Molecular Cloning of the p45 Subunit of Pyruvate Dehydrogenase Kinase*

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Purified preparations of rat heart pyruvate dehydrogenase kinase have two polypeptides with molecular weights of 48,000 (p48) and 45,000 (p45). Recently, we reported the primary structure of p48 (Popov, K. M., Kedishvili, N. Y., Zhao, Y., Shimomura, Y., Crabb, D. W., and Harris, R. A. (1993) J. Biol. Chem. 268, 26602-26606) and presented evidence that (i) it exhibits kinase activity for pyruvate dehydrogenase and (ii) it belongs to a family of mitochondrial protein kinases unique from other eukaryotic protein kinases. Here, we report the molecular cloning and deduced amino acid sequence of p45. The protein sequence of p45 has 70% identity to the protein sequence of p48. Minor differences exist throughout the protein sequences with the greatest difference occurring at the amino termini. Recombinant p45 protein, expressed in Escherichia coli and purified to homogeneity, catalyzed the phosphorylation and inactivation of kinase-depleted pyruvate dehydrogenase complex, indicating that p45 and p46 correspond to different isoforms of pyruvate dehydrogenase kinase. Northern blot analysis revealed a single hybridizing species of 2.5 kilobases. The highest level of p45 message expression was found in heart and skeletal muscle and the lowest in spleen and lung. Liver, kidney, brain, and testis express intermediate amounts of p45 mRNA. In contrast, p48 mRNA is predominantly expressed in heart, with other tissues expressing only a modest amount of this message. Tissue-specific expression of isoforms of pyruvate dehydrogenase kinase may indicate the existence of tissue-specific mechanisms for the regulation of pyruvate dehydrogenase activity.

The mitochondrial multienzyme complex pyruvate dehydrogenase (PDH) catalyzes an irreversible step in the degradation of glucose. Rapid adjustment of flux through PDH is regulated by reversible phosphorylation and dephosphorylation of the E1 component of the complex (3,4). Phosphorylation leads to inactivation of E1 (3,4) and, as a consequence, the holocomplex (4,5). Dephosphorylation and reactivation of PDH is catalyzed by a highly specific pyruvate dehydrogenase phosphatase (4), loosely associated with the complex (6). It is generally believed that the overall activity of PDH is determined by the activity of an intrinsic PDK, which itself is regulated by the products and substrates of the PDH reaction (4,7-9). In general, the products of the dehydrogenase reaction (acetyl-CoA and NADH) stimulate the kinase (4,8), whereas the substrate (pyruvate) and the coenzyme (thiamine pyrophosphate) are inhibitory (4,7,9). Besides this short-term regulation, stable changes in activity of PDK have been observed during starvation and diabetes (10,11). Recent evidence suggests that starvation increases the specific activity of the kinase, implicating the involvement of covalent modification of the kinase as a potential regulatory mechanism (12). However, the exact mechanism of long-term regulation of PDK is still obscure.

The first successful purification of PDK from bovine kidney mitochondria was reported by Stepp et al. (13). They reported that the enzyme exists as a dimer of two nonequivalent subunits of 48 and 45 kDa, designated as α and β. The kinase activity was reported to reside in the α subunit, with the function of the β-subunit unknown, although it was suggested that it may be involved in regulation of kinase activity and/or the attachment of the kinase to the complex (13). Recently, we reported the purification of PDK from rat heart (14). Like the enzyme from bovine kidney, rat heart PDK appears to consist of two subunits that differ in electrophoretic mobility (M, 48 kDa (p48) and 45 kDa (p45), respectively). Amino-terminal sequence analysis of individual subunits electrophoretically separated on polyvinylidene fluoride membrane yielded two different sequences: ASD-SASGSPASE5SV for p48 and KNASLAGPKI6FSKFS for p45, indicating that the polypeptides found in highly purified preparations of PDK may be products of different genes or alternative splicing. In an attempt to further characterize the structure of the subunits of PDK, we cloned a cDNA encoding p45 (15). The analysis of the deduced protein sequence revealed high homology to another mitochondrial protein kinase, the branched chain α-ketoacid dehydrogenase kinase (16), an enzyme functionally similar to PDK (17). Especially high homology was found within putative kinase domains located near the carboxyl termini of the proteins (18). This finding established p48 as a catalytic subunit of PDK.

In this paper, we report the molecular cloning of the cDNA of p45 of rat heart PDK. An analysis of the deduced protein sequence of p45 revealed 70% amino acid identity with p48.

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These results, along with evidence that the recombinant p45 protein catalyzes an ATP-dependent inactivation of the activity of the PDH complex, indicate that the p45 cDNA encodes for another isoform of PDK. We also report here the tissue distribution of both isoforms based on Northern blot analysis.

**EXPERIMENTAL PROCEDURES**

**Polymerase Chain Reaction**—The downstream gene-specific oligonucleotide primer (AAAGTGTCTCAGTGCTAGATGTACCTCTTCG) was designed according to the amino-terminal sequence of p45 (APKGCYF), and primers were used in the third position of codons with a degeneracy of four. Upstream primers (one for each insert orientation) were synthesized to correspond to bases 4266-4289 (GGTGGCGGACGACCCCGGCGG) and bases 4325-4352 (TTGACCGAGGCAACTGTAAATGGGACG) of the Echerichichia coli Lac operon flanking the EcorI site in agt11 plasmid (18). agt11 plasmid DNA from 1 ml of stock (5.1010 plaque forming units of a Agt10 rat heart cDNA library (Clontech) essentially as described by Sambrook et al. (18). Hybridization conditions were as follows: 6 x SSC (1 x SSC = 150 mm sodium chloride, 15 mm sodium citrate, pH 7.5), 5 x Denhardt's solution (0.1% (w/v) bovine serum albumin, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) Ficoll 400), 0.1 mg/ml denatured salmon sperm DNA, and the radiolabeled probe (2 x 106 cpm/ml) at 70°C for 17 h. The filters were washed with 0.1% (w/v) SDS four times at room temperature and one time with 2 x SSC, 0.1% (w/v) SDS at 55°C for 5 min. Two positive plaques were purified through four more cycles of plating and screening. cDNAs were cut out of a phage with EcoRI and religated in EcoRI-digested M13 mp18 for sequencing.

**Rapid Amplification of 5'-End of p45 cDNA (5'RACE)—Rat heart 5'RACE-ready templates were obtained from Clontech. The templates were amplified with a gene-specific primer (CCGGCGGAAACCAGCAT-CTCTC) corresponding to bases 115-156 of the p45 cDNA and the anchor primer (GTGGCGGACGACCCCGGCGG) obtained from Clontech. Each reaction contained 50 pmol of the gene-specific primer, 50 pmol of the anchor primer, and 100 ng of templates. dNTPs, buffer, and Tq polymerase were added according to the manufacturer's instructions (Clontech). 40 cycles of PCR were performed by using 1 min at 94°C for denaturation and 1 min at 60°C for annealing. PCR products were separated by electrophoresis and analyzed by Southern blotting with a label-end labeled oligonucleotide probe corresponding to bases 93-110 (GGCAGGGGAACCGCAT) of p45 cDNA. A hybridizing PCR product of 136 bp was purified and subcloned in M13 mp18 for sequencing.

**Expression of p45 cDNA in E. coli—Sac1 and XhoI restriction sites flanking the coding region of p45 cDNA were constructed by PCR. The resulting 1.2-kilobase Sac1XhoI fragment was ligated in a pET28a expression vector (Novagen, Madison, WI), cut with Sac1 and XhoI, and dephosphorylated with alkaline phosphatase to produce an in-frame amino-terminal fusion with a carboxyl-terminal stretch of 6 histidine residues (His-Tag) and polypeptide MAMTGGCGQG (‘Tag’-Tag). The resulting plasmid was confirmed by direct sequencing. The p45 cDNA was expressed in E. coli HMS 174 (DE3) (Novagen) as previously described (15). Recombinant protein was purified by metal chelation chromatography (18). ATP-dependent Inactivation of Kinase-depleted PDH by Recombinant p45—ATP-dependent inactivation of kinase-depleted PDH by Recombinant p45 was assayed essentially as previously described (15). Phosphorylation reactions (total volume, 600 ml) contained 20 mM Tris-HCl (pH 7.4), 5 mM MgCl2, 60 mM KC1, 5 mM dithiothreitol, 0.5 mM ATP, 10 pmol purified kinase-depleted PDH (14) and 0.5 pmol purified p45. Phosphorylation reactions were initiated by ATP, and incubations were conducted at room temperature. Aliquots (50 ml) were withdrawn at indicated times to measure residual activity of PDH spectrophotometrically as previously described (14).

**RESULTS AND DISCUSSION**

**Amplification of the 5'-End of p45 cDNA—Available amino-terminal sequence of p45 (KNASLAGAPYIEFSKFS) allowed the design of an oligonucleotide gene-specific primer with fairly low degeneracy. The degeneracy was further decreased by incorporation of inosines in the third positions of codons for alanine and proline, as described under “Experimental Procedures.” This primer was used in combinations with primers specific for agt11 DNA sequences to amplify a rat heart cDNA library. One set of primers gave rise to a PCR product of 119 bp (data not shown), which was subcloned in M13 mp18 and sequenced. Analysis of the deduced protein sequence of the PCR product yielded a sequence exactly matching the amino-terminal sequence of p45 (data not shown). This information was used to design a perfect oligonucleotide probe (see “Experimental Procedures”), corresponding to the amino terminus of p45 (KNASLAG), for library screening.

**agt10 Rat Heart cDNA Library Screening—**A rat heart cDNA library was screened with the perfect oligonucleotide probe described above. Approximately 0.5 x 106 clones were screened, and two positive clones were isolated. The first contained an insert of 716 bp, and the second gave two fragments of 286 and 1834 bp, indicating that the p45 cDNA has an internal EcoRI site. All fragments were subcloned in M13 mp18, and both strands were sequenced.

**Nucleotide and Predicted Amino Acid Sequences of p45—**Sequence analysis of the three cDNAs revealed that the 1834-bp fragment contained the complete sequence of p45 cDNA, while the 286-bp fragment contained a partial sequence for the 5'-end. The 716-bp cDNA contained the complete sequence of the 286-bp fragment as well as that of the 119-bp product obtained by agt11 cDNA library amplification. It also contained, however, 330 bp of extraneous sequence at its 5'-end. The latter was found from the DNA data banks available through GenInfo to correspond to a portion of the sequence of the β-subunit of cytochrome oxidase C. To obtain more information about the sequence of the 5'-end of the p45 cDNA, a partial cDNA was generated by the 5'RACE protocol (see “Experimental Procedures”). Amplification of rat heart templates gave rise to a 136-bp PCR product lacking the 330 bp at the 5'-end of the 716-bp cDNA, indicating that the latter sequence is most likely an artifact produced during construction of the library.

The resulting 2216-bp composite cDNA for p45 was constructed by aligning the 136-bp 5'RACE product with the 286- and 1834-bp cDNA fragments (Fig. 1). The cDNA has one open reading frame defined by an ATG triplet at base 93 and an in-frame stop codon at position 714 (15). A unique PCR primer was confirmed by perfect matches of the three peptides encoded by the open reading frame to the sequences of two tryptic peptides derived from purified preparations of p45 (14) as well as the amino-terminal sequence of the mature protein (underlined in Fig. 1). The sequence coding for the mature protein begins at nucleotide 117. It is 399 residues in length with a calculated molecular weight of 45,031, in good agreement with the estimated molecular weight of the p45 subunit of...
Alignment of the deduced protein sequences of p48 and p45

Unlike other members of the mitochondrial protein kinase family (15), p45 has a putative kinase domain of approximately 150 amino acid residues, located in the carboxyl-terminal part of the protein sequence. If this is the proteolytic cleavage site, the catalytic domain is defined by an invariant asparagine residue occurring within a highly conserved region with the sequence KNAMRAT (subdomain V). The central core of the catalytic domain consists of subdomains I1 (consensus sequence Asp282-X-Gly284-X-Gly286), II (defined by invariant His296), III (defined by invariant Asp306), and IV (defined by invariant Phe316). Invariant residues of subdomains I, II, III, IV, and V are presumably involved in ATP binding and catalysis, but their exact functions are currently unknown. As proposed earlier (15), a phosphohistidine intermediate may be involved in the catalysis by mitochondrial protein kinases. The only histidine residue conserved among all members of the family occurs in subdomain I, located within the amino terminus of p45 and separated by a

PDH kinase (13, 14). The ATG codon at nucleotides 93–95 corresponds to the first methionine in an open reading and therefore is the most probable start of the protein sequence. If this is the case, the protein has a 8-residue-long mitochondrial entry sequence.

Alignment of the Deduced Protein Sequence of p48 and p45

Alignment of the deduced protein sequences of p48 and p45 revealed 70% amino acid identity between the two sequences, indicating that the p45 cDNA codes for another isoform of PDH kinase. The amino terminal, the catalytic domain is defined by an invariant asparagine residue (Asn247) occurring within a highly conserved region with the sequence RNASMAT, occurring within a highly conserved region with the sequence RNASMAT

**Fig. 1. Nucleotide and deduced protein sequences of the p45 subunit of PDH.** The amino-terminal and internal tryptic peptide sequences determined for the purified protein are underlined.
in cardiac muscle during starvation is different from other tissues (21, 22). Starvation for 48 h decreases the proportion of PDH activity by metal chelation chromatography (data not shown). Recombinant p45 was purified to homogeneity with concomitant phosphorylation of the α subunit of E3 (data not shown). This observation, together with the above analysis of the p45 sequence relative to that of p48, provides convincing evidence that the cloned cDNA for p45 also encodes an isofrom of PDH kinase. Taking into account the demonstration that both p45 and p48 are catalytic subunits of PDH (15) be designated PDH kinase I (or PDK I) and the second isoform discovered in this study be designated pyruvate dehydrogenase kinase I1 (PDK I1).

Northern Blot Analysis—Tissue distribution of both forms of PDH was characterized by Northern blot analysis (Fig. 4). The mRNA of PDH I corresponded to a single hybridizing species of approximately 2.5 kilobases in all tissues tested (Fig. 4A). The highest amount of message was found in heart and skeletal muscle and the lowest amount in spleen and lung. Testis, liver, brain, and kidney expressed an intermediate amount of PDK I1 mRNA markedly differs from PDH I. The message for PDH I was predominantly expressed in heart with only modest levels of expression in other tissues (Fig. 4B). This observation is in accord with previous studies, indicating that regulation of PDH in cardiac muscle during starvation is different from other tissues (21, 22). Starvation for 48 h decreases the proportion of PDH in the active form in all tissues tested. In heart, this inhibition occurs in part as a result of activation of β-oxidation of long-chain fatty acids (22). The inhibitor of fatty acid β-oxidation, 2-tetradecylglycidic acid, reverses the effect of 48-h starvation in heart but has no effect in most other tissues (22), suggesting that in contrast to other tissues, the activity of heart
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PDK may be tightly regulated by metabolites of fatty acid oxidation.

Relationship of PDK II to the Kinase Activator Protein and the PDH Kinase β Subunit—Recently, Priestman et al. (12) reported purification and characterization of rat liver pyruvate dehydrogenase kinase activator protein (KAP), which appears to be a free catalytic subunit of PDK (23, 24). Amino-terminal sequencing of KAP (12) revealed two overlapping sequences: KAP I, KNASLAGAIE; and KAP II, SLXGAPKY. These sequences demonstrate remarkable similarity to the sequence of the amino terminus of p45 (KNASLAGAPKYIE) reported in the designated tissues. Positions of RNA ladder are indicated on the right.

FIG. 4. Northern blot analysis. 32P-labeled cDNAs of PDK II (A) and PDK I (B) were used to detect the corresponding mRNAs on a rat multiple tissue Northern blot obtained from Clontech (lanes correspond to approximately 2 μg of poly(A)+ RNA electrophoresed and blotted from the designated tissues). Positions of RNA ladder are indicated on the right.

state may affect the distribution of PDK II without changing the amount of the expressed kinase.

We are not certain how the p45 subunit of the rat heart PDK purified (14) and now cloned by this laboratory relates to the 45-kDa regulatory β-subunit of PDK purified from bovine kidney by Stepp et al. (13). It does not appear that our rat heart preparations of PDK contain the β-subunit described by Stepp et al. (13), unless it has a blocked amino terminus. The procedure we used to purify heart PDK is quite different from the one described by Stepp et al. (13). Perhaps only catalytic subunits of PDK are released from the PDH complex by our procedure. A better appreciation of the structure/function relationships of PDK will likely result from successful cloning of the β-subunit of PDK.

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REFERENCES

1. Randle, P. J. (1986) Biochem. Soc. Trans. 14, 799–806
2. Yeaman, S. J. (1989) Biochem. J. 257, 625–632
3. Linn, T. C., Pettit, F. H., and Reed, L. J. (1969) Proc. Natl. Acad. Sci. U. S. A. 63, 234–241
4. Linn, T. C., Pettit, F. H., Hocho, F., and Reed, L. J. (1969) Proc. Natl. Acad. Sci. U. S. A. 63, 227–234
5. Walsh, D. A., Cooper, R. H., Denton, R. M., Bridges, B. J., and Randle, P. J. (1976) Biochem. J. 157, 41–47
6. Teague, W. M., Pettit, F. H., Wu, T.-L., Silberman, S. R., and Reed, L. J. (1982) Biochemistry 21, 5585–5592
7. Pratt, M. L., and Roche, T. E. (1979) J. Biol. Chem. 254, 7191–7196
8. Rahmatullah, M., and Roche, T. E. (1985) J. Biol. Chem. 260, 10146–10152
9. Cooper, R. H., Randle, P. J., and Denton, R. M. (1974) Biochem. J. 143, 625–641
10. Kerbey, A. L., Radcliffe, P. M., and Randle, P. J. (1977) Biochem. J. 164, 509–519
11. Denyer, G. S., Kerbey, A. L., and Randle, P. J. (1986) Biochem. J. 230, 347–354
12. Priestman, D. A., Mistry, S. C., Kerbey, A. L., and Randle, P. J. (1992) FEBS Lett. 308, 83–86
13. Stepp, L. R., Pettit, F. H., Yeaman, S. J., and Reed, L. J. (1983) J. Biol. Chem. 258, 9454–9458
14. Popov, K. M., Shimomura, Y., and Harris, R. A. (1991) Protein Expression Purif. 2, 278–286
15. Popov, K. M., Kodishvili, N. Y., Zhao, Y., Shimomura, Y., Crabb, D. W., and Harris, R. A. (1993) J. Biol. Chem. 268, 26602–26606
16. Popov, K. M., Zhao, Y., Shimomura, Y., Kunz, M. J., and Harris, R. A. (1992) J. Biol. Chem. 267, 13129–13130
17. Fatania, H., Leu, K. S., and Randle, P. J. (1981) FEBS Lett. 132, 285–288
18. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 8.46–8.47, 17.3–17.41, 18.40–18.41, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Hoffman, A., and Roeder, R. G. (1991) Nucleic Acids Res. 19, 6337–6338
20. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
21. Kerbey, A. L., Randle, P. J., Cooper, R. H., Whitehouse, S., Paak, H. T., and Denton, R. M. (1976) Biochem. J. 154, 327–348
22. Catterman, J. D., Fuller, S. J., and Randle, P. J. (1982) Biochem. J. 208, 53–60
23. Mistry, S. C., Priestman, D. A., Kerbey, A. L., and Randle, P. J. (1991) Biochem. J. 275, 775–779
24. Jones, B. S., and Yeaman, S. J. (1991) Biochem. J. 275, 781–784