγ vector as a template to generate a substitution of Ser84 to Ala. An initial denaturation step was performed at 95 ºC for 2 min. and followed by 20 cycles at 95 ºC for 1 min, annealing at 57 ºC for 1 min, and extension at 68 ºC for 7.5 min. A final extension phase was performed at 68 ºC for 7 min. The DNA sequence was confirmed by the Tufts University Core Facilities (www.tucf.org). PPARγ sequence inserts were double digested with Kpn I and Xba I and subcloned into the pTracerA/Bsd vector utilizing the Quick Ligase Kit according to the manufacturer’s instructions, at a 3:1 ratio of PPARγ insert to pTracer. Stable cell lines expressing pTracer, pTracerFlag-wtPPARγ, or pTracerFlag-mutPPARγ were generated by blasticidin selection (5 µg/mL) and confirmed by Western analysis using the mouse monoclonal anti-PPARγ antibody and the mouse monoclonal anti-green fluorescent protein (GFP) antibody, and by fluorescent light microscopy.

Cell Culture. The human CRC cell line DLD-1 and the mouse CRC cell line MC-26 were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Grand Island, NY)
supplemented with 10% fetal bovine serum (FBS) (GIBCO) and 1% penicillin/streptomycin (GIBCO). The human CRC cell line SW48 was maintained in McCoy’s medium (GIBCO) supplemented with 10% FBS and 1% penicillin/streptomycin. All cell lines were kept in a humidified, 5% CO₂ environment. 24 h prior to experimentation, serum-containing media were replaced with serum-free media.

[^H]-Thymidine Incorporation. Equal amounts of cells (3 x 10⁵ cells/well) were plated in 6-well plates. 24 h of serum starvation in 0.5 mM thymidine-containing media, cells were gently washed with phosphate-buffered saline (PBS) and then incubated for 22 h in fresh serum-free media in the presence of G17 or vehicle alone. In addition, to examine the involvement of the CCK-2R in DNA synthesis, 500 nM L-365,260, a specific CCK-2/gastrin receptor antagonist, was added to the culture medium. 4 h prior to termination of the experiment, cells were pulsed with [³H]-thymidine at a final concentration of 1 µCi/well. At the end of the incubation period, the radioactive media were aspirated and cell monolayers were gently rinsed with PBS at 4 °C. Ice-cold 10% trichloroacetic acid was then added and cells were gently rocked at 4 °C for 30 min. Cell monolayers were washed again with PBS, followed by incubation for 30 min. at 25 °C in 1 N NaOH. Scintillation fluid was added and samples were transferred into scintillation vials for measurement of [³H]-thymidine using an automatic Beckman liquid scintillation counter.

Cell Counting. Equal amounts of cells (3 x 10⁵ cells) were plated onto 10-cm plates. After 24 h of serum starvation in 0.5 mM thymidine-containing media, cells were gently washed with PBS and the media were replaced with 1% FBS containing media with or without G-17. After a 6-day incubation period, cells were trypsinized, harvested, and resuspended in equal volumes. Cell number was determined by counting with the aid of a hemacytometer under an inverted light microscope.

MTT Assays. MTT (3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide) assays (Promega Corp., Madison, WI) were also performed to measure cell growth. This method measures the quantity of the formazan product, as measured by the ratio of 490/630 nm absorbance, which is proportional to the number of living cells in culture. Cells were seeded onto 96-well plates at a density of 5 x 10⁴ cells per well and incubated overnight in 10% FBS media. The media was then replaced with serum free media the following day and incubated for 24 h. To determine the effect of gastrin on cell growth, the media were then replaced with media containing 1% FBS and increasing concentrations of G-17 or Gly-G, and cells were grown for an additional 3 days. To determine the effects of gastrin on cell growth in the presence of PPARγ ligands, DLD-1 cells were pre-incubated with gastrin peptides for 12 h prior to addition of ciglitazone or rosiglitazone. At the end of each experiment, 20 µl of CellTitre96® Aqueous Solution Reagent were added to each well, and the plate was incubated for 30 min. The absorbance ratio (490/630 nm) was recorded using a 96-well Elx800 universal plate reader (BIO-TEK Instruments, Inc., Winooski, VT).

Transient Transfection and Luciferase Assays. DLD-1 cells (4 x 10⁴ cells/ well) were plated in 24-well plates. After an overnight incubation, cells were transiently transfected with the phd(x3)luc vector in Opti-MEM for 16 h. To normalize for transfection efficiency, the cells were co-transfected with a pCMV-ß-gal reporter construct. FuGENE 6 was used according to the manufacturer’s instructions and a FuGENE6 to DNA ratio of 3:1 was used in each transfection experiment. After a 16-h transfection period, the media was replaced with media containing 1% FBS in the presence or absence of 200 nM gastrin for 12 h. The cells were then treated with various concentrations of ciglitazone for an additional...
16 h, after which cell lysates were collected and PPARγ reporter activity measured using the luciferase assay system. Values were normalized to β-galactosidase activity.

RNA Extraction and RT-PCR Analysis. DLD-1 cells (1.50 x 10⁶ per plate) were plated onto 10-cm diameter plates and incubated overnight. After 24 h of serum starvation, DLD-1 cells were incubated in the presence or absence of G-17. At the indicated time points, RNA was extracted using the RNAeasy Mini Kit. Total RNA was measured and 1 μg of total RNA was reverse transcribed using the ThermoScript Reverse Transcriptase. The reverse transcriptional reaction was carried out at 50 ºC for 60 min and 85 ºC for 5 min. To quantify the amount of PPARγ cDNA, all samples were subjected to PCR amplification using the QuantiTect SYBR Green PCR Kit (Qiagen). The forward primer PPARγ−F, 5'-TCTCTCCTGAATGGAAGACC-3' and reverse primer PPARγ-R, 5'-GCATTATGAGCATCCCATC3', were used according to the method of Terashita et al. (37). The PCR protocol was as follows: initial denaturation at 95 ºC for 15 min, followed by 35 cycles at 94 ºC for 15 s, annealing at 55 ºC for 30 s, and extension at 72 ºC for 30 s. The PCR product was quantified by the intensity of SYBR Green I fluorescence at 83 ºC.

Western blot analysis. Cell monolayers were rinsed twice with 1X PBS, directly lysed in the plate on ice with RIPA buffer containing Tris-HCl (50 mM, ph 7.4), NaCl (150 mM), NP-40 (1%), sodium deoxycholate (0.5%), SDS (0.1%), 1 μM phenylmethlysulfonyl fluoride, and complete Protease Inhibitor Cocktail mix. Cell debris was pelleted by centrifugation at 14,000 rpm for 15 min, and the supernatant was collected for protein quantification. The bicinchoninic acid protein assay (Pierce, Rockford, IL) was used to estimate protein concentration according to the manufacturer’s instructions. 50 μg of protein were diluted with 4X SDS sample loading buffer, boiled for 5 min., and separated by SDS-polyacrylamide gels. Following electrophoresis, separated proteins were transferred onto nitrocellulose membranes. The membranes were then blocked with 5% milk/PBS and incubated with the indicated primary antibodies. After incubation with the primary antibodies, membranes were washed thoroughly in TBS-Tween buffer (25 mM Tris, pH 8.0, 125 mM NaCl, 0.1% Tween 20). Appropriate secondary antibodies conjugated with horseradish peroxidase were used to detect the primary antibodies. Immunoreactive bands were visualized by chemiluminescence in signaling solution (Pierce).

ERK1/2 activation, EGF receptor activation, PPARγ phosphorylation. To investigate the effects of gastrin on ERK1/2 activation, EGFR transactivation, or PPARγ phosphorylation, DLD-1 cells were incubated in the presence or absence of 200 nM G-17. At different time points, whole cell lysates were collected, and Western analysis was performed with specific antibodies. For identification of the active form of ERK1/2, an antibody specifically recognizing ERK1 and ERK2 phosphorylated at Thr202 and Tyr204, respectively, was utilized. To determine the involvement of the EGFR in ERK1/2 activation, DLD-1 cells were preincubated for 30 min with the EGFR antagonist AG1478 prior to the addition of G-17. To evaluate EGFR transactivation by gastrin, Western analysis was performed with a monoclonal antibody specifically immunoreactive with the EGFR phosphorylated at Tyr1173 to detect the activated form of the EGFR. To examine PPARγ phosphorylation, Western analysis was performed using a rabbit polyclonal antibody specifically immunoreactive with PPARγ phosphorylated at Ser84. Protein loading was normalized by the measurement of β-actin.

PPARγ stability. To examine the effects of gastrin on PPARγ protein stability, DLD-1 cells were incubated with the protein synthesis inhibitor cycloheximide (50 μg/mL) in the presence or absence of 200 nM G-17. At different time points, whole cell lysates were collected, and Western analysis was
performed using the anti-PPARγ antibody to detect PPARγ, with β-actin measured as a loading control. Linear regression analysis was performed to determine the half-life of PPARγ in the presence and absence of gastrin. Values were normalized to β-actin and plotted on a log vs. time scale. To ascertain whether PPARγ protein was degraded through the proteasomal pathway, cells were preincubated with the proteasomal inhibitor, MG132 (20 µM) or lactacystin (5 µM), for 30 min prior to the addition of 200 nM G-17. To determine whether ERK1/2 and EGFR activation might affect PPARγ half-life, DLD-1 cells were preincubated with 40 µM PD98059, a potent MEK1 inhibitor, to inhibit the downstream activation of ERK1/2, with 100 nM AG1478, or with 10 µM SB20358, the p38 MAPK inhibitor, for 30 min prior to addition of cycloheximide.

Statistical analysis. All results are expressed as the mean ± SD. Statistical analysis was performed using ANOVA and Student's t-test. A P value <0.05 was considered to be statistically significant.

RESULTS

Effects of PPARγ and gastrin on cellular proliferation. To determine the effect of gastrin on cellular proliferation, DLD-1 cells were incubated in the presence and absence of 50 or 200 nM G-17 for 24 h and [3H]-thymidine uptake was measured. [3H]-thymidine uptake was increased by 35.4±7.3% (p<0.05) and 51±12.4% (p<0.01) by 50 nM and 200 nM G-17 treatment, respectively. In the presence of the CCK-2R antagonist, L-365,260, the increase in cell proliferation induced by 200 nM G-17 was attenuated by approximately 60% to 20±9.2% of control values (p<0.01 compared to 200 nM G-17, p<0.01 compared to vehicle treatment alone) (Fig. 1). Similar results were obtained from cell counting experiments and the MTT proliferation assay (Data not shown).

In separate experiments, DLD-1 cells were incubated in the presence of increasing concentrations of two known PPARγ agonists, ciglitazone and rosiglitazone, to determine the effect of PPARγ activation on cellular proliferation. Both ciglitazone and rosiglitazone treatment suppressed cell growth in a concentration-dependent manner. Rosiglitazone treatment decreased cellular proliferation at concentrations as low as 1 µM (by 18.4±6.7%, p<0.05), and maximal inhibition began to plateau from 5 µM (69.4±10.2% of control, p<0.01) to 10 µM (64.8±8.6% of control, p<0.01) (Fig. 2a). Similarly, ciglitazone treatment decreased cellular proliferation at concentrations as low as 1 µM (by 13.8±6.6%, p<0.05), with maximal inhibition achieved at a concentration of 10 µM (71.3±3.4% of control, p<0.01) (Fig. 2b). In the presence of 200 nM G-17, PPARγ growth suppression induced by 10 µM ciglitazone and 10 µM rosiglitazone was significantly attenuated to 93.8±5.3% of control (p<0.05) (Fig. 2a) and 89.9±6.8% of control (p<0.05) (Fig. 2b), respectively.

Effects of gastrin on PPARγ activity. Because gastrin attenuated the inhibitory effects of PPARγ activation on the growth of DLD-1 cells, we hypothesized that PPARγ activity might likewise be affected. To examine this possibility, we measured PPARγ transcriptional activity using the pHD(x3)Luc vector, a construct that has been previously used to assess PPARγ activity (38). DLD-1 cells were transiently transfected with the luciferase reporter construct and the pCMV-β-galactosidase vector as a control. Following transfection, DLD-1 cells were incubated with ciglitazone in the presence or absence of 200 nM G-17. As shown in Fig. 3, ciglitazone treatment enhanced PPARγ activity in a concentration-dependent manner. A 1.6-fold increase (p<0.001) in PPARγ transcriptional activity was observed in the presence of 1 µM ciglitazone, and maximum activity was achieved with 10 µM ciglitazone (2.9-fold, p<0.001). This ligand-dependent PPARγ activation correlated with the growth inhibition observed with ciglitazone treatment. However, when cells were preincubated with G-17, PPARγ activation induced by
ciglitazone was markedly attenuated at all concentrations. In the presence of 200 nM G-17, PPARγ activation by 10 µM ciglitazone was significantly attenuated by ~50% to a 1.42-fold increase (P<0.01) (Fig. 3).

Effects of gastrin on PPARγ expression. We next examined whether gastrin might affect PPARγ protein expression. DLD-1 cells were incubated in the presence or absence of G-17 (50 nM or 200 nM) for 12, 24, and 48 h, at which time PPARγ protein levels were measured by Western analysis. As shown in Fig. 4a, in response to the incubation of DLD-1 cells in the presence of G-17, PPARγ protein levels decreased when compared to control levels at all indicated time points. In addition, the decrease in PPARγ protein levels was concentration-dependent, with more pronounced reductions detected in the presence of 200 nM G-17. To determine whether the gastrin-promoted decrease in PPARγ protein concentrations occurred as a result of a decrease in PPARγ gene expression, DLD-1 cells were incubated in the presence of 50 nM and 200 nM G-17 or vehicle alone for 3, 6, 12, and 24 h, at which time total RNA was extracted and quantitative PCR analysis performed. No significant differences in PPARγ gene expression were detected in the presence of G-17 when compared to vehicle treatment at any of the above time points (Data not shown).

Two additional CRC cell lines, SW48 and MC-26, were employed to determine whether the reduction in PPARγ protein levels induced by G-17 was cell-specific. As shown in Fig. 4b, 24-h incubation with 200 nM G-17 significantly diminished PPARγ protein levels in both SW48 and MC-26 CRC cells. Therefore, the effects of gastrin on PPARγ expression observed in DLD-1 cells, a human CRC cell line possessing an APC mutation, appear to extend to a murine CRC cell line (MC-26) and to a human CRC cell line possessing the wild-type APC phenotype (SW48).

Because gastrin decreased PPARγ protein levels in the absence of exogenous PPARγ ligands, we next examined the effect of gastrin on PPARγ protein levels in the presence of the PPARγ ligand, rosiglitazone. When DLD-1 cells were incubated with 5 µM rosiglitazone, PPARγ protein levels slightly increased when compared to untreated cells (Fig. 4c). The addition of 200 nM G-17 to the culture medium containing 5 µM rosiglitazone significantly diminished PPARγ protein levels, while PPARγ levels decreased to a greater extent when cells were incubated in the presence of 200 nM G-17 alone (Fig. 4c).

Proteasomal degradation of PPARγ. Recent studies have demonstrated that the treatment of 3T3-L1 adipocytes with interferon γ (IFNγ) decreases PPARγ half-life (39,40). In addition, Spiegelman et al. have demonstrated that PPARγ is degraded by the proteasome in adipocytes. To further define the mechanisms mediating the gastrin-stimulated decrease in PPARγ protein levels, we investigated the possibility that gastrin might promote PPARγ proteasomal degradation. DLD-1 cells were incubated in the presence of the protein synthesis inhibitor, cycloheximide (50 µg/mL), with or without G-17. As depicted in Fig. 5a, after a 12-h incubation, G-17 induced PPARγ degradation, with the maximal effect observed using 200 nM G-17. Furthermore, the half-life of PPARγ in DLD-1 cells incubated in the presence of 200 nM G-17 decreased from ~11.3 h to ~7.1 h (P<0.05) (Fig. 5b).

To further investigate the role of the proteasome in PPARγ degradation, DLD-1 cells were incubated in the presence of two 26S proteasomal inhibitors, lactacystin or MG132. In the presence of 5 µM lactacystin or 20 µM MG132, both basal and gastrin-induced PPARγ degradation were markedly inhibited (Fig. 6). These results are consistent with the hypothesis that a decrease in PPARγ protein levels following gastrin treatment is mediated by targeting PPARγ for proteasomal degradation.

Gastrin transactivation of the EGF receptor. Earlier studies have demonstrated the involvement of many growth factors in the
transactivation of the EGFR (41-49). However, gastrin transactivation of the EGFR has not been previously examined in CRC. To evaluate the role of EGFR transactivation by gastrin in CRC, DLD-1 cells were incubated in the presence of 200 nM G-17 for 2 h, and whole cell lysates were collected at various time points. The lysates were evaluated by Western analysis using an antibody against the phosphorylated, active form of the EGFR. As shown in Fig. 7, G-17 promoted a biphasic activation of the EGFR. An initial increase in EGFR phosphorylation was observed after 5 min, which then declined after 30 min to a level slightly above control. The second component of the biphasic activation of the EGFR was detected at 60 min and remained steadily up-regulated for up to 2 h.

Gastrin-stimulated activation of ERK1/2 and the involvement of EGF receptor transactivation. The Ras/Raf/Mek/ERK1/2 pathway comprises a known signaling pathway for the promotion of cell proliferation. To investigate the possibility that gastrin activates the MAPK pathway in CRC, we incubated DLD-1 cells in the presence or absence of 200 nM G-17 for 2 h, and whole cell lysates were collected at various time points. Western analysis was subsequently performed using antibodies specific for the active, phosphorylated forms of the MAPK extracellular signal-regulated protein kinases, ERK1 and ERK2. As seen in Fig. 8, ERK1 and ERK2 (ERK1/2) were dramatically activated in the presence of 200 nM G-17 by 5 min, as determined by ERK1/2 phosphorylation. This activation declined to baseline levels after 15 min, which was followed by a second rise in ERK1/2 activation after 60 min treatment. ERK1/2 activation by 200 nM G-17 remained elevated at 120 min. ERK1/2 activation by G-17 exhibited a similar profile to gastrin-induced EGFR transactivation (Fig. 7).

Past studies have demonstrated that ERK1/2 activation may occur, in part, through transactivation of the EGFR by G-protein coupled receptors (10-18). Because ERK1/2 activation by gastrin nearly paralleled gastrin-stimulated EGFR transactivation in our study, we hypothesized that gastrin may promote activation of ERK1/2 through transactivation of the EGFR. To examine the role of gastrin-stimulated transactivation of the EGFR in ERK1/2 activation, DLD-1 cells were pretreated with the EGFR kinase inhibitor AG1478, which inhibited gastrin-stimulated EGFR phosphorylation (Data not shown). Moreover, in the presence of AG1478, activation of ERK1/2 by G-17 was nearly abolished at all observed time points when compared to the activated ERK1/2 levels in cells treated with G-17 alone (Fig. 8). A slight up-regulation of ERK2 activation still remained after 5 min and 120 min when compared to the levels evaluated at 0 min (Fig. 8). These results suggest that gastrin-induced EGFR transactivation may play a role in ERK1/2 activation.

Involvement of EGFR transactivation and ERK1/2 activation in gastrin-stimulated phosphorylation of PPARγ at the Ser84. Numerous studies have demonstrated that MAPK phosphorylates PPARγ in adipocytes (19,20,22,23). Because gastrin promoted MAPK activation, the peptide may have, in turn, also promoted PPARγ phosphorylation. To examine this possibility, DLD-1 cells were incubated in the presence of 200 nM G-17 for 1 h, after which Western analysis was performed using a phospho-specific antibody recognizing Ser84 phosphorylation of PPARγ. As depicted in Fig. 9a, G-17 promoted PPARγ phosphorylation starting at 5 min and persisting up to 60 min. Furthermore, G-17-induced PPARγ phosphorylation coincided with ERK1/2 activation. β-actin was used as a loading control as previous studies have shown that the total PPARγ protein levels are not significantly affected over a 60 min time period.

Because gastrin stimulated ERK1/2 activation and appeared to promote EGFR transactivation, we examined the possibility that these two pathways may also play a role in gastrin-stimulated PPARγ phosphorylation by utilizing AG1478 and PD98059 to inhibit EGFR and ERK1/2 activity, respectively. DLD-1 cells were pretreated individually with
these inhibitors for 45 min prior to addition of 200 nM G-17. As depicted in Fig. 9b, in the presence of AG1478, gastrin-stimulated PPAR\(\gamma\) phosphorylation at Ser84 was significantly diminished. Similarly, PPAR\(\gamma\) phosphorylation was abolished by co-incubation with PD98059 (Fig. 9b). Moreover, gastrin-induced ERK1/2 activation was abolished by the co-incubation of DLD-1 cells with either AG1478 or PD98059 (Fig. 9b). These results suggest that gastrin-enhanced PPAR\(\gamma\) phosphorylation at Ser84 is mediated, in part, through EGFR transactivation and ERK1/2 activation.

**The Roles of EGFR and ERK1/2 activation in PPAR\(\gamma\) degradation by gastrin.** Stephens and co-workers have demonstrated that ERK1/2 activation is involved in IFN\(\gamma\)-promoted PPAR\(\gamma\) degradation in adipocytes (40). However, the role of MAPK phosphorylation in the regulation of PPAR\(\gamma\) has not been evaluated in neoplasia. To examine the roles of gastrin-stimulated EGFR and ERK1/2 activation in PPAR\(\gamma\) degradation, we employed PD98059 to inhibit ERK1/2 activation and AG1478 to inhibit EGFR activity and used Western analysis to measure PPAR\(\gamma\) protein levels. We also used SB203580, a p38 MAPK inhibitor, as a negative control since this MAPK has not been demonstrated to play a major role in PPAR\(\gamma\) phosphorylation (20). DLD-1 cells were pretreated with the inhibitors individually for 45 min prior to the addition of cycloheximide and vehicle alone or 200 nM G-17. In the presence of PD98059, G-17-induced PPAR\(\gamma\) degradation was abrogated when compared to G-17 treated cells alone (Fig. 10). In addition, EGFR inhibition by AG1478 also abolished gastrin-induced PPAR\(\gamma\) degradation (Fig. 10). As predicted, SB203580 had no significant effect on gastrin-stimulated PPAR\(\gamma\) degradation (Fig. 10). These results suggest that gastrin may promote PPAR\(\gamma\) degradation through activation of ERK1/2 and transactivation of the EGFR, without involvement of the p38 MAPK pathway.

**Mutation of the Ser84 phosphorylation site reverses the attenuation of ligand-dependent PPAR\(\gamma\) activity by gastrin.** Ser84 phosphorylation of PPAR\(\gamma\) has been demonstrated to inhibit ligand-dependent PPAR\(\gamma\) activation in adipocytes (19). Because gastrin promoted PPAR\(\gamma\) phosphorylation at Ser84, we examined the possibility that gastrin might attenuate ligand-dependent PPAR\(\gamma\) activation through this mechanism. A phosphorylation mutant of PPAR\(\gamma\) was generated by mutating the Ser84 residue into an alanine residue, and stable lines of DLD-1 cells expressing GFP-tagged pTracer, pTracer-Flag-wtPPAR\(\gamma\), or pTracer-Flag-mutPPAR\(\gamma\)S84A were created. Protein expression was confirmed by Western analysis (Fig. 11a), and PPAR\(\gamma\) transcriptional activity in these cell lines was measured using the pHD(x3)Luc vector. Cells were transiently transfected with the luciferase reporter contract and the pCMV-\(\beta\)-galactosidase vector as a control. Following transfection, DLD-1 cells were incubated with 0, 5, or 10 \(\mu\)M rosiglitazone in the presence or absence of 200 nM G-17. As shown in Fig. 11b, no significant differences were observed in basal PPAR\(\gamma\) activity between the cell lines overexpressing wild-type PPAR\(\gamma\) (wtPPAR\(\gamma\)) and those overexpressing mutant PPAR\(\gamma\)S84A. Rosiglitazone incubation increased PPAR\(\gamma\) activity in both cell lines either overexpressing wtPPAR\(\gamma\) or the mutant PPAR\(\gamma\)S84A. Maximal activity began to plateau at a rosiglitazone concentration of 5 \(\mu\)M in both the wild-type (2.4-fold vs. 0 \(\mu\)M rosiglitazone, \(p<0.001\)) and mutant (2.2-fold vs. 0 \(\mu\)M rosiglitazone, \(p<0.001\)) PPAR\(\gamma\) overexpressing cell lines. However, whereas co-incubation with G-17 markedly attenuated PPAR\(\gamma\) activity stimulated by rosiglitazone at all concentrations in DLD-1 cells overexpressing wtPPAR\(\gamma\), gastrin had no effect in cells overexpressing the mutant PPAR\(\gamma\)S84A (Fig. 11b).

**Mutation of the Ser84 phosphorylation site attenuates gastrin-promoted degradation of PPAR\(\gamma\) protein.** Because attenuation of...
ligand-dependent PPARγ activity by gastrin was abrogated by alteration of the Ser84 phosphorylation site, we next determined whether this mutation might affect gastrin enhanced PPARγ protein degradation. DLD-1 cells stably overexpressing either wtPPARγ or mutPPARγS84A protein were incubated in the presence of cycloheximide (50 µg/mL), with or without G-17. After 12 h, PPARγ protein levels were measured by Western analysis. While wtPPARγ protein levels were significantly diminished in the presence of gastrin, no significant effect of gastrin on mutPPARγS84A protein levels was observed (Fig. 11c). These observations indicate that gastrin may promote PPARγ protein degradation through the phosphorylation of Ser84.

Mutation of the Ser84 phosphorylation site attenuates the effects of gastrin on ligand-dependent PPARγ growth suppression. We next determined whether mutation of the Ser84 phosphorylation site of PPARγ might also affect the attenuation of PPARγ-induced growth suppression by gastrin. 5 µM rosiglitazone decreased DLD-1 cell proliferation to 64% of control (p<0.001) in DLD-1 cells overexpressing wtPPARγ, and to 66% (p<0.001) in cells overexpressing mutant PPARγS84A, when compared to their respective controls (Fig. 11d). Moreover, in response to gastrin incubation, rosiglitazone-induced growth suppression was diminished by approximately 64% (p<0.001 vs. 5 µM rosiglitazone alone) in wtPPARγ overexpressing cells. In contrast, in the presence of 200 nM G-17, PPARγ growth suppression was minimally attenuated in cells overexpressing mutPPARγS84A (Fig. 11d). These results indicate that gastrin appears to attenuate rosiglitazone-induced PPARγ growth suppression, at least in part, through phosphorylation of PPARγ at Ser84.

Because gastrin reversed the effects of rosiglitazone on PPARγ growth suppression in part by the phosphorylation of PPARγ at Ser84, we next examined whether the overexpression of mutPPARγS84A might attenuate gastrin-stimulated DLD-1 cell proliferation. As seen in Fig. 11e, 200 nM G-17 promoted a 49.3±8.4% increase in DLD-1 cell proliferation when compared to control conditions, an effect that was diminished by ~38% in response to the overexpression of mutPPARγS84A (Fig. 11e).

Glycine-extended gastrin promotes proliferation by decreasing PPARγ protein levels. Studies were next performed to determine whether, similar to α-amidated gastrin, glycine-extended gastrin might also promote DLD-1 cell proliferation by decreasing PPARγ protein levels. DLD-1 cells were incubated in the presence of 1 nM - 100 nM Gly-G. As shown in Fig. 12a, Gly-G promoted a concentration-dependent increase in DLD-1 cell proliferation, as determined using the MTT proliferation assay. DLD-1 cell growth increased by 15.0±2.2% and 26.1±2.2% in the presence of 1 nM and 10 nM Gly-G, respectively, with maximal proliferation detected with 50 nM Gly-G (49.5±1.5%) and 100 nM Gly-G (50.1±1.8%). To determine whether the mitogenic properties of Gly-G might be mediated, in part, by decreasing PPARγ levels, DLD-1 cells overexpressing the empty vector, wild-type PPARγ, or mutPPARγS84A were incubated in the presence of 50 nM Gly-G for 24 h. As depicted in Fig. 12b, 50 nM Gly-G decreased PPARγ protein levels in the cell lines overexpressing the empty vector or wild-type PPARγ. In contrast, no change in PPARγ protein levels was observed in the cells overexpressing mutPPARγS84A. These observations suggest that Gly-G may likewise promote the growth of DLD-1 colorectal cancer cells, in part, by decreasing PPARγ protein levels through the promotion of PPARγ phosphorylation at Ser84.

Finally, studies were performed to determine whether Gly-G affects ligand-dependent PPARγ-induced growth suppression. As shown in Fig. 12c, 50 nM Gly-G attenuated rosiglitazone-induced growth suppression by approximately 45% (p<0.001 vs. 5 µM rosiglitazone alone) in DLD-1 cells overexpressing wtPPARγ. In
contrast, similar to the above studies examining α-amidated gastrin (G-17), the effects of PPARγ growth suppression were not significantly attenuated by Gly-G in cells overexpressing mutPPARγS84A (Fig. 12c).

**DISCUSSION**

Recent reports have indicated that both hypergastrinemia and decreased expression of PPARγ are associated with an increased risk for the development of CRC (10,37,50,51). However, the possibility of a link between gastrin and PPARγ has not been reported previously. In the present study, we have reported for the first time that ligand-dependent PPARγ growth suppression is attenuated by gastrin, at least in part, through attenuation of PPARγ activity and through an increase in PPARγ protein degradation. In addition, gastrin-induced EGFR transactivation and ERK1/2 activation appear to play a central role in this process.

Gastrin has been implicated as a trophic factor for various neoplasms of GI origin, including CRC and those of pancreatic, gastric, and esophageal origin (52-62). Gastrin and gastrin receptors are expressed aberrantly in colorectal adenomas and malignant tumors and have been implicated in malignant progression (17,18). While Smith et al. (17) and Guo et al. (46) reported that a majority (up to 80%) of all colorectal adenomas express the CCK-2R, others have detected the receptor in only 11-38% of such tumors (15)(63). These discrepancies may be explained, at least to some extent, by the detection of splice variants of the CCK-2R and by differences in methods utilized by various investigators.

The results of the present study demonstrate that gastrin and its precursor, Gly-G, stimulated the growth of DLD-1 cells in a concentration-dependent manner. Interestingly, the specific CCK-2 receptor antagonist L-365,260 only partially attenuated G-17-stimulated growth of DLD-1 cells. These findings suggest that gastrin-stimulated cell growth in these cells may be mediated, at least in part, by other receptors that bind gastrin peptides. In addition to the “classical” gastrin receptor binding site, several alternative binding sites on DLD-1 cells have been proposed. Ahmed et al. recently identified two gastrin binding sites with nanomolar and micromolar affinities, respectively, on the cell membrane, both of which played key roles in cell proliferation stimulated by amidated and precursor gastrin peptides (64). Furthermore, Yang et al. identified a micromolar affinity site for both G-17 and the non-amidated glycine-extended gastrin precursors on DLD-1 cells (65). Thus, it appears that the activation of alternative gastrin receptors, in addition to the “classical” CCK-2R, might mediate gastrin-stimulated proliferation of DLD-1 cells.

The activation of ERK1/2 is a known pathway by which gastrin stimulates cell proliferation (42,46,66,67). Although several studies have demonstrated that gastrin promotes ERK1/2 activation through transactivation of the EGFR, many of them have been performed in cell lines ectopically overexpressing the CCK-2R (42,45,46,60,62). The present study represents the first to demonstrate that gastrin promotion of ERK1/2 activation involves EGFR transactivation in human CRC cells expressing endogenous gastrin receptors. Our findings corroborate an earlier study by Guo et al., who reported that gastrin activates ERK1/2 in a biphasic manner in rat intestinal epithelial (RIE) cell lines ectopically expressing the CCK-2R (46). However, in their study, EGFR transactivation appeared to promote only the later phase of ERK1/2 activation. In our study, EGFR transactivation played a key role in both early and late ERK1/2 activation, as demonstrated by significant inhibition of both phases in the presence of the EGFR kinase inhibitor, AG1478. In addition, the biphasic activation of ERK1/2, although not identical, paralleled that of EGFR transactivation in the presence of gastrin, further suggesting the involvement of the EGFR in the early and late phases of ERK1/2 activation. Moreover, these results are consistent with the hypothesis that gastrin-stimulated EGFR transactivation promotes ERK1/2 activation, which in turn appears to
play a critical role in mediating the trophic properties of gastrin in CRC.

Nevertheless, the possibility of the involvement of additional pathways independent of the EGFR in the regulation of gastrin-enhanced phosphorylation of ERK1/2 cannot be excluded. As indicated above, gastrin-stimulated phosphorylation of the EGFR exhibited a similar profile to that of ERK1/2, but was not identical. For example, at 15 min, ERK1/2 phosphorylation was diminished while EGFR phosphorylation was increased. Furthermore, residual ERK1/2 activation was evident at 5 and 120 min the presence of AG1478. Thus, it is likely that gastrin promotes ERK1/2 phosphorylation by additional pathways not involving the EGFR, including direct stimulation of MAP kinase pathways (42,46,66,67).

Although not examined in the present study, several mechanisms responsible for gastrin-stimulated EGFR transactivation have been suggested in other cell lines overexpressing the CCK-2R. For example, CCK-2R activation by gastrin increases the shedding of several membrane-bound EGFR ligands, including HB-EGF, TGFα, FGF, and amphiregulin (44,45,60,62,68). Shedding of these ligands may promote autocrine/paracrine stimulation of the EGFR. In RIE cells ectopically overexpressing the CCK-2R, gastrin transactivation of the EGFR was reported to be mediated through an intracellular signaling pathway involving activation of the adaptor protein Src (46). Src activation promotes the formation of a multiprotein complex containing EGFR, Src, and other adaptor proteins, which leads to activation of the EGFR (69). An alternative suggested mechanism involves gastrin-stimulated expression of EGFR ligands. In AGS human gastric carcinoma cells, gastrin stimulated the expression of HB-EGF through PKC- and EGFR-dependent mechanisms (60). Future studies will be necessary to determine whether these pathways likewise mediate gastrin-stimulated EGFR transactivation in DLD-1 and other CRC cells.

Activation of ERK1/2 plays a key role in promoting the mitogenic effects of gastrin, and PPARγ, a tumor suppressor, has been identified as a substrate for phosphorylation by the various MAPKs (19,20,22,23,70), including ERK1/2. Because gastrin is known to increase ERK1/2 activation, we sought to determine whether PPARγ phosphorylation might also be involved in promoting the mitogenic effects of gastrin. Our findings indicate that activation of ERK1/2 by gastrin was associated with the phosphorylation of PPARγ at Ser84, a putative MAPK phosphorylation site located within the ligand-independent transactivation domain (AF-1). Previous studies have demonstrated that phosphorylation of PPARγ in the AF-1 domain downregulates its activity (19,20,22,23,70). Interestingly, the EGFR kinase inhibitor AG1478 only partially attenuated gastrin-stimulated PPARγ phosphorylation, while the MEK inhibitor PD98059 abolished PPARγ phosphorylation. These observations provide additional evidence that both EGFR-dependent and -independent activation of ERK1/2 may promote gastrin-stimulated PPARγ phosphorylation.

In the present studies, gastrin also decreased ligand-dependent PPARγ activity. The elimination of the Ser84 phosphorylation site prevented the attenuation of ligand-dependent PPARγ activity by gastrin. Thus, gastrin-promoted phosphorylation at Ser84 appears to play an integral role in the downregulation of ligand-enhanced PPARγ activity. It has been previously shown that phosphorylation of PPARγ may diminish PPARγ activity by activating the proteasome. Floyd and coworkers demonstrated that IFNγ treatment of 3T3-L1 adipocytes enhanced ERK1/2 activation which coincided with the ubiquitination and subsequent proteasomal degradation of PPARγ, thus suggesting a role for phosphorylation as a signal for PPARγ degradation (40). In our studies, gastrin promoted the phosphorylation of PPARγ at Ser84 and enhanced the proteasomal degradation of PPARγ. Moreover, the mutation of the Ser84 phosphorylation site stabilized PPARγ protein in the presence of gastrin and was associated with attenuation of ligand-dependent PPARγ growth suppression.
Therefore, gastrin-stimulated phosphorylation of PPARγ at Ser84 may promote subsequent proteasomal degradation of PPARγ, leading to a decrease in PPARγ protein expression and ligand-dependent activity, thereby attenuating the ligand-dependent PPARγ growth suppression of DLD-1 cells.

Although Hauser et al. previously demonstrated that PPARγ ligands target PPARγ for degradation (71), in the present study, PPARγ protein levels increased in response to the incubation of DLD-1 cells with rosiglitazone. This discrepancy may be explained in part by different cell types used during the course of the studies. Whereas Hauser and colleagues employed NIH-3T3 fibroblasts and fibroblast-derived adipocytes (71), we performed our studies using colorectal cancer cell lines. Similar discrepancies have been observed in studies examining the estrogen receptor, in which incubation with estradiol led to the rapid degradation of the estrogen receptor α in MCF-7 breast cancer cells, but stabilization in Ishikawa uterine-derived cells (72).

Proper maintenance of PPARγ activity and protein levels is critical for the suppression of tumorigenesis, as demonstrated by recent reports showing that PPARγ+/- mice are more susceptible to CRC (73). Moreover, Sarraf et al. recently reported that 8% of CRC tumors harbor a loss of function mutation in the PPARG gene (36), which results in decreased functional PPARγ protein levels. As evident by the partial attenuation of gastrin-induced cell proliferation in cells overexpressing the PPARγS84A mutant (Fig. 11e), our studies suggest that gastrin appears to exert its trophic effects in part through phosphorylation, inactivation, and degradation of PPARγ.

In conclusion, the present study is consistent with the following proposed model (Fig. 13). Gastrin peptides appear to promote cell proliferation, in part, by inducing PPARγ degradation. Initially, gastrin stimulates ERK1/2 activation, at least in part, through transactivation of the EGFR, which, in turn, phosphorylates PPARγ at the Ser84 residue. Although not examined in the present study, previous studies have demonstrated that gastrin also stimulates ERK1/2 activation directly (42,46,66,67). The phosphorylation of PPARγ at Ser84 decreases PPARγ transactivational activity and signals PPARγ for proteasomal degradation. As a result, PPARγ protein levels are diminished, thereby attenuating the tumor suppressive effects of PPARγ, resulting in enhanced cellular proliferation. Additional studies will be necessary to fully elucidate the relationship between gastrin peptides and PPARγ and to determine their precise roles in the pathogenesis CRC and other malignancies of the GI tract.

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FIGURE LEGENDS

FIG. 1. **Gastrin promotes DLD-1 CRC cell proliferation.** *A*, Gastrin stimulates DNA synthesis, as measured by \(^{3}\text{H}\)-thymidine uptake. Following serum starvation, DLD-1 cells were incubated in the presence of 50 nM G-17, 200 nM G-17, or vehicle alone for 22 h. To determine the involvement of the CCK-2R, DLD-1 cells were preincubated with L-365,260 30 min. prior to addition of 200 nM G-17. Values are expressed as a mean percent of control (vehicle) ± SD of at least three independent experiments performed in triplicate. \(*P<0.05, \overset{\dagger}{P}<0.01\)

*FIG. 2. Ligand-dependent PPAR\(\gamma\) growth suppression is attenuated by pretreatment with gastrin.* DLD-1 cells were serum starved for 24 h followed by 12 h incubation in media containing 200 nM G-17 or vehicle alone. Following the 12 h incubation period, rosiglitazone (0-20 \(\mu\)M) in *A*, or ciglitazone (0-20 \(\mu\)M) in *B*, was added and incubated for 72 h, at which time cell proliferation was measured by the MTT assay method, as described under “Experimental Procedures”. Rosiglitazone in *A*, and ciglitazone in *B*, dose dependently suppressed DLD-1 cell proliferation. In the presence of 200 nM G-17, PPAR\(\gamma\) growth suppression was attenuated. Data are represented as mean percent of control (no rosiglitazone or ciglitazone) ± SD of three independent experiments performed in sixteen replicates. \(*P<0.05\) compared to control, \(\overset{\dagger}{P}<0.01\) compared to control. \(\overset{\ddagger}{P}<0.05\) compared to 10 \(\mu\)M rosiglitazone or ciglitazone.

FIG. 3. **Gastrin attenuates ligand-dependent PPAR\(\gamma\) activation.** DLD-1 cells were co-transfected with the pHD(x3)Luc and pCMV-\(\beta\)-gal. Then DLD-1 cells were incubated for 12 h in the presence or absence of 200 nM G-17 in media containing 1% FBS. Ciglitazone (0-10 \(\mu\)M) was then added and incubated for 16 h, after which luciferase and \(\beta\)-galactosidase activities were measured. Ciglitazone promoted a dose-dependent increase in luciferase activity, which was attenuated by G-17 pretreatment. Data are expressed as mean-fold activation ± SD compared to control (no ciglitazone) of three independent experiments performed in triplicate. \(*P<0.001\) compared to control, \(\overset{\dagger}{P}<0.01\) compared to respective ciglitazone concentrations (1 \(\mu\)M, 5 \(\mu\)M, 10 \(\mu\)M).

FIG. 4. **Gastrin promotes a concentration-dependent decrease in PPAR\(\gamma\) protein expression.** *A*, Following serum starvation, DLD-1 cells were incubated in the presence of vehicle alone, 50 nM G-17, or 200 nM G-17 for the indicated time periods. *B*, Following serum starvation, SW48 and MC-26 cells were incubated in the presence of vehicle alone or 200 nM G-17 for 24 h. *C*, Following serum starvation, DLD-1 cells were incubated in the presence of vehicle alone or 200 nM G-17 and in the presence of absence of 5 \(\mu\)M rosiglitazone for 24 h. At the indicated time points, cell lysates were collected, resolved by SDS-PAGE, and immunoblotted with anti-PPAR\(\gamma\) antibody. \(\beta\)-actin was used as a control for loading. Above immunoblots are representative of at least three independent experiments.

FIG. 5. **Gastrin decreases the stability of PPAR\(\gamma\) protein.** *A*, Following serum starvation, DLD-1 cells were incubated with vehicle alone or 50 nM, 100 nM, or 200 nM G-17 in the presence of 50 \(\mu\)g/mL cycloheximide for 12 h. Whole cell lysates were then collected, resolved by SDS-PAGE, and immunoblotted for PPAR\(\gamma\). \(\beta\)-actin levels were measured as a control for loading. G-17 increased PPAR\(\gamma\) protein degradation in a concentration-dependent manner. *B*, Following serum starvation, DLD-1 cells were incubated over a 12-h period in the
presence or absence of 200 nM G-17 in media containing 50 µg/mL cycloheximide. Whole cell lysates were collected at the indicated time points, resolved by SDS-PAGE, and immunoblotted with the anti-PPARγ antibody. β-actin levels were measured as a control for loading. As shown in the lower panel, gastrin decreased the half-life of PPARγ when compared to untreated cells represented in the upper panel. C, Linear regression analysis of data from six experiments was plotted on a log scale to determine the half-life of PPARγ in the presence (*P<0.05) or absence of 200 nM G-17. The t1/2 of PPARγ protein is decreased from ~11.3 h (sl-G-17=-4.43, r²=0.993) in the absence of G-17 to ~7.1 h in the presence of 200 nM G-17 (sl+G-17=-7.02, r²=0.981, P<0.05 vs. sl-G-17).

FIG. 6. Gastrin promotes PPARγ degradation through the proteasomal pathway. Following serum starvation, DLD-1 cells were preincubated with or without the proteasomal inhibitors, MG132 and lactacystin. Cells were then treated with 200 nM G-17 or vehicle alone in media containing cycloheximide for 12 h. Above immunoblot is representative of three independent experiments.

FIG. 7. Gastrin promotes transactivation of the EGF receptor. A, Shown is a time course of EGFR transactivation by 200 nM G-17. Following serum starvation, DLD-1 cells were incubated in the presence of 200 nM G-17 over a 2 h period. At the indicated time points, whole cell lysates were collected, separated by SDS-PAGE, and immunoblotted with the phospho-specific anti-EGFR (pTyr1173) antibody. β-actin protein levels were measured as a control for loading. B, Graphical representation of data from three experiments depicted in panel A. Values are normalized to β-actin and are expressed as fold-activation ± SD of control (vehicle). *P<0.05 compared to control, N.S. = not significant compared to 200 nM G-17 treatment alone.

FIG. 8. Gastrin-stimulated ERK1/2 activation is mediated, in part, through the EGF receptor. After serum starvation, DLD-1 cells were incubated with 200 nM G-17 over 2 h. At the indicated time points, whole cell lysates were collected, separated by SDS-PAGE and immunoblotted with the phospho-specific ERK1/2 monoclonal antibody (pThr202/Tyr204). To determine the involvement of the EGFR, cells were preincubated with 100 nM AG1478 prior to addition of 200 nM G-17. β-actin was measured as a control for loading. Above immunoblot is representative of at least three independent experiments.

FIG. 9. Gastrin-stimulated PPARγ phosphorylation is mediated, in part, through transactivation of the EGF receptor and activation of ERK1/2. A, Following 24 h serum starvation, DLD-1 cells were incubated with 200 nM G-17 over a 60-min period. B, To determine the EGFR and ERK1/2 involvement, DLD-1 cells were preincubated with 100 nM AG1478 or 40 µM PD98059, respectively, for 30 min prior to addition of 200 nM G-17. After 5 min incubation with G-17, whole cell lysates were collected for a determination of phospho-PPARγ and phospho-ERK1/2 protein levels. At the indicated time points, whole cell lysates were collected, separated by SDS-PAGE and immunoblotted with the phospho-specific PPARγ antibody (pSer84) and the phospho-specific ERK1/2 antibody (pThr202/Tyr204). β-actin was measured as a control for loading. Immunoblots are representative of at least three independent experiments.

FIG. 10. Inhibition of ERK1/2 activation and EGF receptor transactivation attenuates gastrin-induced PPARγ degradation. A, After serum starvation DLD-1 cells were
pre-incubated with 100 nM AG1478, 40 µM PD98059, or 10 µM SB203580 for 30 min. 200 nM G-17 or vehicle alone was then added in the presence of the above inhibitors in media containing 50 µg/mL cycloheximide. After 12 h, cell monolayers were lysed, resolved by SDS-PAGE, and immunoblotted with the anti-PPARγ antibody and the phospho-specific ERK1/2 antibody (pThr202/Tyr204).  

B, Graphic representation of data of five experiments depicted in panel A. Values are normalized to β-actin and are expressed as a mean percent ± SD of control (no treatment).  *P<0.001 compared to control, ‡P<0.001 compared to 200 nM G-17 treatment alone, N.S.=not significant compared to 200 nM G-17 treatment alone.

**FIG. 11.** Mutation of the Ser84 phosphorylation site reverses the attenuation of ligand-dependent PPARγ activity and growth suppression by gastrin.  
A, Confirmation of stable expression of PPARγ variants. 1 = parental DLD-1; 2 = DLD-1 pTracer mutPPARγS84A; 3 = DLD-1 pTracer wtPPARγ; 4 = DLD-1 pTracer empty vector.  B, DLD-1 cells were co-transfected with the pHD(x3)Luc and pCMV-β-gal, after which DLD-1 cells were incubated for 12 h in the presence or absence of 200 nM G-17. Rosiglitazone (0-10 µM) was then added and incubated for 16 h, after which luciferase activity was measured. β-galactosidase activity was measured as a control. Rosiglitazone promoted an increase in luciferase activity, which was attenuated by G-17 pretreatment in cells overexpressing wtPPARγ but not in cells overexpressing mutPPARγS84A. Data are expressed as mean-fold activation ± SD compared to control (no rosiglitazone) of three independent experiments performed in triplicate.  
C, DLD-1 cells overexpressing wtPPARγ or mutPPARγS84A protein were serum-starved for 24 h and then incubated in the presence of 50 µg/mL of cycloheximide with or without 200 nM G-17. Cell monolayers were lysed, resolved by SDS-PAGE, and immunoblotted with the anti-PPARγ antibody and β-actin was measured as a loading control. Immunoblot is representative of three independent experiments.  
D, DLD-1 cells were serum starved for 24 h, followed by 6-h incubation in media containing 200 nM G-17 or vehicle alone. Rosiglitazone (0-10 µM) was then added and incubated for 72 h, at which time cell proliferation was measured by the MTT assay method, as described in “Materials and Methods”. Rosiglitazone suppressed DLD-1 cell proliferation. In the presence of 200 nM G-17, PPARγ growth suppression was significantly attenuated in cells overexpressing wtPPARγ, but to a much lesser extent in cells overexpressing mutPPARγS84A. Data are represented as a mean percent of control (no rosiglitazone) ± SD of three independent experiments performed in 24 replicates. *P<0.001 compared to control, †P<0.001 compared to 5 µM rosiglitazone.  
E, DLD-1 cells overexpressing pTracer alone or mutPPARγS84A were serum-starved for 24 h and then incubated with or without 200 nM G-17. Cell proliferation was measured by the MTT assay method, as described in “Materials and Methods”. Overexpression of PPARγ attenuated G-17-induced cell proliferation. Data are represented as a mean percent of control (vehicle) ± SD of three experiments performed in 16 replicates. *P<0.01 compared to vehicle treatment, #P<0.01 compared to 200 nM G-17 alone.

**FIG. 12.** Glycine-extended gastrin (Gly-G) promotes cell proliferation, in part, by attenuation of PPARγ.  
A, Gly-G stimulates DLD-1 cell proliferation, as measured by the MTT assay. Following serum starvation, DLD-1 cells were incubated in the presence of 1 nM – 100 nM Gly-G, and proliferation was measured by the MTT assay. Values are expressed as a mean percent of control (vehicle) ± SD of at least three independent experiments performed in 24 replicates. *P<0.001 compared to vehicle.  
B, DLD-1 cells overexpressing wtPPARγ or mutPPARγS84A protein were serum-starved for 24 h and then incubated in the presence or absence of 50 nM Gly-G. Cell monolayers were lysed, resolved by SDS-PAGE, and immunoblotted with the anti-PPARγ antibody and β-actin was measured as a loading control.  
C, DLD-1 cells were serum starved for 24 h, followed by a 6-h incubation in media containing 50 µg/mL cycloheximide.
nM Gly-G or vehicle alone. Rosiglitazone (0-10 µM) was then added and incubated for 72 h, at which time cell proliferation was measured by the MTT assay method, as described in “Materials and Methods”. Rosiglitazone suppressed DLD-1 cell proliferation. In the presence of 50 nM Gly-G, PPARγ growth suppression was significantly attenuated in cells overexpressing wtPPARγ, but to a much lesser extent in cells overexpressing mutPPARγS84A. Data are represented as a mean percent of control (no rosiglitazone) ± SD of two independent experiments performed in 24 replicates. *P<0.01 compared to control, ‡P<0.01 compared to 5 µM rosiglitazone, N.S.=not significant compared to 5 µM rosiglitazone.

FIG. 13. Proposed model of gastrin-stimulated cell proliferation by enhancement of PPARγ protein degradation. Gastrin promotes activation of ERK1/2, in part, through activation of the EGFR. Although not examined in the present study, gastrin may also stimulate ERK1/2 activation directly. ERK1/2 activation leads to phosphorylation of PPARγ at Ser84. Ser84 phosphorylation decreases ligand-dependent PPARγ activity and signals PPARγ for degradation. As a result, PPARγ levels are diminished, which, in turn, stimulates DLD-1 cell proliferation.
Figure 1
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Figure 5

**a**

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12 h Incubation

**b**

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<td>β-actin</td>
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**c**

![Graph showing the remaining PPARγ (%)(Normalized to β-actin) over time.](image)

- G17: $t_{1/2} \approx 11.3$ h
- G17: $t_{1/2} \approx 7.1$ h
Figure 6

<table>
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<tr>
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<th>β-actin</th>
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</tbody>
</table>
Figure 7
Figure 8
Figure 9

- **a**
  - Time (min) 0 5 15 30 60
  - pPPARγ
  - pERK1/2
  - β-actin

- **b**
  - G-17: - - + + + - + -
  - AG1478: - - - - + + - -
  - PD98059: - - - - - - + +
  - pPPARγ
  - pERK1/2
  - β-actin

5 min incubation
Figure 10
**Figure 11**

(a) Western blot analysis of PPARγ, GFP, and β-actin expression.

(b) Luciferase activity assay showing the effect of Rosiglitazone on pTracer-wtPPARγ and pTracer-mutPPARγ with or without G-17.

(c) Western blot analysis of PPARγ and β-actin expression with G-17 treatment.

(d) Relative growth assay showing the effect of Rosiglitazone on wtPPARγ and mutPPARγS84A with or without G-17.

(e) Relative growth assay showing the effect of Gastrin-17 on mutPPARγS84A with or without 200 nM G-17.

**Key Points:**
- PPARγ and β-actin expression levels are analyzed using Western blot.
- Rosiglitazone and G-17 affect luciferase activity.
- Growth inhibition is observed with Gastrin-17 and G-17.
Figure 12
Figure 13
Attenuation of peroxisome proliferator activated receptor gamma (PPARγ) mediates gastrin-stimulated colorectal cancer cell proliferation
Albert J. Chang, Diane H. Song and M. Michael Wolfe

J. Biol. Chem. published online March 30, 2006

Access the most updated version of this article at doi: 10.1074/jbc.M602623200

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