Cell-specific Expression of the Glucose-dependent Insulinotropic Polypeptide Gene in a Mouse Neuroendocrine Tumor Cell Line*

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Glucose-dependent insulinotropic polypeptide (GIP), a 42-amino acid gastrointestinal regulatory peptide that, in the presence of glucose, stimulates insulin secretion. GIP is expressed in K cells of the small intestine and in cells of the submandibular salivary gland. Using a rat GIP cDNA as a specific probe, we screened a number of established cell lines for the expression of GIP mRNA. STC-1 cells, a cell line derived from a mouse neuroendocrine tumor, were found to express high levels of GIP mRNA. GIP-specific transcripts were not detected in other cell lines tested, which included cells of intestinal, salivary, and endocrine origin. Analysis of GIP-luciferase fusions identified two promoters, a distal and a proximal promoter, upstream of the translation initiation codon for GIP. The distal promoter, located upstream of position +1, corresponds to the principal promoter of the GIP gene and can promote cell-specific transcription. Sequential deletion and site-directed mutational analysis of the distal promoter demonstrated that the sequence between –193 and –192 determines cell-specific expression of GIP. Contained in this region is a consensus GATA motif, suggesting that a member of the GATA family of DNA-binding proteins is involved in the cell-specific regulation of the GIP gene.

Glucose-dependent insulinoactive polypeptide (GIP), first isolated in 1969 from porcine small intestine, was originally named gastric inhibitory polypeptide, on the basis of its ability to inhibit gastric acid secretion in dogs (1). The primary structure of GIP was described in 1971 (2), and its amino acid sequence placed it in the secretin family of gastrointestinal regulatory peptides. GIP immunoreactivity has been demonstrated in the cytoplasmic granules of K cells in the mucosa distributed throughout the length of the small intestine (3). After intestinal glucose perfusion, the primary site of endogenous GIP release is the duodenum and proximal jejunum (4). Although several studies have supported the role of GIP as a physiological inhibitor of acid secretion (5–8), some investigators have challenged this notion (9, 10). Moreover, subsequent studies of the physiological properties of GIP demonstrated that, in addition to its inhibitory effects in the stomach, when...
Tissue Preparation, RNA Extraction, and Northern Hybridization Analysis—Cells were grown to 80% confluence, after which they were aspirated, and the cells were washed twice with ice-cold phosphate-buffered saline prior to RNA isolation. Intestinal mucosa was prepared for RNA extraction by surgically removing an 8–10-cm segment of duodenum immediately distal to the pylorus and scraping the mucosa with a glass slide. Total RNA from duodenal mucosa or STC-1 cells was extracted using the acid/phenol method of Chomczynski and Sacchi (22).

Northern hybridization analysis was done as described previously, using the EcoRI fragment of the rat GIP cDNA (17) radiolabeled with [α-32P]deoxycytidine triphosphate.

Genomic Library Screening, Mapping, and Sequencing—A rat genomic DASH® library, purchased from Stratagene, was screened by plaque hybridization with the rat GIP cDNA clone, as described previously (17). The hybridization was carried out using standard methods (23). The DNA from two positive clones was digested with a variety of enzymes and subjected to Southern analysis using the oligonucleotide probe. Two overlapping restriction fragments, a 2.8-kilobase pair (kb) BamHI-HindIII fragment and a 5.9-kb SacI fragment, were identified and subcloned into pBSKII (Strategene) to form the plasmids pBSBH and pBSSac3, respectively (Fig. 1A).

GIP-specific inserts were then sequenced using double-stranded template and synthetic oligonucleotides.

The GIP-specific probe. Two overlapping restriction fragments, a 2.8-kilobase pair (kb) BamHI-HindIII fragment and a 5.9-kb SacI fragment, were identified and subcloned into pBSKII (Strategene) to form the plasmids pBSBH and pBSSac3, respectively (Fig. 1A).

GIP-Luciferase Plasmids—The promoterless plasmid pGL2-basic (Promega, Madison, WI), containing the firefly luciferase reporter gene, was used to examine the transcriptional regulation of the GIP gene. Two series of chimeric clones were constructed (Fig. 1, B and C). In the first series, which included pGL-943i, pGL-425i, pGL-397i, pGL-363i, pGL-259i, pGL-203i, pGL-193i, pGL-182i, and pGL-143i, all constructs contained GIP-specific inserts with the same 3' end at base pair +8, ligated to the BgIII site in the pGL2-basic polycloning region. In the second series, which included pGL-943, pGL-425, pGL-397, pGL-363, pGL-259, pGL-203, pGL-193, pGL-182, and pGL-143, the GIP-specific inserts extended 3' to include sequences for exon 1. Each of these clones ends with the AluI site at base pair +781, ligated to an EcoRV site that was placed immediately in front of the HindIII site in the polycloning region of pGL2-basic.

Site-directed Mutagenesis—GIP promoter-specific primers containing various base substitutions were synthesized by Life Technologies, Inc. Mutations were introduced by polymerase chain reaction using forward primers with the following sequences: 5'-GGGGTACCCAGATGACACTGACACTGAGATACCCAAA-3' (mut1), 5'-GGGGTACCCAAAAAACACACTGCAGATACCCAAA-3' (mut2), 5'-GGGGTACCCAGATGACACGTGACACTGAGATACCCAAA-3' (mut3), and 5'-GGGGTACCCAAAAAACACACTGCAGATACCCAAA-3' (mut4).

The luciferase-specific primer GLprimer2 (Promega, Madison, WI) was used as the reverse primer with the wild type pGL193 DNA as the template for each reaction. The polymerase chain reaction products were digested with KpnI and HindIII before ligation into pGL2-basic.

Transient Transfection Assays—One day prior to transfection, cells were plated at a density of ~3 × 10^5 cells per 60-mm dish in the appropriate growth medium. A mixture containing 2.5 μg of pGL2 reporter plasmid, 14 μl of lipofectamine (Life Technologies, Inc.), 0.5 μg of pCMVβ-gal DNA (a control for transfection efficiency), and 600 μl of serum-free medium was incubated at room temperature. After 15 min, 2.4 ml of media was added, and the DNA mixture was added to cells previously washed twice with serum-free medium. After 5 h, 3 ml of medium containing twice the normal concentration of serum was added, and the incubation was continued for 48 h, after which the cells were harvested. For luciferase assays, the cells were first washed twice with phosphate-buffered saline and then were lysed in 500 μl of lysis buffer.
following the manufacturer's instructions (Analytical Luminescence, San Diego, CA).

Luciferase and β-Galactosidase Measurements—To assay luciferase activity, 100 μl of the cell lysate was mixed with 100 μl of luciferase substrate solution A (Analytical Luminescence). Using a luminometer with automatic injection, 100 μl of substrate solution B (Analytical Luminescence) was then added, and luciferase activity was measured as the light emission over a 30-s period. β-Galactosidase activity in 40 μl of the cell lysate was determined after a 5–30-min incubation at 37 °C with 2 m M chlorophenol red β-galactopyranoside (Boehringer Mannheim) in 2 m M MgCl₂, 0.1 m M MnCl₂, 45 m M mercaptoethanol, and 100 m M NaHPO₄, pH 8.5. The reactions were stopped by adding 500 μl of 0.5 m M EDTA, pH 8.0, and the absorbance at 570 nm was measured using a spectrophotometer. Within each experiment, luciferase activity was determined in duplicate and normalized to β-galactosidase activity for each dish. Each plasmid was tested at least six times in two separate experiments.

RESULTS

Initially, a number of established cell lines were screened for the expression of immunoreactive GIP. Because GIP expression was initially detected in both intestinal and salivary tissues, cells of intestinal origin, including STC-1 and intestinal 407, and cells derived from the salivary gland, including SCA-9 and Hs124, were screened. In addition, three endocrine cell lines, HIT T15, Rin5AH, and GH₄, were also screened for expression of immunoreactive GIP. Expression of GIP was demonstrated only in the mouse neuroendocrine tumor cell line STC-1 and the human embryonic intestinal 407 cell line (data not shown). The level of immunoreactive GIP expression was significantly greater in STC-1 cells than in 407 cells. Consistent with this observation, Northern hybridization analysis using a GIP cDNA probe demonstrated a specific band in RNA isolated from STC-1 cells with the same mobility as rat duodenal GIP mRNA, while no hybridization was detectable in RNA isolated from any of the other cell lines screened, including 407 cells; comparable levels of actin transcripts were detected in the same samples (Fig. 2).

To characterize the tissue-specific transcriptional activity of the GIP promoter, the GIP gene was isolated from a rat genomic library after screening 10⁶ plaques with a rat intestinal GIP cDNA. Two overlapping clones, each having inserts of approximately 20 kbp, were identified. Restriction fragments that contained upstream sequences were identified by restriction endonuclease digestion and Southern hybridization analysis using a synthetic oligonucleotide homologous to the 5′-end of the GIP cDNA sequence. Two fragments, a 5.9-kbp SacI–SacI fragment and a 2.8-kbp BamHI–HindIII fragment, were identified, subcloned into pBSKII, and partially sequenced (Fig. 1A). The sequence of the GIP gene was found to be identical to a previously published sequence (24) with the exception of a one-base pair substitution (G for T) at position 800. This substitution introduces an AluI site.

To define DNA regions upstream to the GIP start site responsible for basal transcriptional activity (24), a series of restriction fragments of varying sizes were placed upstream to the promoterless luciferase gene of the reporter plasmid pGL2-basic. Two series of GIP-Luc chimeric plasmids were constructed. One series contained sequences upstream of position +8 with respect to the putative transcription start site (Fig. 1B), and the second series of clones contained sequences upstream of position +781 and included intron 1 (Fig. 1C).

After transient transfection of the GIP-Luc constructs into STC-1 cells, transcriptional activity was determined by measuring luciferase activity in cell extracts. All GIP-Luc constructs produced luciferase activities higher than the control plasmid, pGL2-basic (Fig. 3). Transfection of STC-1 cells with the plasmids pGL-943, pGL-425, pGL-397, pGL-363, pGL-259, pGL-203, and pGL-193 resulted in activities approximately 200-fold over control (Fig. 3A). In contrast, transfection of pGL-182 induced only an approximately 30-fold increase in luciferase activity when compared with pGL2-basic (Fig. 3A). When the construct pGL-173 was compared with pGL2-basic, only an 11-fold enhancement was observed. The GIP-specific insert in the plasmid pGL-173 contains a TATA box sequence (−27 to −24), a consensus sequence of an enhancer core (−133 to −126), and two CAAT box sequences (−158 to −154 and −171 to −167) and may represent the minimal promoter of the GIP gene. This minimal promoter activity was almost completely abolished when sequences from −173 to −144 were deleted from pGL-173, producing the plasmid pGL-143 (Fig. 3A).

The differences in the transcriptional activities observed between constructs pGL-182 and pGL-193 indicated the possible presence of a specific element(s) that can enhance basal GIP gene expression is present in the region of base pairs −193 to −182. Analysis of the DNA sequence in this region revealed a potential GATA binding motif starting at position −190, with the sequence AGATAA. This sequence conforms to the consensus sequence, (A/T)GATA(A/G), for a GATA binding motif (25). A mutational analysis was performed to determine the importance of this site, as well as another site located 6 base pairs downstream that shares five of the six bases with the consensus sequence for a GATA motif having the sequence AGATAC. The sequences of the wild type (WT) and four mutants (Mut1–4) in the region of interest are shown in Fig. 4B. An A to G substitution at position −186 (Mut1) yielded a 40% reduction in promoter activity (Fig. 4C). The enhancer effect was abolished by mutating two sites within the upstream GATA motif, a G to A and a T to A substitution at positions −189 and −186 (Mut2), respectively (Fig. 4C). In contrast, a similar mutation in the downstream GATA motif (Mut3) produced only a 35% reduction in promoter activity from the wild type sequence (Fig. 4C). A construct with two mutated GATA motifs...
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Fig. 3. Transcriptional activity of GIP-Luc chimeric genes in STC-1 cells. Chimeric constructs, containing various lengths of rat GIP gene 5'-flanking sequences, were transfected into STC-1 cells. After incubation for 48 h, the cells were harvested for analyses of luciferase and β-galactosidase activities. The data represents mean activity ± S.E. of six separate transfections that are normalized to the activity of the promoterless construct pGL2-basic after correcting for differences in transfection efficiencies by measurement of β-galactosidase activity. A, constructs with GIP specific sequences of GIP extending 3' to +8. B, constructs with GIP-specific sequences extending 3' to +781 and including intron 1.

Fig. 4. Mutational analysis of the rat GIP promoter. A, sequence of the 5'-flanking region of the rat GIP gene. The numbers above the sequence indicate the position of the base relative to the transcription start site (+1). Potential TATA and CAAT boxes as well as transcription factor binding motifs are underlined. B, sequence of wild-type (WT) promoter and mutants in the region between base pair positions −194 and −168. All mutants are derivatives of pGL-193. The two potential GATA binding motifs are underlined and the bases within these motifs that were mutated are in boldface type. C, transcriptional activity of wild type and mutant GIP promoter in STC-1 cells. Derivatives of pGL-193 containing wild type and mutant sequences were transfected into STC-1 cells. After incubation for 48 h, the cells were harvested for analyses of luciferase and β-galactosidase activities. The data represents mean activity ± S.E. of six separate transfections that are corrected for differences in transfection efficiencies by measurement of β-galactosidase activity and plotted as a percentage of the activity of the wild type promoter.

(Mut4) behaved the same as the construct with only the upstream motif (Mut2) altered (Fig. 4C), suggesting that the upstream site is more important in determining promoter activity.

As described previously, consensus sequences for cis-acting elements, including a TATA box and a CAAT box, are present within the first intron of the rat GIP gene (24). To examine the properties of these sequences, a second series of GIP-Luc chimeric constructs containing intron 1 sequences (Fig. 1C) was transfected into STC-1 cells. Transfection of STC-1 cells with the plasmid pGL+111i, which does not contain sequences upstream of the putative transcriptional start site, did increase luciferase activity over control (Fig. 3B), suggesting that intron one sequences may possess promoter activity. The transcriptional activity of pGL+111i is equivalent to the activity of pGL-182i that, in addition to the intron one sequences, includes exon 1 and upstream sequences that are responsible for the low level of promoter activity observed with pGL-182. Similar to the first series of GIP-Luc constructs, enhancement of transcriptional activity could be seen when the sequences from −190 to −182 were included (Fig. 3B). Interestingly, transcription rates did not differ significantly between GIP-Luc fusions that contained intron 1 from those GIP-Luc fusions that lacked these sequences (Figs. 3, A and B), suggesting that the intron 1 sequences do not contribute to the activity of the upstream promoter. To determine whether the putative enhancer located between −190 and −182 might influence transcription initiated within intron 1, the plasmids pGL-Δ363i and pGL-Δ425i were constructed by deletion of sequences between −177 and +111 from the plasmids pGL-363i and pGL-425i, respectively. When pGL-Δ363i and pGL-Δ425i were transfected into STC-1 cells, no further increase in luciferase activity, over that for pGL+111i, was detected (Fig. 3B).

To determine whether changes in transcriptional activity represent cell-specific expression, luciferase activity was measured in a number of additional cell lines transfected with GIP promoter constructs (Fig. 5). All cell lines examined demonstrated a high level of luciferase activity following transfection with pRSV-LUC (data not shown). As demonstrated in Fig. 5, only minimal GIP promoter activity was detected in these cell lines. Moreover, the enhancer activity observed in STC-1 cells was greatly diminished in all the other cell lines (Fig. 5). Interestingly, GIP promoter activity was not enhanced at all in the human embryonic intestinal 407 cell line, which expressed detectable levels of immunoreactive GIP. These results are consistent with Northern hybridization analysis that demonstrated GIP-specific transcripts only in STC-1 cells (Fig. 2). In addition, with the exception of the NIH 3T3 cells, minimal
promoter activity elicited from constructs containing intron 1 sequences was similar to activity resulting from constructs containing sequences +8 to −182 to (Fig. 5). In the NIH 3T3 cell line, reduced promoter activity was detected in cells transfected with constructs containing intron 1 sequences only.

**Discussion**

Because of their presumed importance in the pathogenesis of noninsulin-dependent diabetes mellitus (NIDDM), renewed interest in incretins, such as GIP and its closely related peptide glucagon-like peptide 1 (GLP-1), has been evident in recent years. Elevated circulating levels of GIP have been found in some patients with NIDDM (26, 27), and recent clinical trials have demonstrated the therapeutic potential of GLP-1 in the treatment of some forms of NIDDM (28). Both GIP and GLP-1 are synthesized in the small intestine and augment insulin secretion after binding to specific G-coupled protein receptors present on pancreatic β-cell membranes (29).

Although substantial information has been accrued concerning tissue-specific expression of GLP-1, relatively little information is available regarding the biosynthesis of GIP. The gene encoding GLP-1 is transcribed in pancreatic α-cells, in L cells of the small intestine, and in the brain (30, 31). The tissue-specific expression of GLP-1 is regulated at both the transcriptional and posttranslational levels. GLP-1 results from limited proteolysis of proglucagon, a process restricted to the L cells of the intestine and possibly the brain (32). Like GLP-1, the GIP gene is expressed in more than one tissue. We have previously demonstrated GIP immunoreactivity in K cells of the small intestine and in ductal cells of the rat submandibular salivary gland (17, 33). GIP-(1–42) is the main functional proteolytic product of proGIP, the primary product of the GIP gene in the upper small intestine. Recently, GIP-(7–42) was purified from the upper part of the porcine intestine and was shown to have antibacterial activity (34). It is unknown whether the immunoreactivity detected in the salivary gland represents either GIP or GIP-(7–42) or whether it is a closely related peptide derived by alternate posttranslational processing of proGIP.

In the present study, we have used a combination of Northern hybridization analysis and cell-mediated transcription assays to identify an established cell line that can be used to examine tissue-specific regulation of GIP. The identification of a surrogate cell line was necessary to enable investigation of the regulation of GIP gene expression because of difficulties encountered when trying to isolate and culture intestinal K cells. Canine K cells, which represent less than 0.1% of duodenal mucosal cells, have been enriched as much as 100-fold; however, the viability of these cells in culture has been limited to less than 2 days (35).

Only one cell line we tested, STC-1, expressed detectable levels of GIP-specific transcripts. The mRNA had an electrophoretic mobility identical to GIP mRNA previously identified in rat duodenal mucosa (17). STC-1 cells were derived from an intestinal endocrine tumor isolated from a transgenic mouse carrying two oncogenes, SV40 large T antigen and polyoma small T antigen, both of which were linked to the rat insulin promoter. Like the tumors that developed in these same transgenic mice, STC-1 cells are plurihormonal and, in addition to GIP, express GLP-I, GLP-II, glicentin, secretin, somatostatin, gastrin-CCK, neurotensin, and pancreatic polypeptide. Rindi et al. (20) suggested that the plurihormonal nature of these tumors arises from the ability of their proliferating cells to switch to multiple differentiated states, a quality that has apparently prevailed in the establishment of the STC-1 cell line (20).

Therefore, like many tumor-derived cell lines, the STC-1 cell line must be viewed as a mixture of different cell types (36, 37), and interactions among the various cell types must be taken into consideration when studying the regulation of hormone expression. Interestingly, while attempting to clone a pure GIP-expressing cell from parental STC-1 cells, Kieffer et al. (21) found that only 30% of the expanded clone still expressed GIP immunoreactivity, suggesting that dedifferentiation of STC-1 cells was possible.

Despite the heterogeneous nature of the STC-1 cells, they have been used as a convenient model to study both gastrointestinal peptide release and transcriptional regulation (21, 38, 39). We have used variable length restriction fragments, representing the 5′-flanking region of the rat GIP gene, fused to the luciferase reporter gene in transient transfection assays to investigate GIP gene transcription in STC-1 cells. Two separate regions of the GIP gene were able to promote gene transcription in STC-1 cells. The major promoter located upstream has been previously identified by primer extension analysis and RNase protection analysis of GIP mRNA expressed in rat duodenal tissue (24). The first 173 base pairs of this promoter encode minimal promoter activity that is lost if base pairs −144 to −173 are deleted. Interestingly, two putative CCAAT boxes are located in this sequence. In addition, the sequence from −154 to −161, TCACCCAT, is similar to the sequence, TCACCA, that is located from −152 to −159 in the human GIP promoter and that confers CAMP responsiveness in a hamster insulinoma cell line (40).

In the present study, the addition of base pairs −183 to −193 resulted in a significant increase in promoter activity, suggesting the presence of an enhancer sequence in this region. The further addition of sequences 5′ to position −193 up to −963 did not significantly change the level of promoter activity. We have also found similar rates of transcription with a sequence that extends to approximately position −2500 of the rat GIP gene. This observation does not preclude the presence of additional tissue-specific enhancers as well as repressors upstream of position −193. Earlier studies of the proglucagon promoter demonstrated that sequences that were capable of promoting gene expression in STC-1 cells could not promote intestine-specific gene expression in a transgenic mouse model. A 5′-flanking segment extending only to position −238 promoted enhanced transcriptional activity in STC-1 cells, while a seg-

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**Figure 5.** Cell-specific gene transcription promoted by the rat GIP 5′-flanking sequences. Three plasmids containing GIP-Luc chimeric genes were co-transfected with pCMVβ-gal into a number of established mammalian cell lines. Cells were harvested 48 h after transfection, and cell lysates were analyzed for luciferase and β-galactosidase activities. The data represent the mean activity ± S.E. of six separate transfections that are normalized to the activity of the promoterless construct pGL2-basic after correcting for differences in transfection efficiencies by measurement of β-galactosidase activity.
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...extending past position −1400 was required for intestine-specific promoter activity in the transgenic model (39).

The sequence between base pairs −173 and −193 contains two consensus GATA binding motifs. In the present study, a mutational analysis of this region demonstrated the upstream motif to be more important than the downstream sequence in enhancing expression of the GIP gene. Disrupting this motif resulted in a loss of 90% of the activity. Interestingly, of the two GATA motifs, only the sequence of the upstream motif is conserved between rats and humans (41). DNA regulatory elements conforming to the sequence (A/T)GATA(A/G) were first identified to play a role in the transcriptional regulation of erythroid genes (42). Since then, GATA binding motifs have been implicated in the tissue-specific expression of numerous other genes. (43–45). Intestinal K cells that express GIP are thought to be derived from the endomembrane during development like other neuroendocrine tissue. Since GATA-binding proteins, including GATAA/5 and 6 (25, 46, 47), have been found in a number of different endodermally derived tissues, it is possible that the upstream GATA motif in the GIP gene could represent a target for one of these transcription factors.

The second GIP promoter, located within the first intron, conferred only minimal promoter activity in STC-1 cells. This more proximal promoter does contain a TATAA box, as well as a putative CCAAT box, but does not include an enhancer core consensus sequence (24). The rate of transcription of this promoter was not affected by putative enhancers located between base pairs −173 and −193. This was demonstrated by the removal of sequences required for the initiation of transcription of the primary (or distal) promoter (+111 to +781) that conferred minimal gene transcription. The resulting GIP-Luc chimera produced the same transcriptional activity as the GIP-10. Yamagishi, T., and Debas, H. T. (1980) Gastroenterology 78, 537–541.

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