Identification of Two Missense Mutations in the GIP Receptor Gene: A Functional Study and Association Analysis with NIDDM

No Evidence of Association with Japanese NIDDM Subjects

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Gastric inhibitory polypeptide (GIP) potently stimulates insulin secretion from pancreatic islets in the presence of glucose as an incretin. Because the insulinotropic effect of GIP is reduced in NIDDM, it should be clarified whether defects in the GIP receptor gene contribute to the impaired insulin secretion in NIDDM. Using genomic DNA samples from Japanese NIDDM and non-NIDDM subjects, we have investigated the entire coding region of the GIP receptor gene by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP). We have identified two missense mutations, Gly^{198}→Cys (Gly198Cys) in exon 7 and Glu^{354}→Gln (Glu354Gln) in exon 12. Investigation of the function of GIP receptor with either of these mutations reveals a half-maximal stimulation value of GIP-induced cAMP response in Chinese hamster ovary cells expressing the GIP receptor with Gly198Cys of 6.3 ± 1.2 × 10^{-10} mol/l (*n* = 3), which was considerably higher than that of the normal GIP receptor, 9.4 ± 3.8 × 10^{-12} mol/l GIP (*n* = 3), whereas that of the GIP receptor with Glu354Gln was not significantly different from that of the normal GIP receptor. To assess the possible role of the GIP receptor gene in genetic susceptibility to NIDDM, we have examined the allelic frequencies of Gly198Cys and Glu354Gln in NIDDM and control subjects. Association studies show no relationship between NIDDM and either of the two mutations. *Diabetes* 45:1701–1705, 1996

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CHO, Chinese hamster ovary; ED_{50}, half maximal stimulation; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; IBMX, 3-isobutyl-1-methylxanthine; α MEM, α minimal essential medium; PCR, polymerase chain reaction; SSCP, single strand conformational polymorphism; VIP, vasoactive intestinal peptide.

NIDDM is a heterogeneous metabolic disorder characterized by defects in insulin secretion as well as in insulin action (1–3). Genetic factors play a well-known, important role in the development of NIDDM (4). Investigation of the candidate genes that are involved in maintaining the glucose homeostasis has been intense, and mutations in the genes encoding glucokinase (5–7), insulin (8), insulin receptor (9), glucagon receptor (10), and mutations in mitochondrial DNA (11,12) have been identified so far. These mutations, however, account for a cause of NIDDM in only a small percentage of NIDDM subjects, and the etiology of most NIDDM remains unknown.

Gastric inhibitory polypeptide (GIP) is a 42–amino acid hormone belonging to the vasoactive intestinal peptide (VIP)/glucagon/secretin family (13–16). It was first isolated from porcine small intestine on the basis of its ability to inhibit gastric acid secretion (13). Subsequent studies of the physiological roles of GIP have demonstrated that GIP also potently stimulates insulin secretion in the presence of elevated glucose as an incretin (17,18). Thus, GIP is now referred to as glucose-dependent insulinotropic polypeptide. Stimulation of specific receptors on pancreatic β-cells by GIP activates adenylyl cyclase, which increases the intracellular cAMP concentration and results in the increased secretion of insulin. A recent study demonstrated that insulinotropic effect of GIP was selectively reduced in diabetic patients, whereas that of glucagon-like peptide-1 (GLP-1) was preserved (19), suggesting that a dysfunction of GIP may be involved in the pathogenesis of diabetes mellitus. The GIP receptor gene, therefore, is one of the candidate genes in the pathogenesis and development of NIDDM. Recently, GIP receptor cDNAs of several species have been isolated (20–23). Since we isolated the human GIP receptor gene (22), we have analyzed the coding region of GIP receptor gene in NIDDM patients by polymerase chain reaction-single strand conformational polymorphism (PCR-SSCP) analysis and have identified two missense mutations in the GIP receptor gene.

TABLE 1
Oligonucleotide primers for PCR amplification of GIP receptor gene

exon 2	5′-TGGCACGAACCAGACCCTTCG-3′ 5′-GAGCTCCTCTGGCTCCTCG-3′	exon 9	5′-TGAGCGCGCTGACAGCTGC-3′ 5′-TCACAACCCGCCACAGTGC-3′
exon 3	5′-TGGGCCACATCGGACCTAGCA-3′ 5′-CTCTCAGCCTCAGGTCCTC-3′	exon 10	5′-CTCTTAAGCTCTACTCCGCCT-3′ 5′-CAGCCCACTTCCCAGAATGG-3′
exon 4	5′-AGTAGGTCCACTGGGCACC-3′ 5′-GGGGCAAAGGTTAGCAGTTG-3′	exon 11	5′-TGGCTCAGCCCTTAATCTCTC-3′ 5′-CCTCGGTCCCCAACGCATG-3′
exon 5	5′-GTGCCTTAATTCCCTCTCTT-3′ 5′-CTGGACTCCTAACCTGAAG-3′	exon 12	5′-TGCGGGATCACTGCTGCCG-3′ 5′-GAAGGATGGAGCCCCTGCA-3′
exon 6	5′-GAGCCAAGCCTTGTTCCCTT-3′ 5′-ATCTCTGCCGCCACCACTCA-3′	exon 13	5′-ACCAGCGATGTAACCTCCG-3′ 5′-TGAGACGATGGCGCTCAGA-3′
exon 7	5′-ACACTGCCTTCCAAATTCCC-3′ 5′-CCAGAGAAGCAAGATTCCCA-3′	exon 14	5′-TCTGAGCGCCATCGTCTCA-3′ 5′-AATCCATGCTAACTGAACAG-3′
exon 8	5′-GCTGACTACCCCTCTACCG-3′ 5′-GGGGACGGGATCGGAGCT-3′		

RESEARCH DESIGN AND METHODS

Subjects. Twenty unrelated Japanese patients with NIDDM diagnosed according to World Health Organization criteria and 20 nondiabetic Japanese control subjects contributed samples for primary screening by SSCP. For association studies, 280 patients with NIDDM and 210 control individuals matched for age and sex were used.

PCR-SSCP. Genomic DNA was extracted from peripheral blood cells using DNA extractor WB kit (Wako, Osaka, Japan). The protein-coding regions of exon 2 through 14 of the GIP receptor gene were amplified by the PCR method and analyzed by the PCR-SSCP procedure (24). The PCR reaction was performed in a volume of 10 µl containing 0.1 µg of genomic DNA; 50 mmol/l KCl; 10 mmol/l Tris-HCl (pH 9.0 at 25°C); 2.0 mmol/l $MgCl_2$; 0.1% Triton X-100; 200 µmol/l each of dATP, dCTP, dGTP, and dTTP; 500 nmol/l Cy5-labeled oligonucleotide primers (Pharmacia Biotech, Tokyo, Japan), and 0.25 U Tao DNA polymerase (Toyota, Osaka, Japan). After an initial denaturing at 94°C for 3 min, the samples were subjected to 40 cycles of amplification with a Perkin-Elmer Setups DNA Thermal Cycle, denaturation at 94°C for 15 s, and annealing at 55°C for 15 s with extension at 72°C for 30 s. An aliquot of the reaction was mixed with formamide/blue Dextran gel-loading buffer, heated at 94°C for 3 min, and separated on 6% Long Ranger gels with or without 5% glycerol at 35 W constant power at 25°C using the ALF DNA sequencer (Pharmacia, Uppsala, Sweden). For initial screening of the polymorphisms, the reaction was carried out in a solution containing unlabeled primers and [^{32}P]dCTP and was electrophoresed as described previously (25).

Direct sequence analysis. Samples that showed aberrant bands were amplified by PCR, and the PCR products were subjected to direct dideoxy sequence analysis using Δ Tth polymerase autosequencer core kit (Toyota). Direct sequence analyses were carried out in both sense and antisense strands.

Establishment of Chinese hamster ovary (CHO) cells expressing normal or mutant GIP receptors. CHO cells were grown in monolayers in α minimal essential medium (α MEM, GIBCO/BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in 5% CO_2 at 37°C. In nontransfected CHO cells, no GIP receptor was detected (21). Full-length GIP receptor cDNA having Cys198 or Gln354 was constructed by the PCR-based site-directed mutagenesis method. The cDNA fragments were subcloned into the expression vector, pCMV6b (26), and the resulting constructs were cotransfected with pRSVneo into the CHO cells using Lipofectin reagent (GIBCO/BRL). Stable transfectants were selected in α MEM containing 400 µg/ml of G418.

Measurements of cAMP formation. cAMP formation was measured as previously described (26). Briefly, CHO cells were grown to confluency in 12-well plates. The cells were washed twice, and the reaction was started in 500 µl of Krebs-Ringer buffer containing 1 mmol/l 3-isobutyl-1-methylxanthine (IBMX) with or without GIP (Peptide Institute, Osaka, Japan) at the

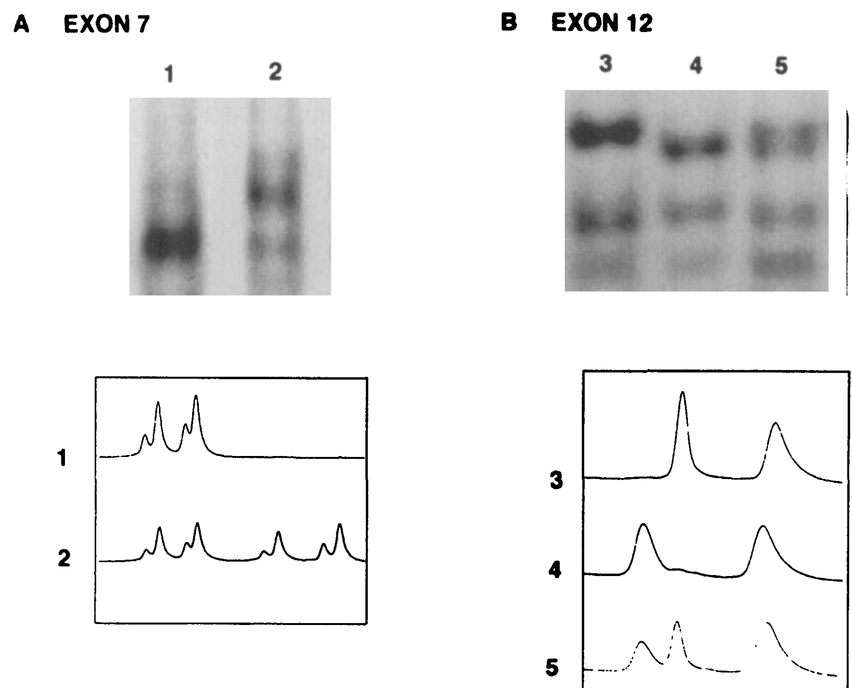

FIG. 1. PCR-SSCP analyses of GIP receptor gene. The results of SSCP analyses of exon 7 (*A*) and exon 12 (*B*) by two different methods (upper panel, autoradiography of SSCP analysis using [^{32}P]dCTP; lower panel, SSCP analysis with the use of Cy5-labeled primers) of the same samples are shown. *A*: *lane 1* shows a SSCP pattern for a normal homozygous subject; *lane 2* shows a SSCP pattern for a heterozygous subject for codon 194 mutation. *B*: *lane 3* shows a SSCP pattern for a normal homozygous subject; *lane 4* corresponds to a homozygous subject; and *lane 5* corresponds to a heterozygous subject of codon 354 mutation.

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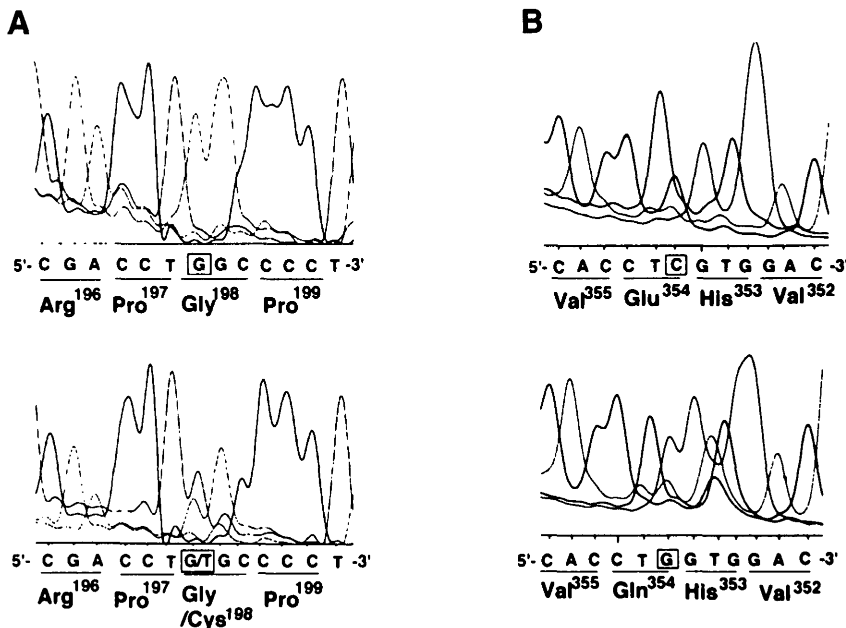

FIG. 2. Direct sequence analyses of PCR-amplified DNA of exon 7 (*A*) and exon 12 (*B*). Direct sequence analyses were carried out in both sense and antisense strands of each exon. *A*: sequencing of sense strands of a normal subject (upper panel) and a heterozygous subject (lower panel) are shown. In codon 194, a missense mutation from GGC to TGC was detected in a heterozygous subject. The same mutation was confirmed in the sequencing of the antisense strand of this subject. *B*: sequencing of antisense strands of a normal subject (upper panel) and a homozygous subject (lower panel) are shown. In codon 354, a missense mutation from CTC to CTG was detected in a homozygous subject. The same mutation was confirmed in the sequencing of the sense strand of this subject.

indicated concentration and incubated at 37°C for 30 min. The reaction was stopped by adding 100 μl of 30% trichloroacetic acid, and cAMP levels were determined with a radioimmunoassay kit (Yamasa, Chiba, Japan).

Statistical analysis. Statistical analyses were performed with the unpaired *t* test for the functional studies and with χ^2 test for the association study.

RESULTS

Identification of two missense mutations in the GIP receptor gene. We have previously reported that the human GIP receptor gene is composed of 14 exons spanning about 13.8 kb (22). According to the exon-intron data, we have analyzed the coding sequence of the GIP receptor gene for mutations by PCR-SSCP. Primer pairs for SSCP were designed in intron sequences to amplify each exon and its exon-intron boundaries (Table 1). Genomic DNA samples derived from the 20 NIDDM patients were submitted for the primary screening, which revealed two SSCP variants in the GIP receptor gene. Figure 1*A* shows the SSCP analysis of exon 7. In *lane 2*, an aberrant band was detected. Direct sequence of this fragment revealed the heterozygote of a normal allele and a mutant one that has a missense mutation from $\underline{G}GC^{198}$ to $\underline{T}GC^{198}$ (Fig. 2*A*), resulting in an amino acid substitution from glycine to cysteine (Gly198Cys). Another SSCP variant was detected in exon 12 (Fig. 1*B*). Sequence analysis of the antisense strand revealed a missense mutation from $CT\underline{C}^{354}$ to $CT\underline{G}^{354}$ (Fig. 2*B*), which also results in an amino acid substitution from glutamic acid to glutamine (Glu354Gln). No aberrant band was detected in any other exon.

Functional analysis of GIP receptors. To elucidate the functional properties of the GIP receptor with these amino acid substitutions, we have constructed two recombinant GIP receptor cDNAs (GIPRM7, Gly198Cys; GIPRM12, Glu354Gln) as described in METHODS. The normal and two mutant GIP receptor cDNAs were subcloned into the expression vector, and each clone was transfected into CHO cells with pRSVneo. More than 12 clonal cell lines were selected by G418 for each GIP receptor clone, and cell lines expressing high levels of normal or mutant GIP receptors were screened by measuring their intracellular cAMP response to the stimulation of 1 μmol/l GIP. Because GIP exerts its biological effects through elevating the intracellular cAMP level, we have analyzed the cAMP response in CHO cells expressing the receptors. Dose-response analyses of GIP-stimulated cAMP formation in CHO cells expressing the normal or either of the two mutant GIP receptors are shown in Fig. 3. In CHO cells expressing the normal GIP receptor, GIP-stimulated cAMP formation in a dose-dependent manner and the maximal stimulation was observed at the concentration of 10^{-10} mol/l GIP. Half-maximal stimulation (ED_{50}) of cAMP formation occurred at $9.4 \pm 3.8 \times 10^{-12}$ M

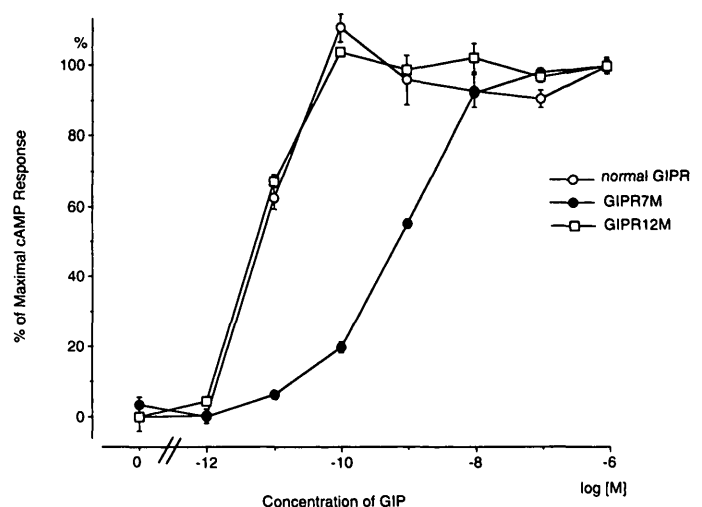

FIG. 3. Dose-response analyses of GIP-induced cAMP formation in CHO cells expressing GIP receptor cDNAs. CHO cells stably expressing normal GIP receptor (○), GIPRM7 (●), and GIPRM12 (□) were incubated with varying concentrations of GIP and intracellular cAMP levels were determined. One representative result of three independent experiments is shown. Values are means ± SE of triplicate determinations

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TABLE 2
Genotype and allelic frequencies of the mutations of GIP receptor gene identified in exon 7 and 12

		Genotype (NN:NMu:MuMu)		Allelic frequencies (N:Mu)	
		NIDDM	Control	NIDDM	Control
Exon 7	Gly^{198}→Cys	257(96.6):8(3.0):1(0.4)	192(96.0):8(4.0):0(0)	98.1:1.9	98.0:2.0
Exon 12	Glu^{354}→Gln	188(68.1):74(26.8):14(5.1)	138(67.7):57(27.9):9(4.4)	81.5:18.5	81.6:18.4

Genotype data are n (%); allelic frequencies are %. N, normal allele; Mu, mutant allele.

GIP (n = 3). In contrast to the normal GIP receptor, the cAMP response was lowered in CHO cells expressing GIPRM7, and the ED_{50} value was $6.3 \pm 1.2 \times 10^{-10}$ (n = 3), which was significantly different from that of the normal GIP receptor ($P < 0.01$). In CHO cells expressing GIPRM12, the ED_{50} value was $7.0 \pm 1.0 \times 10^{-12}$ (n = 3), which was not significantly different from that of the normal GIP receptor. The unstimulated cAMP levels were not significantly different among the three cell lines.

Association studies of the GIP receptor gene and NIDDM. To elucidate the prevalence of these mutations and their association with NIDDM, we have analyzed the frequencies of these mutations using fluorescence-labeled primers and a DNA sequencer in a large population of unrelated NIDDM patients and a control group. Genotype frequencies of normal homozygotes, heterozygotes, and mutant homozygotes of Gly198Cys mutation were 96.6, 3.0, and 0.4% in NIDDM subjects and 96.0, 4.0, and 0% in control subjects, respectively (Table 2). Allelic frequencies of the Gly198Cys mutation were 1.9% in NIDDM and 2.0% in control subjects with no significant difference between two groups. Genotype frequencies of normal homozygotes, heterozygotes, and mutant homozygotes of the Glu354Gln mutation were 68.1, 26.8, and 5.1% in NIDDM subjects and 67.6, 27.9, and 4.4% in control subjects. Allelic frequencies of the Glu354Gln mutation were 18.5% in NIDDM and 18.4% in control subjects with no significant difference between the two groups.

DISCUSSION

The GIP receptor gene is one of the candidate genes in the pathogenesis and development of NIDDM. This is the first report of the screening of the coding regions of the GIP receptor gene in NIDDM patients. In this study, we have identified two missense mutations in the GIP receptor gene, both of which result in an amino acid substitution of GIP receptor protein: Gly^{198}→Cys in exon 7 and Glu^{354}→Gln in exon 12. The hydropathy plot of the GIP receptor protein shows the presence of seven possible transmembrane domains (22), which is a feature of G protein-coupled receptors. Gly^{198} is located in the first extracellular loop, and Glu^{354} is in the sixth transmembrane domain of the receptor (Fig. 4). Both Gly^{198} and Glu^{354} are conserved among human, rat, and hamster GIP receptors (20–23). Because the substitution of thiol-including Cys residue for Gly and that of uncharged Gln residue for acidic Glu might be expected to affect the protein structure and thereby affect the function of the receptor, we subsequently examined the functional properties of the GIP receptor with Gly^{198}→Cys or Glu^{354}→Gln. We have constructed recombinant the GIP receptor cDNA with each of the two missense mutations from the normal GIP receptor cDNA that we previously isolated (22). Using cell lines stably transfected with normal or mutant GIP receptor cDNAs, we have analyzed GIP-stimulated cAMP formation. In CHO cells expressing the normal GIP receptor, GIP stimulates cAMP formation in a dose-dependent manner with an ED_{50} of $9.4 \pm 3.8 \times 10^{-12}$ mol/l. In contrast, cAMP response in CHO cells expressing GIPRM7 is considerably lower than that in CHO cells expressing normal GIP receptor, whereas that of GIPRM12 is not different from that of normal GIP receptor, suggesting that the Gly198Cys substitution may significantly affect the conformation of the GIP receptor protein.

The Gly198Cys substitution strikingly impairs the GIP-induced cAMP elevation, suggesting that it might cause a reduction of GIP-induced insulin secretion. To assess the possible role of the GIP receptor gene in susceptibility to NIDDM, we have examined the allelic frequencies of Gly194Cys and Glu354Gln in NIDDM and control sub-

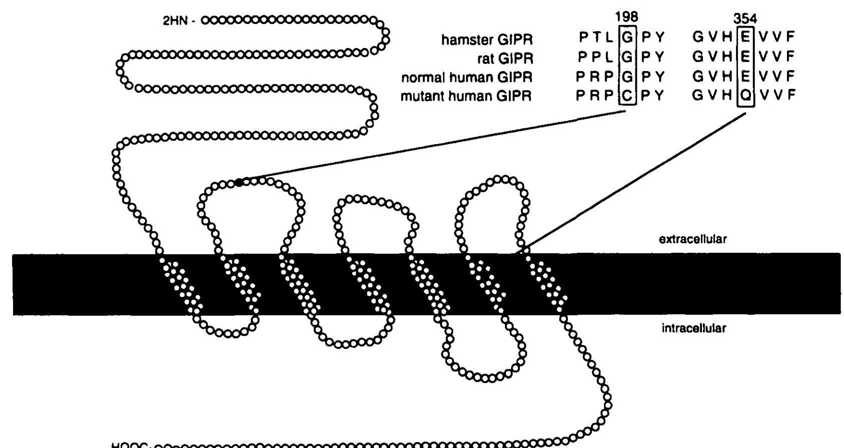

FIG. 4. Structure model of the GIP receptor. Mutated amino acids (●) of the GIP receptor are shown.

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jects. However, neither genotype nor allelic frequency of this mutation is different between Japanese NIDDM patients and non-NIDDM subjects, which suggests that this mutation is not likely to contribute significantly to the loss of GIP-induced insulin secretion in NIDDM patients. We also examined the clinical profiles of the NIDDM subjects with the Gly198Cys substitution, but no common characteristic features were found among them. Because NIDDM is polygenic, the possibility that this mutation is a risk factor in a small population of NIDDM cannot be excluded. The physiological significance of this mutant GIP receptor is unknown. Because a recent study has demonstrated that the GIP receptor also is expressed in α-cells (27), glucagon secretion might also be affected. Additional research is needed to clarify how this mutation affects the islet function.

Possibly, genetic defects in the promoter region of GIP receptor gene might impair the expression of the gene. The promoter region of the GIP receptor gene, however, has not yet been characterized, so we are unable to address the hypothesis that genetic defects in the promoter region might contribute to the loss of GIP-induced insulin secretion in NIDDM patients. In addition, the possibility that a large deletion involving an entire exon, which PCR-SSCP would miss, might be present in NIDDM patients requires additional study.

We have identified two missense mutations in the GIP receptor gene. Although $Gly^{198} \rightarrow Cys$ in exon 7 impairs the function of the GIP receptor, studies find no significant association between this mutation and NIDDM. Another mutation, $Glu^{354} \rightarrow Gln$ in exon 12, which does not alter the function of the receptor and has no association with NIDDM, may be only a silent polymorphism.

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