Primary Structure of Pyruvate Dehydrogenase Kinase Establishes a New Family of Eukaryotic Protein Kinases*  

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We recently reported molecular cloning of the branched chain α-ketoacid dehydrogenase kinase, the first mitochondrial protein kinase to be cloned (Popov, K. M., Zhao, Y., Shimomura, Y., Kuntz, M. J., and Harris, R. A. (1992) J. Biol. Chem. 267, 13127-13130). From a search for proteins related to the branched chain α-ketoacid dehydrogenase kinase, a cDNA encoding the 434 amino acid residues corresponding to pyruvate dehydrogenase kinase has been cloned from a rat heart cDNA library. Evidence that the clone codes for pyruvate dehydrogenase kinase includes: (a) the deduced amino acid sequence is identical to the partial sequence of the kinase determined by direct sequencing; (b) expression of the cDNA in *Escherichia coli* resulted in synthesis of a protein that phosphorylated and inactivated the pyruvate dehydrogenase complex; (c) kinase activity of the recombinant protein is sensitive to inhibition by a specific inhibitor of pyruvate dehydrogenase complex; and (d) antiserum raised against the recombinant protein recognized the protein subunit known to correspond to pyruvate dehydrogenase kinase in a highly purified preparation of the pyruvate dehydrogenase complex. Like the branched chain α-ketoacid dehydrogenase kinase, pyruvate dehydrogenase kinase lacks motifs usually associated with eukaryotic Ser/Thr-protein kinases. Considerable sequence similarity exists between these mitochondrial protein kinases and members of the prokaryotic histidine kinase family, a diverse set of sensing and response systems important in the regulation of bacterial processes. Thus, molecular cloning of these proteins establishes a new eukaryotic family of protein kinases that is related to a prokaryotic family of protein kinases.

Activities of mammalian pyruvate dehydrogenase (PDH) and branched chain α-ketoacid dehydrogenase (BCKDH) complexes are regulated by phosphorylation/dephosphorylation (1, 2). These complexes, both located in the mitochondrial matrix space, play central roles in the oxidative disposal of pyruvate and the branched chain amino acids. Regulation of these complexes is achieved by highly specific protein kinases and phosphoprotein phosphatases encoded in the nucleus but located exclusively in the mitochondrial matrix space. BCKDH kinase (EC 2.7.1.115) was cloned recently in this laboratory (3). The deduced amino acid sequence of BCKDH kinase lacks sequence motifs usually associated with eukaryotic Ser/Thr-protein kinases (4). We report here the molecular cloning of PDH kinase (EC 2.7.1.99). It lacks sequence similarity with all eukaryotic Ser/Thr-protein kinases other than BCKDH kinase. Like BCKDH kinase, however, it has considerable sequence homology with prokaryotic histidine protein kinases.

Since previous work indicates that long term regulatory mechanisms are involved in control of PDH kinase activity (5, 6) and, therefore, the proportion of the PDH complex in the active, dephosphorylated state, the availability of a PDH kinase cDNA will facilitate future work on the molecular mechanisms responsible for regulation of expression of this enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**—Labeled nucleotides were obtained from DuPont NEN; αgt10 rat heart cDNA library from Clontech, Palo Alto, CA; DNA-modifying enzymes from Life Technologies, Inc.; polymerase chain reaction (PCR) reagents from Perkin-Elmer Cetus; pET expression system from Novagen, Madison, WI; site-directed mutagenesis reagents from Pharmacia LKB Biotechnology Inc.; and Sequenase version 2.0 sequencing reagents from U. S. Biochemical Corp. Other materials and reagents were of analytical grade.

**Isolation of PDH Kinase cDNA Clones by Using the 3′-RACE Protocol**—The primer AA(A/G)AA(C/T)GC(N)ATGAGWG)GC(N)AC was designed on the basis of the amino acid sequence of BCKDH kinase within the conserved region II (KNMARMAT) (3). cDNA synthesis and PCR were performed with rat heart mRNA according to the 3′-RACE (rapid amplification of cDNA ends technique) protocol (7). The 650-nucleotide product was isolated from an agarose gel, subcloned into M13 mp18, and sequenced.

**cDNA Library Screening**—A rat heart αgt10 library was screened with the PCR product as a probe using standard molecular biology protocols (8). Of the 10th bacteriophage screened, four contained inserts that hybridized with the probe. The inserts from these αgt10 phages were subcloned and sequenced using Sequenase kits (U. S. Biochemical Corp.) with either dUTP or d-deaza-GTP according to protocols of the manufacturer.

**Expression of Rat Heart PDH Kinase in Escherichia coli**—For the purpose of generating recombinant protein suitable for the production of an antisera, the C-terminal domain of PDH kinase was expressed in E. coli as follows. BstBHI fragment of PDH kinase cDNA was inserted in the BamHI site of pET 15b expression vector to produce N-terminal in-frame fusion with His-Tag. Resulting plasmid was transfected in *Escherichia coli* to express recombinant PDH kinase. The recombinant protein was purified to homogeneity by the following steps: (i) eluting from the His-Tag affinity column, (ii) desalting on a Sephadex G-25 column, and (iii) separating by SDS-PAGE.

**Expression of cDNA Clones in Recombinant Bacteria**—Isolated cDNA clones were ligated into the expression vector, pET 15b, to produce recombinant molecules. Bacterial cells were transformed with cDNA expression vector or control vector. Expression of cDNA was induced with IPTG, the recombinant protein was purified to homogeneity by the same steps as described above, and the purified protein was subjected to SDS-PAGE and Western blotting.

**Purification of Recombinant Protein**—Recombinant protein was purified to homogeneity by the following steps: (i) eluting from the His-Tag affinity column, (ii) desalting on a Sephadex G-25 column, and (iii) separating by SDS-PAGE and Western blotting.

**SDS-PAGE**—Proteins were resolved by SDS-PAGE (14% gel) and stained with silver as described.

**Western Blotting**—Proteins were separated by SDS-PAGE and transferred to nitrocellulose sheets, and the blotted membranes were probed with polyclonal antiserum raised against the recombinant protein.
HMS 174 (DE3) cells. IPTG-induced transformants synthesized a protein of 36,000 Da. Subcellular fractionation revealed that greater than 90% of the recombinant protein accumulated in an insoluble form. Inclusion bodies were purified by using standard techniques (8). Protein was solubilized with 7 M urea and purified by metallochelate chromatography (9). The yield was approximately 20 mg/liter of culture.

An antiserum raised in New Zealand rabbits against the recombinant protein was highly specific toward PDH kinase and suitable for detection of 0.1-1.0 ng of kinase protein in Western blot analysis.

For the purpose of generating recombinant PDH kinase with enzymatic activity, M13 mp18 carrying PDH kinase cDNA was cut with XcmI and SacI, blunt-ended, and religated to make a cDNA encoding the mature protein. A SalI/NarI (blunt) fragment was cut out from the resulting plasmid and ligated between SalI and XhoI (blunt) sites of the pET 21d expression vector (Novagen) to produce an N-terminal in-frame fusion with T7-Tag.

Western and Northern Blot Analysis—PDH (5 ng) and BCKDH (3 pg) purified according to Ref. 10 were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with guinea pig antibodies against BCKDH kinase (dilution 1:300) or rabbit antibodies against recombinant PDH kinase (dilution 1:500). Staining procedures were as described previously (10).

**FIG. 1. Cloning of PDH kinase cDNA.** a, schematic representation of the subdomains conserved in bacterial histidine protein kinases and BCKDH kinase. Amino acids conserved in subdomains I, II, III, and IV of bacterial protein kinases are indicated by boldface letters; amino acid sequences and the spacing between subdomains correspond to that of BCKDH kinase. The degenerate 3'-RACE primer was based upon the amino acid sequence of subdomain II. b, nucleotide and deduced amino acid sequence of PDH kinase. The N-terminal and internal tryptic peptide sequences determined for the purified protein are underlined.

| a | b |
|---|---|
| ![DNA Sequence](image) | ![Peptide Sequence](image) |

**PDH Kinase Activity Assay—** For phosphorylation experiments 0.2 millimolar of kinase-depleted porcine heart PDH was combined with 0.1 mg of *E. coli* cytosol protein in a final volume of 100 μl containing 20 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 50 mM KCl, 0.05% (w/v) Triton X-100, 50 mM dithiothreitol, and 0.2 mM (γ-32P)ATP (specific activity 250 cpm/nmol). At the indicated times, 10-μl aliquots were withdrawn, and the reaction was stopped with 10 μl of SDS-denaturating mixture. Samples were separated on 10% SDS-PAGE and autoradiographed.

ATP-dependent inactivation experiments were performed essentially as described above, except that an ATP regeneration system (25 mM creatine phosphate and 0.5 unit/ml creatine kinase) was included and labeled ATP excluded. Residual activity of the multienzyme complex was assayed as described (10).
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Sequence analysis of BCKDH kinase showed that, in contrast to all other mammalian Ser/Thr-protein kinases, this protein has a completely different structure of the kinase domain that resembles bacterial histidine protein kinases (3, 11). Structurally, the family of bacterial histidine protein kinases is defined by short regions of common sequences, similarly arranged, but variably spaced. The first region, the most variable of the four, is located in the N-terminal half of the kinase and includes a histidine residue that serves as the site of autophosphorylation. The other regions are located in the C-terminal half of the molecule. An invariant asparagine residue occurs in the second region; glycine-rich loops characteristic of nucleotide-binding domains (12) appear in the third and fourth regions. BCKDH kinase contains the four conserved regions, all arranged exactly as they are in bacterial histidine protein kinases (Fig. 1a).

In a search for proteins related to BCKDH kinase, degenerate gene-specific oligonucleotide primers were designed corresponding to conserved sequences of BCKDH kinase and used to amplify rat heart mRNA using the 3'-RACE protocol (7). One primer, corresponding to the sequence of conserved region 11, generated two PCR products, one of which was BCKDH kinase, whereas the second encoded a clearly different protein sharing significant sequence similarity with BCKDH kinase within the putative kinase domain. This PCR product was used to screen a rat heart cDNA library. Four positive clones were obtained. Three of them appeared to be partial clones, whereas the fourth one contained a cDNA of 1.5 kilobases (Fig. 1b). The transcript of this clone has an open reading frame defined by an ATG triplet at base 36 and an in-frame stop at position 1338. It encodes a polypeptide of 434 amino acids with a calculated molecular weight of 49,136. The sequence of the first 26 amino acids corresponds to a typical mitochondrial leader peptide (13), i.e. this stretch of amino acids is devoid of negatively charged residues and enriched in hydrophobic and positively charged residues. The mature protein, which we know from direct amino acid sequencing, starts with amino acid number 1 in Fig. 1a and is a polypeptide of 408 amino acids with a calculated molecular weight of 48,000 estimated for the α subunit of PDH kinase by SDS-PAGE (10, 14). Alignment with BLAST and TFASTA algorithms revealed 30% amino acid iden-
Pyruvate Dehydrogenase Kinase

PDHK
PKCE
BCKI
TRBP

PDH kinase sequence comparison with an especially high degree of similarity within the C-terminal region. All four conserved motifs defining the putative kinase domain were found within the open reading frame, suggesting that the cDNA encodes a protein homologous to BCKDH kinase. The sequences of four polyptides encoded by the open reading frame correspond exactly to the sequences of three tryptic polypeptides derived from purified preparations of PDH kinase (10) as well as the N-terminal sequence of the mature protein (underlined in Fig. 1b).

To gain further proof that the cloned cDNA encodes PDH kinase, the cDNA encoding the mature protein was expressed in E. coli. A recombinant protein of apparent molecular weight 48,000 recognized by anti T7-Tag antibodies appeared in IPTG-induced transformants of E. coli (data not shown). Reconstitution of the recombinant protein with kinase-depleted PDH restored ATP-dependent phosphorylation (Fig. 2a) and inactivation (Fig. 2b) of the enzyme complex. A highly specific inhibitor of PDH kinase, dichloroacetate (17), abolished ATP-dependent inactivation of the reconstituted complex. As further confirmation, antiserum raised against the C-terminal half of the recombinant protein recognized a single polypeptide of molecular weight 48,000 in preparations of highly purified rat heart PDH (Fig. 3), consistent with a large body of evidence (14, 18) that the kinase tightly associates and co-purifies with PDH through several chromatographic steps. No cross-reactivity of PDH kinase antiserum with BCKDH kinase nor of BCKDH kinase antiserum with PDH kinase was observed (Fig. 3), further confirming that unique protein kinases are associated with these closely related enzyme complexes (10). These observations taken together provide convincing evidence that the cloned cDNA represents PDH kinase.

Northern blot analysis of rat tissue RNA using the PDH kinase cDNA as a probe revealed three transcripts of about 1.2, 1.8, and 3.5 kilobases in heart, kidney, liver, and skeletal muscle (Fig. 4). These results suggest that alternative polyadenylation sites may be utilized or that alternatively spliced isoforms of PDH kinase may be expressed in different tissues. The relative abundance of all PDH kinase transcripts gradually decreased from heart to skeletal muscle: heart > kidney > liver > skeletal muscle, correlating well with the relative activity of PDH kinase measured in these tissues. A search of the current GenBank database has revealed two hypothetical proteins with considerable sequence similarity to PDH kinase and BCKDH kinase (Fig. 5). Hypothetical protein ZK 370.5 from C. elegans (19) shows remarkable similarity to PDH kinase (up to 49% identity and 64% similarity, when conserved substitutions are allowed) and may correspond to the PDH kinase of this organism. A second protein, hypothetical phosphoprotein 3 from Trypanosoma brucei (20), has around 20% overall positional identity with PDH and BCKDH kinases but lacks one glycine-rich loop (region 111). The latter protein is a subject of special interest because (a) the protein phosphorylated by this putative protein kinase is unknown and (b) very specific patterns of expression occur during the developmental cycle of T. brucei (20).
The results of this study suggest that eukaryotic cells contain a novel family of protein kinases sharing a high degree of similarity with bacterial histidine protein kinases. BCKDH kinase, PDH kinase, and similar molecules apparently participate in signaling within the mitochondria matrix space, controlling fluxes through different metabolic pathways. Further analysis of already identified kinases and additional searches for other members of this family should provide new insight into basic mechanisms regulating mitochondrial processes.

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