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Protein Targeting to Glycogen/PPP1R5: Screening of Coding and Flanking Genomic Regions for Polymorphisms and Association Analysis with Insulin Action in Pima Indians

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Insulin resistance, a major predictor of type 2 diabetes mellitus, is genetically inherited in Pima Indians, a population with a high prevalence of the metabolically complex disease. Protein targeting to glycogen/PPP1R5 has recently been identified as a potential regulator of glycogen synthase, the rate-limiting enzyme of the insulin-induced glycogenesis. The gene is located on chromosome 10q23-24, where there is a suggestive linkage to insulin action in this population, establishing it as a functional and positional candidate gene. In this study, we discovered 2 novel polymorphisms upstream of the 5′UTR of the gene, with only one found in Pima Indians, but no polymorphism in the coding sequence. The genotype frequencies of the polymorphism and transcript levels of the gene in skeletal muscle do not correlate with insulin action in the subjects. These results exclude any significant role of protein targeting to glycogen/PPP1R5 in insulin resistance in Pima Indians. © 1999 Academic Press

Type 2 diabetes mellitus is a major public health problem that is increasing in prevalence in the developed world. The Pima Indians of Arizona have the highest reported prevalence of the disease; more than half of the population over 35 is affected [1, 2]. Insulin resistance is manifested as a reduced rate of insulin-stimulated glucose conversion to glycogen in skeletal muscle, a major target tissue of insulin action. This intermediate metabolic state predicts type 2 diabetes [3] and has strong genetic determinants in this population [4]. Since insulin resistance is likely to be modulated by fewer genes than the type 2 diabetes syndrome is, it may be less complicated to evaluate potential contributions of particular genes in this metabolic precursor of the disease.

Insulin resistant Pima Indians have decreased activity of glycogen synthase (GS), the rate-limiting enzyme in the insulin-stimulated glycogenesis [5]. Despite a significant association between genotypes of the GS encoding gene, *GYS1*, with type 2 diabetes and a 25% reduction of the immunoreactive protein, there were no alterations in the structure and expression of the gene [6]. The reduced level of GS activity in insulin resistant Pima Indians is partly explained by a concomitant decrease of activity of protein phosphatase 1 (PP1), which dephosphorylates and activates GS [7]. Nevertheless, there are no polymorphisms in genes encoding PP1 catalytic subunits that can account for this defect [8, 9].

The attention has since shifted to analyze PP1 inhibitors [10] as well as regulatory subunits [11] that also serve to localize the enzyme to its appropriate subcellular locations and modulate its substrate specificity [12]. Investigation of the gene encoding the skeletal muscle glycogen-targeting subunit of PP1 (*PPP1R3*) revealed a common variant at the 3′-untranslated region (UTR) that is associated with transcript and protein levels in vivo, and, yet, only partly contributes to insulin resistance and type 2 diabetes in Pima Indians [13].

Recently, a subunit of PP1 was discovered by its homology to previously identified subunits, and was called PPP1R5 [14] or PTG (Protein Targeting to Glycogen) [15]. Even though this protein is present in insulin sensitive tissues and can complex PP1 with its targets, such as phosphorylase kinase and GS, it is apparently insensitive to insulin action [15]. The *PPP1R5* gene lies within a region on chromosome

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TABLE 1

Clinical Characteristics of Pima Indian Subjects

	Insulin sensitive	Insulin resistant	*P* value
Males/Females	13/4	7/10	
Age (years)	30 ± 5	27 ± 5	
% Body fat	34 ± 6	32 ± 4	
Low dose M* ($mg \cdot min^{-1} \cdot kg\ EMBS^{-1}$)	3.3 ± 1.0	1.8 ± 0.2	<0.001
High dose M* ($mg \cdot min^{-1} \cdot kg\ EMBS^{-1}$)	12.2 ± 0.8	5.1 ± 1	<0.00001

* M: insulin-stimulated glucose uptake rates at insulin infusion doses of 40 (low dose) or 400 (high dose) $mU \cdot m^{-2} \cdot min^{-1}$; EMBS: estimated metabolic body size determined from resting metabolic rate = fat free body mass + 17.7 [4]. Values are means ± SD. *P* values between groups are analyzed by Student's *t* test.

10q23-24 [14] that shows evidence for linkage to insulin action (glucose disposal rate during the low dose insulin infusion of 40 $mU \cdot m^{-2} \cdot min^{-1}$ during the glucose clamp) in Pima Indians [16], establishing PPP1R5 as a positional and functional candidate gene for insulin resistance.

In the present study, we have investigated the potential role of PPP1R5 in insulin resistance in non-diabetic Pima Indians by searching for allelic variations in its genomic structure. We have also performed RT-PCR analysis to measure the transcript levels in selected subjects.

MATERIALS AND METHODS

Patients. The subjects were full-blooded, non-diabetic Pima Indians, who are members of the Gila River Indian community, and some Caucasians for comparison. The Pima Indians have been participating in a longitudinal study of the development of type 2 diabetes and were classified as either insulin sensitive or resistant based on the mean value of their insulin-mediated glucose uptake rates as measured by a two-step hyperinsulinemic euglycemic clamp [5] on one or more occasions over the course of several years. Clinical characteristics of these subjects are tabulated in Table 1.

cDNA synthesis. Skeletal muscle biopsies were obtained from a subset of Pima Indian subjects (n = 9) with a broad range (from 4.3 to 14 $mg \cdot min^{-1} \cdot kg\ EMBS^{-1}$) of insulin-stimulated glucose uptake rates measured at high dose insulin infusion (400 $mU \cdot m^{-2} \cdot min^{-1}$). The procedure was as described previously [6]. Briefly, percutaneous muscle biopsies were taken from the quadriceps femoris muscle using Bergstrom needles (Depuy), and were immediately frozen in liquid nitrogen. Total RNA was isolated from the frozen tissues homogenized in Trizol Reagent (Gibco BRL). Oligo dT-primed cDNA was subsequently synthesized using the Superscript cDNA Synthesis Kit (Gibco BRL).

Extraction of genomic DNA. Individual genomic DNA from Pima subjects was prepared from transformed lymphocyte cultures or peripheral white blood cells [17]. All Caucasian DNAs (n = 34) were obtained from the NIGMS repository (Coriell Institute, Camden, NJ).

Reverse-transcription PCR. RT-PCR amplifications were performed using PTG specific primers (forward: 5′-CACGTCCTTTGACAAGTTCG-3′ and reverse: 5′-TGGAGTCAGCAAACACAACG-3′) and human B-actin specific primers (forward: 5′-CTGACTGACTACCTCATGAAGAT and reverse: 5′-CGTCATACTCCTGCTTGCTGAT-3′). After 24 cycles, aliquots were collected every two subsequent cycles to determine the exponential phase of amplification. The products were resolved on 2.5% Nusieve GTG gel (FMC) containing ethidium bromide. The image of the gel was taken using Polaroid 665 film (Polaroid Corporation) and the relative concentrations of PCR products were scanned by scanning densitometry (Scanmaster 3+) and analyzed using Bioimage Image Analyzer (Millipore).

Polymerase chain reaction (PCR) and cycle sequencing of PCR products. PCR and sequencing primers were designed based on the genomic sequence of *PPP1R5* that include the coding and flanking genomic regions that contain 3′- and 5′UTR (kindly provided by Drs. J. Printen and A. Saltiel; Genbank submission number AF110824). The whole ~4 kb region was amplified as 2 overlapping fragments with similar lengths using primer sequences as listed in Table 2. PCR amplifications were performed on 100 ng of genomic DNA using Gene Amp XL PCR kit (Perkin Elmer) according to the manufacturer's instructions. Purified PCR products were subsequently sequenced with sequencing primers listed in Table 2, using Big Dye Terminator chemistry (Applied Biosystems) in ABI 377 automated sequencer according to the manufacturer's recommendations. Most regions, including the one containing the polymorphisms, were se-

TABLE 2

Sequences of PCR and Sequencing Primers

Primers	Orientation	Fragment A*	Fragment B*
PCR	Forward	5′-AAGCTTTAAGAACATACCCATCC-3′	5′-CAGTGACCCACAAAGCTATTCC-3′
PCR	Reverse	5′-GCTCCGGAGAATCAAGATCT-3′	5′-AGGTAGACATAGAAGATC-3′
Sequencing	Forward	5′-AAGCTTTAAGAACATACCCATCC-3′	5′-CAGTGACCCACAAAGCTATTCC-3′
Sequencing	Forward	5′-CTTGGAGATAATCAAATCGAA-3′	5′-TCTTATCACAGTTTGCCATAGC-3′
Sequencing	Forward	5′-GGCCTCTTATCGATGAATTAAGC-3′	5′-GGCCTGGAGCTGTGGTTCG-3′
Sequencing	Forward	5′-CCTTAATGATATCTCCTCTGC-3′	5′-TTCTTGGTTTAATATTGCTGAGC-3′
Sequencing	Reverse	5′-AGGTAGACATAGAAGATC-3′	5′-CCTCTCTGCCTAATGAGCTG-3′
Sequencing	Reverse	5′-GGTCTTGACTTGTCATATTCC-3′	5′-TTGGCATTTTCTAATAGAC-3′
Sequencing	Reverse	5′-TGGAGTCAGCAAACACAACG-3′	5′-CCAGGAATTGGAAAGAAAGAAG-3′
Sequencing	Reverse	5′-GTATCACTATCTGTGCCACC-3′	5′-AGGTAGACATAGAAGATC-3′

* Fragment A spans a region 535 bp upstream to 1552 bp downstream of the start codon, whereas fragment B covers a region 164 bp to 2575 bp upstream of the start codon.

TABLE 3

Genotype Frequencies of a Single Nucleotide Polymorphism Located 2353 bp from the Start Codon of *PPP1R5*

Genotype	C/C	C/G	G/G
Insulin sensitive Pima Indians	10 (29.4)	7 (20.6)	0 (0)
Insulin resistant Pima Indians	12 (35.3)	5 (14.7)	0 (0)
Caucasians	23 (67.6)	9 (26.5)	2 (5.9)

Note. Data are n (% of the respective population).

quenced from both forward and reverse directions. The resulting data was analyzed using ABI Sequencer program.

RESULTS AND DISCUSSION

We have investigated the *PPP1R5* gene as a functional and positional candidate gene for insulin resistance in Pima Indians. There was no polymorphism found in the coding region of *PPP1R5,* indicating that amino acid variants in the protein do not contribute to insulin resistance in the representative subjects of the population. During the preparation of this manuscript, a study on insulin resistant Caucasian patients was published, revealing 3 silent polymorphisms in the coding region of this gene with low allelic frequencies [18].

The above finding did not exclude the potential abnormal regulation of transcription. Thus, we carried out RT-PCR on cDNA from skeletal muscle biopsies of selected Pima Indian subjects with a broad range of insulin-stimulated glucose uptake as a measure of insulin sensitivity. When analyzed against this metabolic parameter, the ratios of the PPP1R5/actin transcripts did not reach statistical significance, indicating absence of abnormal transcriptional regulation that correlates with insulin action.

Comparison of the genomic sequence with the published cDNA sequence [14] reveals a ~2 kb intron in the 5′-UTR which does not contain any polymorphisms in any subjects. In the region further upstream of the 5′ UTR, we discovered 2 novel single nucleotide polymorphisms at 2353 and 2381 base pairs (bp) upstream of the starting codon. These polymorphisms may modify sites regulated by Th1E47 or NF1 and IK2 transcription factors respectively (research in genetic data banks by Matinspector v2.2, [19]). Pima Indian subjects only have the first polymorphism, and its genotype frequencies did not correlate with insulin resistance (Table 3).

In conclusion, despite its function in regulating GS activity and position in a region with linkage to insulin action in Pima Indians, there is no polymorphism in the coding region or aberrant transcriptional regulation of *PPP1R5* gene that contributes to insulin resistance in this population. Pima subjects have only one of the two novel single nucleotide polymorphisms upstream of the 5′UTR found in Caucasian subjects, and its genotype distribution does not indicate any significant role of this gene in insulin resistance. Thus, the linkage to insulin action on chromosome 10q23-24 may result from a different nearby locus.

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