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GATA-4 upregulates glucose-dependent insulinotropic polypeptide expression in cells of pancreatic and intestinal lineage

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Abstract

A thorough examination of glucose-dependent insulinotropic polypeptide (GIP) expression has been hampered by difficulty in isolating widely dispersed, GIP-producing enteroendocrine K-cells. To elucidate the molecular mechanisms governing the regulation of GIP expression, 14 intestinal and pancreatic cell lines were assessed for their suitability for studies examining GIP expression. Both STC-1 cells and the pancreatic cell line β TC-3 were found to express GIP mRNA and secrete biologically active GIP. However, levels of GIP mRNA and bioactive peptide and the activity of transfected GIP reporter constructs were significantly lower in β TC-3 than STC-1 cells. When β TC-3 cells were analyzed for transcription factors known to be important for GIP expression, PDX-1 and ISL-1, but not GATA-4, were detected. Double staining for GIP-1 and GATA-4 in mouse duodenum demonstrated GATA-4 expression in intestinal K-cells. Exogenous expression of GATA-4 in β TC-3 cells led to marked increases in both GIP transcription and secretion. Lastly suppression of GATA-4 via RNA interference, in GTC-1 cells, a subpopulation of STC-1 cells with high endogenous GIP expression resulted in a marked attenuation of GIP promoter activity. Our data support the hypothesis that GATA-4 may function to augment or enhance GIP expression rather than act as an initiator of GIP transcription.

Keywords

Glucose-dependent insulinotropic polypeptide; GIP; Insulin; Gene expression; GATA-4; PDX-1; ISL-1

1. Introduction

Glucose-dependent insulinotropic polypeptide (GIP), like glucagon-like-peptide-1 (GLP-1), is an incretin, a mediator of the enteroinsular axis that helps to maintain glucose homeostasis under physiological conditions (Murphy et al., 1995). In the presence of elevated blood glucose, incretins stimulate insulin secretion from pancreatic β -cells (O'Harte et al., 1998; Usdin et al., 1993; Yip et al., 1998). Although GLP-1 appears to be the more potent pharmacological stimulator of insulin release (Gutniak et al., 1992; Nauck et al., 1997), using GIP and GLP-1 specific receptor antagonists, it has been demonstrated that GIP is the major physiological incretin, accounting for ~80% of nutrient induced enteroinsular pancreatic β -cell stimulation (Gault et al., 2003; Tseng et al., 1996). GIP is a 42-amino acid peptide that is produced in

specific enteroendocrine cells, termed K-cells, dispersed primarily within the small intestinal mucosa (Buchan et al., 1978; Cheung et al., 2000; Yeung et al., 1999) and is released into circulation in response to the ingestion of nutrients (Cataland et al., 1974; Pederson et al., 1975).

We have begun to elucidate the mechanisms by which GIP expression is regulated. Characterization of the rat GIP gene has demonstrated that transcription initiates from a promoter located in the 5'-flanking region of the gene (Boylan et al., 1997). Furthermore, deletional analysis of the GIP promoter revealed that the first 193 bp upstream of the transcription initiation site are sufficient to direct specific expression of the GIP gene in the neuroendocrine cell line STC-1 (Boylan et al., 1997). A subsequent mutation analysis of this region demonstrated that GIP expression requires the binding of three transcription factors (Boylan et al., 1997; Jepeal et al., 2003, 2005).

The region located between base pairs -193 and -182 with respect to the transcription initiation site (+1) of the GIP promoter contains a consensus binding motif for the GATA family of DNA binding proteins, WGATAR (Jepeal et al., 2003; Orkin, 1992). Previous studies in our laboratory have shown that binding of GATA-4 to this site is essential for high GIP promoter activity in STC-1 cells (Jepeal et al., 2003). A second *cis*-regulatory region located between base pairs -156 and -151 of the GIP promoter also has been shown to be essential for transcription of the GIP gene in STC-1 cells (Jepeal et al., 2003). The transcription factors ISL-1 and PDX-1 were found to bind to this region and appear to function in conjunction with GATA-4 to regulate GIP gene expression (Jepeal et al., 2003, 2005).

Unfortunately, the molecular analysis of GIP expression and K-cell function has been hampered by the inability to adequately purify populations of GIP-producing K-cells, and consequently has required the identification and use of surrogate cell lines as models for such *in vitro* studies. Most of what is known about the regulation of GIP expression has been determined using a single cell line, STC-1 (Boylan et al., 1997; Jepeal et al., 2003, 2005; Kieffer et al., 1995). STC-1 cells were derived from an intestinal tumor isolated from a transgenic mouse expressing a viral oncogene under control of the insulin promoter (Rindi et al., 1990). These cells express multiple peptides, including GIP, glucagon, somatostatin, CCK, chromogranin, and amylin, but not insulin (Boylan et al., 1997; Jepeal et al., 2003; Kieffer et al., 1995; Rindi et al., 1990). This plurihormonal pattern of gene expression is consistent with previous studies demonstrating the expression of multiple hormones in cells of endocrine neoplasms (Brubaker et al., 1998; Philippe et al., 1987, 1988; Sidhu et al., 2000).

In addition to intestinal tumors, the transgenic mouse line that gave rise to STC-1 cells also developed pancreatic neoplasms. The cell line β TC-3 was derived from one such pancreatic β -cell tumor. Because β TC-3 cells were found to possess many of the characteristics of native differentiated β -cells, including the synthesis and secretion of considerable amounts of insulin, they have been used extensively to study insulin regulation and β -cell function (D'Ambra et al., 1990; Matsuoka et al., 2003). However, β TC-3 cells do not behave entirely like native islet β -cells by virtue of their capacity to synthesize proglucagon-derived peptides in addition to insulin (Efrat et al., 1988).

In the present study, we have demonstrated the expression and secretion of biologically active GIP from β TC-3 cells. In addition, we have examined the transcriptional regulation of GIP expression in these cells and have specifically evaluated the role of the transcription factors GATA-4, ISL-1 and PDX-1.

2. Methods

2.1. Cell culture

STC-1 cells (mouse neuroendocrine tumor derived) (Boylan et al., 1997), GTC-1 cells (a high GIP-expressing subpopulation of STC-1 cells) IEC-6 cells (normal rat small intestine derived immature intestinal stem cells [ATCC, Manassas, VA]), β TC-3 cells (mouse pancreatic β -cell tumor [ATCC]) and LGIPR2 cells (provided by Dr. Ted B. Usdin, Bethesda, MD) (Usdin et al., 1993) were grown in Dulbecco's minimal essential medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS) at 37 °C in an atmosphere of 5% (v/v) CO₂/95% O₂, in the presence of 100 U/ml penicillin G, 100 μ g/ml streptomycin.

2.2. Plasmids

The GIP/Luc reporter constructs used for this study are described in detail elsewhere (Boylan et al., 1997; Jepeal et al., 2003). Briefly, in the plasmid pGL-193M2, the GATA-4 binding site located between base pairs –156 and –151 of the GIP promoter is mutated from GATAA to AAAAA. To generate the GATA-4 expression plasmid GATA-4/pcDNA3, the GATA-4 cDNA (a generous gift from Dr. Michael Parmacek, University of Pennsylvania, Philadelphia, PA) was cloned into the KpnI–EcoRI site of the eukaryotic expression vector pcDNA3.1/Zeo (Invitrogen Corp., Carlsbad, CA).

2.3. GIP bioassay

LGIPR2 cells plated at a density of 4×10^{-4} cells per well were grown overnight on 24-well plates in DMEM containing 10% (v/v) FBS. The following day, the cells were washed with phosphate-buffered saline (PBS) and then incubated with either GIP standards (10^{-9} to 10^{-13} M GIP diluted in 500 μ l DMEM containing 10% (v/v) FBS) or 500 μ l of conditioned media. Conditioned media was obtained from nearly confluent plates of either STC-1 or β TC3 cells that had been incubated overnight in DMEM containing 10% (v/v) FBS. After a 5-h incubation, the cells were washed twice with PBS and placed at –80 °C for 10 min. Plates were removed from the freezer and incubated for 10 min at 37 °C in 0.01 M sodium phosphate pH 8.0, 0.2 mM MgSO₄ and 0.01 mM MnCl₂. After the addition of 200 μ l PM2 buffer (0.4% Triton X-100, 0.1 M sodium phosphate pH 8.0, 2 mM MgSO₄, 0.1 M MnCl₂), cell lysates were mixed by tapping the plate, followed by the addition of 50 μ l of a 5 mg/ml solution of chloramphenicol red- β -D-galactopyranoside (CRPG) (Roche Molecular Bioanalytics, Indianapolis, IN) diluted in PM2. Plates were incubated at 37 °C until the color was sufficiently developed. To stop the reactions, 500 μ l of cold 0.5 M EDTA were added, and the triplicate samples read with a spectrophotometer at a wavelength of 570. The concentration of bioactive GIP in the conditioned media was standardized to the total protein content of the cells from which it originated.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Primers were synthesized by Invitrogen. Primer sequence, size of the amplified fragments and optimal annealing conditions of amplification are detailed in Table 1.

Total RNA was extracted from cells grown to 80% confluence using an RNeasy® kit, as recommended by the manufacturer (Qiagen, Valencia, CA). cDNA was synthesized from 5 μ g of mRNA and 50 pmole of oligo (dT) primer using the ThermoScript RT-PCR system (Invitrogen). One tenth of the single-stranded cDNA was then amplified using the gene specific primers in Table 1 in a total volume of 50 μ l containing; 50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl₂, 0.2 mM dNTPs and 25 U Taq DNA polymerase (Roche, Mannheim, Germany). After an initial denaturation step at 94 °C for 2 min, the PCR cycles included: 94 °C for 1 min, primer annealing at 50–65 °C for 1 min, and extension at 72 °C for 2 min. After

30 cycles, a terminal elongation step of 5 min at 72 °C was performed. All primer pairs produced a specific single band. PCR products were analyzed by electrophoresis on a 1.5% agarose gel with ethidium bromide staining and photographed under UV transillumination.

2.5. Western blot analysis

Cells grown on 6-well plates to 80% confluence were washed with 1× phosphate-buffered saline suspended in 200 µl of RIPA buffer (Boston Bioproducts, Ashland, MA), containing Complete™ protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), and incubated on ice. After 15 min, the lysed cells were centrifuged at 14,000 rpm at 4 °C for 5 min, and the supernatant was collected. Total protein content of the lysates was determined using a BCA Protein Assay kit (Pierce, Rockford, IL). Proteins in the lysates (50 µg) were subjected to SDS-PAGE electrophoresis and transferred to Protran nitrocellulose membranes (PerkinElmer Life Sciences, Boston, MA). Membranes were blocked in 5% nonfat milk, 1× PBS, 0.5% Tween-20 at room temperature (RT) for 2 h, and then incubated with the appropriate primary antibody (rabbit anti-PDX-1; 1:1000 (Brissova et al., 2002); goat anti-GATA-4, 1:1000, Santa Cruz, Biotechnology, Santa Cruz, CA; mouse anti-ISL-1, 1:750, Chemicon International, Temecula, CA; or mouse anti-β-actin; 1:20,000, BD Biosciences, Palo Alto, CA) diluted in 5% nonfat milk in 1× PBS. After a 1-h incubation, membranes were washed 3 times for 5 min at room temperature with wash buffer (1× PBS, 0.5% Tween-20) and incubated with the appropriate horseradish peroxidase-conjugated secondary antiserum diluted in 5% nonfat milk in 1× PBS. Membranes were then rinsed three times for 5 min in wash buffer, soaked in chemiluminescence reagent, as instructed by the manufacturer (Super Signal West Pico Chemiluminescent substrate, Du Pont, Wilmington, DE), and exposed to X-ray film for 0.1–5 min.

2.6. GIP ELISA

Total GIP (both biologically active and inactive cleaved peptide metabolite) secreted by each cell line was quantified using a commercially available GIP ELISA kit (Linco Research, St. Charles, MO), following the manufacture's instructions. For each cell line, a minimum of four samples of conditioned media was tested in triplicate using the ELISA kit. 10 µl samples were assayed and the GIP concentration was determined by comparison to provided standards. Regular media was used a negative control, and control samples provided in the kit were included as positive controls.

2.7. Northern blot analysis

Total RNA (20 µg), extracted from cells grown to 80% confluence using a RNeasy® kit as recommended by the manufacturer (Qiagen) was size-fractionated on a 0.8% agarose gel containing 2.2 M formaldehyde and transferred to a Gene screen Plus® nylon membrane (New England Nuclear, Boston, MA) by capillary blotting in 10× SSC (15 mM sodium citrate, 150 mM sodium chloride, pH 7.4). The RNA was cross-linked to the membrane using a UV-Stratalinker (Stratagene, La Jolla, CA), followed by prehybridization for 2 h at 65 °C in PerfectHyb Plus hybridization buffer (Sigma, St. Louis, MO), containing 10 µg/ml denatured herring sperm DNA, and hybridization overnight at 65 °C in PerfectHyb Plus hybridization buffer, 20 µg/ml herring sperm DNA and ~10⁷ cpm of probe. After hybridization, the blots were washed once at RT in 1× SSC, 1% sodium dodecyl sulfate (SDS) for 15 min, once at RT in 0.5× SSC, 0.5% SDS for 15 min, twice at RT in 0.1× SSC, 0.1% SDS for 15 min, and once at 65 °C in 0.1× SSC, 0.1% SDS for 30 min. Autoradiographs were developed after exposure to X-ray film for 18–36 h at –70 °C, using a Cronex intensifying screen (Dupont, Wilmington, DE).

2.8. Immunohistochemistry

The duodenum was dissected from adult female BalbC mice, and the tissue was fixed in ice-cold 5% paraformaldehyde at 4 °C for 2 h. The tissue was then immersed overnight in a solution consisting of 30% sucrose in 1× PBS and then embedded in O.C.T. (Sakura Finetek USA, Torrance, CA) and flash frozen using methylbutane and dry ice. Frozen fixed tissue was cut into 7-µm sections and stored at −80 °C.

For immunofluorescence labeling, frozen sections were thawed at RT for 15 min, then fixed in 4% PFA for 15 min, permeabilized in 0.25% Triton X-100/PBS for 10 min, washed again 3× with PBS, and then blocked with 10% normal donkey serum for 60 min at RT. The primary antibodies were diluted in PBS containing 5% normal donkey serum and incubated with the sections overnight at 4 °C. Slides were washed in PBS and incubated with the appropriate secondary antibodies diluted in PBS containing 5% normal donkey serum for 2 h at RT. Slides were washed in PBS and then mounted with UltraCruz Mounting Medium containing 15 µg/ml DAPI for DNA counterstaining (Santa Cruz). Slides were examined and photographed using a fluorescence microscope with appropriate filters.

Antibodies to the following proteins were used at the listed concentrations: GIP, goat polyclonal (1:200 dilution; Santa Cruz); GATA-4, rabbit polyclonal (1:200 dilution, Santa Cruz). The secondary antibodies used were anti-goat immunoglobulin conjugated to FITC (for GIP) (1:200 dilution, Jackson ImmunoResearch Lab Inc., West Grove, PA) and anti-rabbit immunoglobulin conjugated Cy3 (for GATA-4) (1:200 dilution, Jackson ImmunoResearch Lab Inc.).

2.9. Short hairpin RNA (shRNA)

ShRNAs, purchased from Origene (Rockville, MD), were directed against the following sequences: pRS-GATA-4-18 (G18), 5'-GACTTCTCAGAAGGCAGAGAGTGTGTGCAA-3'; and pRS-GATA-4-20 (G20), 5'-CGTTAACATTGTCTTAAGGTGAAATGGCTGT-3'. pRS-shGFP (GFP), used as a negative control, contains a non-effective shGFP sequence cassette. The empty vector pRS was used as a second negative control.

2.10. Transient transfection

One day prior to transfection, cells were plated onto 6-well plates at a density of $\sim 1-2 \times 10^5$ cells per well. A mixture containing a total of 1 µg plasmid DNA, 4.5 µl lipofectamine (Invitrogen Corp.), and 600 µl serum free media was incubated at RT. For functional assays, the DNA consisted of 1.0 µg of the GIP/LUC reporter plasmid and 16 ng pRL-CMV (Promega, Madison, WI), which are included in order to control for transfection efficiency. For the overexpression experiments, 0.5 µg of the GIP/LUC reporter plasmid, 16 ng pRL-CMV, and 0.5 µg of the GATA-4 expression plasmid, GATA-4/pcDNA3.1, or the empty vector, pcDNA3.1, was used. For shRNA experiments, the transfection DNA consisted of 0.7 µg of the appropriate shRNA plasmid, 0.4 µg of the GIP/Luc reporter plasmid, and 20 ng of pRL-CMV. After 15 min, 0.8 ml of media was added, and the mixture was added to cells previously washed twice with serum free medium. After 5 h, 1 ml of media containing 20% serum was added, and the incubation was continued for 48 h. Media was removed and stored at −80 °C for analysis of secreted GIP using the bioassay described above. The cells were washed twice with PBS, lysed in the appropriate buffer for analysis of functional activity by luciferase assay or mRNA levels by northern blot analysis.

2.10.1. Luciferase assay—For luciferase assays, 200–400 µl lysis buffer were added to the transfected cells, which were subjected to two freeze thaw cycles, according the manufacturer's instructions for the dual-luciferase reporter assay system (Promega). To

measure luciferase (LUC) and renilla (REN) activities, 20 μ l of each sample were measured in duplicate using an Optocomp 1 luminometer (MJ Research Inc, Waltham, MA). LUC activity, expressed in light units, was standardized to REN activity to control for variation in transfection efficiency.

2.11. Statistical analysis

All constructs were analyzed in triplicate in at least three separate experiments. Data were analyzed using Student's *t*-test for unpaired samples, and statistical significance was assigned for $P < 0.05$.

3. Results

3.1. GIP is secreted by the pancreatic cell line β TC-3

Biologically active GIP peptide was measured reliably using a sensitive and specific *in vitro* bioassay. The LGIPR2 cells used in this assay are mouse L-cells cells that express both the human GIP receptor and a β -galactosidase (β -gal) reporter coupled to the cyclic adenosine monophosphate (cAMP) responsive vasoactive intestinal polypeptide (VIP) promoter (Usdin et al., 1993). When LGIPR2 cells are incubated with samples containing active GIP, the peptide present binds to and activates the GIP receptors, which in turn, increases intracellular cyclic adenosine monophosphate. The cAMP translocates to the nucleus, where it induces the VIP promoter resulting in a concentration-dependent increase in the expression of β -gal. As illustrated by the standard curve shown in Fig. 1A, the GIP bioassay measures bioactive GIP in a concentration-dependent manner, with a lower detection limit of 10^{-13} M.

We initially assessed the utility of the GIP bioassay by measuring GIP released from STC-1 cells. STC-1 conditioned media was generated by incubating a 10-cm plate of STC-1 cells grown to 75% confluence in 10 ml of DMEM containing 10% FBS for 24 h. When the STC-1 conditioned media was assayed using the bioassay, the concentration of GIP was found to be approximately 10^{-10} M (Fig. 1B). To determine whether the activity measured in the bioassay was due solely to GIP in the media, a GIP-specific antagonist, (7–30)GIP-NH₂ (ANTGIP) was included with the samples. As shown in Fig. 1B, the inclusion of 10^{-5} M ANTGIP in both the 10^{-10} M GIP peptide control sample and the STC-1 conditioned media sample resulted in suppression of activity by 94 and 97%, respectively.

The bioassay was also used to determine whether β TC-3 cells express GIP. STC-1, β TC-3, and IEC-6 cells (an intestinal stem cell line that does not express GIP) plated at 75% confluence were incubated with media containing 10% FBS. After 24 h, conditioned media were collected and the concentration of GIP determined using the bioassay. After removal of the conditioned media, the STC-1, β TC-3, and IEC-6 plates were lysed and their protein content determined. The concentrations of GIP in the media were normalized to protein content in the cell lysates to correct for variations in cell plating densities. For each cell line, the relative concentrations of bioactive GIP secreted per μ g of total cellular protein were calculated and are graphed in Fig. 1C. As shown, both STC-1 and β TC-3 cells were found to secrete measurable levels of GIP, while GIP secretion was undetectable in conditioned media obtained from IEC-6 cells. However, β TC-3 cells were found to secrete approximately 75% less GIP than STC-1 cells. The presence of GIP in the conditioned media of β TC-3 cells was further confirmed using a commercially available GIP Elisa (Linco Research Inc., St. Charles, MO, Data not shown).

3.2. GIP transcripts are detected in β TC-3 cells

Using northern blot analysis, we sought to demonstrate the presence of GIP mRNA in β TC-3 cells. As seen in the representative northern blot shown in Fig. 2A, using a GIP cDNA probe, we detected the presence of a 0.8 kb transcript in both STC-1 and β TC-3 cells, but not in IEC-6

cells. To quantify the differences in GIP expression levels between the cell lines, densitometric measurements from four independent experiments were obtained and the ratio of GIP to β -actin was calculated. As seen in Fig. 2B, GIP mRNA levels were 2.7-fold lower in β TC-3 cells than in STC-1 cells ($P < .001$).

3.3. GIP/LUC constructs are active in β TC-3 cells

In addition to comparing endogenous GIP mRNA levels, we assessed the relative activity of our full-length GIP/LUC reporter construct (pGL-2569) in β TC-3 and STC-1 cells by a transient transfection assay (Fig. 3). For each transfection, either pGL-2569 or the empty vector pGL2 was co-transfected with the control plasmid pRL-CMV. The REN activity of pRL-CMV was used to standardize for differences in transfection efficiency both between the samples and between the two cell lines. As depicted in Fig. 3, the GIP promoter construct was found to be active in β TC-3 cells, displaying a 4.8-fold increase in activity when compared to the promoterless vector pGL2 ($P = 1.2 \times 10^{-9}$). However when the relative activities of the reporter constructs in β TC-3 and STC-1 cells were compared, the GIP promoter construct pGL-2569 was found to be 12.9-fold more active in STC-1 cells than in β TC-3 cells ($P < 10^{-11}$).

3.4. β TC-3 cells express the transcription factors PDX-1 and ISL-1, but do not express GATA-4

We postulated that the differences in GIP expression levels between the two cell lines occur as a direct result of the inherent differences in the complement of transcription factors present in each cell line. Consequently, we next examined the expression of the transcription factors known to be important for GIP expression. To date, three transcription factors—ISL-1, PDX-1, and GATA-4 have been shown to be required for GIP expression in STC-1 cells (Jepeal et al., 2003, 2005).

When β TC-3 cells were screened for the presence of ISL-1, PDX-1, and GATA- mRNA by RT-PCR analysis, transcripts for ISL-1 and PDX-1 were readily detectable in both STC-1 cells and β TC-3 cells (Fig. 4A). However, when primers specific for GATA-4 were used, whereas RT-PCR analysis of mRNA from STC-1 cells generated a robust product, mRNA from β TC-3 produced only a very faint band (Fig. 4A).

We next used Western analysis to compare the protein levels of these three transcription factors in, β TC-3, IEC-6, and STC-1 cells. Whole cell lysates were probed with PDX-1, ISL-1, and GATA-4 specific antibodies (Fig. 4B). The PDX-1 and ISL-1 antibodies detected a 46-kDa band and a 45-kDa protein, respectively, in samples derived from STC-1 and β TC-3. In contrast, neither PDX-1 nor ISL-1 was detected when whole cell lysates from IEC-6 cells were examined. Moreover, when the blots were probed with the GATA-4 specific antibody, a 53-kDa band was detected in the STC-1 lysates but not in either β TC-3 or IEC-6 cells. Incubation of the blot with antiserum specific for β -actin did confirm the presence of comparable amounts of protein in all three lanes).

To further examine GATA-4 expression, we analyzed GATA-4 mRNA levels by northern blot analysis. As shown in Fig. 4C, a GATA-4 specific, radiolabeled probe readily detected GIP transcripts in STC-1 cells but not in β TC-3 cells. Further hybridization of the blot with an β -actin specific probe confirmed the presence of comparable mRNA in both lanes.

3.5. Expression of GATA-4 in native K-cells

To analyze the *in vivo* expression of GATA-4 in GIP-producing K-cells, we performed immunohistochemistry on intestinal mucosal tissue from 10-week-old BalbC mice (Fig. 5). In all cells, the nuclei were counterstained with DAPI and are shown in blue (A–F). As illustrated in Fig. 5A and D, GATA-4 positive nuclei (red) were found in a subpopulation of epithelial

cells. In Fig. 5B and E, GIP immunoreactivity is detected in the cytoplasm of K-cells. Merged images are depicted in Fig. 5C (for Fig. 5A and B) and in Fig. 5F (for Fig. 5D and E), respectively. The white arrows point to GATA-4 positive cells (red) that also stain for GIP immunoreactivity (green).

3.6. Exogenous expression of GATA-4 leads to an increase in GIP mRNA levels

Previous experiments from our laboratory have demonstrated that the transcription factor GATA-4 is required for high levels of GIP expression in STC-1 cells. We postulated that the absence of GATA-4 expression in β TC-3 cells could be responsible in part for the lower level of GIP expression in β TC-3, compared to STC-1, cells. To test this hypothesis, we first modulated GATA-4 expression and then evaluated its effect on the expression of GIP. To perform these studies, we prepared RNA from plates of β TC-3 cells that had been transfected 48 h earlier with either the GATA-4 expression plasmid, GATA-4/pcDNA3, or the vector alone, and used northern blot analysis to compare the relative levels of endogenous GIP mRNA. As shown in Fig. 6A and B, β TC-3 cells transfected with the GATA-4 expression plasmid demonstrated a significant increase in the level of GIP transcripts ($P = 9.2 \times 10^{-4}$).

3.7. GATA-4 overexpression induces an increase in GIP secretion

Because exogenous GATA-4 expression upregulated GIP transcription, we next examined its effect on GIP secretion using our GIP bioassay described above. We prepared conditioned media from plates of β TC-3 cells that had been transiently transfected 24 h earlier with either the GATA-4 cDNA expression construct or the empty vector pcDNA3.1. Fig. 7 depicts the relative amounts of GIP secreted into the media and demonstrates that overexpression of GATA-4 in β TC-3 cells resulted in a 2.2-fold increase in GIP secretion ($P = 2.74 \times 10^{-6}$).

3.8. Suppression of GATA-4 by shRNA attenuates GIP promoter activity

To confirm the direct correlation between expression of the transcription factor GATA-4 and the expression of GIP, we examined their relationship in GTC-1 cells, a STC-1 cell subpopulation that is enriched with GIP-producing cells. Using silencing RNA technology, we examined whether a reduction in GATA-4 expression would lead to an attenuation of GIP expression. As shown in Fig. 8, the co-transfection of two GATA-4-specific shRNA constructs (Origene) and a luciferase reporter plasmid containing 193 base pairs of the GIP promoter, which includes the GATA-4 binding site, led to a significant decreases in transcriptional activity ($P < 10^{-10}$). In contrast control shRNA plasmids containing either a non-effective shRNA cassette or vector alone did not affect the expression of the GIP/LUC reporter gene. In addition, none of the shRNA plasmids affected activity of the promoterless luciferase reporter plasmid pGL3. The REN activity of pRL-CMV was used to standardize for differences in transfection efficiency.

4. Discussion

GIP expression *in vitro* had previously been detected only in the intestinal-like cell line STC-1 (Boylan et al., 1997; Jepeal et al., 2005; Rindi et al., 1990) and cell lines derived from STC-1 cells such as GTC-1 (Cheung et al., 2000), STC6-14 (Kieffer et al., 1995), and GIP-INS (Ramshur et al., 2002). Although numerous other intestinal cell lines have been screened by northern blot analysis, GIP bioassay and RT-PCR (Boylan et al., 1997), none have been found to express GIP. However, because the STC-1 cell line was derived from an intestinal tumor isolated from a transgenic mouse expressing a viral oncogene under control of the insulin promoter (Rindi et al., 1990), we explored the possibility of GIP expression in insulin expressing pancreatic β -cell lines and detected GIP expression in the cell line β TC3.

While mature β cells do not normally express GIP nor do mature K-cells express insulin, it is nevertheless not surprising that we observed GIP and insulin co-expression in β TC3 cells. Both GIP and insulin are peptide hormones that function in concert to promote efficient nutrient storage. Regulation of the expression of both the GIP and insulin genes involves common transcription factors, such as PDX-1 (Ahlgren et al., 1998; Jepeal et al., 2005) and ISL-1 (Jepeal et al., 2003; Peers et al., 1994) and, GIP-producing intestinal K-cells and insulin-producing pancreatic β -cells share a common developmental lineage.

Previous studies have suggested that the four principal cell types in the intestinal epithelium – enteroendocrine cells (which include K-cells), goblet cells, enterocytes, and Paneth cells – arise from a relatively small number of multipotent stem cells located near the base of the crypts of Lieberkühn (Rindi et al., 1999; Traber and Silberg, 1996). It is thought that progenitor cells, derived from these stem cells, cease dividing and migrate up (or down in the case of Paneth cells) the crypt–villus axis and, during this process, terminally differentiate into their respective lineages (Marshman et al., 2002). These stem cells, like other neuroendocrine cells, such as those that comprise the pancreatic islets, appear to originate developmentally from the primitive endoderm.

Recent studies have provided important insight into the lineage relationship between intestinal and pancreatic epithelial cells and have found that many of the transcription factors involved in the differentiation of the various pancreatic cell types are likewise involved in intestinal epithelial cell differentiation. It has been shown that the basic helix–loop–helix (bHLH) transcription factor neurogenin3 (*ngn3*) is required for the specification of the endocrine lineage both in uncommitted progenitors in the developing pancreas and in multipotent intestinal progenitor cells (Jenny et al., 2002; Kayahara et al., 2003; Rindi et al., 1999).

In addition to its role in insulin and GIP expression, PDX-1 has also proven to be a key factor in both pancreatic and intestinal development. An analysis of PDX-1 knockout mice have demonstrated that animals lacking PDX-1 exhibit both pancreatic agenesis, and an altered architecture of the rostral duodenum leading to a reduction in enteroendocrine cells including a near abolishment of GIP expressing K-cells (Jepeal et al., 2005; Jonsson et al., 1994; Offield et al., 1996). Lastly, the Notch signaling pathway of cellular differentiation has been shown to be critical for the determination of endocrine and progenitor cell fate (Skipper and Lewis, 2000). The transcriptional repressor Hes-1, a downstream target in the Notch signaling pathway, acts as a general negative regulator of endocrine differentiation, both in the intestine and in the pancreas, by modulating the expression or activity of a wide range of transcriptional activators, including Math1, *Ngn3*, ISL-1, PAX-4, and PAX-6 (Jensen et al., 2000).

Enhancers are known to play an important role in regulating the tissue-specific expression of eukaryotic genes by binding multiple transcription factors that act in a combinatorial fashion (Mitchell and Tjian, 1989). These factors may be expressed in a tissue-restricted pattern, may be widely expressed, or may be ubiquitous. Factors binding to the enhancer sites are thought to function by forming a higher order complex termed an “enhancersome” (Carey, 1998).

Previous studies in our laboratory identified an enhancer region located between base pairs –193 and –145 of the rat GIP promoter that is highly conserved across rat, mouse, and human species, exhibiting 85.4% homology among all three species and 94% homology between rat and mouse sequences. Furthermore, using STC-1 cells, we have shown that this region is required for high levels of transcriptional activity and that activation of the GIP promoter was dependent on presence of sequences that specifically bind the transcription factors PDX-1, ISL-1, and GATA-4 to this region (Boylan et al., 1997; Jepeal et al., 2003, 2005).

We have also provided evidence for the involvement of PDX-1 in the regulation of GIP expression within the enteroendocrine K-cells of the proximal duodenum, and we demonstrated

the co-expression of GIP and PDX-1 within the K-cells at both the embryonic and adult stage (Jepeal et al., 2005). In addition, a careful analysis of GIP expression in both wild-type and PDX-1(-/-) mice showed that the loss of PDX-1 expression nearly abolished GIP-producing cells in the small intestinal mucosa (Jepeal et al., 2005). This finding is further corroborated by a study by Boyer et al. in which they performed transgene-based complementation experiments on *PDX-1* null mice (Boyer et al., 2006). In these mice, the expression of PDX-1 in the duodenum of the transgenic rescue mice was significantly lower than that of wild-type mice. The authors reported that the decrease in PDX-1 expression levels resulted in a reduction in the number of GIP-expressing cells, but not a decrease in GIP expression levels within individual K-cells.

In the present study, we have detected both GIP biosynthesis and secretion in a cell line that lacks the transcription factor GATA-4. By showing that the exogenous expression of GATA-4 in these cells results in an increase in endogenous GIP transcription and a corresponding increase in GIP secretion, we have demonstrated that although GATA-4 is not required for GIP gene expression, it is capable of modulating the level of GIP expression.

GATA-4 belongs to a family of transcription factors in which members are classified into two subfamilies (GATA-1, -2, and -3 and GATA-4, -5, -6) based upon similarities in both their amino acid sequences and patterns of expression. Members of the latter subfamily are expressed in the gastrointestinal tract in distinct overlapping patterns along the longitudinal axis (Dusing et al., 2003). GATA transcription factors have been found to play critical roles in regulating tissue specific gene expression, and GATA-4 in particular has been shown to play an important role in regulating the transcription of numerous intestinal genes (Boudreau et al., 2002; Divine et al., 2004; Dusing et al., 2003; Fang et al., 2001; Kiela et al., 2003).

Consistent with our results, others have suggested that in some intestinal cells, GATA-4 may function more to modulate gene expression rather than control tissue-specific expression of the gene. Dusing et al. (2003) reported that the purine metabolic gene adenosine deaminase (ADA) gene is expressed at high levels in absorptive enterocytes in the villous epithelium of the proximal small intestine. They reported that a duodenum specific enhancer, which binds both transcription factors PDX-1 and GATA-4, controls this pattern of expression. The authors demonstrated that PDX-1, whose expression is limited to principally to the duodenum and pancreas, is likely to contribute significantly to the tissue specificity of the duodenal enhancer (Dusing et al., 2001). However their results suggest that although GATA-4 serves as a strong activator of the duodenal specific enhancer, GATA-4 may not be involved in determining duodenal specificity (Dusing et al., 2001).

The hypothesis that GATA-4 may function to upregulate GIP expression in terminally differentiated K-cells is further supported by the work of Gao et al. (1998). Upon characterizing the expression patterns of the GATA-4, -5 and -6 transcription factors in the intestinal epithelium, this group of investigators found GATA-6 to be the predominant GATA family member expressed in the less differentiated, proliferating progenitor cell population. However, they found GATA-4 and GATA-5 to be expressed abundantly in the differentiated epithelium. They suggested that GATA-6 might function primarily within the proliferating progenitor population, while GATA-4 may function during differentiation to activate terminal-differentiation genes including IFABP. It is plausible that GATA-4 may serve a similar function in the K-cell, by increasing GIP expression when progenitor cells terminally differentiate into mature K-cells. GATA-4 may function to increase GIP expression. Future investigation including the complete characterization of GIP expression will require a thorough evaluation of other regulatory regions of the promoter, as well as downstream and intronic sequences. Such studies will be necessary to enable the ultimate determination of the physiologic and pathologic significance if this important metabolic regulatory peptide.

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Fig. 1.

Measurement of secreted GIP using *in vitro* bioassay. (A) Representative standard curve in which standards, ranging from 10^{-9} to 10^{-13} M GIP diluted into DMEM containing 10% FBS, was assayed using the GIP bioassay. The results are expressed as the mean $\pm$ S.E. ($n = 3$). (B) Effect of GIP specific antagonist on GIP bioassay. Media containing 10^{-10} M GIP (positive control) or conditioned media from STC-1 cells was assayed using the GIP bioassay with (black) or without (striped) the addition of 10^{-5} M AntGIP, a GIP specific antagonist. The results are expressed as the mean $\pm$ S.E., * $P = 6.5 \times 10^{-4}$ ($n = 4$), ** $P = 4.8 \times 10^{-3}$ ($n = 6$). (C) Relative levels of secreted GIP per μ g protein. The concentrations of secreted GIP in conditioned media from an overnight incubation of STC-1, β TC-3 and IEC-6 cells were assayed using the bioassay, and were normalized to protein content of test cell plates. The relative expression levels, compared to STC-1 cells, were calculated and the results were expressed as the mean $\pm$ S.E. ($n = 6$), * $P = 1.45 \times 10^{-8}$ vs. STC-1 cells.

Fig. 2.

Analysis of GIP transcript levels in β TC-3 cells. (A) Representative northern blot. Total RNA was extracted from STC-1, β TC-3 cells, and IEC-6 cells, as described in Section 2. After electrophoresis through a 1.0% agarose gel, the RNA was transferred to a nylon membrane, hybridized to a 600-bp GIP cDNA probe, and exposed overnight to X-ray film (top panel). The blot was washed and then probed with an actin specific cDNA probe as a positive control for RNA loading (bottom panel). (B) Quantification of GIP expression levels by densitometry measurements. Data are expressed as a percentage of the average GIP mRNA levels in STC-1 cells and are normalized to β -actin expression levels. For each cell line, values are expressed as the mean $\pm$ S.E. ($n = 4$). * $P = 1.45 \times 10^{-8}$ vs. STC-1.

Fig. 3.

Analysis of GIP-LUC reporter construct activity in β TC-3 cells. The relative activities of GIP/LUC reporter constructs were analyzed by transient transfections assays. Co-transfections into STC-1 cells (solid black bar) or β TC-3 cells (striped bar) were performed using control plasmid pRL and full-length GIP promoter construct (pGL2569) or the promoterless vector pGL2. The data are expressed as the mean $\pm$ S.E. ($n = 18$) of results obtained from three independent experiments. All values were normalized to Renilla activity to control for differences in transfection efficiencies both between samples and between cell lines. * $P < 10^{-11}$, ** $P = 1.2 \times 10^{-9}$.

Fig. 4.

Expressions levels of ISL-1, PDX-1 and GATA-4 in βTC-3 cells. (A) RT-PCR analysis of mRNA from derived STC-1 (lanes 1 + 2), BTC-3 (lanes 3 + 4) and IEC-6 (lanes 5 + 6) cells. The +/- indicates the presence or absence of reverse transcriptase in the RT reaction. NT denotes the absence of template in the PCR reaction. Using specific primers outlined in Table 1, cDNA was screened for the presence of ISL-1 (404 bp), PDX-1 (565 bp) and GATA-4 (404 bp). Primers for β-actin were used to confirm the integrity of the cDNA. (B) Protein lysates from βTC-3 cells, IEC-6 cells and STC-1 cells were screened by Western blot for the presence of the transcription factors PDX-1, ISL-1, and GATA-4. (C) Northern blot analysis of GATA-4 mRNA in STC-1 and βTC-3 cells. The blot was probed with a 449-bp fragment corresponding to bp -481 to -930 of the published cDNA sequence. Blots representative of three independent experiments shown.

Fig. 5.

Double staining for GIP-1 and GATA-4-1 in mouse duodenum demonstrates GATA-4 expression in intestinal K-cells. Tissue sections of 10-week-old BalbC mice were probed with rabbit anti-GATA-4 antibody (red; A and D) and goat anti-GIP antibody (green; B and E). Nuclei (blue; A–F) were counterstained with DAPI (Santa Cruz). Merged images are shown in C (for A and B) and F (for D and E). White arrows indicate the location of a GATA-4 and GIP double-positive cell, and orange arrows show GATA-4 positive cells that do not express GIP.

Fig. 6.

Effect of GATA-4 overexpression on endogenous GIP transcription in β TC-3 cells. (A) Northern blot analysis of GIP mRNA from β TC-3 cells that had been transfected 48 h earlier with either the GATA-4 expression plasmid, GATA-4/pcDNA3 (lanes 1 and 2), or the vector alone (lanes 3 and 4). (B) Data from 6 independent transfections were pooled and the relative increase of GIP transcripts compared to vector alone was plotted. ($P = 9.2 \times 10^{-4}$).

Fig. 7.

Effect of GATA-4 overexpression on GIP secretion in β TC-3 cells. Conditioned media from plates of β TC-3 cells that had been transiently transfected 24 h earlier with either the GATA-4 cDNA expression construct or the empty vector pcDNA3.1 was assayed for functional GIP by bioassay. The amounts of GIP secreted into media were standardized to total cellular protein content, and the relative increase of GIP secretion as compared to vector alone was plotted. ($P = 2.74 \times 10^{-6}$, $n = 16$).

Fig. 8.

Effect of GATA-4 inhibition on GIP promoter activity in GTC-1 cells. GTC cells were transiently co-transfected with the reporter constructs 193/LUC (lanes 1–4) or pGL3 basic (lanes 5–8) and the shRNA plasmids pRS (vector control, lanes 1 and 5), GFP (nonspecific shRNA, lanes 2 and 5), G18 (GATA-4 shRNA plasmid, lanes 3 and 7), or G20 (GATA-4 shRNA plasmid, lanes 4 and 8). The plasmid pRL-CMV was included in all transfections to allow for standardization of transfection efficiencies. The experiment was repeated 3 times with an n of 6 per experiment. * $P < 10^{-10}$.

Table 1
Gene-specific primers used for RT-PCR

Gene	Sense primer (5'–3')	Antisense primer (5'–3')	T _a (°C)	Size (bp)	Accession No.
GIP	GAAGACCTGCTCTCTGTGTGCTGGT	CAGAGCTCTGCTTGGTCCACCATC	60	404	MN08119.2
GATA-4	CCGGGCTGTCACTCACTAT	GTTTGAAACAACCCGGAAACAC	55	404	MN008092.2
PDX-1	CCATGAACAGTGAGGAGCAGTACT	CTCGGTCAAAGTTCAACATCACTGC	65	565	MN008814.2
ISL-1	CCAAAGTGCAGCATAGGCTTC	CTAGTTGCTCCTTCATGAGCGCAT	65	404	MN021459.2
β-Actin	ACCACACCTTCTACAATGAGC	GTGCCCAATAGTGATGACCTG	55	496	MN007393.1

T_a: Annealing temperature.