Report

A Variation in 3′ UTR of *hPTP1B* Increases Specific Gene Expression and Associates with Insulin Resistance

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Protein tyrosine phosphatase 1B (*PTP1B*) inhibits insulin signaling and, when overexpressed, plays a role in insulin resistance (Ahmad et al. 1997). We identified, in the 3′ untranslated region of the *PTP1B* gene, a 1484insG variation that, in two different populations, is associated with several features of insulin resistance: among male individuals, higher values of the insulin resistance HOMA$_{IR}$ index ($P = .006$), serum triglycerides ($P = .0002$), and total/HDL cholesterol ratio ($P = .025$) and, among female individuals, higher blood pressure ($P = .01$). Similar data were also obtained in a family-based association study by use of sib pairs discordant for genotype (Gu et al. 2000). Subjects carrying the 1484insG variant showed also *PTP1B* mRNA overexpression in skeletal muscle ($6,166 \pm 1,879$ copies/40 ng RNA vs. $2,983 \pm 1,620$; $P < .01$). Finally, *PTP1B* mRNA stability was significantly higher ($P < .01$) in human embryo kidney 293 cells transfected with 1484insG *PTP1B*, as compared with those transfected with wild-type *PTP1B*. Our data indicate that the 1484insG allele causes *PTP1B* overexpression and plays a role in insulin resistance. Therefore, individuals carrying the 1484insG variant might particularly benefit from *PTP1B* inhibitors, a promising new tool for treatment of insulin resistance (Kennedy and Ramachandran 2000).

The insulin resistance/metabolic syndrome—characterized by the variable coexistence of hyperinsulinemia, dislipidemia, obesity, and hypertension—is influenced by both environmental and genetic background, the latter being mostly unknown (Trischitta et al. 1997; Virkamaki et al. 1999). Protein tyrosine phosphatase 1B (*PTP1B*) is a major regulator of insulin sensitivity and body fat (Ahmad et al. 1995; Kenner et al. 1996; Ahmad et al. 1997; Elchebly et al. 1999; Goldstein et al. 2000; Klaman et al. 2000). In fact, *PTP1B* directly interacts with and dephosphorylates the activated insulin receptor (Seely et al. 1996; Bandyopaddhyay et al. 1997), thus inhibiting insulin signaling and action. In addition, type

2 diabetes and obesity have been linked to markers on human chromosome 20q13.1 (Lembertas et al. 1997; Lee et al. 1999; Klupa et al. 2000), which harbors *PTP1B*. Also, the mouse *PTP1B* region (i.e., the distal arm of chromosome 2, syntenic with human chromosome 20) is likely to harbor a gene for obesity (Lembertas et al. 1997). These data indicate that *PTP1B* is a candidate gene for insulin resistance/metabolic syndrome.

We searched for polymorphisms in both the regulatory and coding regions of the human *PTP1B* gene (Forsell et al. 2000). Table 1 shows the primer sets used for the screening by PCR and SSCP. Because of an alternative splicing in intron 9, two different 3′ UTRs are transcribed for *PTP1B* (Forsell et al. 2000). Both 3′ UTRs were screened. Samples carrying different electrophoretic patterns were automatically sequenced after cloning (at least five clones) in pCR II TOPO vector (Invitrogen).

Several single-nucleotide polymorphisms (SNPs) were identified in 100 unrelated subjects from the Gargano area (on the east coast of central Italy) (table 2). Because

Table 1

Primers and PCR Conditions

Exon	Primers	Annealing Temperature (°C)	Amplimer Size (bp)
PR1 (−594/−431)	Forward: 5′-TGCAGCACCCAAGTGGAAATTC-3′	60	229
	Reverse: 5′-TCATGCACCTCCTTTCCTGCAG-3′		
PR2 (−510/−215)	Forward: 5′-TGATACTCCTGAGTTTCAT-3′	58	338
	Reverse: 5′-AGCACCGCGAGATATCTAATAGC-3′		
PR3 (−261/−55)	Forward: 5′-GTCGCCTAGGCAACAGGCGCG-3′	60	262
	Reverse: 5′-TAGCCGCTGCTCTTCTTCATG-3′		
PR4 (−68/+108)	Forward: 5′-GCAGGCGTGATGCGTAGTTC-3′	60	218
	Reverse: 5′-GACTTGTCGATCTGCTCGAA-3		
Exon 1	Forward: 5′-CTGGCAGGCGTGATGCGTAG- 3′	68	300
	Reverse: 5′-GATGTTCAAGCGGCCTAAGCG-3′		
Exon 2	Forward: 5′-GTCTTCCTCAGTGTCTGACGGTC-3′	68	192
	Reverse: 5′-GCCTGCAGCAAAGGAAGAGC-3′		
Exon 3	Forward: 5′-TGTACACATTCAGCTTTTCC-3′	58	226
	Reverse: 5′-TGAGGTGTGGATAACAGC-3′		
Exon 4	Forward: 5′-TCGTTAGCTGACTGCAGAAGG-3′	60	244
	Reverse: 5′-ATGGTAACTATATGGAGTGG-3′		
Exon 5	Forward: 5′-TCATGAAGCTTGTGGGATGTGC-3′	60	316
	Reverse: 5′-TGACATTAGGTAATATCACC-3′		
Exon 6	Forward: 5′-GAAGGTGACTCTGTGTGTAC-3′	64	349
	Reverse: 5′-TCACAGCAGAGCAGGTAGAGGAGC-3′		
Exon 7	Forward: 5′-TGAGAATTGGACCTGGC-3′	62	283
	Reverse: 5′-TACAACTGACAGCCTCCTTC-3′		
Exon 8	Forward: 5′-TGACAAACCAGCCGAAGTGAAC-3′	62	354
	Reverse: 5′-TCAGTACCAGCGTGTGTTTC-3′		
Exon 9	Forward: 5′-GAGTACCCATCTCTGCCCTCTG-3′	62	324
	Reverse: 5′-CAGATGCACCACAGAACTGAATCC-3′		
Exon 10[a]	Forward: 5′-CATGAGGCGACAGCACTGC-3′	62	350
	Reverse: 5′-CTTCCATTCCCAGTACTACCTGA-3′		
3′ UTR A	Forward: 5′-AGGACGGTTGTAAGCAGTTGTT-3′	62	329
	Reverse: 5′-GGAACCACAGCCAGTTTATGAT-3′		
3′ UTR B	Forward: 5′-TCTCTGCTTACTAATGTGCCCC-3′	60	351
	Reverse: 5′-TCAAGAGTGTCGACTTGGA-3′		
3′ UTR C	Forward: 5′-TCTGGACATGATTTAGGGAAGC-3′	62	320
	Reverse: 5′-TGCCGTGTTTTTCATGTTAAAA-3′		
3′ UTR D	Forward: 5′-AAAGGGAACTGAAGACCTCCAC-3′	64	314
	Reverse: 5′-GGAGGTTAAACCAGTACGTCCA-3′		
3′ UTR E	Forward: 5′-ATTCTGAGCTGGCTTGTTGTTT-3′	62	234
	Reverse: 5′-GGTTTATTCCATGGCCATTGTA-3′		
Intron 9 A	Forward: 5′-CTGGTCAACATGTGCGTGG-3′	62	267
	Reverse: 5′-CTTGGGACCAGAGGGCTC-3′		
Intron 9 B	Forward: 5′-TTAAGGATCGATGCACTGGG-3′	62	324
	Reverse: 5′-TTGGGATTCCTTCCCTGGG-3′		
Intron 9 C	Forward: 5′-CCTTAGGTGATGTAATCAGCC-3′	62	350
	Reverse: 5′-AGGCCTCGAGGACACCC-3′		
Intron 9 D	Forward: 5′-CCTGTGACAGCCATCTTGC-3′	62	327
	Reverse: 5′-CATCTGATGTACTCAGATGCC-3′		
Intron 9 E	Forward: 5′-ACTAGCCTCAGAGCTCTGG-3′	58	348
	Reverse: 5′-GTGGAGGTGGAGTGGAGG-3′		

[a] Proximal region of the 3′ UTR.

of a low allele frequency (AF) (i.e., <2%) and/or the nature of sequence variations (i.e., either silent or intronic), only the SNP localized in the 3′ UTR (1484insG SNP, according to the published sequence [GenBank accession number M33689]) was considered for association studies with insulin resistance/metabolic syndrome.

For this purpose, RFLP analysis was used, because a *Sac*II restriction site was created by 1484insG.

To minimize the inclusion of genetic determinants of β-cell failure, rather than insulin resistance, and to avoid the confounding effect of hyperglycemia on insulin-resistance–related abnormalities, we analyzed only non-

Table 2

SNPs in the *PTP1B* Gene

Designation	Location	Type	Restriction Enzyme	AF (%)
−105delA	5′ UTR	Noncoding	…	1
A669G	Exon 6	Synonymous	…	3
C981T	Exon 8	Synonymous	…	7
C1207T	Exon 9	Missense[a]	…	.9
delT	Intron 3		…	1.1
A/C	Intron 5			50
1484insG	3′ UTR	Noncoding	*Sac*II	7.7
1737insG	3′ UTR	Noncoding	…	1.9
T2251C	3′ UTR	Noncoding	…	1
A2261G	3′ UTR	Noncoding	…	1

[a] Leu379Phe.

diabetic subjects. First, 477 normoglycemic (fasting plasma glucose <126 mg/dl), unrelated white subjects from the Gargano area were studied. Also, 335 normoglycemic individuals from Sicily were analyzed, to replicate the association study. In all subjects, the following parameters were assessed: BMI; waist and hip circumference; blood pressure; and fasting glucose, insulin, and lipid profiles. Plasma glucose (mmol/l), serum insulin (pmol/l), and lipid profile (total serum cholesterol, HDL cholesterol, and serum triglycerides) were measured by use of commercially available enzymatic kits, as described elsewhere (De Cosmo et al. 1997). The insulin-resistance index $HOMA_{IR}$ (homeostasis model assessment) was calculated as fasting serum insulin (μU/ml) $\times$ fasting plasma glucose (mmol/l)/22.5 (Bonora et al. 2000). In individuals from Sicily, glucose and insulin levels were also measured before, 60 min after, and 120 min after a 75-g oral-glucose load was given. Values are given as mean $\pm$ SEM. Mean

values of unrelated individuals from the two genotype groups were compared by Student t-test or Mann-Whitney U test, as appropriate. Informed consent was obtained from participants before they entered the study, which was approved by the local research ethics committee. Among the 477 unrelated individuals from the Gargano region (165 male subjects, aged 37.9 $\pm$ 0.9 years, and 312 female subjects, aged 38.2 $\pm$ 0.6 years; 1484insG AF 7.7%, in Hardy-Weinberg equilibrium), male subjects carrying the 1484insG allele ($n = 15$) showed, compared with wild-type individuals, higher BMI values (29.2 $\pm$ 1.1 kg/m² vs. 25.7 $\pm$ 0.3; $P = .001$), fasting plasma insulin (10.0 $\pm$ 1.1 mU/L vs. 7.8 $\pm$ 0.3; $P = .048$), serum triglycerides (168 $\pm$ 39 mg/dl vs. 118 $\pm$ 6; $P = .034$), total/HDL cholesterol ratio (5.2 $\pm$ 0.4 vs. 4.4 $\pm$ 0.1; $P = .035$) and the insulin-resistance $HOMA_{IR}$ index (Bonora et al. 2000) (2.4 $\pm$ 0.3 vs. 1.7 $\pm$ 0.1; $P = .025$). Female subjects carrying the 1484insG allele ($n = 56$, including 3 homozygous subjects) showed higher values of systolic (114 $\pm$ 1.6 mm Hg vs. 110 $\pm$ 0.7; $P = .024$) and diastolic (75 $\pm$ 0.9 mm Hg vs. 73 $\pm$ 0.5; $P = .038$) blood pressure but not of other insulin-resistance–related parameters. As mentioned above, 335 nondiabetic Sicilians (170 male subjects, aged 38.5 $\pm$ 0.9 years, and 165 female subjects, aged 35.2 $\pm$ 0.9 years; 1484insG AF 5.2%) were studied, to replicate the data in a population that, although geographically close to the first population, is known to be of different ethnicity (Piazza et al. 1988). This would minimize the risk of false-positive results due to "population stratification" (Altshuler et al. 1998; "Freely Associating" 1999). Male subjects carrying the 1484insG allele ($n = 18$, including 1 homozygous subject) showed higher levels of fasting

Table 3

Clinical Features of Subjects from the Two Different Populations Pooled Together

	MEAN $\pm$ SEM VALUES FOR SUBJECTS			
	Male		Female	
FEATURE	Wild Type ($n = 302$ [90%])	1484insG ($n = 33$ [10%])	Wild Type ($n = 401$ [84%])	1484insG ($n = 76$ [16%])
Age (years)	38.2 $\pm$ .7	38.1 $\pm$ 1.8	36.7 $\pm$ .6	39.8 $\pm$ 1.3[a]
BMI (kg/m²)	28.2 $\pm$ .4	29.4 $\pm$ 1.3	26.5 $\pm$ .3	25.7 $\pm$ .7
Fasting plasma glucose (mg/dl)	91.6 $\pm$ .6	93.4 $\pm$ 1.3	88.7 $\pm$ .5	88.9 $\pm$ .9
Fasting plasma insulin (mU/l)	9.2 $\pm$ .3	12.5 $\pm$ 1.8[b]	8.9 $\pm$ .3	8.1 $\pm$ .6
$HOMA_{IR}$	2.1 $\pm$ .1	3.0 $\pm$ .5[b]	2.0 $\pm$ .1	1.8 $\pm$ .1
Fasting serum cholesterol:fasting serum HDL cholesterol ratio	4.6 $\pm$ .1	5.2 $\pm$ .3[a]	3.7 $\pm$ .1	3.7 $\pm$ .1
Fasting serum triglycerides (mg/dl)	118.0 $\pm$ 4.0	169.8 $\pm$ 20.3[c]	83.7 $\pm$ 2.6	87.2 $\pm$ 5.1
Systolic blood pressure (mm Hg)	119.1 $\pm$ .7	117.6 $\pm$ 1.9	111.1 $\pm$ .6	114.2 $\pm$ 1.5[a]
Diastolic blood pressure (mm Hg)	78.7 $\pm$ .5	76.7 $\pm$ 1.1	73.0 $\pm$ .4	75.7 $\pm$ .8[b]

NOTE.—All differences—except fasting serum cholesterol/fasting serum HDL cholesterol, in male subjects, and systolic blood pressure, in female subjects—remained significant after correction for multiple comparisons.

[a] $P < .05$ versus wild type.
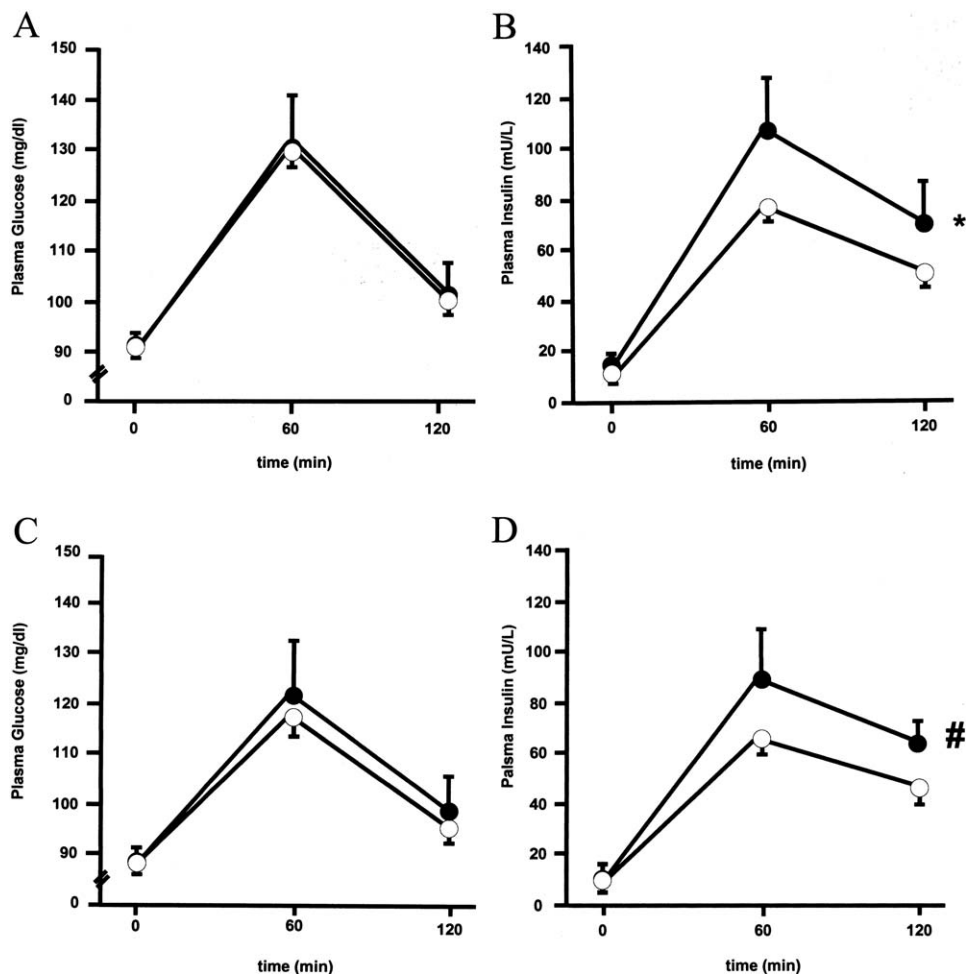[b] $P < .01$ versus wild type.
[c] $P < .001$ versus wild type.

Figure 1 Glucose (*A* and *C*) and insulin (*B* and *D*) plasma levels before (time 0), 60 min after, and 120 min after a 75-g oral-glucose load was given to 170 male subjects (*A* and *B*) and 165 female subjects (*C* and *D*) from Sicily. Blackened circles indicate subjects carrying the 1484insG. Unblackened circles indicate subjects not carrying the 1484insG. Data are mean ± SEM. An asterisk (*) denotes a *P* value <.01, by two-way ANOVA, versus subjects not carrying 1484insG. A pound sign (#) denotes a *P* value <.05, by two-way ANOVA, versus subjects not carrying 1484insG.

plasma insulin (14.6 ± 3.1 μU/L vs. 10.6 ± 0.6; $P = .041$) and serum triglycerides (171 ± 19 mg/dl vs. 118 ± 5; $P = .001$) as compared to individuals carrying the wild-type genotype. No difference between the two groups was observed in BMI, HOMA$_{IR}$ index, or total/HDL cholesterol ratio. Also, no difference in any of the above-mentioned variables was observed between female subjects carrying the 1484insG allele ($n = 20$) and those not carrying that allele. In this second population, glucose and insulin levels during oral-glucose–tolerance test (OGTT) were also available. Although glucose levels were similar between the two genotype groups (fig. 1*A* and 1*C*), insulin levels during OGTT were higher both in male subjects ($P = .005$, by two-way analysis of variance [ANOVA]) and, to a lesser extent ($P = .024$), in female subjects carrying the 1484insG allele, as compared with wild-type individuals (fig. 1*B* and 1*D*) (i.e., compensatory hyperinsulinemia, a typical feature of insulin resistance in normoglycemic individuals). Then the two populations were pooled and were examined together (table 3). Male subjects carrying the 1484insG allele ($n = 33$ [10%]) had higher values of insulin resistance HOMA$_{IR}$ index ($P = .006$), total/HDL cholesterol ratio ($P = .025$), and serum triglycerides ($P = .0002$) and had a 3.5-fold (95% CI 1.64–7.47) higher ($P = .001$) risk to show a cluster of insulin-resistance–related metabolic abnormalities (i.e., values in two or three of the three above-mentioned parameters were in the highest quartile of the entire cohort). Female subjects carrying the 1484insG allele ($n = 76$ [16%]) had higher systolic ($P = .03$) and diastolic ($P = .007$) blood pressure, the latter parameter remaining significantly ($P = .01$) different also when adjusted
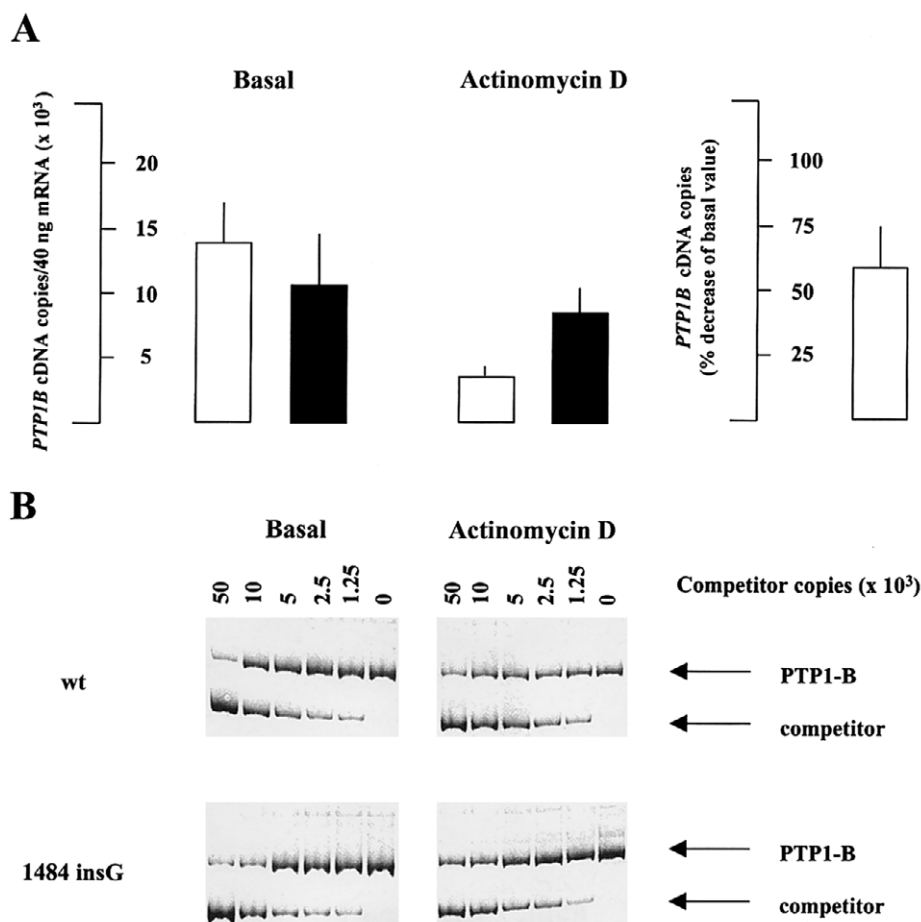
Figure 2 *A, left,* specific mRNA content (by competitive PCR before and after transcription inhibition for 40 h with 5 μg/ml actinomycin D) in human embryo kidney 293 cells transfected with either wild-type (*white columns*) or 1484insG (*black columns*) cDNA. *A, right,* data from the left panel are recalculated as % decrease after treatment with actinomycin D. Data are mean ± SEM of the results of three independent experiments. *B,* Representative competitive PCR for both wild-type (wt) and 1484insG transfected cells.

(by analysis of covariance) for the slightly different observed age (table 3). The proportion of individuals carrying the 1484insG allele was higher ($P = .02$, by χ^2 test) in female subjects (16%) than in male subjects (10%) (table 3). This may be the consequence of the apparently stronger association between the 1484insG allele and insulin resistance observed in male subjects than is observed in female subjects. One could, in fact, speculate that male 1484insG carriers are more likely to have been removed from the cohort of normoglycemic subjects we recruited, because of a more rapid progression to type 2 diabetes and/or early mortality for cardiovascular disease—both events being possible outcomes of insulin resistance/metabolic syndrome. To further minimize the risk of population-stratification bias, sib pairs concordant for sex and discordant for genotype from the Gargano region were also studied. Of 181 sib pairs concordant for sex, 13 were discordant for the *PTP1B* genotype. The differences in continuous variables between the siblings were estimated by use of a permutation test for paired replicates, as de-

scribed elsewhere (Gu et al. 2000). The permutation test does not make any assumptions about the normality, the homogeneity of the variance, or the precise form of the underlying distribution. In the permutation test for 13 pairs, there are 2^{13} equally likely outcomes for each variable, under the assumption of no difference between the paired siblings. Because of computational limitations, the two-tailed P values were estimated by use of a very large (10^7) random sample from all possible permutations. If the observed sum of differences (OSD) entered the 5% region of rejection, the differences between pairs was considered significant. The differences in phenotypic values were computed as the value in the sibling with the 1484insG variant minus the value in the sibling with the wild-type genotype. Sibs carrying the 1484insG allele showed higher BMI, total/HDL cholesterol ratio, triglycerides, and diastolic blood pressure (table 4). All together, these data show that the 1484insG variant of the *PTP1B* gene 3′ UTR associates with several features of insulin resistance/metabolic syndrome. This association seems to

be stronger among male subjects than among female subjects. This is not surprising, because a sex-specific effect of *PTP1B* (Klaman et al. 2000) and other insulin-resistance genes (Bruning et al. 2000) has been reported in animal models. In several instances, the 3′ UTRs may regulate gene expression through the modulation of mRNA stability (Day and Tuite 1998; Xia et al. 1998; Frittitta et al. 2001). Accordingly, *PTP1B* mRNA levels were measured in skeletal-muscle specimens by competitive PCR, as described elsewhere (Frittitta et al. 2000). For this purpose, a competitor was created. A *PTP1B* cDNA portion containing nt 662–1251, according to the published sequence (GenBank accession number M33689), was amplified from the pAD.CMVPTP1B plasmid. An internal *Ear*I fragment (nt 931–1073) was removed and the deleted cDNA, cloned in pCR II TOPO vector (Invitrogen), was used as the competitor. Constant amounts of *PTP1B* reverse-transcription first-strand products were coamplified with increasing copy-number amounts of competitor, and the equivalence point was determined after PCR and electrophoretic analysis.

To assess the reproducibility of competitive PCR, samples were analyzed in triplicate, with a mean coefficient of variation of 15%. *PTP1B* mRNA levels were higher in five muscle samples from 1484insG carriers than in 11 age- and sex-matched wild-type individuals (6,166 ± 1,879 copies/40 ng RNA vs. 2983 ± 1620; $P < .01$).

To investigate whether the 1484insG variation may be responsible for changes in *PTP1B* mRNA stability, human embryo kidney 293 cells were transiently transfected (Chen and Okayama 1987) with either 1484insG or wild-type cDNA. Specific *PTP1B* mRNA level (by competitive PCR) before and after 40 h of 5 μg/ml actinomycin D pre-exposure (i.e., inhibition of transcription) was then measured. The decrease of mRNA level after transcription inhibition was significantly ($P < .01$) lower for 1484insG *PTP1B* transfected cells as compared to wild-type *PTP1B* transfected cells (fig. 2). This indicates that the G insertion at position 1484 stabilizes *PTP1B* mRNA. The 3′ UTR

sequence has an essential role for the regulation of mRNA stability, and variants in this region have been associated with insulin resistance (Xia et al. 1998; Maegawa et al. 1999). The 3′ UTR may regulate mRNA stability through the binding with specific proteins, which occurs mostly but not exclusively at AU-rich regions (Conne et al. 2000; Day and Tuite 1998). The 3′ UTR of the *PTP1B* gene does not contain adenylate/uridylate–rich elements. Therefore, the 1484insG variation is likely to play a role in *PTP1B* mRNA stability through the modulation of protein binding to not-yet-identified 3′ UTR elements.

In conclusion, the 1484insG variation increases *PTP1B* mRNA stability and associates with several features of insulin resistance/metabolic syndrome. This association has been validated (Altshuler et al. 1998; "Freely Associating" 1999) by replication of data in unrelated individuals of different ethnicity and in a family-based study.

Screening for the 1484insG variation may, therefore, identify those subjects in whom *PTP1B* overexpression can be recognized as a molecular cause of insulin resistance. These individuals might particularly benefit from *PTP1B* inhibitors, a promising new tool for treatment of insulin resistance (Kennedy and Ramachandran 2000).

Acknowledgments

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Table 4

Clinical Features of 13 Sib Pairs Discordant for the *PTP1B* Genotype

| | MEAN ± SEM VALUES FOR SIBS | | | |
FEATURE	Wild Type	1484insG	OSD	P^a
Age (years)	35.7 ± 2.6	36.1 ± 2.9	22.0	NS
BMI (kg/m²)	23.7 ± .88	26.3 ± 1.14	34.2	.02
Fasting plasma glucose (mg/dl)	88.2 ± 1.16	88.2 ± 1.9	.50	NS
Fasting plasma insulin (mU/L)	7.5 ± 1.0	7.8 ± .7	4.45	NS
HOMA$_{IR}$	1.63 ± .2	1.72 ± .2	1.07	NS
Fasting serum cholesterol:fasting serum HDL cholesterol ratio	3.5 ± .3	4.35 ± .4	11.3	.02
Fasting serum triglycerides (mg/dl)	92.4 ± 14.5	116.6 ± 16.6	315.0	.03
Systolic blood pressure (mm Hg)	109.6 ± 4.2	113.4 ± 4.5	50.0	NS
Diastolic blood pressure (mm Hg)	73.3 ± 2.5	77.1 ± 2.8	50.0	.05

[a] NS = not significant.

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