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Molecular cloning and chromosomal localization of a human skeletal muscle PP-1γ1 cDNA

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Abstract. Type-1-protein phosphatase (PP-1) activity is reduced in skeletal muscle from human subjects with insulin resistance (Kida et al. 1990). This reduced phosphatase activity probably leads to the abnormal insulin action for glucose storage observed in insulin-resistant subjects. In the present study, a human homolog of rat liver PP-1γ1 cDNA was isolated from human skeletal muscle. The nucleotide sequence contains a 957-nucleotide open reading frame encoding an amino acid sequence identical to that encoded by rat liver PP-1γ1 cDNA. Northern blot analysis shows PP-1γ1-specific mRNA is expressed in human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. PP-1γ1 was localized to human Chromosome 12.

Introduction

Two major classes of serine/threonine protein phosphatases have been identified in eukaryotes, PP-1 and PP-2 (Cohen 1989). PP-1 is distinguishable biochemically from PP-2 by its sensitivity to inhibition of activity by okadaic acid and by the cellular proteins inhibitor 1 and inhibitor 2. PP-1 participates in many functions of cell metabolism, for example, protein synthesis, muscle contractility, cell growth, and glycogen metabolism (Bollen and Stalmans 1992).

Five PP-1 catalytic subunit cDNAs encoding different isoforms have been isolated from mammals (Bollen and Stalmans 1992). Both PP-1α (Cohen 1988) and PP-1β (Berndt et al. 1987), the latter also referred to as PP-1δ by Sasaki and coworkers (1990), have been identified in mammalian muscle. In humans, a cDNA for PP-1α has been isolated from liver (Barker et al. 1990), which encodes an amino acid sequence identical to that encoded by rabbit skeletal muscle PP-1α (Berndt et al. 1987; Bai et al. 1988). Evidence has also been presented for a PP-1γ isoform in a human teratocarcinoma cell line (Alessi et al. 1993). However, no cDNA sequences have been reported identifying the PP-1 catalytic subunit isoforms present in human skeletal muscle.

Recent observations suggest that abnormal PP-1 activity in human muscle (Kida et al. 1992) contributes to the reduced insulin-stimulated glycogen synthesis associated with insulin resistance for glucose disposal in man (Bogardus et al. 1984; Shulman et al. 1990). The cause of this abnormal PP-1 activity is yet unknown. One possible explanation for this abnormal activity may be a mutation in the nucleotide sequence of PP-1. Identification and characterization of the isoforms for the PP-1 catalytic subunit in human muscle are needed for an understanding of the causal role that abnormal PP-1 catalytic subunit has in the mechanism for insulin resistance for glucose disposal.

In the present study, we report the isolation and characterization of a novel human PP-1 cDNA which appears to be the human homolog of the rat PP-1γ1 (Sasaki et al. 1990).

Materials and methods

Subjects

Subjects were admitted to the Clinical Diabetes and Nutrition Section, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health. Physical examinations were given to and written consent forms were obtained from all subjects in this study. The subjects

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number L07395.

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were healthy and not taking medication. Muscle biopsies were done as described by Kida and associates (1990).

RNA isolation

Total RNA was isolated from human skeletal muscle biopsies by a modification of the method of Chomczynski and Sacchi (1987). Briefly, frozen skeletal muscle was homogenized in a solution containing 4 M guanidine thiocyanate, 23 mM Na citrate (pH 7.0), 0.5% (v/v) sarkosyl, and 0.1 M β-mercaptoethanol. Sodium acetate (pH 4.0) was added to a final concentration of 2 M along with an equal volume of phenol and 1/5 volume of chloroform. The solution was incubated for 15 min at 0°C. Following centrifugation at 10,000 *g* for 20 min at 4°C, the aqueous layer was precipitated in 1 volume of isopropanol. RNA was pelleted by centrifugation at 10,000 *g* for 10 min at 4°C, and the pellet was resuspended in 0.3 ml of homogenization solution, precipitated with isopropanol, and the final pellet was resuspended in DEPC-treated water. Poly $(A)^+$ RNA was isolated from total RNA by oligo(dT) cellulose chromatography (Aviv and Leder 1972).

Reverse transcription and PCR amplification

The polymerase chain reaction (PCR) product used as a probe for screening human skeletal muscle libraries was generated from human skeletal muscle RNA by reverse transcription PCR. Reverse transcription of cDNA from poly $(A)^+$ RNA was performed with the GeneAmp RNA PCR kit (Perkin Elmer Cetus) according to the manufacturer's instructions except that the reaction was initiated with 200 ng of poly $(A)^+$ RNA. The resulting cDNA was then amplified in a Gene Machine II Thermal Controller (USA Scientific) with PP-1α-specific (Barker et al. 1990) oligonucleotide primer sequences 5′-CTGAGCCAGCCCATTCTTCTGGAG-3′ and 5′-AATCTGCTCCATAGACTGCAG-3′ at a final sequence of 0.2 μM. PCR amplification was performed under the following conditions: 2 min at 95°C followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C with a final extension cycle of 7 min at 72°C. Oligonucleotides were synthesized on a PCR-MATE EP DNA synthesizer (Applied Biosystems) and purified on OPC purification columns (Applied Biosystems).

cDNA library screening

Custom cDNA libraries made from human skeletal muscle were purchased from Clontech; 3.6×10^5 recombinant phage were screened in a human skeletal muscle cDNA library with the human skeletal muscle PP-1α PCR product as a probe. The 420-bp probe was labeled with α^{32}P dCTP by random primed labeling (Feinberg and Vogelstein 1983) to a specific radioactivity of 6×10^8 dpm/μg (see Results). A partial human PP-1γ1 cDNA clone (IRPP-1GAM) was subsequently used to screen 1.0×10^6 recombinant phage in a second human skeletal muscle cDNA library. IRPP-1GAM was labeled to a specific radioactivity of 6×10^8 dpm/μg with α^{32}P dCTP by the random primed labeling method.

DNA sequence analysis

DNA sequences were determined by the method of Sanger et al. (1977) with Sequenase DNA polymerase (United States Biochemical Corporation). Sequences were analyzed with the IBI Pustell sequence analysis program.

Chromosomal localization

Human/hamster somatic cell hybrid DNA panels (Bios Laboratories) were used to determine the chromosome location of human PP-1γ1 by Southern blot analysis. The genomic DNA panels were probed with a partial PP-1γ1 cDNA clone, IRPP-1GAM (see results), radiolabeled with α^{32}P dCTP to a specific radioactivity of 6×10^8 dpm/μg.

Northern analysis

A Northern blot containing 2 μg poly A^+ RNA from human tissues—brain, kidney, liver, placenta, lung, heart, pancreas, and skeletal muscle—was purchased from Clontech and hybridized to an oligonucleotide probe under the following conditions: the blots were incubated at 60°C in prehybridization solution containing 2 × SSPE, 2% SDS, 2 × Denhardts, 200 μg/ml yeast tRNA, and 200 μg/ml salmon testes DNA for 2–4 h. The RNA was then hybridized to the radiolabeled PP-1γ-specific oligonucleotide probe 5′-TTATTCTGCGGTGAAGTTGAGGCTTATAAGTTAAAACAAAGGAA-3′ (5×10^6 cpm/ml) for 18–20 h at T_m-8°C (60°C). Following hybridization, the blots were washed twice in 2 × SSPE, 0.1% SDS for 15 min at 25°C, twice in 1 × SSPE, 0.1% SDS for 15 min at 60°C, with a final wash in 0.1 × SSPE, 0.1% SDS at 60°C.

Results

cDNA cloning and sequencing

A 420-bp PP-1α product was generated from human skeletal muscle by reverse transcription PCR amplification with primers corresponding to the human liver PP-1α sequence. The PCR product contains nucleotide sequences spanning the nucleotide positions + 139 to +558 of the human liver PP-1α cDNA (Barker et al. 1990) with a single nucleotide difference (A → C) at position +237, resulting in a silent mutation (S. Norman, unpublished data). This PCR product was used as a probe to screen a human skeletal muscle cDNA library (Clontech). Initially four positive clones were isolated by screening 3.6×10^5 plaques under medium stringency conditions (final wash was in 1 × SSPE, 0.1% SDS at 65°C). Three of the four isolates were identified as PP-1α by their sequence similarity to human liver PP-1α cDNA sequence. A FASTA search (Pearson and Lipman 1988) of the GenBank and EMBL nucleotide sequence databases revealed that the fourth clone, IRPP-1GAM, was a partial cDNA clone similar to the rat liver PP-1γ1 (Sasaki et al. 1990). IRPP-1GAM was subsequently used to probe 1×10^6 plaques from a second human skeletal muscle cDNA library under high stringency conditions (final wash was in 0.2 × SSPE, 0.1% SDS at 65°C). The composite nucleotide sequence of IRPP-1GAM and one clone isolated from the second library screen (14a2-211) is shown in Fig. 1. The sequence contains a 957-nucleotide open reading frame followed by a 1.266-kb 3′ untranslated region. A polyadenylation signal sequence AATAAA (Proudfoot and Brownlee 1976) is located at nucleotide position 2205–2210. A composite nucleotide sequence for PP-1γ1 has been deposited with GenBank under accession number L07395.

A comparison of the human PP-1γ1 and rat liver PP-1γ1 nucleotide sequences shows an 86% sequence similarity overall and a 93% sequence similarity for the coding region. The predicted amino acid sequence of human PP-1γ1 is 100% identical to amino acids 4–330 of rat liver PP-1γ1.

Tissue distribution of human PP-1γ1 mRNA

A Northern blot with poly A^+ mRNA from human tissues (heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas) was probed with a synthetic oligonucleotide complementary to nucleotides 1932–1975 of the human PP-1γ1 cDNA (Fig. 1). A PP-1γ1 homologous mRNA approximately 2.6 kb in length was expressed in all tissues

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GATAAACTCAACATCGACAGCATTATCCAACGGCTGCTGGAAGTGAGAGGGTCCAAGCCT   60
 D  K  L  N  I  D  S  I  I  Q  R  L  L  E  V  R  G  S  K  P

GGTAAGAATGTCCAGCTTCAGGAGAATGAAATCAGAGGACTGTGCTTAAAGTCTCGTGAA  120
 G  K  N  V  Q  L  Q  E  N  E  I  R  G  L  C  L  K  S  R  E

ATCTTTCTCAGTCAGCCTATCCTACTAGAACTTGAAGCACCACTCAAAATATGTGGTGAC  180
 I  F  L  S  Q  P  I  L  L  E  L  E  A  P  L  K  I  C  G  D

ATCCATGGACAATACTATGATTTGCTGCGACTTTTTGAGTACGGTGGTTTCCCACCAGAA  240
 I  H  G  Q  Y  Y  D  L  L  R  L  F  E  Y  G  G  F  P  P  E

AGCAACTACCTGTTTCTTGGGGACTATGTGGACAGGGGAAAGCAGTCATTGGAGACGATC  300
 S  N  Y  L  F  L  G  D  Y  V  D  R  G  K  Q  S  L  E  T  I

TGCCTCTTACTGGCCTACAAAATAAAATATCCTGAGAATTTTTTTCTTCTCAGAGGGAAC  360
 C  L  L  L  A  Y  K  I  K  Y  P  E  N  F  F  L  L  R  G  N

CATGAATGTGCCAGCATCAACAGAATTTATGGATTTTATGATGAATGTAAAAGAAGATAC  420
 H  E  C  A  S  I  N  R  I  Y  G  F  Y  D  E  C  K  R  R  Y

AACATTAAACTATGGAAAACTTTCACAGACTGTTTTAACTGTTTACCGATAGCAGCCATC  480
 N  I  K  L  W  K  T  F  T  D  C  F  N  C  L  P  I  A  A  I

GTGGATGAGAAGATATTCTGCTGTCATGGAGGTTTATCACCAGATCTTCAATCTATGGAG  540
 V  D  E  K  I  F  C  C  H  G  G  L  S  P  D  L  Q  S  M  E

CAGATTCGGCGAATTATGCGACCAACTGATGTACCAGATCAAGGTCTTCTTTGTGATCTT  600
 Q  I  R  R  I  M  R  P  T  D  V  P  D  Q  G  L  L  C  D  L

TTGTGGTCTGACCCCGATAAAGATGTCTTAGGCTGGGGTGAAAATGACAGAGGAGTGTCC  660
 L  W  S  D  P  D  K  D  V  L  G  W  G  E  N  D  R  G  V  S

TTCACATTTGGTGCAGAAGTGGTTGCAAAATTTCTCCATAAGCATGATTTGGATCTTATA  720
 F  T  F  G  A  E  V  V  A  K  F  L  H  K  H  D  L  D  L  I

TGTAGAGCCCATCAGGTGGTTGAAGATGGATATGAATTTTTTGCAAAGAGGCAGTTGGTC  780
 C  R  A  H  Q  V  V  E  D  G  Y  E  F  F  A  K  R  Q  L  V

ACTCTGTTTTCTGCGCCCAATTATTGCGGAGAGTTTGACAATGCAGGTGCCATGATGAGT  840
 T  L  F  S  A  P  N  Y  C  G  E  F  D  N  A  G  A  M  M  S

GTGGATGAAACACTAATGTGTTCTTTTCAGATTTTAAAGCCTGCAGAGAAAAAGAAGCCA  900
 V  D  E  T  L  M  C  S  F  Q  I  L  K  P  A  E  K  K  K  P

AATGCCACGAGACCTGTAACGCCTCCAAGGGGTATGATCACAAAGCAAGCAAAGAAATAG  960
 N  A  T  R  P  V  T  P  P  R  G  M  I  T  K  Q  A  K  K  -

ATGTCGTTTTGACACTGCCTAGTCGGGACTTGTAACATAGAGTATATAACCTTCATTTTT 1020
AAGACTGTAATGTGTACTGGTCAGCTTGCTCAGATAGATCTGTGTTTGTGGGGGCCCTTC 1080
CTTCCATTTTTGATTTAGTGAATGGCATTTGCTGGTTATAACAGCAAATGAAAGACTCTT 1140
CACTCCAAAAAGAAAAGTGTTTTGTTTTTTAATTCTCTGTTCCTTTTGCAAACAATTTTA 1200
ATGATGGTGTTAAAGCTGTACACCCCAGGACAGTTTATCCTGTCTGAGGAGTAAGTGTAC 1260
AATTGATCTTTTTTAATTCAGTACAACCCATAATCATGTAAATGCTCATTTTCTTTAGGA 1320
CATAAAGAGAGCCCTAGGGTGCTCTGAATCTGTACATGTTCTTGTCATAAAATGCATACT 1380
GTTGATACAAACCACTGTGAACATTTTTTATTTGAGAATTTTGTTTCAAAGGGATTGCTT 1440
TTTCCTCTCATTGTCTTGTTATGTACAAACTAGTTTTTATAGCTATCAACATTAGGAGTA 1500
ACTTTCAACCTTGCCAGCATCACTGGTATGATGTATATTTAATTAAAGCACACTTTTCCC 1560
CGACCGTATACTTAAAATGACAAAGCCATTCTTTTAAATATTTGTGACTCTTTCCTAAAG 1620
CCAAAGTTTCTGTTGAATTATGTTTTGACACACCCCTAAGTACAAGGTGGTATGGTTGTA 1680
TACACATGCTGCCTTCTTGGGGATTCAAAAACAGGTTTTTGATTTTGAATAGCAATTAGT 1740
GATATAGTGCTGTTTAAGCTACTAACGATAAAAGGTAATAACATTTTATACAATTTCCAT 1800
ATAGTCTATTCATTAAGTAATCTTTTTACAGTTGCATCAGGCCTGAACCCGTCCATTCAG 1860
AAAGCTTCAAATTATAGAAACAATACTGTTCTATACGAGTGACCGATTATGCTTTCTTTG 1920
GCCTACATTCTTTATTCTGCGGTGAAGTTGAGGCTTATAAGTTAAAACAAAGGAACTAAC 1980
TTACTGTCCACCAGTTTATACAGAACTCACAGTACCTATGACTTTTTTAAACTAAGATCT 2040
GTTAAAAAAGAAATCTGTTTCAACAGATGACCGTGTACAATACCGTGTGGTGAAAATGAA 2160
TTCAGACTTATTAAATGATGAACTTGTTAAATCTTCTCAGTGTCTATTTATCAGCACAAT 2160
ACACACAGGAGAACTGTTGATGGCATATTGAATAGATTTTCCTGAATAAATTGCTCTGGA 2220
AACCAC
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Fig. 1. Nucleotide sequence and predicted amino acid sequence of human skeletal muscle PP-1γ1 cDNA. The termination codon and polyadenylation signal site are **underlined.** The **boxed sequence** is complementary to the synthetic oligo probe used for Northern analysis (Fig. 2).

examined (Fig. 2). The variability in signal is due to differences in RNA loading between lanes, as evidenced by differences in amount of RNA visible on the ethidium bromide-stained gel used for the Northern blot (data supplied by Clontech). An additional mRNA approximately 1.65 kb in length was also detected in heart and skeletal muscle (Fig. 2, lanes 2 and 7).

Chromosomal localization

The chromosomal localization of PP-1γ1 was determined by Southern blot analysis of *Pvu*II-digested genomic DNA from human/hamster somatic hybrid cell lines (Bios Laboratories) with IRPP-1GAM as a probe (Table 1). Genomic DNA from hybrid cell lines 683, 507, and 904 hybridized to fragments identical in size to those in control lanes containing total human genomic DNA, indicating the presence of human PP-1γ sequences in these cell lines. These results are summarized in Table 1. A weak signal was also seen for hybrid cell line 756, but only after an extended exposure (8 days). Since recent karyotyping showed Chromosome (Chr) 12 is now present in only 5%–30% of the cells (Clontech, personal communication), the weak hybridization signal was apparently due to the loss of this human chromosome in the majority of cells in hybrid line 756. The only human chromosome shared among the IRPP-1GAM-positive cell lines is Chr 12. This chromosome is not present in the remaining cell lines of the panel. Therefore, this result is consistent with a location of the PP-1γ gene on human Chr 12.

The Southern blots were also probed with a human ge-

Table 1. Chromosomal localization of PP-1γ1 by Southern analysis of *Pvu*II-digested genomic DNA from human/hamster somatic cell lines (Bios Laboratories). Abbreviation: "●" indicates the presence of a specific human chromosome in >75% of the corresponding human/hamster cell line population; a percentage number is given for cell populations containing <75% of a given chromosome; + indicates a human specific hybridization pattern was observed by Southern analysis with a PP-1γ1 probe. **"D"** indicates Chr 5 was deleted at 5p15.1–5p15.2 and **"Dq"** indicates multiple deletions in 5q (Bios Laboratories Chromosome Panel Blot instruction manual).

PP1γ	Cell line	Human Chromosome																						
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
	867	60%				●								●	●				●	●					
	852		●																						
	423			●																					
	860			30%		●	●				15%									35%		●			
	803				●	●			●			15%											●	●	
	909					D	●		●						●									●	
	1006				55%	●		●	●					●		●				●		●			
	811								●									65%	65%						
	967					●			●								●								
	734					●				●									●						
	968					●				●				●										65%	
+	683					●							●		●					●		●	●		
+	507			●		●							●		65%						40%		25%		●
	750					D								●	●	●				●					
	1099	65%				D								●						40%		●	●		
	324																		●						
	940					●															●				
	983					●					15%														
	937	●				●									●	●		●				●			
	854		5%			●																			
	1079			●		●					45%					10%	●								
	756					D	65%	●					●	65%	45%					●	●	●			●
+	904					D	●						●				5%					●			●
	862					●				●															
	1049					●						●													
	212					Dq																			●

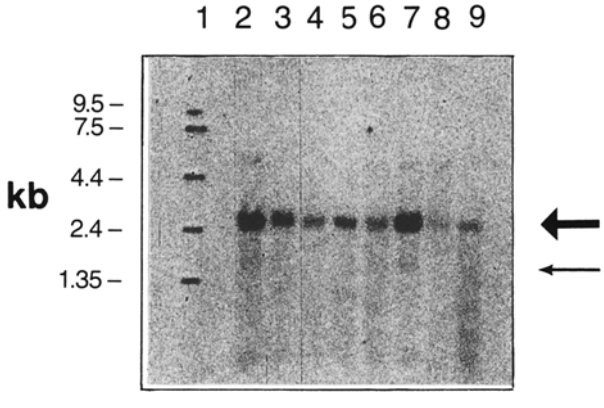

Fig. 2. Northern blot analysis of mRNA from multiple human tissues was probed with an oligo corresponding to nucleotides 1932–1975 of the human PP-1γ1 sequence (Fig. 1 boxed nucleotide sequence). The oligo hybridized to a 2.6-kb mRNA in all tissues examined **(large arrow).** Lane 1, size standard; lane 2, heart; lane 3, brain; lane 4, placenta; lane 5, lung; lane 6, liver; lane 7, skeletal muscle; land 8, kidney; lane 9, pancreas. A 1.6-kb mRNA was also detected in heart and skeletal muscle **(small arrow).**

nomic myf-5 clone containing the first exon and 2 kb of 5′ untranslated sequence (Dr. Bruce Thompson, personal communication). Myf-5 was used as a positive control because it has previously been localized to human Chr 12 (Braun et al. 1989). The Myf-5 probe gave a hybridization pattern identical to that observed with IRPP-1GAM.

Discussion

We have isolated a human homolog of rat liver PP-1γ1 from skeletal muscle cDNA libraries. The nucleotide sequence in the coding region is highly conserved (93% sequence similarity) between rat and human, with all nucleotide differences encoding silent substitutions in amino acid sequence. The overall nucleotide sequence is highly conserved with 86% sequence similarity between the two species.

A 2.6-kb PP-1γ1 homologous mRNA was detected in heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas. In addition, a 1.65-kb mRNA was also de-

tected in heart and skeletal muscle. The 1.65-kb mRNA may also be expressed in other tissues but was below the level of detection of our Northern analysis. Both of these mRNA sizes are in agreement with those reported for rat PP-1γ hybridizing mRNA expressed in kidney, heart, brain, and testis (Sasaki et al. 1990). In addition to the rat PP-1γ1 cDNA clone, Sasaki and coworkers (1990) isolated a clone from a rat testis cDNA library, PP-1γ2, which was produced from the same gene as PP-1γ1 by alternative splicing. Their Northern analysis results suggest that PP-1γ1 corresponds to a 2.6-kb mRNA while PP-1γ2 corresponds to a 1.8-kb mRNA species. The oligo probe used in our Northern analysis has nucleotide sequence corresponding to the PP-1γ-specific sequence used to identify PP-1γ1 and PP-1γ2 homologous mRNA in rat tissues (Sasaki et al. 1990). The 1.65-kb mRNA species present in human heart and skeletal muscle may also be an alternative splice product.

We have localized human PP-1γ1 to Chr 12 by Southern analysis of human/hamster hybrid cell line genomic DNA. The previous report that PP-1α is located at Chr 11q13 (Barker et al. 1990) indicates that PP-1γ and PP-1α are distinct members of a PP-1 gene family.

The identification of PP-1γ1 cDNA clones in human skeletal muscle libraries further describes the complexity of regulation of PP-1 activity in human muscle which apparently involves at least three isoforms (α, β, and γ). We are interested in determining whether a mutation in the PP-1γ gene is responsible for the reduced PP-1 activity previously observed in insulin-resistant Pima Indian subjects (Kida et al. 1992). The cDNA sequence provides information needed for the single-strand conformational polymorphism (SSCP) analysis for possible abnormalities of the PP-1γ1 isoform in insulin-resistant subjects.

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