The Sp transcription factors are involved in the cellular expression of the human glucose-dependent insulino tropic polypeptide receptor gene and overexpressed in adrenals of patients with Cushing’s syndrome

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Abstract

The best characterized effect of glucose-dependent insulino tropic polypeptide (GIP) is its stimulatory effect on insulin secretion by pancreatic β-cells. Recently, it was demonstrated that some cases of primary adrenal Cushing’s syndrome were secondary to the ectopic expression of non-mutated GIP receptor (GIP-R) in bilateral adrenal hyperplasias or unilateral adrenal adenomas, resulting in food-dependent steroidogenesis. Using a human multiple-expression tissue array, GIP-R was found to be expressed in a large number of human adult and fetal tissues, but not in the adrenal gland. The analysis of the promoter region of human (h) GIP-R gene revealed six consensus sequences important in regulating the reporter gene activity and capable of binding to Sp1 and Sp3 transcription factors. Data obtained by gene array and semi-quantitative RT-PCR showed an increase in the expression of Sp3 and CRSP9 (co-regulator of Sp1 transcription factor, subunit 9) in the adrenal adenomas or bilateral macronodular hyperplasias of patients with GIP-dependent Cushing’s syndrome; they were, however, also increased in some patients with non-GIP-dependent cortisol-secreting adenomas or with ACTH-dependent Cushing’s disease. This study represents the first step in our understanding of the mechanisms involved in the regulation of the expression of the hGIP-R gene.

Journal of Molecular Endocrinology (2005) 35, 61–71

Introduction

Glucose-dependent insulino tropic polypeptide (GIP) regulates the secretion of insulin by pancreatic β-cells (Dupré et al. 1973). In rat, GIP receptor (GIP-R) mRNA is expressed not only in pancreatic β-cells, but also in various other tissues, including the gut, heart, pituitary and brain (Usdin et al. 1993). GIP-R was also detected in the rat adrenal cortex where it is coupled to corticosterone synthesis (Mazzocchi et al. 1999). In human tissues, GIP-R distribution has not been widely studied, but GIP-R mRNA is expressed in the pancreas (Gremlich et al. 1995) and brain (Chabre et al. 1998). In contrast to rat, GIP-R is not expressed or functionally coupled to steroidogenesis in the human fetal or adult adrenal cortex (Chabre et al. 1998, Lebrethon et al. 1998, Luton et al. 1998, N’Diaye et al. 1998, 1999). Several groups have shown that some cortisol- and other steroid-producing unilateral adrenal tumors or bilateral macronodular adrenal hyperplasia may be controlled by the aberrant expression of a diversity of membrane hormone receptors (Lacroix et al. 2001, 2004, Bertagna et al. 2003).

Hamet et al. (1987) were the first to identify food-dependent cortisol production in a patient with unilateral adrenal adenoma and Cushing’s syndrome. Further studies demonstrated the involvement of ectopic GIP-R expression in the adrenal tissues of patients with GIP-dependent Cushing’s syndrome and bilateral macronodular hyperplasia (Lacroix et al. 1992, Reznik et al. 1992). GIP-dependent Cushing’s syndrome has now been identified in at least 17 patients with adrenocorticotropin (ACTH)-independent macronodular adrenal hyperplasia (Chabre et al. 1998, Lebrethon et al. 1998, N’Diaye et al. 1999, Pralong et al. 1999, Croughs et al. 2000, Gerl et al. 2000, Lacroix et al. 2001, Groussin et al. 2002, Bertagna et al. 2003) and seven with unilateral adenoma (Hamet et al. 1987, De Herder et al. 1996, Chabre et al. 1998, Luton et al. 1998, Tsagarakis et al. 2001). Sequence analysis of the full-length cDNA of GIP-dependent adrenal tissues revealed no GIP-R mutation in the affected adenomas or macronodular hyperplasia of these patients (N’Diaye et al. 1998). Recent analysis also showed that no mutation was present in the promoter region of the human (h) GIP-R gene in the adrenal tissues of patients.
with GIP-dependent Cushing’s syndrome compared with normal controls (Antonini et al. 2004). This suggests that abnormalities in transcription factors or co-factors could be responsible for the ectopic GIP-R expression in these patients.

The recently cloned rat promoter does not have TATA or CAAT boxes but contains potential consensus sequences for Sp1, CREB and Oct-1 transcription factors (Boylan et al. 1999). The molecular mechanisms responsible for human GIP-R expression are still unknown. To identify some of the transcription factors involved in the expression of the hGIP-R gene, we partially characterized its proximal promoter region.

**Experimental procedures**

**Human multiple-expression tissue array**

A membrane containing mRNA from a large number of adult and fetal tissues was purchased from Clontech (BD Bioscience, Palo Alto, CA, USA). An hGIP-R cDNA probe corresponding to nucleotides 387–1195 and a ubiquitin probe (Clontech) were labeled with [α-32P]dCTP, using the Klenow fragment (Invitrogen, Carlsbad, CA, USA). Hybridization was carried out under conditions described in the protocol from Clontech. Detection was conducted with a PhosphorImager after a 24 h exposure.

**Functional analysis of the hGIP-R gene promoter**

Deleted fragments of the hGIP-R gene promoter were amplified by genomic PCR. Mutated fragments were prepared by two rounds of PCR as described by Chen & Przybyle (1994). The fragments were sub-cloned into a pGL-3 Basic vector (Promega Biosciences, San Luis Obispo, CA, USA) and the constructs were verified by sequencing. Human gastric HGT-1 (kindly gifted by Dr C L Laboisse, INSERM, Nantes, France) and mouse adrenocortical Y1 (American Type Culture Collection, Manassas, VA, USA) cell lines were plated in 60 mm dishes in DMEM+ (Invitrogen) supplemented with 10% fetal bovine serum (BioMedia, Drummondville, QC, Canada) and containing 100 µg/ml penicillin/streptomycin (Sigma-Aldrich). The different promoter reporter gene constructs (2·5 µg) were transiently co-transfected with an RSV β-gal-containing plasmid (2·5 µg) by the calcium phosphate precipitation method. The media were changed 24 h after transfections. β-Gal and luciferase activities were measured 48 h after transfection. Luciferase activity was normalized with respect to β-gal activity.

**Nuclear extracts and electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were prepared according to the method of Andrews & Faller (1991). The probes (see Fig. 3) were labeled with [α-32P]dCTP, using the Klenow fragment. Binding reactions were prepared in a final volume of 30 µl (24 mM Hepes, 3 mM MgCl2, 70 mM KCl, 24% glycerol, 3 mM dithiothreitol (DTT), 600 µg/µl BSA, 1 µg/µl poly(dI-dC), 5 µg nuclear extracts, 150 000 c.p.m. labeled probe) and incubated for 30 min at room temperature. For competition assays, unlabeled specific or non-specific (5’ TCTGGAGGGGTACGTGTAC ACAGGAAGTGACAGTTTTC 3’) fragments were added simultaneously with the labeled fragment. When antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was included in the reaction, nuclear extracts and antibody were pre-incubated for 15 min at room temperature before addition of the probe. Complexes were separated on non-denaturing 4% polyacrylamide gel in 0·5 × Tris–borate–EDTA buffer. Detection was undertaken with a PhosphorImager after a 24 h exposure.

**Human adrenal tissues studied**

Adrenal tissues were collected for this study from patients with Cushing’s syndrome associated with either GIP-dependent Cushing’s syndrome with confirmed ectopic GIP-R expression (one patient with unilateral adenaoma and six patients with bilateral macronodular adrenal hyperplasia), non-GIP-dependent unilateral adrenal adenoma (seven patients), ACTH-dependent Cushing’s disease (seven patients) or adrenal carcinoma (two patients), and from three normal adrenal of patients undergoing radical nephrectomy. Commercially available RNA (from a pool of 52 adrenals and from two other normal individuals) was also used as normal control (Clontech and Ambion (Austin, TX, USA) respectively). Informed consent was obtained from each patient and this study was approved by the institutional ethics committee. Tissue specimens obtained at surgery were immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Total RNA was extracted with Trizol reagent (Invitrogen). RNA (3 µg) was reverse-transcribed using M-MLV RT (Invitrogen) in a final volume of 20 µl (1 × first-strand buffer; 10 mM DTT; 0·5 mM dNTPs; 0·01 µg/µl random primers; 1 U/µl RNase out; 1 U/µl M-MLV RT). PCR was performed using TAAQ DNA polymerase (Invitrogen) in a final volume of 50 µl (1× TAAQ DNA polymerase buffer; 0·2 mM specific sense and antisense probes; 0·2 µM 18S probe (Ambion); 0·2 mM dNTPs; 2 mM MgCl2; 2 µl cDNA; 0·05 U/µl TAAQ DNA polymerase). The linear range for PCR amplification was determined as described in the Quantum RNA 18S internal control (Antonini et al. 2004). This suggests respect to
The number of cycles used for each PCR amplification are indicated in the figure legends. PCR products were separated in a 2% agarose gel and detected using a PhosphorImager. Data are expressed as means ± s.d. Student’s t-test was used in the statistical analysis and a $P<0.05$ was considered statistically significant.

**Results**

**GIP-R expression in human tissues**

Distribution of GIP-R mRNA in human tissues was determined by a multiple-expression tissue assay with a blot containing mRNA from 65 adult and seven fetal tissues. In adult tissues (Fig. 1A, lanes A–I), maximal expression was found in the pancreas and trachea. GIP-R was also detected, at a lower level, in the brain, heart, gut, spleen, thymus, blood cells, lung and kidney, whereas it was not seen in the liver, placenta, testis, uterus and adrenal gland. In fetal tissues (Fig. 1A, lane J), the messenger for GIP-R was found in the lung, heart and kidney, but not in the brain, liver, spleen and thymus. Specificity of the probe was confirmed by the absence of signal in the control samples (Fig. 1A, lanes K1–K6). Some of these results were also confirmed by semi-quantitative RT-PCR (Fig. 1B). The expected bands for GIP-R were clearly present in pancreas, brain, trachea, kidney and small intestine, but were only faintly detected in adrenal gland.

**Transfection of deleted fragments of the hGIP-R gene promoter**

The sequences for the hGIP-R mRNA and promoter are available from GeneBank (NM000164 and AC006132 respectively). The exact localization of the transcription start site is still unknown but we used the 5’-end of the published cDNA sequence as the −1 nucleotide to identify the constructs.

To identify regions of interest, we transfected deleted fragments of the hGIP-R promoter up to 2 kb in HGT-1 and Y1 cells expressing respectively high and low levels of GIP-R mRNA (Fig. 2A). Transfection of HGT-1 cells with deleted fragments from −2068 to −595 bp showed an increase of ~5 fold of the control vector in relative luciferase activity whereas, transfection of Y1 cells with these fragments failed to elevate the luciferase activity higher than the control vector. These results suggest that different transcription factors could be able to bind that region of the promoter depending of the cell lines. Deletion of the region between −595 and −473 bp led to a decrease in relative luciferase activity to levels comparable with the control luciferase vector in HGT-1 cells. This observation suggests the presence of a potential response element for an activating factor between −595 and −473 bp. In Y1 cells, no modification of the relative luciferase activity was found. Further deletion until −336 bp induced significant elevation of relative luciferase activity in HGT-1 and Y1 cells. The binding of an inhibitor between −473 and −336 bp is probably responsible for that effect. Our hypothesis to explain the effect observed between −336 and −595 bp is that the loss of inhibition with the construct GIPR−595/+73 is due to the binding of an activator that interferes with the factors capable of interaction with the inhibitory binding site. Relative luciferase activity was maintained with fragments up to −100 bp, but was not detected with the −49 bp fragment. To ensure that the differences observed in the promoter activity in HGT-1 and Y1 cell lines was not due to the species used, we also transfected some of the constructs in the human HeLa cell line that expresses a low level of GIP-R mRNA. Relative luciferase activity was similar in Y1 and HeLa cell lines.

**Characterization of the basal activity of the hGIP-R promoter**

The proximal promoter region of the hGIP-R gene revealed a GC-rich region without TATA or CAAT boxes, but contains multiple GC ($−237/−242$, $−77/−82$, $−65/−70$, $−48/−53$) or GT ($−223/−229$, $−150/−156$) boxes (Fig. 3). The sites are potential response elements for transcription factor Sp1.

To better characterize this region of the promoter, we transfected constructs that contained an increasing number of GC/GT boxes in HGT-1 cell lines (Fig. 2B). We also transfected constructs with punctual mutations within the consensus sequences (Fig. 2C). The short 49 bp fragment of the hGIP-R promoter, which does not contain any consensus for transcription factors, did not confer transcriptional activity compared with the control. Minimal basal luciferase activity was observed with the construct GIP-R−66/+73 that includes the GC-4 box. We also observed that single mutation of GC-4 led to the abolition of transcriptional activity of the promoter. Taken together, these results suggest that the GC-4 site is essential but not sufficient to confer high luciferase activity. Maximal luciferase activity is detected with the addition of the GC-3 box (construct GIP-R−75/+73). Mutation of the GC-3 binding site also led to the abolition of transcriptional activity of the promoter showing that GC-3 is also necessary to confer luciferase activity. The presence of other GC and GT boxes caused only a small change in the promoter activity. Surprisingly, an inactivating mutation of GC-1, GC-2, GT-1 and GT-2 increased the luciferase activity of the promoter. Interactions between protein complexes could be an explanation of these two discordant observations.
Figure 1 Expression of GIP-R gene in human adult and fetal tissues. (A) Multiple-expression tissue array. Hybridization was carried out under conditions described in the protocol from Clontech with an hGIP-R probe (upper panel) and a ubiquitin probe (lower panel). Detection was performed with the PhosphoImager after 3 days of exposure for the hGIP-R probe and 2 h for the ubiquitin probe. (B) Semi-quantitative RT-PCR. RNA from human adult tissues was reverse-transcribed and then amplified using specific primers for hGIP-R (sense: 5'GGGACAGGCCTGATCGCCCCT3'; antisense: 5'TGTAGCCGCCCCTGAACAACTC3') (upper panel) and 18S gene (lower panel) for 30 cycles (95°C/15 s, 55°C/10 s, 72°C/1 min). PCR products were run on a 2% agarose gel and detection was performed using a PhosphorImager.
Figure 2 Functional analysis of the hGIP-R gene promoter. Fragments of the hGIP-R promoter were transiently co-transfected with a β-gal-containing vector. All measurements were taken in duplicate from three independent experiments. Error bars represent S.D. and the results expressed as fold of promoter-less reporter gene vector activity or as percentage of wild-type. (A) Deleted fragment up to 2 kb. (B) Fragments containing an increasing number of binding sites for transcription factor Sp1. (C) Mutated fragments.

Figure 3 Nucleotide sequence of the hGIP-R gene promoter. The numbers beside the sequence indicate the nucleotide position relative to the 5'-end of the cDNA. Potential transcription factors are in bold. Sequences corresponding to EMSA probes are boxed and mutations introduced in the sequence are described.
To identify proteins capable of binding to the GC/GT boxes, we performed EMSAs. Nuclear extracts from HGT-1 cells were incubated with DNA fragments containing one of the GC or GT boxes. The resulting complexes were resolved on non-denaturing polyacrylamide gel (Fig. 4). We observed the formation of four complexes in the presence of the GT-1 probe (Fig. 4D, lane 1) and GC-4 probe (data not shown), five complexes with the GC-2 probe (Fig. 4B, lane 1) and six complexes with the GC-1 (Fig. 4A, lane 1), GC-3 (Fig. 4C, lane 1) and GT-2 probes (Fig. 4E, lane 1). The specificity of binding was demonstrated by competition assay. The complexes were displaced by an increasing amount of unlabeled probe (data not shown). A 100-fold excess of unlabeled specific probe completely abolished the DNA–protein interactions (lane 2). A 100-fold excess of an unlabeled non-specific probe had no effect (lane 3) on the complexes. GC/GT boxes are potential binding sites for the Sp1 transcription factor. To determine if Sp1 was capable of binding to these probes, we incubated nuclear extracts with Sp1 antibody (lane 4). A supershifted band was produced with a parallel decrease in the abundance of slowly migrating C1 complex using the GC-1, GC-3 and GT-2 probes, whereas the complex C1 disappeared when the Sp1 antibody was pre-incubated with nuclear extracts prior to addition of the GC-2 and GT-1 probes. Transcription factor Sp3 also binds GC/GT-rich element (lane 5). Pre-incubation with Sp3 antibody blocked the formation of the C2 and C3 complexes for the GC-2, GC-3 and GT-1 probes, whereas only the complex C2 was lost in the presence of the GT-2 probe. Pre-incubation of the nuclear extracts with the Sp3 antibody prior to incubation with the GC-1 probe leads to loss of C2 and C4 complexes. Sp1 and Sp3 antibodies were unable to displace the complexes observed in the presence of the GC-4 probe. Pre-incubation of the nuclear extracts with pre-immune serum had no effect on migration of the complexes showing the specificity for the interactions between the antibodies and the shifted complexes (lane 6). Incubating recombinant Sp1 protein in the presence of the wild-type GIP-R promoter probes leads to the formation of only one complex that corresponds to the complex C1 shifted by the Sp1 antibody (data not shown). These data confirm the

**Figure 4 EMSAs.** Nuclear extracts from HGT-1 were incubated in the presence of radiolabeled probe each containing one GC or GT boxes. For competition assays, unlabeled fragment was added simultaneously with the labeled fragment, while antibodies were pre-incubated for 15 min at room temperature before addition of the labeled probe. Complexes were separated on non-denaturing 4% polyacrylamide gel. Detection was performed by PhosphoImager after a 24 h exposure. (A) DNA fragment containing GC-1; (B) DNA fragment containing GC-2; (C) DNA fragment containing GC-3; (D) DNA fragment containing GT-1; (E) DNA fragment containing GT-2. Lane 1: incubation with the labeled probe; lanes 2 and 3: competition with 100×excess of unlabeled specific and non-specific probes respectively; lanes 4 and 5: incubation with antibody against Sp1 and Sp3 respectively; lane 6: incubation with pre-immune serum.
The presence of the Sp1 transcription factor in the slowly migrating complex.

**Expression of Sp3 and CRSP9 (co-regulator of Sp1 transcription factor, subunit 9) in Cushing’s syndrome patients**

To identify whether Sp transcription factor family genes are regulated in adrenal tissues of patients with Cushing’s syndrome, we performed further analysis by gene array of samples from patients with macronodular adrenal hyperplasia previously described (Bourdeau et al. 2004). We found overexpression of the transcription factor Sp3 and the co-factor CRSP9 in adrenal tissues from three patients with GIP-dependent Cushing’s syndrome compared with a pool of normal adrenal tissues. These preliminary results were extended by semi-quantitative RT-PCR in a larger number of adrenal tissues from patients with GIP-dependent Cushing’s syndrome compared with a normal control (Fig. 5A, Fig. 6A). We also observed a 1.5-fold increase in the expression of Sp3 and CRSP9 in four out of seven cases of Cushing’s disease (Fig. 5B, Fig. 6B). In non-GIP-dependent cortisol-secreting adrenal adenoma, Sp3 mRNA was increased 1.3-fold in three out of seven patients (Fig. 5C), while the level of CRSP9 mRNA was not changed (Fig. 6C). No significant difference was observed in two adrenal carcinomas (Fig. 5D, Fig. 6D). GIP-R was not expressed in non-GIP-dependent Cushing’s syndrome patients overexpressing CRSP9 and/or Sp3 (Fig. 7).

**Discussion**

GIP-R is a G protein-coupled receptor widely expressed in rat tissues (Usdin et al. 1993). We now demonstrate that GIP-R mRNA is more widely distributed in human
tissues than expected from its relatively restricted known biological activities. Identification of GIP-R mRNA in the pancreas is consistent with the role of GIP in the regulation of insulin secretion by the pancreatic β-cells (Dupré et al. 1973). As previously described in rat (Usdin et al. 1993) and in human (Chabre et al. 1998) we also identified GIP-R mRNA in several regions of the brain. GIP-R mRNA was also detected in the trachea, heart, gut, spleen, thymus, blood cells, lung, kidney and thyroid gland. In fetal tissues, GIP-R mRNA was found in lung, heart and kidney. The identification of the messenger for GIP-R in these tissues suggests a number of unknown actions for GIP. However, it remains to be established whether GIP-R mRNA is also translated into functional protein in all these tissues.

GIP-R has been identified and shown to be coupled to steroidogenesis in rat adrenocortical cells (Mazzocchi et al. 1999). In this study, we show that GIP-R mRNA is not or very weakly expressed in the normal human adult adrenal gland. The faint band detected after PCR amplification is not or very weakly expressed in the normal human adult adrenal gland. Data result from three independent experiments (n=3) performed in duplicate and are expressed as means±s.d. Student’s t-test was used on the statistical analysis and a P value less than 0.05 was considered statistically significant. *P≤0.05, **P≤0.01, ***P≤0.001. (A) GIP-dependent Cushing’s syndrome (‘GIP’); (B) Cushing’s disease (‘CD’); (C) adrenal adenoma (‘A’); (D) adrenal carcinoma (‘C’).
Figure 7 Expression of GIP-R gene in Cushing’s syndrome patients. RNA was reverse-transcribed and then amplified using specific primers for hGIP-R (sense: 5’-GGGACAGGCCGCTGATCGCCCTC3’; antisense: 5’-TGTAGCGCCGCTGAACAAACTC3’) and 18S gene for 30 cycles (95°C/15 s, 55°C/10 s, 72°C/1 min). PCR products were run on a 2% agarose gel and detection was performed using Phosphorlmager. (A) Level of hGIP-R mRNA in adrenal tissues from seven patients with Cushing’s disease (‘CD’) as compared with one patient with GIP-dependent macronodular adrenal hyperplasia (‘GIP1’) and a normal adrenal gland. (B) Level of GIP-R mRNA in seven patients with cortisol-secreting adrenal adenoma (‘A’) compared with one patient with GIP-dependent macronodular adrenal hyperplasia (‘GIP1’) and normal adrenal gland. (C) Level of 18S mRNA.

In conclusion, the hGIP-R gene promoter is TATA-less and contains multiple Sp1/Sp3 binding sites, which appear to be involved in the cellular expression of the receptor. This is similar to previous studies of other members of this family of seven trans-membrane hormone receptors such as VIP, glucagon, PTH and GLP-1 receptors. Further studies will be necessary to identify the tissue-specific transcription factors that are involved in the normal and ectopic expression of hGIPR resulting in GIP-dependent Cushing’s syndrome.

Acknowledgements

The authors acknowledge the editorial assistance of Mr O DaSilva, Editor, Research Support Office, Research Center, Centre Hospitalier de l’Université de Montréal.
This work was supported by Grant MT-13189 from the Canadian Institutes of Health Research. P-O D was supported by Fonds de Recherche en Santé du Québec. The authors declared that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 6 March 2005
Accepted 18 April 2005