

## Genetic Analysis of Human Type 1 Protein Phosphatase Inhibitor 2 in Insulin-Resistant Pima Indians

PASKASARI A. PERMANA<sup>1</sup> AND DAVID M. MOTT*Clinical Diabetes and Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Phoenix, Arizona 85016*

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**The rate-limiting enzyme in insulin-mediated nonoxidative glucose disposal, glycogen synthase, has reduced activity in insulin-resistant subjects at risk for developing non-insulin-dependent diabetes mellitus (NIDDM). The synthase-activating enzyme, type 1 protein phosphatase (PP1), also has an abnormally low level of activity. Inhibitor 2 (I-2) reversibly inhibits and facilitates the proper conformation of free catalytic subunits of PP1. This study investigates whether genetic alteration(s) in the I-2 coding locus (*PPP1R2*) could contribute to insulin resistance in Pima Indians. We determined that the authentic *PPP1R2* gene is located on chromosome 3q29 and consists of six exons. The previously reported homologue of *PPP1R2* on chromosome 5 is identified as an intronless pseudogene. Comparative sequencing of *PPP1R2* exons and splice junctions revealed no mutations in insulin-resistant Pima Indians. The information on the genomic structure of *PPP1R2* is important for exploring this gene as a potential candidate contributing to insulin resistance and NIDDM in other populations.** © 1997

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Inhibitor 2 (I-2) of type 1 protein phosphatase is a thermostable cytosolic protein of 203 residues that forms a heterodimer (PP1I) with the catalytic subunit of protein phosphatase 1 (PP1C). The isolated cytosolic PP1I complex is inactive and can be activated upon reversible phosphorylation of Thr72 on I-2 by glycogen synthase kinase 3 (GSK3) (2). I-2 can also be phosphorylated on Ser86, Ser120, and Ser121 *in vivo*, as well as by casein kinase II *in vitro* (9). Ser86 phosphorylation enhances the rate of Thr72 phosphorylation by GSK3 (7).

The prevalence of non-insulin-dependent diabetes mellitus (NIDDM) in Pima Indians and other ethnic groups points to a strong genetic component in the development of the disease (3). Longitudinal studies of

the Pima Indian population suggest that resistance to insulin-mediated glucose disposal (insulin resistance) is a good predictor of the development of NIDDM (4). A reduced activation of the rate-limiting enzyme in insulin-mediated nonoxidative glucose disposal, glycogen synthase (GSY1), has been associated with the pathogenesis of insulin resistance (5). Insulin stimulation of type 1 protein phosphatase (PP1) results in dephosphorylation and activation of GSY1. PP1 activity in skeletal muscle is decreased in insulin-resistant compared to insulin-sensitive Pima Indians (10). The genetic structures of both GSY1 (12) and the predominant  $\beta$  isoform of PP1C (15) appear to be normal in the Pima Indian population.

Several proteins that bind and regulate PP1C have been characterized (6). I-2 is particularly interesting as a candidate enzyme responsible for insulin resistance, because upon binding to PP1C, it induces a conformational change and activation of the catalytic subunit (1). A recent study of altered PP1C activity in insulin-resistant Pima Indians suggests that conformational states of PP1C could explain their abnormal phosphatase activity (13). An unusually larger proportion of inactive conformational states of PP1C can result from abnormal I-2 function, which, in turn, can be a consequence of mutations in the gene coding for I-2, the *PPP1R2* locus.

To investigate the potential genetic contribution of *PPP1R2* as a candidate gene for insulin resistance, we have isolated the human gene and verified its authenticity. Subsequent information on its genetic structure enabled us to perform comparative sequencing of its coding regions in genomic DNA from insulin-resistant and insulin-sensitive Pima Indians.

Primers 5'-TGACTATAGTACGTTGCTTCTT-3' and 5'-ATATCCTGTTGTTCAATTGGCT-3', which hybridize specifically to the 3' noncoding region of human *PPP1R2* gene (8), were used to screen the human foreskin fibroblast genomic library (Genome Systems, St. Louis, MO). Two resulting positive P1 clones were sequenced using primers designed from the I-2 cDNA sequence (8, 17). Individual exons were PCR amplified and sequenced using the solid phase Sequenase dye

Sequence data from this article have been deposited with the GenBank Data Library under Accession Nos. U68106-U68111.

<sup>1</sup>To whom correspondence should be addressed at CNDS/NIH, 4212 North 16th Street, Phoenix, AZ 85016. Telephone: (602) 200-5341. Fax: (602) 200-5335.

**TABLE 1**  
**Primers Used to Amplify *PPP1R2* Exons and to Investigate Potential *PPP1R2* Locus on Human Chromosome 5 (Fig. 1)**

Amplified region	Primer sequences	Orientation	PCR product (bp)
Exon 1	5'-GGCTCTGGCGTCGGGGTCG-3'	Forward	313
	5'-GGTTCCTGGGTAGGTTACGG-3'	Reverse	
Exon 2	5'-CATTTGTGAATTTTGATATTCAGG-3'	Forward	241
	5'-CGTTCATATGAAATGCAAAATGG-3'	Reverse	
Exon 3	5'-TTTCTCCCTTGTTTTGTGGC-3'	Forward	213
	5'-GCTGCTTTCAACTCTGCACG-3'	Reverse	
Exon 4	5'-TAAGAATCAACAGTTGAATAAGG-3'	Forward	226
	5'-CTTAGCAAAATTAAGTACTAGCACC-3'	Reverse	
Exon 5	5'-AAACAAATGAGCATCACTGTTTC-3'	Forward	312
	5'-AGACATGACAAAGTCTCAGTG-3'	Reverse	
Exon 6	5'-GTCATGCATGCTAATAGAAGG-3'	Forward	620
	5'-ACAGACAAATCAAGTTGAAGAC-3'	Reverse	
Fragment a	5'-AAGAACAAGACCTCTACGACTTC-3'	Forward	496
	5'-ATTCATGCTTTCTCCATCTGC-3'	Reverse	
Fragment b	5'-AAGAACAAGACCTCTACGACTTC-3'	Forward	700
	5'-TCTTGGCAATATATAATAACTGG-3'	Reverse	
Fragment c	5'-AAGAACAAGACCTCTACGACTTC-3'	Forward	997
	5'-ATATCCTGTTGTTCAATTGGCT-3'	Reverse	
Fragment d	5'-CAGTTGCGGCCGCTTTAGC-3'	Forward	931
	5'-TCTTGGCAATATATAATAACTGG-3'	Reverse	
Fragment e	5'-GGTTTATCTTTTCTGCATTGG-3'	Forward	219
	5'-CGTTCATATGAAATGCAAAATGG-3'	Reverse	

terminator DNA sequencing kit (Applied Biosystems) on an ABI Model 373A automated DNA sequencer (Perkin-Elmer). DNA sequences were analyzed with the Sequencher 2.1 program (Gene Codes Corp., Ann Arbor, MI). The primer sets are tabulated in Table 1. Both clones contained the complete human *PPP1R2* gene with coding sequences identical to that previously reported (8, 17). A total of six exons and the exon-intron boundaries were identified (Table 2). The sequences of the splice junctions show that the introns begin and end with the conserved GT-AG motif (Table 2).

To determine the chromosomal localization of the *PPP1R2* gene in the P1 clones, purified DNA of one clone, P1-PPP1R2, was sent to Bios Laboratories (New Haven, CT) for fluorescence *in situ* hybridization. The DNA was dUTP-labeled and hybridized to normal human metaphase chromosomes. Specific labeling of the distal long arm of a group A chromosome consistent with chromosome 3 showed up in 78 of 80 metaphase chromosomes. The identification was confirmed by co-hybridization of a chromosome 3 centromere-specific probe (D3Z1) with the labeled P1-PPP1R2 clone (data not shown). Measurements of the hybridized chromosomes showed that P1-PPP1R2 probe hybridized to an area corresponding to band 3q29. Higher stringency

using *PPP1R2* genomic probe eliminated the probability of cross-hybridization to potential pseudogene locations.

Previously reported *in situ* hybridization using an I-2 cDNA probe gave positive signals on chromosomes 3q29, 5q33, and 6p21.31 (17), and there have been suggestions that the locus on chromosome 6 corresponds to a *PPP1R2* pseudogene (8, 17). To investigate the genetic structure of the putative *PPP1R2* gene homologue on human chromosome 5, PCR amplifications were carried out using primers (Table 1) that yielded specific bands (Fig. 1, fragments b-d) from skeletal muscle I-2 cDNA (data not shown). A primer set that hybridized to the first and the last exons gave rise to a DNA fragment of 496 bp (Fig. 1, fragment a) from both genomic DNA and human/rodent somatic cell hybrid DNA containing human chromosome 5 (NA 10114, Coriell Institute, Camden, NJ), but not from the P1-PPP1R2 clone or the rodent DNA control (Fig. 1, bottom left). This fragment size was expected if there were no intronic sequences between the primer hybridization sites. To investigate the presence of 5' and 3' UTRs of I-2 cDNA sequence on human chromosome 5, PCR amplifications of different fragments (Fig. 1, fragments b-e) were carried out. The longest contiguous region amplified in NA 10114 (Fig. 1, bottom right, fragment

**TABLE 2**  
**Exon–Intron Organization of the Human *PPP1R2* Gene**

5' exon	Size (bp)	Splice junctions	3' exon	
1	122 <sup>a</sup>	AlaG1 CTGAG/ <b>gt</b> gagcgcctg-----ttaatttt <b>ag</b> /CAAAAAA HisSe	uLysLys rMet	2
2	108	CATAG/ <b>gt</b> aatgttac-----gtcactact <b>ag</b> /TATG ArgLy	sLeu	3
3	78	AGGAA/ <b>gt</b> aagtacta-----tggtgcat <b>ag</b> /ATTA ArgG	luLys	4
4	95	CGAG/ <b>gt</b> agtttatt-----catcttat <b>ag</b> /AAAAA GlnG	lySer	5
5	168	CAAG/ <b>gt</b> tagatgta-----atctttg <b>cg</b> /GATCT Ser Ser stop		6
6	44 <sup>a</sup>	AGT TCA tag		

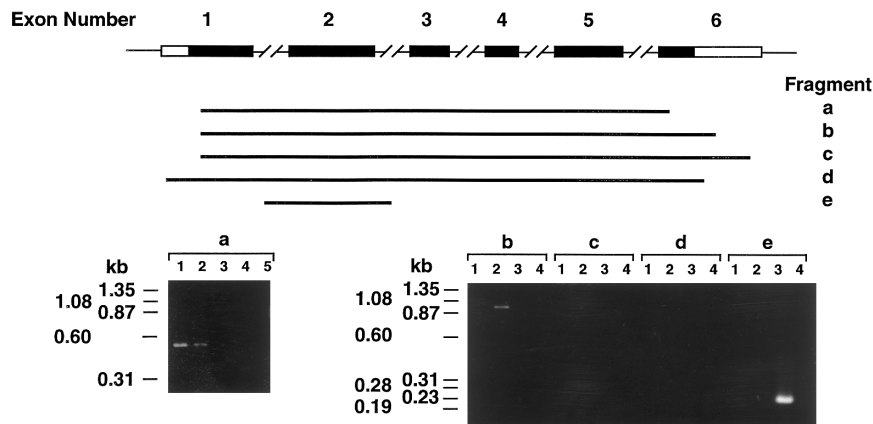
*Note.* The splicing boundaries (denoted by "/") separate the coding sequences (capital letters) from the intronic sequences (lowercase). Splice donor and acceptor sites are in boldface. The complete coding regions and their respective flanking intronic sequences can be accessed through GenBank under Accession Nos. U68106–U68111.

<sup>a</sup> Sizes of the coding regions only.

b, lane 2) extended from the first exon to the beginning of the 3' UTR (998 bp), suggesting that human chromosome 5 contains a *PPP1R2* intronless pseudogene lacking the 5' UTR and part of the 3' UTR.

Thus, there are at least two *PPP1R2* pseudogenes in the human genome localized to chromosome 6p21.31 (8, 17) and to chromosome 5q33 (17; this report). Coupled with the information that the *PPP1R2* gene contained in the P1-*PPP1R2* clone localizing to chromosome 3q29 has intronic sequences and 5' and 3' UTRs (see above), these data indicate that the P1-*PPP1R2* clone used in further analysis contained the authentic human *PPP1R2* gene.

To search for polymorphic markers that could be beneficial for association studies of the *PPP1R2* gene to different parameters of insulin action, including insulin resistance, *Alu*-PCR amplification and direct sequencing procedures were performed on the P1-*PPP1R2* clone. The amplification utilized either 5'-C<sub>4</sub>(CA)<sub>11</sub>-3' or 5'-G<sub>4</sub>(GT)<sub>11</sub>-3' primers (14) in combination with one of the *Alu*-specific primers (18). Direct sequencing of the P1-*PPP1R2* clone using degenerate CA repeat primers (16, 19) as well as OligoScan probes (Bios Laboratories) was performed with a double-stranded cycle sequencing kit (GIBCO BRL, Gaithersburg, MD). The degree of variation of the longest re-



**FIG. 1.** Characterization of the *PPP1R2* locus on human chromosome 5. The top half represents a schematic diagram of the *PPP1R2* gene as characterized on chromosome 3q29 (upper line; not drawn to scale), in which closed blocks indicate coding regions, open ones untranslated regions (UTRs); and thin lines intronic sequences. Fragments a–e (lower lines) were selected for PCR amplification with the primers tabulated in Table 1. The primer sets have been successfully tested on I-2 cDNA from skeletal muscle total mRNA to give the expected fragments (data not shown). The PCR products were separated on 2.5% NuSieve GTG agarose gel. The bottom left shows that the size of fragment a amplified in genomic DNA (lane 1) and in human/rodent somatic cell hybrid DNA containing human chromosome 5 (NA 10114; lane 2) was 496 bp, as expected from contiguous coding exons. This fragment was not seen in parent rodent DNA control (NA 10908; lane 3) or P1-*PPP1R2* clone (lane 4). Lane 5 contained the negative control in which the template DNA was substituted with water. The bottom right shows experiments to investigate the presence of *PPP1R2* 5' and 3' UTRs as well as intronic sequences on chromosome 5. PCR was carried out to amplify fragments b–e in a set of four samples using different templates: (1) parent rodent DNA (NA 10908), (2) human/rodent somatic cell hybrid DNA containing human chromosome 5 (NA 10114), (3) P1-*PPP1R2* clone, and (4) water as the negative control.

TABLE 3

**Clinical Characteristics of Nondiabetic Pima Indians Selected for Comparative Analyses**

	Insulin resistant	Insulin sensitive	<i>P</i> value
Males/females	8/6	6/7	NS
Age (years)	31 ± 8	26 ± 4	<0.05
% Fat of body weight	38 ± 4	31 ± 9	<0.05
Fasting glucose (mmol/L)	5.6 ± 0.6	4.7 ± 0.4	0.0001
2-h glucose (mmol/L) during OGTT	8.5 ± 1.7	6.4 ± 1.7	<0.05
Log <sub>10</sub> fasting insulin (pmol/L)	2.6 ± 0.9	2.2 ± 1.0	<0.0001
Low-dose M (mg · min <sup>-1</sup> · kg EMBS <sup>-1</sup> )	1.8 ± 0.3	4.5 ± 1.0	<0.0001
High-dose M (mg · min <sup>-1</sup> · kg EMBS <sup>-1</sup> )	6.4 ± 1.3	10.8 ± 2.6	0.0001

Note. *P* values of the differences between groups are analyzed by Student's nonpaired *t* test. NS, nonsignificant. OGTT, oral glucose tolerance test; M, insulin-stimulated glucose uptake rates at insulin infusion rates of 40 (low dose) or 400 (high dose) mU × m<sup>-2</sup> × min<sup>-1</sup>; EMBS, estimated metabolic body size determined from resting metabolic rate = fat free body mass + 17.7 (11). Values are means ± SD.

peat, (AAAG)<sub>5</sub>, was evaluated in 20 Pima Indians, and two alleles (157 and 161 bp) were identified with a heterozygosity of 5% (data not shown). This low level of polymorphism and the absence of other informative markers in the vicinity of the *PPP1R2* gene underlined the importance of direct sequence analysis in searching for potential genetic alterations in the coding region.

Automated sequencing of the *PPP1R2* exons was performed on genomic DNAs from 17 insulin-resistant and 13 insulin-sensitive Pima Indians, as well as on a control Caucasian. The Pima Indian nondiabetic subjects were grouped as either insulin sensitive or insulin resistant according to their insulin-mediated glucose uptake rates as determined by a two-step hyperinsulinemic euglycemic clamp as previously described (5). Characteristics of the two groups are summarized in Table 3. Genomic DNA was obtained as previously described (15). PCR amplification of *PPP1R2* exons utilized primers in Table 1. The products were sequenced and analyzed as above. Sequencing of the genomic DNA from all subjects did not detect any mutation in the coding or the splice junction regions of the *PPP1R2* gene that might implicate the role of the gene in insulin resistance (data not shown). Nevertheless, the gene transcription and translation control may still be abnormal in insulin-resistant subjects.

This study has determined that the authentic *PPP1R2* gene consists of six exons and is localized on chromosome 3q29. The previously reported cross-hybridizing locus on chromosome 5 is a pseudogene. No mutations of the *PPP1R2* gene are detected in insulin-resistant Pima Indians tested in this study. The information provided in this paper describing the molecular structure of *PPP1R2*, as well as the screening condi-

tions for sequence variants, will make comparative studies for possible *PPP1R2* mutations in other populations feasible.

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