Cloning and Characterization of \textit{PDK4} on 7q21.3 Encoding a Fourth Pyruvate Dehydrogenase Kinase Isoenzyme in Human\textsuperscript{*}

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Different isoenzymes of pyruvate dehydrogenase kinase (PDK) inhibit the mitochondrial pyruvate dehydrogenase complex by phosphorylation of the E1α subunit, thus contributing to the regulation of glucose metabolism. By positional cloning in the 7q21.3-q22.1 region linked with insulin resistance and non-insulin-dependent diabetes mellitus in the Pima Indians, we identified a gene encoding an additional human PDK isoform, as evidenced by its amino acid sequence identity (>65%) with other mammalian PDKs, and confirmed by biochemical analyses of the recombinant protein.

We performed detailed comparative analyses of the gene, termed \textit{PDK4}, in insulin-resistant and insulin-sensitive Pima Indians, and detected five DNA variants with comparable frequencies in both subject groups. Using quantitative reverse transcription polymerase chain reaction, we found that the variants identified in the promoter and 5′-untranslated region did not correlate with differences in mRNA level in skeletal muscle and adipose tissue. We conclude that alterations in \textit{PDK4} are unlikely to be the molecular basis underlying the observed linkage at 7q21.3-q22.1 in the Pima Indians. Information about the genomic organization and promoter sequences of \textit{PDK4} will be useful in studies of other members of this family of mitochondrial protein kinases that are important for the regulation of glucose metabolism.

Non-insulin-dependent diabetes mellitus (NIDDM)\textsuperscript{3} is a disorder of glucose homeostasis with complex etiology involving environmental and genetic factors (1). To search for the genetic components, we are conducting a genome-wide scan of DNA microsatellite markers in the Pima Indians of Arizona, who have the highest known prevalence of NIDDM in the world. More than half of this population over the age of 35 years is affected (2, 3), and prospective studies show that the onset of the disease in the Pima Indians is predicted by manifestations of impaired insulin action (insulin resistance; Ref. 4). Recently, we detected linkage and association of insulin resistance and NIDDM with a cluster of microsatellite markers at the cytogenetic band q21.3-q22.1 on chromosome 7 (5, 6), and one of these markers (D7S479) was also linked with NIDDM in Caucasian families living in Utah. Because these results are consistent with the presence of an NIDDM susceptibility locus at 7q21.3-q22.1, we have begun to search for potential candidate genes using a positional cloning approach.

Several genes have been identified and physically positioned within this region (7–9), including a novel transcription unit encoding a cDNA with more than 65% identity to the sequences of two isofoms of rat pyruvate dehydrogenase kinase, PDK1 (10) and PDK2 (11). PDKs are serine/threonine protein kinases that selectively inhibit the activity of pyruvate dehydrogenase (PDH), a mitochondrial multienzyme complex that catalyzes the first irreversible step in glucose oxidation (reviewed in Ref. 12). Rat PDK1 and PDK2 belong to a newly recognized family of mitochondrial protein kinases (PDH), which catalyzes the first irreversible step in glucose metabolism, and based on our previous linkage results, the \textit{PDK4} gene at 7q21.3 was an excellent candidate for further analyses with respect to insulin resistance and NIDDM in the Pima

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\textsuperscript{6} The abbreviations used are: NIDDM, non-insulin-dependent diabetes mellitus; PDH, pyruvate dehydrogenase; PDK, pyruvate dehydrogenase kinase; bp, base pair(s); kb, kilobase(s); Mb, megabase(s); PCR, polymerase chain reaction; RT, reverse transcription; YAC, yeast artificial chromosome; UTR, untranslated region; RACE, rapid amplification of cDNA ends; SSCP, single-strand conformation polymorphism; RFLP, restriction fragment length polymorphism; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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Indians. Here we report cloning and characterization of PDK4 and show that the corresponding recombinant protein phosphorylates and inactivates PDH. We also performed a comparative molecular analysis of the gene in insulin-sensitive and insulin-resistant Pima Indians.

EXPERIMENTAL PROCEDURES

Subjects—The subjects are members of the Gila River Indian Community who have participated in longitudinal studies of NIDDM since 1965 (3, 4). Measurements of clinical parameters of insulin action and classification of NIDDM (4), percutaneous biopsies of skeletal muscle (16) and adipose tissue (17), and preparation of genomic DNA samples (18) in this population have been described previously. Subjects selected for inclusion in the study were free of known cardiac or renal disease and were not first degree relatives and were divided into an insulin-resistant and insulin-sensitive group according to their insulin-mediated glucose uptake rates determined by the two-step hyperinsulinemic euglycemic clamp (4) using criteria described in Ref. 19.

Polymerase Chain Reactions—Amplifications were performed in GeneAmp 9600 thermal cycler (Perkin-Elmer, Norwalk, CT) as described previously (20). The PCR amplification of exon 1 was not first degree relatives and was divided into an insulin-resistant and insulin-sensitive group according to their insulin-mediated glucose uptake rates determined by the two-step hyperinsulinemic euglycemic clamp (4) using criteria described in Ref. 19.

Direct cDNA Selection and Isolation of the Full-length cDNA Clone—The direct cDNA selection experiments were conducted as described previously (20). Four YAC clones (HSC7E1275, HSC7E1301, HSC7E1170, and HSC7E1180) that encompass approximately 3 Mb of DNA and 10 microsatellite markers (7cen-AMFA1573b-AMFA283x1-D7S527-D7S1812-D7S821-D7S2553-D7S479-D7S476-D7S491-D7S1796-Tqter) at 7q13-q22.1 (7) were used. The YAC DNA was purified away from the endogenous yeast chromosome by pulsed-field gel electrophoresis, transferred to nylon membrane, and used as substrate for exhaustive hybridization with PCR-amplified cDNA pools made from RNA isolated from 10 tissues.

A 1.7-kb cDNA clone (designated FC4–1#1) was isolated from a frontal cortex cDNA library purchased from Stratagene (La Jolla, CA). Since the 5′ end of the transcript was not represented in this clone, a two-step PCR protocol utilizing 5′-RACE-ready cDNA from skeletal muscle (Clontech) was used. Primary PCR was performed in a 25-μl volume with 0.5 μg of the cDNA using antisense primer PDHK-13R (5′-CAGGTTTTAACCATTGACCTG-3′) located in exon 2 in combination with the anchor primer provided with the cDNA (Clontech). Because we experienced difficulties in amplification of the 5′-UTR and of the first exon using standard PCR conditions (possibly due to the high GC content of the 5′ region), dGTP was reduced to 50 μM, and 150 μM of either 7-deaza-dGTP or dITP were added in separate PCR experiments to facilitate structure-independent amplification (22). Furthermore, AmpliTaq DNA polymerase was not included in the master mix, but was added to each tube after an initial denaturation at 98 °C for 3 min. Cycling parameters included 30 cycles of 98 °C for 20 s, 57 °C for 30 s, and extension for 2 min (at 72 °C for the first 5 cycles, and at 72 °C for the remaining 25 cycles). A 0.5-μl aliquot of the primary PCR was used for a secondary PCR performed under the same conditions with the nested antisense primer PDHK-2R (5′-GTAAGCCTGCTTCTAGGAA-CAGCG-3′) and the same anchor primer. Under both conditions (with 7-deaza-dGTP or dITP), we amplified a specific product as verified by sequencing (see below).

The remaining part of 3′-UTR was isolated by 3′-RACE using the Marathon cDNA amplification kit (Clontech) in combination with the Expand long template PCR system (Boehringer Mannheim). The sense primer PDHK-7 (5′-CCTCAGTTTTTCCATCTGTTTTT-3′) from the 3′ end of FC4–1#1 was used in combination with the adaptor primer APL supplied with the Marathon kit. The 5′- and 3′-RACE products were subcloned into pcRII vector using the TA cloning kit (Invitrogen, San Diego, CA), and sequenced with the DyeDeoxy terminator cycle sequencing kit or with the Sequenase terminator sequencing kit (both from United States, Inc.) for sequencing with various 5′-3′ end-labeled primers. The sequences were confirmed by sequencing an internal segment of the PCR-amplified 3′-UTR using the DyeDeoxy terminator cycle sequencing kit (Perkin Elmer) according to the manufacturer’s recommendations, except that the post-preamplification washes were performed at 37–50 °C to minimize nonspecific background. Samples were then sequenced on an ABI automated sequencer model 373A (Perkin Elmer).

Quantitative RT-PCR—Total RNA was isolated from skeletal muscle or abdominal subcutaneous fat biopsies as described previously (19). For quantitative mRNA analyses in skeletal muscle, oligo(dT)-primed cDNAs were amplified using PCR in standard mapping. PDHK-6 (5′-TGGATT- TCTCGTCTGTTATG-3′) and PDHK-7R (5′-AAAAACAGATGGAAAAACT- GAGG-3′) were used as sense and antisense primers, respectively. The amplified PCR products were labeled with [32P]dCTP and hybridized onto a nitrocellulose filter. The amount of specific radioactivity was determined by liquid scintillation counting. The amount of 3′-UTR RNA was determined in randomly primed cDNA samples amplified with an initial amplification with primer PDHK-9 (5′-GAGACCTTGGGAAGGG-3′) and PDHK-7R (5′-GAGACCTTGGGAAGGG-3′) in the second exon, or PDHK-13R (5′-CAGGTTTTAACCATTGACCTG-3′) in the third exon. After 35 PCR cycles, 0.2 pmol of 3′-32P-end-labeled antisense primer PDHK-1R (5′-CCTCAGTTTTTCCATCTGTTTTT-3′) were added to each sample, and one additional cycle (96 °C for 20 s, 57 °C for 30 s, 72 °C for 5 min) was performed to synthesize the complementary strand encompassing the variant site. After denaturation, the single-stranded labeled products were resolved on a 6% sequencing gel to differentiate between the 73- and 75-base-long allelic forms. To assess the possibility of allele-specific differences in expression, the variant site was sequenced. Southern and Northern Blot Analyses—DNA samples from CEPH Caucasian subjects were obtained from the NIGMS repository (Correll...
Institute, Camden, NJ), and non-human genomic DNAs (for the “zoo” blot) were obtained from Clontech. Southern blots were developed with 10 μg of EcoRI-digested genomic DNA, or with 50 ng of purified cosmid DNA transferred to a nylon membrane, and hybridized as described previously (25). The final post-hybridization wash of human DNA blots was at 65 °C in 0.1 × SSC/0.1% SDS, and the “zoo” blot was washed at 55 °C in 0.5 × SSC/0.1% SDS.

A human multiple tissue Northern blot containing approximately 2 μg of poly(A)+ RNA from heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas was obtained from Clontech. Hybridization with 32P-labeled FC4–1#1 insert was performed according to manufacturer’s recommendations, and the final wash was at 65 °C in 0.1 × SSC/0.1% SDS. To confirm that all lanes contain comparable amounts of intact mRNA, the blot was stripped and rehybridized with a β-actin cDNA probe provided by the manufacturer.

Cloning of PDK4 into the Baculovirus Transfer Vector—The cDNA clone FC4–1#1 contains a 1748-bp insert with the initiating methionine and stop codon at positions 173 and 1406, respectively. By PCR methodology, a tag encoding for 6 histidine residues was incorporated at the 3′ end of the open reading frame immediately before the stop codon. This modified cDNA (also lacking 5′- and 3′-UTR regions) was ligated into pESCRP (Stratagene) and the amplified regions were sequenced to confirm that no errors were introduced. The cDNA was then ligated into the baculovirus transfer vector pVL1393 (Invitrogen) for transfection into Sf9 cells. This construct is referred to as PDK4-his/pvl.

Transfection of Sf9 Cells—Sf9 cells were maintained in Grace’s medium, pH 6.2 (Life Technologies, Inc.), supplemented with 3.3 g/liter TC yeast extract, 3.3 g/liter l-glutamate, 0.35 g/liter NaHCO3, 0.1% Pluronic F-68, and 10% fetal bovine serum. Cells were grown as a suspension culture at 27 °C at 110 rpm, and passed twice weekly to a concentration of 0.5 × 106 cells/ml. Methods for transfection of the cDNA and plaque purification of recombinant virus were essentially as described in Ref. 26. Briefly, 1 μg of PDK4-his/pvl and 200 ng of linearized AcBP23acZi (Pharmpingen) were mixed with 5 μl of Cellfectin (Life Technologies, Inc.) and transfected into Sf9 cells. Two days later, serial dilutions of the medium were applied to Sf9 cells for plaque assay. Plaques were visualized with an overlay of 0.5% SeaKem-agarose (FMC BioProducts) containing 0.05 mg/ml Neutral Red (Sigma) and 0.25 mg/ml 5-bromo-4-chloro-3-indoyl β-n-galactoside (Life Technologies, Inc.). One day later clear plaques were picked and used to infect Sf9 cells. When noticeable infection was present (4–6 days), the cells were screened for PDK activity by incorporation of 32Pi into recombinant E1o protein (see “PDK Assay” below). Virus-containing medium from cells that expressed PDK4 was plaque-purified as described in (26).

PDK Assay—Infected cells were lysed in 10 mM imidazole pH 7.2, 5 mM DTT, 0.1 mM EDTA, 10−7 M pepstatin, 2 mM phenylmethylsulfonyl fluoride, and 2% μg leupeptin with 30 passes in a Thomas homogenizer. The homogenate was centrifuged at 16,000 × g for 20 min, and the supernatant used in the kinase assay. For the kinetic assay, 50 μl of medium contained 20 mM Hepes pH 7.5, 50 mM KCl, 2 mM DTT, 0.1 mg/ml bovine serum albumin, 0.5 mM γ-32PiATP (~500 cpm/pmol), 5 mM MgCl2, 20 μl of supernatant (5–10 μg of protein), and 0.85–2.5 μg of recombinant E1o (a gift from Dr. M. S. Patel, State University of New York, Buffalo). The reaction was assayed for 10 min at room temperature and stopped with 10 μl of 5% SDS buffer. Proteins were resolved by 10% SDS-PAGE and exposed to X-Omat film at −70 °C. In some instances, the band corresponding to E1o was excised from the gel and counted in 2 ml of Omnifluor scintillation solution (ICN Biomedicals).

Cloning and Characterization of a Full-length cDNA—The initial PDK4 cDNA clone (E1301ed24) was isolated by the technique of direct selection (28, 29) from YAC clone HSC7E1301 (7), which encompasses approximately 1 Mb of DNA in the Fq21.5 region. The gene was positioned between ON and D7S527 immediately adjacent to the microsatellite marker APM2A303x.1. The cDNA clone from the YAC clone in this region has been described previously (7, 9). The E1301ed24 fragment (0.4 kb) was used to screen a frontal cortex cDNA library, and a 1.7-kb clone (FC4–1#1) was isolated. The se-
The predicted protein of 411 amino acid residues (Fig. 1) with a calculated molecular weight of 46,466. Data base searches detected a significant similarity of the predicted protein with rat PDK1 (65% identity/81% similarity allowing for conservative substitutions), rat PDK2 (65%/82%), and with the hypothetical protein ZK370.5 from C. elegans (48%/65%). The deduced protein described here, PDK4, has 62%-66% identity/79%-82% similarity with the three human PDK isoforms, and we performed multiple alignment of all four proteins to determine shared regions of homologies. As shown in Fig. 2, PDK4 contains all motifs characteristic for the putative kinase domain of the eukaryotic mitochondrial kinase family as defined previously (11,15), inculding subdomains I–V (with conserved residues underlined) are indicated in Roman numerals (15).

To determine if other species have genomic sequences homologous to PDK4, the FC4–1 probe was used to hybridize to Northern blot containing poly(A)⁺ RNA from eight human tissues. As shown in Fig. 3, a single band approximately 4 kb long was detected in all lanes, and the strongest signal was observed in heart and skeletal muscle. Because the 1.7-kb clone did not represent the full-length transcript, we isolated the corresponding ends by 5'- and 3'-RACE PCR. All four sequenced 5'-RACE clones (see “Experimental Procedures”) begin at the same adenosine residue, that we have designated as the putative transcription start site in Fig. 1 and Fig. 5. By sequencing of a 3'-RACE product, we found that the poly(A) tail is preceded by the typical polyadenylation signal AATAAA (Fig. 1).
sponding to intron number 1, 2, 4, and 5) are located at analogous sites in PDK4 (intron 3, 4, 7, and 8), consistent with a conservation of their genomic structures (data not shown).

To investigate the promoter region, we have sequenced approximately 4 kb of the 5' region preceding the start of the coding sequence. Although the gene does not have a typical TATA box, a TATA-like motif (AAATAAAA) was found beginning at position −26 from the putative transcription start site. Other predicted transcriptional regulatory elements include an inverted CCAAT box at −2114, four putative SP1-binding sites (GGGCGG) at −216, −2143, −2338, and −2561, and an AP2 site (GGCAGCCC) at −1631. As shown in Fig. 5, all of these elements are clustered within a −640-bp segment immediately upstream from the transcription initiation site.

Screening for DNA Variants in PDK4—To search for large variants or rearrangements in the locus, Southern blots of EcoRI-digested genomic DNA from 52 Pima Indians (23 insulin-sensitive and 29 insulin-resistant), and from a Caucasian subject were hybridized with the FC4–1#1 clone. Using stringent washing conditions, four invariant bands were observed in all subjects, consistent with the absence of gross structural alterations of the gene in the Pima Indians (data not shown).

PCR-amplified genomic segments from 30 subjects were screened for small (e.g. single-base) variants as described under “Experimental Procedures.” The only difference detected in the coding sequences was a silent substitution (GCA → GCC) at the Ala341 codon in exon 10, with a nearly identical frequency in the insulin-resistant and insulin-sensitive groups (0.68 and 0.69, respectively; p = 0.88).

The promoter and 5'UTR were analyzed in three segments to include the transcription start site, and the recognized putative transcription factor binding sites described above. As shown in Fig. 5, we detected two variants in the 5'UTR, including a two-base (C/CCC) polymorphism (presumably an insertion or deletion) at position +138 (referred to as C+138 and CCC+138, respectively). The frequency of the CCC+138 allele was 0.28 in the insulin-resistant group, and 0.31 in the insulin-sensitive group (p = 0.64). The CCC+138 variant was also found at a comparable frequency (0.28) in 9 CEPH Caucasian subjects. A less common A → G substitution was detected at position +15 with a frequency 0.11 in the insulin-resistant, and 0.06 in the insulin-sensitive group (p = 0.2).

Two single-base substitutions were also detected in the promoter, including a T → C substitution at −153 located 5 bp upstream from the second SP1 site, and a C → T substitution at −208 (Fig. 5). Both variants create a RFLP at an MspI site (recognition sequence 5'CCGG), including a loss of the restriction site at −208 (CCGG → CTGG; designated site 1), and a gain of the restriction site at −153 (CTGG → CCGG; designated site 2). Because of their close proximity, both variants were typed simultaneously on a single PCR product (see “Experimental Procedures”). We observed three combinations (haplotypes) of the variants at sites 1 and 2 in the Pima Indi-
and imidazole wash. Essentially all lanes 1–22 381 left axis imidazole eluted very little as substrate. Molec-
ular weight markers (Bio-Rad) are: rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

**Fig. 6. Purification of PDK4 from Sf9 cells.** A, autoradiogram of PDK4 activity measured as described under “Experimental Procedures” using recombinant E1α as substrate. Lane 1, Sf9 extract; lane 2, Ni-NTA flow-through; lanes 3–5, the Ni-NTA washes as described under “Experimental Procedures”; lane 6, 100 mM imidazole wash; lanes 7–9, 500 mM imidazole elution. Lanes 8 and 9 contain 1/5 and 1/10 the amount of protein as lane 7, respectively. The radioactive band at 46.5 kDa corresponds to E1α; PDK4, visualized by Coomassie staining, migrated slower, was resolved from E1α protein, and was not phosphorylated under these conditions (data not shown). B, silver-stained gel. Lane 1, Sf9 extract; lane 2, Ni-NTA flow-through; lane 3, Ni-NTA 100 mM imidazole wash; lane 4, Ni-NTA 500 mM imidazole elution. Molecular weight markers (Bio-Rad) are: rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).

**Fig. 7. Time-course inactivation and phosphorylation of kinase-depleted PDH by PDK4.** Kinase-depleted PDH was incubated with 1 μg of PDK4 for 0.5, 1, 2, and 4 min, and the residual PDH activity measured as described under “Experimental Procedures.” Values on the left axis are expressed as % maximal activity, which is defined as the amount of PDH activity measured in the absence of PDK4. The right axis shows the amount of pmol of 32P incorporated into the E1α subunit of the PDH complex over the same time course.
was no inhibition of PDH activity if ATP was omitted from the reaction (data not shown). To determine if PDK4 phosphorylated the E1α subunit of the complex, the phosphorylation part of the two-step assay was repeated using [γ-32P]ATP. The radioactive band migrating at ~46.5 kDa corresponding to the E1α subunit was excised and counted. The results, plotted on the right axis of Fig. 7, indicate a time-dependent increase in the phosphorylation of the E1α subunit that closely coincides with the time course of the inhibition of PDH activity.

**DISCUSSION**

Pyruvate dehydrogenase kinase is important for the regulation of mitochondrial PDH activity which plays a key role in the oxidative metabolism of glucose. Recent studies have described multiple, genetically distinct mammalian PDK isoenzymes, including two forms in rat (10, 11), and three in human (15). Here we describe a fourth human isoform (PDK4) encoded by a gene located at 7q21.3. Our evidence that this protein is an authentic PDK includes: 1) a high degree of identity with all known mammalian PDK isoforms, 2) the ability to phosphorylate the E1α subunit of PDH, and 3) the ATP-dependent inactivation of the PDH complex. The PDK4 transcript was present in all tissues examined, and the highest level was detected in heart and skeletal muscle, as was also found for other PDK isoforms (15). Assuming that the protein level corresponds to mRNA, we expect that the amount of PDK4 varies considerably in different tissues. Development of PDK4-specific antibodies will help to address this issue, and also to determine what proportion of the total PDH protein is controlled by this isoform.

Our Southern blot data indicate that the PDK4 cDNA probe does not cross-hybridize with other members of the PDK family in human, and we predict that the simple direct hybridization patterns detected on the "zoo" blot correspond to conserved homologues of PDK4 in other species. In addition, the similarity of the exon-intron organization between PDK4 and the ZK370.5 gene in C. elegans indicates an evolutionary conservation of their genomic structure.

The PDK4 gene was identified by positional cloning in a region on 7q that is linked with insulin-resistance and NIDDM in the Pima Indians. Based on comparative analyses of the genomic sequences and of the transcript, we conclude that alterations in this gene are unlikely the underlying basis for the linkage of the 7q21.3-q22.1 region with insulin resistance and NIDDM in the Pima Indians. Therefore, other genes in this chromosomal interval need to be investigated as potential candidates. Identification of a fourth PDK isoenzyme in human, and knowledge of the organization of the gene and of its promoter sequence provide new information that should facilitate molecular genetic studies of other members of this mitochondrial kinase family, and of their function in the regulation of glucose metabolism.

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