Menin and GIP are inversely regulated by food intake and diet via PI3/AKT signaling in the proximal duodenum

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BACKGROUND AND AIMS: Ingestion of food stimulates the secretion of incretin peptides glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 to ensure the proper absorption and storage of nutrients. Menin is the 67 kDa protein product of the MEN1 gene recently reported to have a role in metabolism. In this study, we will determine the regulation of menin in the proximal duodenum by food intake and diet in correlation with GIP levels in the proximal duodenum of mice after an 18 h fast followed by 4 and 7 h refeeding and 3 months of high-fat diet.

METHODS: A dual luciferase assay was used to determine GIP promoter activity and ELISA was used to measure the levels of GIP after inhibition of menin through small interfering RNA (siRNA) and exposure to MAPK and AKT inhibitors. Colocalization of menin and GIP were determined by immunofluorescence.

RESULTS: Menin and GIP expression are regulated by fasting, refeeding and diet in the proximal duodenum. Overexpression of menin in STC-1 cells significantly inhibited GIP mRNA and promoter activity, whereas menin siRNA upregulated GIP levels. Inhibition of GIP expression by the PI3/AKT inhibitor, LY294002, was abrogated in STC-1 cells with reduced menin levels, whereas the MAPK inhibitor, U0126, inhibited the expression of GIP independent of menin. Exposure of STC-1 cells to GIP reduced menin expression in a dose-dependent manner via PI3K-AKT signaling.

CONCLUSION: Feeding and diet regulates the expression of menin, which inversely correlates with GIP levels in the proximal duodenum. In vitro assays indicate that menin is a negative regulator of GIP via inhibition of PI3K-AKT signaling. We show menin colocalizing with GIP in K cells of the proximal gut and hypothesize that downregulation of menin may serve as a mechanism by which GIP is regulated in response to food intake and diet.

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INTRODUCTION

Feeding and diet initiate a complex network of hormonal and neural signals for the absorption and storage of nutrients from the gastrointestinal tract. Factors produced by K and L cells located within the intestinal mucosa in response to nutrient ingestion are capable of stimulating the release of hormones from the pancreas for regulation of blood glucose. K cells are located predominately in the proximal duodenum of the intestinal tract, whereas L cells are present in the lower jejunum and terminal ileum.1–3 Incretin peptides are glucose-lowering intestinal derived factors that consist of glucagon-like peptide 1 (GLP-1) secreted by L cells and glucose-dependent insulinotropic polypeptide (GIP) produced by K cells. Both incretin peptides share common actions on the islet beta cells of the pancreas. GIP is a 42-amino acid incretin hormone secreted into the bloodstream in response to the ingestion of carbohydrates, proteins and fat.1,4,5 GIP binds to its receptor (GIP receptor, GIPR) in the pancreas and potentiates glucose-stimulated insulin secretion.

The enteroinsular axis is an essential component of carbohy-
drate metabolism and consists of the pancreas, GLP-1 and GIP. It has been reported that hypersecretion of endogenous GIP is responsible for the hyperfunction of enteroinsular signaling in patients with duodenal ulcers and total gastrectomy,6 supporting GIP’s role as a principal mediator of the enteroinsular axis.7,8 Both GIP and GLP-1 are responsible for the incretin effect in healthy subjects,9 with GIP considered to have a predominant role.10 The GIP repertoire of functions include potentiating glucose-dependent insulin release upon activation of the GIPR.8,11 This leads to nutrient deposition via glucose uptake, fatty acid synthesis and fatty acid incorporation in adipocytes.12 Genetic ablation of GIPR demonstrates a critical role of this receptor in the enteroinsular axis13,14 and genetic variation of GIPR in humans is associated with a reduction in early phase insulin reaction and elevation of blood glucose.15,16 This suggests that defective GIPR signaling might have a critical role in the early pathophysiology of impaired glucose tolerance and type 2 diabetes.

Previous reports have shown that the tumor suppressor, menin, is expressed highest in the proximal duodenum of the gastro-intestinal tract and during fasting17 compared with feeding. Menin is a tumor suppressor protein implicated in the development of tumors that overexpress the AKT and MAPK signaling pathways.8,18 Menin inhibits AKT kinase activation and uncouples Elk-1 from MAP kinase activation sites.20,21 Menin is expressed in the pancreas and when down-regulated is associated

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with increased beta cell proliferation. MEN1 syndrome is associated with the development of gastrinomas in the duodenum and tumors in endocrine organs such as the pancreas and pituitary, but not the liver. The reason for organ-specific preferences still remains unknown.

The serine/threonine protein kinase PI3/AKT has an important role in cell growth, survival, transcripion, differentiation and modulation of insulin signaling for glucose metabolism. Since menin directly inhibits AKT activation and expression, we hypothesized that inhibition of GIP expression by menin may be a consequence of inactivation of the PI3K-AKT kinase signal transduction cascade.

The development of type 2 diabetes and obesity has been linked to an ‘anti-incretin’ theory with the authors postulating the existence of a negative regulatory mechanism to oppose the actions of incretins, such as GIP. An imbalance in the equilibrium between ‘anti-incretin’ factors and incretins could lead to delayed insulin response and impaired insulin action.

In this study, we investigated the presence of menin in K cells of the proximal duodenum and determined if it regulated the expression of GIP via PI3/AKT signaling in response to fasting and refeeding, and high-fat (HF) diet.

**MATERIALS AND METHODS**

Animal maintenance

C57BL/6 wild-type male mice, 5 fasted for 18 h, 4 fasted for 18 h and refed at 4 h, and 5 fasted for 18 h and refed at 7 h, were used for the proposed studies. An additional 3 animals per group, fed regular diet (RD) or HF diet, were utilized for the diet studies. All animals were kept in a 12 h dark/light cycle and control animals were fed RD (ad libitum). All procedures were approved by the University of Toledo Animal Care and Utilization Committee. For the feeding experiments, 3-month-old wild-type mice were fed a RD and a high-fat (HF) diet for 3 months (Research Diets Inc., New Brunswick, NJ, USA; Cat #D12451 and D12079B respectively). The RD contains 69% carbohydrates and 11% fat and the HF diet contains 35% carbohydrates and 45% fat.

**Cell culture**

The enteroendocrine intestinal cell line, STC-1, was obtained from ATCC with permission from Douglas Hanahan (UCSF-Ca) and maintained at 37 °C and 5% CO₂ in DMEM medium containing 1% penicillin and streptomycin. All experiments were performed on cells passaged five times at 80% confluence. LY294002, an inhibitor of the PI3K-AKT kinase signaling pathway, and UO126, a MAP kinase inhibitor, were both obtained from Cell Signaling Technology (Beverly, MA, USA), and were used at concentrations of 10 μM from a 10 mM stock reconstituted in DMSO. Aliquots were stored at −20 °C and vehicle was used for control experiment.

**Transfections**

The 2.9 and 0.210 kb fragments of the human GIP promoter were cloned into the luciferase-containing pGL4 reporter plasmid, as previously described. STC-1 cells were plated in 6-well plates (2 × 10⁵ cells/well) and allowed to adhere for 24 h. Thereafter, cells were transfected with a mixture of 4 μg of plasmid DNA (pBABE) containing menin (Addgene Inc., Cambridge, MA, USA), 0.5 μg of the control plasmid (pGL4) or GIPLuc reporter plasmid using Lipofectamine 2000 reagent according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). After 48 h of incubation with complete media, luciferase and renilla activities were assayed according to manufacturer’s instruction (Promega-Madison, WI, USA). Firefly luciferase activity was normalized to renilla luciferase expression and is presented as percent activity over samples transfected with pGL4. All experiments were analyzed in duplicate in at least three separate experiments. Menin small interfering RNA (siRNA) oligos and the non-targeting control siRNA and reagents were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and the assay was performed according to the manufacturer’s protocol.

**Western blot**

STC-1 cells were plated in 6-well plates in triplicate, serum starved overnight and exposed to 0 or 20 nM GIP for 24 h. The concentrations of proteins from total cell lysates were quantified prior to analysis on 10 or 4–12% gradient SDS-PAGE and immunoprobed with specific antibodies. Rabbit anti-menin (Bethyl Labs/Abcam, Cambridge, MA, USA), phospho-p38 (Cell Signaling, Beverly, MA, USA), goat anti-GIP (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (Sigma-Aldrich, St, Louis, MO, USA) antibodies were used. Proteins were detected using an Odyssey Infrared Imaging System (Lincoln, NE, USA), using corresponding secondary antibodies conjugated to fluorescent dyes of different wavelengths.

**GIP ELISA assay**

STC-1 cells were plated in 6-well plates in triplicate, serum starved overnight and transfected with siRNA oligos for menin or scrambled controls according to manufacturer’s protocol (Santa Cruz Biotechnology). The transfected cells were exposed to LY294002, an inhibitor of PI3K-AKT kinase signaling, or the MAP kinase inhibitor, UO126, overnight prior to harvest and determination of protein concentration. 20 μg of protein in a volume of 10 μl total lysate and 10 μl media were used for a rat/mouse GIP ELISA (Cat. #ZRMGIP-55K, Millipore, Billerica, MA, USA) according to manufacturer’s protocol.

**Real-time PCR**

RNA was extracted using TRIzol (Life Technologies, Grand Island, NY, USA). Following DNAase digestion (DNAfree, Applied biosystems/Ambion, Austin, TX, USA), 1 μg mRNA was transcribed into cDNA in a 20 μl reaction using High-Capacity cDNA achieve kit (Applied Biosystems/Ambion) and amplified (ABI 7900 HT system). PCR was performed in a 15 μl reaction, containing 5 μl cDNA (1/10 diluted), 1 × SYBR Green PCR Master Mix (Applied Biosystems/Ambion) and 300 nM of each primer. Primers are listed in 5’–3’ orientation.

Menin forward primer: TCATTGCTGCCCCCTATGCC
Menin reverse primer: TCCAGTTTGGGCTGCTGTAGT
GIP forward primer: GTGGCCTTTGAAAAGCCTGCT
GIP reverse primer: AAGTGCTCCTGCTGACTT
GAPDH forward primer: CCACCAAGGCCCCAGAAAGAC
GAPDH reverse primer: GCAGGGACCTCCCAACAGT

Ct values (cycle threshold) were used to calculate the amount of amplified PCR product relative to GAPDH and 18S. The relative amount of mRNA was calculated as 2^(-ΔΔCt). Results are expressed as transcript of interest mean normalized to GAPDH and 18S with s.e.m.

**Immunofluorescence**

One-cm tissue starting from the pyloric sphincter including the Brunner’s gland and into the proximal duodenum was taken from the upper section of the gut from 5 C57BL/6 wild-type male mice fasted for 18 h and 5 C57BL/6 wild-type male mice fed ad libitum. Additional 2 sets of mice for each time point were also used for all described studies and consisted of a group of mice fasted for 18 h, refed and sacrificed after 4 h of feeding, and a second set of mice fasted for 18 h, refed and sacrificed after 7 h.

Tissues were harvested and fixed in 4% paraformaldehyde/phosphate-buffered saline for 18–20 h at room temperature followed by embedding in paraffin. Tissue blocks were obtained and 5 μm thick sections were cut and mounted on poly-lysine coated glass slides, blocked with 20% normal donkey serum/phosphate-buffered saline and 0.1% Triton X-100 for 30 min after citrate antigen retrieval. The slides were incubated for 1 h with a 1:50 dilution of primary antibodies (Bethyl labs, Montgomery, TX, USA) and a 1:200 dilution of fluorescein isothiocyanate-conjugated anti-rabbit or goat (Jackson Laboratories, Bar Harbor, ME, USA) used as secondary antibodies for 1 h, and DAPI for blue staining of nuclei. Negative controls were performed on similar slides using secondary antibodies alone without incubation of primary antibodies.

All colocalization studies were performed on the same sections with specific antibodies raised in different species. Incubations were performed with anti-rabbit menin overnight followed by 1 h incubation with fluorescein isothiocyanate-conjugated donkey anti-rabbit-green and anti-goat GIP overnight followed by streptavidin-Texas Red-conjugated donkey anti-goat for 1 h. Control staining included (a) replacement of the first layer of antibody by non-immune serum and by the diluent alone, and (b) secondary antibodies tested in relation to the specificity of the species.

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in which the primary antibodies were raised, with the secondary antibody in question being replaced by secondary antibodies from different animal species.

Sections were examined with an Olympus IX70 inverted fluorescence microscope (Olympus; Tokyo, Japan) equipped with filters (Olympus) giving excitation at wavelengths of 475–555 nm for Texas Red and 453–488 nm for fluorescein isothiocyanate, with a digital camera. Merged images were viewed by superimposing both photographs at 10× and 40× magnification.

Statistical analysis

Data were analyzed with SPSS software (Armonk, NY, USA) using one-factor analysis of variance analysis or Student's t-test as appropriate. P < 0.05 were considered statistically significant.

RESULTS

Menin colocalizes with GIP in the villi of proximal gut

Previous studies have shown that menin is expressed in the duodenum and that K cells express and secrete GIP. To examine whether menin in the gut is expressed in GIP-expressing K cells, we performed double stain fluorescent co-immunohistochemistry on the proximal duodenum, 1 cm from the pylorus. We determined that menin and GIP colocalize in cells on the villi as shown in Figure 1.

Menin and GIP are inversely regulated by fasting and refeeding in the proximal duodenum

We have previously shown that 4 and 7 h of refeeding are the times that induce the highest spikes of serum insulin with the lowest menin levels observed at 7 h of refeeding (Figures 2a and b). However, GIP expression was lowest at fasting and highest at 7 h of refeeding (Figure 2c). Although our data confirms a previous report that menin expression is highest in the gastrointestinal tract during fasting and at feeding, serum GIP is increased, the effect of refeeding on menin levels and the inverse correlation with GIP expression is entirely novel.

Feeding inversely modulates menin and GIP expression at the mRNA level

We investigated if the effect of feeding on menin and GIP expression is at the level of mRNA by analyzing the proximal duodenum after an 18 h fast followed by 4 and 7 h of refeeding by reverse transcripition-PCR. In Figure 3a, we show that menin mRNA levels steadily decline the with length of refeeding after the 18 h fast, consistent with the levels of protein shown in Figures 2a and b. This steady decline in menin mRNA was associated with an increase in GIP mRNA expression peaking at 7 h of refeeding (Figure 3b), and consistent with the ELISA data in Figure 2c.

High-fat diet downregulates menin expression in the duodenum and is associated with increase in GIP expression

To determine the effect of diet on duodenal menin expression in relation to GIP, we subjected C57BL/6 mice to 3 months of HF diet and analyzed menin and GIP expression in the proximal duodenum by western blot analysis and reverse transcription-PCR. In Figure 4a, we show that HF diet downregulates menin protein levels in the proximal gut of these mice. Furthermore, we observed a significant decrease in menin mRNA expression, (Figure 4b) consistent with reduction in protein levels as shown in Figure 4a. The reduction of menin expression inversely correlated with the upregulation of GIP caused by the HF diet (Figure 4c) relative to expression from mice on RD and consistent with reports that GIP is regulated by dietary fat and glucose.

Menin overexpression decreases endogenous GIP mRNA

To study the mechanism underlying the inverse correlation observed between the expression of menin and GIP, we tested the hypothesis that menin is a negative regulator of GIP in vitro.
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Menin regulates GIP promoter activity and expression via AKT signaling

Previously, when a 2.9 kB human GIP promoter luciferase reporter gene construct was transfected into STC-1 cells, there was a 40-fold elevated GIP promoter activity over the response in IEC cells (a cell line derived from the ileum).28 However, the greatest promoter activity was detected with the highly conserved 0.210 kB promoter construct, suggesting that more distal regions may contain repressor elements.28 We therefore compared the effect of menin on both the 0.210 and 2.9 kB GIP promoters to determine if menin is part of a repressor component that regulates GIP expression in STC-1 cells. Using whole cell lysates derived from STC-1 cells overexpressing menin (Figure 5a), we determined the effect of menin on the GIP promoter by luciferase reporter assay. In Figure 6a, we show that the activity of the short 0.210 kB promoter for GIP was not affected by menin overexpression; however, overexpression of menin significantly inhibited the 2.9 kB GIP promoter (Figure 6b), supporting our hypothesis that menin may be part of a repressor component that negatively regulates GIP. These results suggest that menin may negatively regulate the transcription of GIP in cells and explain the in vivo inverse correlation observed with previous results shown.

In line with our hypothesis and consistent with menin’s possible role as a GIP repressor, we found that menin siRNA (si\text{menin}) significantly increased GIP expression as determined by ELISA performed on STC-1 whole cell lysates (Figure 6c). The PI3K-AKT inhibitor LY294002 (LY) caused downregulation of GIP expression; however, the effect was inhibited with menin siRNA (LYsi\text{menin}) (Figure 6c). Furthermore, the MAP kinase inhibitor UO126 (UO) inhibited GIP expression independent of menin, since menin siRNA did not reverse the effect of UO as observed with LY294002. Expression of secreted GIP in the media followed a similar trend as observed in the lysates (Figure 6d). In Figure 6e, we demonstrate that the addition of menin-specific siRNA to STC-1 cells indeed leads to a significant decrease in menin expression and that the changes observed with si\text{menin} and LYsi\text{menin} can be attributed to the significant downregulation of menin expression in the transiently transfected STC-1 cells. With this data, we conclude that the PI3K-AKT-mediated regulation of GIP is dependent on menin whereas MAP kinase-mediated GIP expression is via a menin-independent pathway.

GIP regulates menin expression via PI3K-AKT signaling

GIP is known to suppress p38 MAPK activation via AKT to induce pro-survival responses and inhibit apoptosis in pancreatic beta cells.27 Menin induces apoptosis and when down-regulated has also been associated with proliferation of beta cells.22 In contrast,
GIP directly stimulates proliferation by binding to its receptor on target beta cells. Since it has been reported that GIP exerts its effects on cell survival via an AKT-dependent pathway, we postulated that GIP inhibits menin expression via the PI3K-AKT signaling cascade. In Figure 7a, we show that STC-1 cells exposed to GIP resulted in significant upregulation of pAKT associated with the downregulation of menin protein. This activation was abrogated with LY294002. Since GIP is known to inhibit MAPK signaling via downregulation of phospho-p38, we demonstrated that 20 nM GIP not only downregulates menin expression but also reduces phospho-p38 levels in the STC-1 cells (Figure 7b). Furthermore, we confirm that the ability of PI3K/AKT inhibitor LY294002 (LYg20) to abrogate GIP (g) downregulation of menin protein expression is via inhibition of pAKT (Figure 7a), as phospho-p38 levels did not change with LY treatment as shown in Figure 7b. Indeed the AKT-mediated GIP suppression of p38 is confirmed with the activation of pAKT (Figure 7a) and the significant reduction in phospho-p38 (Figure 7b). This was followed by LY294002’s ability to abrogate the inactivation of p38 by GIP, as demonstrated by the high expression of phospho-p38 in the LYg20 lane compared to g20 in Figure 7b.

DISCUSSION

The incretin effect mediated by GIP and GLP-1 account for 50–70% of total postprandial insulin release in healthy subjects. In addition to its physiological role in regulating endocrine pancreatic secretion, GIP also acts on the adipose tissue by regulating lipid metabolism. Notably the incretin effect in type 2 diabetes is markedly reduced with greater reduction in GIP’s insulinotropic effect on the pancreatic beta cells compared with GLP-1. The homeostasis between anti-incretin factor(s) and incretins has been postulated to be disrupted most likely in the proximal foregut of diabetics. GIP is synthesized and released from K cells of the duodenum and is known to act as a principal mediator of the enteroinsular axis. GIP is also reported to possess insulin-mimetic properties and induce the activation of the AKT pathway resulting in the uptake of glucose by adipocytes. Inhibition of GIP signaling may also prevent obesity and metabolic syndrome.

Although menin has recently been shown to inhibit AKT activation among its already known functions of interacting with transcriptional regulators such as NF-kB, JunD and Smad 3, little is known about its role in regulating GIP expression. Menin is a negative regulator of GIP expression in the duodenum as shown by the inverse relationship in Figure 4a. Menin expression is downregulated after 3 months on a HF diet, opposite of GIP, which is significantly increased after 3 months on a HF diet. Reverse transcription-PCR was used to determine the relative expression of menin and GIP after normalizing with 18S. N = 6 from each feeding group of mice. Values are mean ± s.e. Figure 4b shows menin expression in RD; HF diet (HF) **P < 0.004; (c) is GIP expression in RD and HF diet (HF) * < 0.0005.

Figure 4. Inverse relationship of menin and GIP in proximal duodenum of mice fed a HF diet. Menin expression is downregulated after 3 months on a HF diet opposite of GIP, which is significantly increased after 3 months on a HF diet. (a) is a representative western blot analysis showing downregulation of menin protein expression after 3 months on HF diet. Reverse transcription-PCR was used to determine the relative expression of menin and GIP after normalizing with 18S. N = 6 from each feeding group of mice. Values are mean ± s.e. (b) shows menin expression in RD; HF diet (HF) **P < 0.004; (c) is GIP expression in RD and HF diet (HF) * < 0.0005.

Figure 5. Menin overexpression decreases endogenous GIP mRNA. Overexpression of menin in STC cells shows a resultant decrease in GIP levels. (a) Western blot analysis showing level of menin overexpression using a menin expression vector (meninO/E). (b) Reverse transcription-PCR data showing downregulation of GIP mRNA following menin overexpression (meninO/E).
known about factors that directly regulate this protein. Refeeding after fasting is known to increase serum GIP levels and leads to an increase in serum insulin that downregulates the expression of menin. We previously reported that menin is expressed in the duodenum and Ratineau et al. described higher menin expression in the duodenum of fasted animals compared with that in ad libitum-fed mice. The authors determined that MEN1 transcripts are expressed in the crypts of the intestine and dispersed on cells of the villi, but did not identify the specific cell-type that expressed menin. Since menin is a

Figure 6. Menin regulates GIP promoter activity and expression and abrogates PI3K-AKT regulation in STC-1 cells. Overexpression of menin in the 0.210 kb GIP did not change GIP activity levels, however overexpression in the 2.9 kb promoter significantly inhibited relative GIP activity, supporting our hypothesis that menin may be part of a repressor component that negatively regulates GIP. In (c) and (d), using AKT and MAPK inhibitors, we concluded that menin regulates the expression of GIP through the AKT pathway. (a) represents the % activity of the 0.210 kb construct and (b) is a representation of % activity of the 2.9 kb construct, ***$P = 0.0001$. (c) represents expression of GIP in whole cell lysates. GIP expression in the media of cells described in (c) was also determined by ELISA and is shown in (d). All ELISA results were calculated as means of two separate experiments. *$P < 0.005$; ** and ##$P < 0.0024$. (e) Western blot analysis showing level of menin downregulation using menin specific siRNA oligos (simenin). Protein lysates were prepared and GIP expression in whole cell lysates determined by ELISA with LYsimenin and UOsimenin representing treatment of LY294002 and UO126 on cells transfected with menin siRNA.

Figure 7. GIP is a negative regulator of menin protein expression. STC cells treated with (a) and (b) 5 or 20 nM GIP (g5 or g20), resulted in decreased menin levels. STC cells treated with 10 μM LY294002 (LY) alone or LY294002 with 5 and 20 nM GIP, respectively (LYg5; LYg20 ) resulted in no change in the menin levels. STC cells treated with the MAPK inhibitor, UO126 (UO) alone or with GIP at 5 nM (UOg5), 20 nM (UOg20), respectively did not change menin expression (c). These results indicate GIP negatively regulates menin through pAKT pathway. A, one representative experiment out of three is shown; B and C, one representative experiment out of two is shown.
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**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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