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GLUCOSE-DEPENDENT INSULINOTROPIC POLYPEPTIDE STIMULATES THE PROLIFERATION OF COLORECTAL CANCER CELLS

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Abstract

Although numerous epidemiological studies have provided convincing evidence for an increase in the prevalence of colorectal cancer (CRC) in obese individuals, the precise mechanisms involved have not been elucidated. Glucose-dependent insulintropic polypeptide (GIP) is a gastrointestinal regulatory peptide whose primary physiologic role is to stimulate postprandial pancreatic insulin secretion. Like insulin, GIP has been linked to enhanced nutrient efficiency, which occurred during the course of evolution. Its expression is increased in obesity, and we thus initiated studies to examine whether GIP might contribute to the pathogenesis of obesity-related CRC. RT-PCR and Western analysis demonstrated the presence of the GIP receptor (GIPR) in several human CRC cell lines. GIP stimulated the proliferation of MC-26 cells, a mouse CRC cell line, in a concentration-dependent manner. Western analysis showed that GIP induced the activity of several downstream signaling molecules known to be involved in cellular proliferation in a concentration- and time-dependent manner. These studies indicate that the presence of GIP receptors in CRC may enable ligand binding and, in so doing, stimulate CRC cell proliferation. The overexpression of GIP, which occurs in obesity, might thereby be contributing to the enhanced rate of carcinogenesis observed in obesity.

Keywords

glucose-dependent insulintropic polypeptide; GIP; obesity; colorectal cancer; Akt; mTOR

INTRODUCTION

Despite significant advances in radiotherapy, chemotherapy, and surgery during the past two decades, colorectal cancer (CRC) remains the third most common cause of cancer-related death in the United States, accounting for 10% of all deaths due to malignancy (1). The American Cancer Society estimates that in 2009, approximately 147,000 Americans will be diagnosed with CRC, of whom nearly 50,000 will die. The magnitude of this burden on the

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American health system emphasizes the importance of elucidating the basic mechanisms underlying the disease to permit the design of optimal treatment modalities.

The development of CRC appears to involve a multi-step process of genetic mutations combined with environmental factors, whereby normal epithelial cells undergo dysplastic transformation, followed by proliferation and histological progression to neoplasia (2,3). One such factor is obesity, which represents a global epidemic and is a leading cause of illness and death worldwide (4). An estimated 100 million adults in the United States are considered obese (5), which substantially raises their risk of morbidity. In addition to cosmetic effects, obesity is a known risk factor in the pathogenesis of type 2 diabetes mellitus, hypertension, pulmonary dysfunction, vascular diseases, gastrointestinal (GI) and biliary disorders, osteoarthritis, and various malignancies, including CRC. Although the precise relationship between CRC and obesity has not been elucidated, the American Cancer Society has estimated that obese individuals possess approximately a 1.5-2.0 relative risk for the development of CRC compared with those of normal weight, and that 20.8% and 35.4% of CRC cases may be attributable to weight in women and men, respectively.

Glucose-dependent insulintropic polypeptide (GIP) is a 42-amino acid peptide hormone synthesized in, and released from, K-cells of the duodenum and jejunum after the ingestion of a meal. Upon its release, GIP induces insulin release from pancreatic islet β -cells, thus acting as the principal mediator of the enteroinsular axis and as a hormone that contributes to optimization of nutrient deposition (7). GIP is overexpressed in obese animal models and humans, and an etiologic role for GIP in the development of obesity has accordingly been suggested (8-12).

The present studies were conducted to assess the possibility that enhanced GIP expression, which occurs in obesity, might contribute to the pathogenesis of obesity-related carcinogenesis, and to investigate the intracellular mechanisms that might mediate GIP-induced CRC tumor growth. After first demonstrating the presence of the GIP receptor (GIPR) in CRC cells, we showed that GIP stimulated the proliferation of MC-26 cells, a mouse CRC cell line. Western analysis demonstrated that GIP induced several downstream signaling molecules known to be involved in cellular proliferation. These studies indicate that the presence of the GIPR in CRC may enable ligand binding and, in so doing, stimulate CRC cell proliferation. GIP overexpression might thereby be contributing to the enhanced rate of carcinogenesis observed in obesity.

MATERIALS AND METHODS

Antibodies and cells

Rabbit anti-GIPR primary antibody was kindly provided by Dr. Joel Habener (Massachusetts General Hospital, Boston, MA). Other primary antibodies were purchased from Cell Signaling Technologies (Danvers, MA): rabbit anti-Phospho-AKT (Ser 473), rabbit anti-AKT antibody, rabbit anti-phospho-p70 S6 kinase (Thr 389), and rabbit anti-Phospho-p44/42 MAP Kinase antibody (Thr 202/204). Monoclonal β -actin primary antibody was purchased from Sigma (St. Louis, MO). The mitogen-activated protein (MAP) kinase kinase (MEK) inhibitor PD98059 and PI3 kinase inhibitor Wortmannin were purchased from Calbiochem (San Diego, CA) and Roche (Indianapolis, IN), respectively.

MC-26 cells, a transplantable mouse CRC cell line, was obtained from Dr. K.K. Tanabe (Massachusetts General Hospital, Boston, MA), and HT29 cells, human CRC cell lines, were purchased from American Type Culture Collection (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's Medium (DMEM; Cellgro Mediatech, Manassas, VA)

containing 10% fetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin G, and 100 µg/ml streptomycin at 37° C in a humidified atmosphere of 5% CO₂.

Immunohistochemistry

Paraffin embedded blocks containing clinical colon biopsy specimens from four patients with stage 2-4 CRC were obtained from the Biospecimen Archive Research Core at Boston University School of Medicine and cut into 7-µm sections. Deparaffinized and rehydrated slides were subjected to antigen retrieval by steaming in a 10-mM citric acid buffer (pH 6.0) at 100 °C for 20 min and then were allowed to cool for 20 min at room temperature (RT). Slides were washed in phosphate-buffered saline (PBS) and then blocked with 5% normal donkey serum for 60 min at RT. The primary antibody, rabbit anti-GIPR (Abcam, Cambridge MA) was diluted 1:200 in PBS containing 1% bovine serum albumin (BSA) and incubated with the sections overnight at 4 °C. Slides were washed in PBS and incubated with Alexa Fluor 594 goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA) diluted 1:250 in PBS containing 1% BSA for 1 h at RT. Slides were washed in PBS and the nuclei were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) and the sections mounted using UltraCruz mounting medium (Santa Cruz Biotechnology, Santa Cruz, CA). Sections were examined using a Nikon Deconvolution Wide-field Epifluorescence microscope. TIFF images were processed in Adobe Photoshop.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to determine the relative expression levels of GIPR. Total RNA was extracted from MC-26 and HT29 cells using the Qiagen RNeasy kit (Qiagen Inc., Valencia, CA). For amplification, starting with 2 µg of RNA as template, single-stranded cDNA was synthesized from total RNA using an oligo (dT) primer (50 pmol) and ThermoScript RT-PCR system (Invitrogen). The single-stranded cDNA was then amplified using published human gene-specific primer sequences for GIPR or for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The GIPR 5' forward primer was (5'-CTG CCT GCC GCA CGG CCC AGA T-3'), and the 3' reverse primer (5'-GCG AGC CAG CCT CAG CCG GTA A-3'). For amplification of GAPDH cDNA, the forward primer was (5'-GGG GAG CCA AAA GGG TCA TCA TCT-3'), and the 3' reverse primer was (5'- GAC GCC TGC TTC ACC ACC TTC TTG-3').

The PCR reaction was carried out in a total volume of 50 µl and consisted of PCR mix (50 mM KCl, 10 mM/Tris-HCl, 1.5 mM MgCl₂, 0.2 mM dNTP's and 25 U Taq DNA polymerase, final pH 8.3; Roche, Mannheim, Germany). The amplification reaction involved denaturation at 94 °C for 2 min, followed by cycling as follows: 94 °C for 1 min, primer annealing at 53 °C for 1 min and extension at 72 °C for 2 min. After cycling, terminal elongation of 5 min at 72 °C was performed. Amplification required 30 cycles for GAPDH and 35 cycles for GIPR. PCR products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide staining and photographed under UV transillumination.

Western blot analysis

Western blot hybridization analysis was performed using previously published methods (7). Immunoreactive protein bands were identified by protein size using known molecular weight standards provided by Bio-Rad Kaleidoscope Prestained Standards (Hercules, CA, USA). Ponceau S (Sigma) staining, and immunoblots with monoclonal β-actin antibody were used to confirm equal loading of Western blot membranes. Autoradiographs were scanned with a Bio-Rad GS 700 Imaging Densitometer (Bio-Rad), and photodensitometric analysis was performed with the NIH ImageJ program.

***In-vitro* cell proliferation assay [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay]**

Cell proliferation was assessed using a CellTiter 96 AQ One Solution Cell Proliferation Assay kit (Promega, Madison, WI). MC-26 cells at a density of 3×10^3 cells per well were plated onto a 96-well microtiter plate and incubated in DMEM with 10% FBS overnight. The FBS containing medium was replaced with serum free medium the following day and incubated for 24 h. To determine the effect of GIP on proliferation, cells were treated in triplicate with increasing concentrations of porcine GIP for 24, 48, 72, and 96 h. At the end of each experiment, 20 μ L of CellTiter96 Aqueous solution Reagent were added to each well, and the plate was incubated for 1 h at 37 °C in a humidified 5% CO₂ atmosphere. The absorbance at 490 nm was then recorded using a 96-well ELx800 universal microplate reader (BIO-TEK Instruments, Winooski, VT).

Cyclic Adenosine Monophosphate (cAMP) measurement

Intracellular cAMP was measured using a cAMP HTS Chemiluminescent Immunoassay Kit (Millipore, Billerica, MA). MC-26 cells were seeded at 80% confluency on a 48-well plate in DMEM containing 10% FBS for 24 h for cells to attach, after which they were incubated in serum free DMEM media for an additional 24 h. Cells were pretreated for 1 h with a 1 x isobutyl-1-methylxanthine (IBMX) and then treated with porcine GIP in DMEM for 10 min. At that time, media were removed, and 200 μ L of cold lysis buffer were added to each well and incubated for 10 min at room temperature. Experiments were performed in triplicate on 96-well anti-rabbit coated assay plates, using 50 :1 of each sample to assay. The assay was performed in accordance with the manufacturer's instructions, and the plates were read for 1 sec with a POLARstar OPTIMA luminometer (MG LABTECH, Durham, NC).

Akt, ERK1/2 pathways

To investigate the effects of GIP on Akt and ERK1/2 activation, MC-26 and HT29 cells were serum-starved overnight. Triplicate wells of cells were then incubated in the absence or presence of 100 nM GIP, with and without pretreatment with 20 nM rapamycin, 1 μ M Wortmannin, or 40 μ M PD98059 for 30 min. Whole cell lysates were extracted and Western analysis was performed using specific antibodies to total Akt, phosphorylated ERK1 and ERK2 (pERK1/2), or p70S6K, or a phospho-specific antibody that recognizes serine 473 phosphorylation of Akt (pAkt).

Statistical analysis

The two-way Student's T-test was performed for paired comparisons. Statistical significance was assigned if $P < 0.05$.

RESULTS

Presence of the GIPR in CRC and in CRC cell lines

An immunohistochemical analysis of all four paraffin-embedded CRC specimens demonstrated strongly positive staining for the GIPR; representative slides from one patient with advanced CRC are shown in Fig. 1. Total RNA was extracted from different CRC cell lines, after which cDNA was synthesized and amplified by PCR using GIPR-specific primers. As shown in Fig. 2A, GIPR transcripts were detected in both the control (HEK 293-T cells transfected with the GIPR) and two CRC cell lines. Cellular lysates were then extracted and assessed by Western analysis for the expression of the GIPR using rabbit anti-GIP receptor specific antibodies. As seen in Fig 2B, GIP receptor protein was present in both MC-26 cells and HT29 cells.

GIP stimulates proliferation of CRC lines expressing the GIPR

We next examined the effects of GIP on cell proliferation. MC-26 cells were incubated with various concentrations of GIP for 24-96 h, and cell proliferation was measured using MTT assays. GIP induced a concentration-dependent increase in cell proliferation at all time points examined (Fig. 2C), with maximum levels measured using 100 nM GIP, during which proliferation increased by 29.8% over basal levels ($P<0.001$).

Activation of the GIPR increases intracellular cAMP

Previous studies using various cell lines have demonstrated that GIP signals through adenylyl cyclase, thereby inducing the generation and accumulation of intracellular cAMP (13). Thus, to determine whether GIP likewise induces signaling through this pathway in CRC cells, cAMP assays were performed using a specific ELISA to measure the intracellular cAMP concentration in MC-26 cells. As shown in Fig. 3, intracellular cAMP concentration increased in response to GIP stimulation at 10 min in comparison to untreated cells. These results indicate that GIP may exert its effects in CRC cells by activating adenylyl cyclase and, in turn, increasing the intracellular generation of cAMP.

GIP signals through PI3 Kinase/Akt pathway and its downstream components in CRC cells

Akt plays a central role in mediating critical cellular responses, including cell growth and survival, angiogenesis, and transcriptional regulation (7,14). GIP promoted Akt phosphorylation in a concentration-dependent manner. After 30 min of incubation, phospho-Akt levels increased by ~3.3-fold in cells treated with 100 nM GIP, when compared to control ($P<0.01$, Fig. 4 A,B). The effects of GIP on Akt phosphorylation were attenuated by pretreatment of cells for 30 min with 1 μ M Wortmannin, and observed results were similar in HT29 cells (Data not shown).

As discussed above, past studies have demonstrated that Akt phosphorylation may activate the mTOR pathway, which is a protein kinase that promotes protein synthesis and cell growth (15,16). Because GIP promoted Akt activation in MC-26 cells, we next examined whether mTOR might also play a role in GIP-induced CRC cell proliferation. Cells were pretreated with 20 nM of rapamycin, a known mTOR inhibitor, for 30 min prior to adding 100 nM GIP for an additional 30 min. Cell lysates were then prepared, and Western analysis was performed to measure the phosphorylation of p70S6K, a principal component of the mTOR pathway. GIP promoted p70S6K phosphorylation (Fig. 4C), which was abolished in the presence of rapamycin in both MC-26 and HT29 cells (latter not shown).

We next examined whether Akt activated by GIP could, in turn, activate any of its many substrates. Western analysis was performed using a phospho-Akt substrate-specific antibody that recognizes proteins phosphorylated by Akt exclusively at a conserved serine/threonine motif, which includes an arginine residue at either the -5 or -3 position. GIP activated the substrate-specific for Akt in a concentration-dependent manner in MC-26 cells (Fig. 4D).

GIP activates MAPK pathway

Because previous studies have demonstrated that GIP induces a signaling cascade involving the MAPK pathway in pancreatic islet β -cells (17), we next performed studies to determine whether this pathway might likewise mediate the effects of GIP on CRC cells. Utilizing an antibody specific for the phosphorylated forms of MAPK extracellular signal-regulated protein kinases, ERK1 and ERK2, Western blot analysis was performed on protein extracted from MC-26 cells incubated in the presence of increasing concentrations of GIP for 30 min. A concentration-dependent increase in phosphorylated ERK1 and ERK2 was detected in MC-26 cells treated with GIP for 30 min (Fig. 5), with a maximum relative increase of ~6.3-fold detected with 100 nM GIP ($P<0.05$). The effects of GIP on the levels of ERK1/2

phosphorylation were nearly abolished by the preincubation of cells for 30 min with 40 μ M of the MEK inhibitor PD98059.

Proposed Model of GIP action on CRC

As depicted in Fig. 6, the results of our studies indicate that GIP appears to function through multiple interrelated pathways that lead to cell growth. GIP appears to bind to its G-protein coupled receptor, which then activates multiple intracellular proteins, such as the cAMP and MAPK pathways. In addition, GIP activates PI3K/Akt, a major growth stimulating pathway, as well as its downstream signals, as evident by its effects on mTOR components and proteins phosphorylated by Akt exclusively at a conserved serine/threonine motif.

DISCUSSION

Despite its original description as an inhibitor of gastric acid secretion (18), the primary biological property of GIP appears to be as a mediator of the enteroinsular axis, whereby the peptide stimulates the release of insulin from pancreatic islet β -cells following the ingestion of glucose and fat. The existence of a chemical stimulant of the endocrine pancreas had been suggested by Moore *et al.* in 1906 (19), who coined the term “incretin” as an insulinotropic substance emanating from small intestine released into the circulation following glucose-containing meals. Of the several GI peptides that have been proposed as candidates, only glucagon-like peptide-1 (GLP-1) and GIP have been shown to function as physiological incretins (20,21).

As mentioned above, several previous reports have suggested an etiologic role for GIP in the development of obesity. Postprandial GIP secretion has been shown to be increased in obese subjects compared with age-matched healthy controls (22). Animal models of obesity, represented by leptin-deficient (ob/ob) mice, have also demonstrated that the concentrations of plasma GIP are enhanced when chronically fed a high-fat diet. In addition, diets high in fat content induced K-cell hyperplasia in these mice (23). Miyawaki *et al.* (12) demonstrated that while normal mice became obese and developed insulin resistance and T2DM in response to a high-fat diet, GIPR-deficient (GIPR^{-/-}) mice were protected and remained normal while consuming the identical diet. More recently, Althage *et al.* (24) used regulatory elements for the rat GIP promoter to express an attenuated Diphtheria toxin in transgenic mice. K-cell number, GIP transcripts, and plasma GIP levels were all profoundly diminished, and body weight was reduced by 25% in the transgenic mice.

Although a relationship between obesity and cancer had long been suspected (25), Calle *et al.* (6) conducted a prospective analysis of 900,000 American adults, who were free of cancer at the outset of the study. During the ensuing 16 years, 6.3% (57,145) died from cancer-related illness. From their extensive analysis, they estimated that cancer-related deaths in 14% and 20% of men and women, respectively, were associated with excess weight. The relative risk of mortality due to CRC increased directly with the body-mass index (BMI): 1.20 for overweight male individuals (BMI of 25.0-29.9), 1.47 for men with functional class I obesity (BMI of 30.0-34.9), and 1.84 for men with a BMI of 35.0-39.9. The risk of CRC-related death was somewhat diminished in women compared with men. The relative risk of mortality due to CRC was 1.36 and 1.46 for women with a BMI of 35.0-39.9 and >40, respectively.

The cause of obesity-related CRC is likely multifactorial, and previous studies have suggested that insulin-like peptides likely represent one of the principal biochemical mediators (26-29). The precise mechanisms by which insulin and insulin-like growth factors might contribute to the development of CRC have not been elucidated. Upon binding to the receptor, insulin activates its receptor's intrinsic kinase, leading to autophosphorylation and

tyrosine phosphorylation of several substrates, including members of the insulin receptor substrate (IRS) family (30). IRS phosphorylation, in turn, recruits other signaling molecules, including PI3K (30). One of the downstream targets of PI3K is protein kinase B (Akt), and its activation causes a cascade of cellular responses (31). Akt appears to contribute to the development of carcinogenesis via several downstream pathways, including attenuation of apoptosis and stimulation of cell proliferation (32).

We have previously reported that, like insulin, GIP induces the activation of the Akt in adipocytes via a wortmannin-sensitive pathway, which, in turn, promoted fat cell membrane GLUT-4 accumulation and enhanced [³H]-2-deoxyglucose uptake (7). The results of the present studies provide further evidence for the insulin mimetic properties of GIP. After demonstrating the presence of the GIPR in human and mouse CRC cell lines, we found that GIP stimulated the proliferation of the cells in a concentration-dependent manner. We next showed that GIP promoted Akt phosphorylation in a concentration-dependent manner (Figs. 4A and 4B) and activated the substrate-specific for Akt in a concentration-dependent manner (Fig. 4D).

In addition to its effects on Akt activation, GIP treatment of CRC cells also promoted the phosphorylation of p70S6K, a principal component of the mTOR pathway, which was abolished in the presence of rapamycin (Fig. 4C). Emerging evidence suggests that mTOR plays a key role in several pathways that are involved in human cancer (15), as well as obesity and its sequelae (33,34). Two mTOR complexes, mTORC1 and mTORC2, have been described, each possessing distinct features, including different combinations of interacting components and variable sensitivity to rapamycin; mTORC1, but not mTORC2, is rapamycin sensitive (35). One of the principal downstream targets of mTORC1 is p70S6K, and the p70S6K through phosphorylation represent important events involved in the regulation of protein synthesis.

The notion that GIP might possess proliferative properties has been reported in studies employing β -cells and surrogate insulin-secreting cell lines. GIP has been shown to activate mitogenic signaling, such as the mitogen-activated protein kinase (MAPK) and PI3K/Akt pathways, in β -cells (15-17). Akt is a major mediator of GIP action on pancreatic islets, increasing β -cell mass and function and promoting β -cell survival (36). Trömpner et al. (37) reported that GIP and glucose acted synergistically as anti-apoptotic factors in the well-differentiated β -cell line INS-1 and involved the activation of several pathways, including adenylyl cyclase, MAPK, and PI3K/Akt. Similarly, Kim et al. (13) reported that GIP stimulated the anti-apoptotic *Bcl-2* gene through the adenylyl cyclase pathway in INS-1 cells. Using this same cell line, they later showed that GIP regulated β -cell apoptosis by down-regulating the pro-apoptotic *bax* gene and up-regulating *Bcl-2* via the activation of PI3K (38). Down-regulation of the *bax* gene via PI3K appeared to be mediated by GIP-induced phosphorylation of the Forkhead/winged helix family member Foxo1 (38).

As stated above, Akt activation has been shown enhance cell growth both through the attenuation of apoptosis and the stimulation of cell proliferation (32). Although the effects of GIP apoptosis in CRC were not examined in the present study, we have demonstrated that the proliferation of CRC cells appears to involve several known mitogenic pathways, including the downstream target p70S6K, as well as adenylyl cyclase and MAPK. Moreover, because GIP is overexpressed in obesity, these studies provide another potential link between obesity and CRC and possibly other malignancies. Additional studies will be necessary to determine the effects of GIP on apoptosis, as well as to elucidate more completely the cellular mechanisms involved in mediating the mitogenic properties of GIP in CRC. Finally, *in vivo* experiments and epidemiological studies in obese humans will be

required to provide further evidence linking obesity and enhanced GIP expression with CRC.

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Figure 1. Immunohistochemical demonstration of GIP receptors in human CRC

7- μ m sections from a stage 4 human CRC paraffin-embedded specimen were deparaffinized and rehydrated and were processed as described in the Materials and Methods. Panels A and B show nuclear staining (blue) using DAPI and GIPR staining (red) with Alexa Fluor 594 goat anti-rabbit secondary antibodies, respectively. Panel C depicts a merged image of both nuclear and cytoplasmic staining, while panel D demonstrates the corresponding bright field image.

Figure 2. Expression of GIP receptors in mouse and human CRC and effects of GIP on cell proliferation

(A) RT-PCR analysis of MC-26 and HT29 cells was performed utilizing primers specific for GIPR to determine the presence of transcripts. GAPDH served as a loading control. (B) CRC lines were cultured in DMEM with 10% FBS to 80% confluency. Total cell lysates were extracted and analyzed by Western blot analysis using GIPR-specific antibodies. β -actin was employed as a control for loading. (C) MC-26 cells (3×10^3 cells/well) were cultured for 24 h in DMEM containing 10% FBS, after which they were incubated in serum-free media. After 24 h, cells were incubated for an additional 24-96 h in media containing increasing concentrations of GIP. Absorbance at 490 nm normalized to untreated cells was measured, and data are expressed as the percentage of cell number $\pm$ SE compared to control. Assays were run in triplicate, and data are expressed as the mean of 3 experiments $\pm$ SE. * $P < 0.05$; ** $P < 0.01$, both compared to control.

Figure 3. Intracellular cAMP accumulation in MC-26 cells in response to GIP

MC-26 cells were seeded to 80% confluency in 48-well plates for 24 h. On the day of the experiment, cells were pre-incubated with 1 mM IBMX for 1 h, followed by increasing concentrations of GIP for an additional 10 min and incubation at 37°C. Cells were lysed, and 50 μ L of cell lysate were used for the cAMP analysis. Assays were run in triplicate, and data are expressed as the mean of 3 experiments $\pm$ SE. * $P < 0.05$ compared to control.

Figure 4. Enhanced signaling of Akt and its downstream targets induced by GIP in MC-26 cells (A) MC-26 cells were incubated with GIP for 30 min, after which total protein lysates were extracted and examined by Western analysis for the expression of phospho-Akt and total Akt. The blot is representative of 3 separate experiments. (B) Quantification of total Akt and Akt phosphorylation was performed using an NIH image analysis program and depicts the ratios of phosphorylated Akt to total Akt. (C) MC-26 cells were pretreated for 30 min with 20 nM rapamycin, followed by an additional 30-min incubation with DMEM containing 100 nM GIP. Total protein lysates were extracted and examined by Western analysis for the expression of phospho-phospho-70S6 kinase using antibodies directed at threonine (Thr) 389 and total β -actin (control for loading). (D) Western analysis of MC-26 cells incubated with GIP for 30 min, using a phospho-specific antiserum that recognizes proteins phosphorylated by Akt only at a conserved serine/Thr motif, which includes an arginine residue at either the -5 or -3 position. The levels of β -actin expression are shown as a loading control. The signals of Akt substrate and β -actin were quantified using an NIH image analysis program, and the blot is representative of 3 separate experiments. Data (4B) are expressed as the mean of 3 experiments done in triplicate $\pm$ SE. * $P < 0.05$; ** $P < 0.01$, both compared to control.

Figure 5. Increased phosphorylation of MAPK species ERK1 and ERK2 in MC-26 cells treated with GIP

(A) MC-26 cells were incubated with GIP for 30 min, after which total protein lysates were extracted and examined by Western analysis for the expression of phosphorylated p44 and p42 MAP kinase species (pERK1/2). Membranes were also probed for total ERK1/2. (B) The signals of total ERK1/2 and pERK1/2 were quantified using an NIH image analysis program and depicts the ratios of pERK1/2 and total ERK1/2. Assays were run in triplicate, and data are expressed as the mean of 3 experiments $\pm$ SE. * $P < 0.05$ compared to control.

Figure 6. Proposed model for GIP signaling in CRC

GIP, which is overexpressed in obesity, appears to bind to its G-protein coupled receptor, which signals through multiple intracellular proteins, including the cAMP and MAPK pathways. In addition, GIP activates PI3K/Akt, a major growth stimulating pathway, as well as its downstream signals, as evident by its effects on mTOR components and proteins phosphorylated by Akt exclusively at a conserved serine/threonine motif. The proposed model indicates that GIP activates several intracellular pathways to produce its proliferative effects in CRC.