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Biochemical and Biophysical Research Communications 294 (2002) 136–144

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Cloning and characterization of a testis and brain-specific isoform of mouse 3′-phosphoinositide-dependent protein kinase-1, mPDK-1β☆

Lily Q. Dong,[a,*] Fresnida J. Ramos,[a] Michael J. Wick,[a] Mei Ann Lim,[a] Zhongmao Guo,[b] Randy Strong,[a,c] Arlan Richardson,[b,c] and Feng Liu[a,d]

[a] *Department of Pharmacology, The University of Texas Health Science Center, San Antonio, TX 78229, USA*
[b] *Department of Physiology, The University of Texas Health Science Center, San Antonio, TX 78229 USA*
[c] *Geriatric Research, Education and Clinical Center, South Texas Veterans Health Care System, Audie L. Murphy Division, San Antonio, TX 78284, USA*
[d] *Department of Biochemistry, The University of Texas Health Science Center, San Antonio, TX 78229, USA*

Received 30 April 2002

Abstract

3′-Phosphoinositide-dependent protein kinase-1 (PDK-1) phosphorylates and activates members of the protein kinase AGC family and plays a key role in receptor tyrosine kinase signaling. Here we report the cloning and characterization of a splice variant of mouse PDK-1, mPDK-1β. The cDNA encoding mPDK-1β contains two alternative start codons and translation from these start codons generates proteins that are, respectively, 27 or 51 amino acid residues shorter at the amino-terminus than the previously identified PDK-1 isolated from mouse liver (now renamed mPDK-1α) [J. Biol. Chem. 274 (1999) 8117]. Analysis of mouse tissues shows that mPDK-1β is highly expressed in the testis and various functional regions of the brain. Expression of this isoform is increased in the brain of aged mice. Both mPDK-1α and mPDK-1β are autophosphorylated at both serine and threonine residues in vitro and showed similar levels of tyrosine phosphorylation when co-expressed with either constitutively active Src or Fyn tyrosine kinases in cells. However, the mPDK-1 isoforms showed significant differences in their response to pervanadate- or insulin plus vanadate-stimulated tyrosine phosphorylation. Taken together, our findings suggest that the two PDK-1 isoforms may be differentially regulated in cells. The specific expression of mPDK-1β in mouse testis and brains of aged mice also suggests potential involvement of this kinase in regulating animal spermatogenesis and aging.

In mammalian cells, insulin and IGF-1 regulate a wide spectrum of biological responses such as carbohydrate and lipid metabolism, protein synthesis, gene transcription, and regulation of cell growth and differentiation. The binding of insulin or IGF-1 to their respective receptors leads to tyrosine phosphorylation and activation of their receptor tyrosine kinases, which in turn phosphorylate cellular adaptor proteins such as the insulin receptor substrate (IRS) proteins. Tyrosine phosphorylation of IRS proteins results in activation of phosphatidylinositol 3-kinase (PI 3-kinase) and its downstream kinases such as Akt/PKB [1].

Activation of Akt/PKB is followed by phosphorylation at two conserved sites, Thr^{308} in the activation loop and Ser^{473} at the carboxyl terminus. Phosphorylation at Thr^{308} is catalyzed by the serine/threonine kinase PDK-1 [2]. The current model of Akt/PKB activation suggests that binding of the PI 3-kinase products $PI(4,5)P_2$ and $PI(3,4,5)P_3$ to the PH domain of Akt leads to a conformational change and plasma membrane translocation of the enzyme, which is essential for Akt/PKB interaction with and phosphorylation by the constitutively active and membrane associated PDK-1 [3]. While membrane translocation has been shown to be essential for phosphorylation and activation of Akt/

☆ *Abbreviations:* CHO, Chinese hamster ovary; EST, expressed sequence tag; GST, glutathione *S*-transferase; IGF-1, insulin-like growth factor-1; IR, insulin receptor; PAGE, polyacrylamide gel electrophoresis; PDK-1, 3′-phosphoinositide-dependent protein kinase-1; PH, pleckstrin homology; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PI, phosphatidylinositol.

* Corresponding author. Fax: +1-210-567-4303.
E-mail address: dongq@uthscsa.edu (L.Q. Dong).

0006-291X/02/$ - see front matter
PII: S0006-291X(02)00449-7

PKB by PDK-1, much less is known about the mechanisms by which PDK-1 phosphorylates and activates its other substrates such as p70 S6 kinase and the serum and glucocorticoid regulated kinase (SGK), which are localized mainly in the cytosol and nucleus. Thus, additional mechanisms may exist by which PDK-1 phosphorylates and activates its various cellular substrates.

There is some evidence suggesting the existence of PDK-1 isoforms. In *Drosophila*, the PDK-1 homolog dPDK-1 or DSTPK61 gene produces multiple transcripts that encode for at least four different protein isoforms with variant N-termini [4]. Additionally, four PI(3, 4, 5) P_3 binding proteins with kinase activity towards Akt have been identified in sheep brain [5]. Our recent studies suggest that PDK-1 isoforms may also exist in mice. We have identified two different PDK-1 mRNA species in mouse testis by Northern blot analysis. In addition, we have detected multiple protein bands in various mouse tissues by Western blot with an affinity-purified PDK-1 polyclonal antibody [6]. However, it remains to be shown whether all of these anti-PDK-1 antibody immunoreactive proteins are true mPDK-1 isoforms and if so, whether these isoforms are regulated differently or play distinct roles in receptor tyrosine kinase signaling.

In this study, we report the cloning and characterization of an isoform of mPDK-1, mPDK-1β, from mouse testis. mPDK-1β is highly expressed in mouse testis and various functional regions of the mouse brain such as hippocampus, striatum, cortex, hypothalamus, middle brain, forebrain, and cerebellum. In addition, the expression level of mPDK-1β is increased in the brain of aged mice. Co-expression of constitutively active Src or Fyn tyrosine kinase led to a marked but similar increase in the tyrosine phosphorylation of both mPDK-1 isoforms in Chinese hamster ovary cells overexpressing the insulin receptor. However, the two isoforms displayed a significant difference in insulin and vanadate- or pervanadate-stimulated tyrosine phosphorylation. Taken together, our results suggest that these PDK-1 isoforms may be differentially regulated in cells. The tissue-specific expression of mPDK-1β in mouse testis and brain of aged mice also suggests that this isoform may play important roles in regulating spermatogenesis and aging.

Experimental procedures

Materials. A mammalian expression vector encoding hemagglutinin (HA)-tagged mPDK-1α was described previously [6]. Mammalian expression vectors encoding Myc-tagged mPDK-1 isoforms were produced by subcloning PCR amplified cDNAs encoding either mPDK-1α or mPDK-1β into pcDNA 3.1 (Invitrogen, CA), in-frame at their C-termini with a sequence encoding the Myc-tag. Mammalian expression vectors containing the constitutively active Src and Fyn (Src^{Y527F}, Fyn^{Y531F}) or dominant negative Src and Fyn (Src^{K-}, Fyn^{K-}) kinases were gifts of Dr. S.A. Courtneidge (Sugen, CA) and have been described previously [7]. Monoclonal antibody to the HA-tag was obtained from BABCO. Monoclonal antibody to Fyn, polyclonal antibodies to Src, and the β-subunit of the IR were purchased from Santa Cruz Biotechnology, Inc. An antibody to phosphotyrosine was acquired from Transduction Laboratories (Lexington, KY). A phospho-specific antibody to Ser^{241} of human PDK-1, which also recognizes Ser^{244} of mouse PDK-1, was obtained from Cell Signaling Technology. A rabbit polyclonal antibody against the COOH terminus of PDK-1, which recognizes both PDK-1α and PDK-1β, was described previously [6]. A goat anti-mPDK-1α polyclonal antibody, which recognizes both mPDK-1α and mPDK-1β in immunoprecipitation experiments, was generated by immunizing goats with full-length mPDK-1 fused to glutathione *S*-transferase. To generate an mPDK-1α-specific antibody, we synthesized a peptide corresponding to the extreme N-terminal sequence of PDK-1α (MARTTSQLYDA VPIQSSV) and conjugated it at its COOH terminus to KLH (peptide synthesis and conjugation were done by Research Genetics). Polyclonal anti-peptide antibody was produced by immunizing rabbits using this peptide antigen and purified by absorption of the antiserum to the peptide-immobilized Affi-Gel 10 beads (Biorad), according to the protocol similar to that described previously [6].

5′ Rapid amplification of cDNA ends (RACE) and cloning of mPDK-1β. 5′ RACE was performed using a Marathon-cDNA library derived from mouse testis (Clontech), according to supplier's protocol. The gene-specific primer was 5′-AGA TGG CTG GCA GGT GGT CTG GAA G-3′. The RACE products were cloned into a PCR TOPO vector (Invitrogen) and the nucleotide sequence of mPDK-1β was determined by DNA sequencing. To generate HA-tagged mPDK-1β, we amplified mPDK-1β cDNA by PCR using the TOPO-mPDK-1β as template and subcloned the amplified fragment into the mammalian expression vector pBEX [6] in-frame with a sequence encoding a 9 amino acid HA-tag (YPYDVPDYA) at the C-terminus.

Isolation of genomic clones. A mouse 129 genomic DNA library (provided by C.X. Deng, NIH) was screened with a [^{32}P]α-dCTP-labeled probe derived from a cDNA encoding the COOH terminus of mouse PDK-1α (residues 265–559) [6] according to standard protocol [8]. The isolated mouse genomic DNA was digested with various restriction enzymes and subcloned into pBluescript (Stratagene) for further analysis.

Cell culture, *transfection*, *immunoprecipitation*, and *Western blot*. A Chinese hamster ovary cell line overexpressing the human IR (CHO/IR) and CHO/IR cells stably expressing PDK-1α were described previously [6]. CHO/IR cells stably expressing mPDK-1β were generated using similar protocol as described [6]. Cells were maintained in Ham's F12 medium supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Transient transfection of cells was performed by using LipofectAMINE (GibcoBRL). Cells were lysed in buffer A (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% TritonX-100, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM microcystin-LR and 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 10 min, the lysates were centrifuged (10,000*g*, 4 °C, 10 min) and the supernatants were used for immunoprecipitation or Western blot analysis. For immunoprecipitation studies, cell lysates were incubated with specific antibodies bound to protein A or G Sepharose (Amersham Pharmacia Biotech) overnight at 4 °C with gentle rotation. The bound protein complexes were washed four times with ice-cold buffer B (50 mM Hepes, pH 7.6, 150 mM NaCl, 0.1% TritonX-100) and eluted by heating at 95 °C for 4 min in SDS–PAGE sample loading buffer. The eluted proteins were separated by SDS–PAGE (10% or 15% gels) and transferred onto nitrocellulose membrane. The phosphorylation or expression levels of various proteins were detected by Western blotting with specific antibodies.

Expression of PDK-1 isoforms in mouse tissues. Male C57BL/6 mice at 6 or 24 months of age were obtained from the animal colonies maintained by the National Institution on Aging (Bethesda, MD). The mice had free access to the diet (NIH-31, Purina Mills, Richmond, IN) and were maintained on a 12-h light/dark cycle. The mice were sacri-

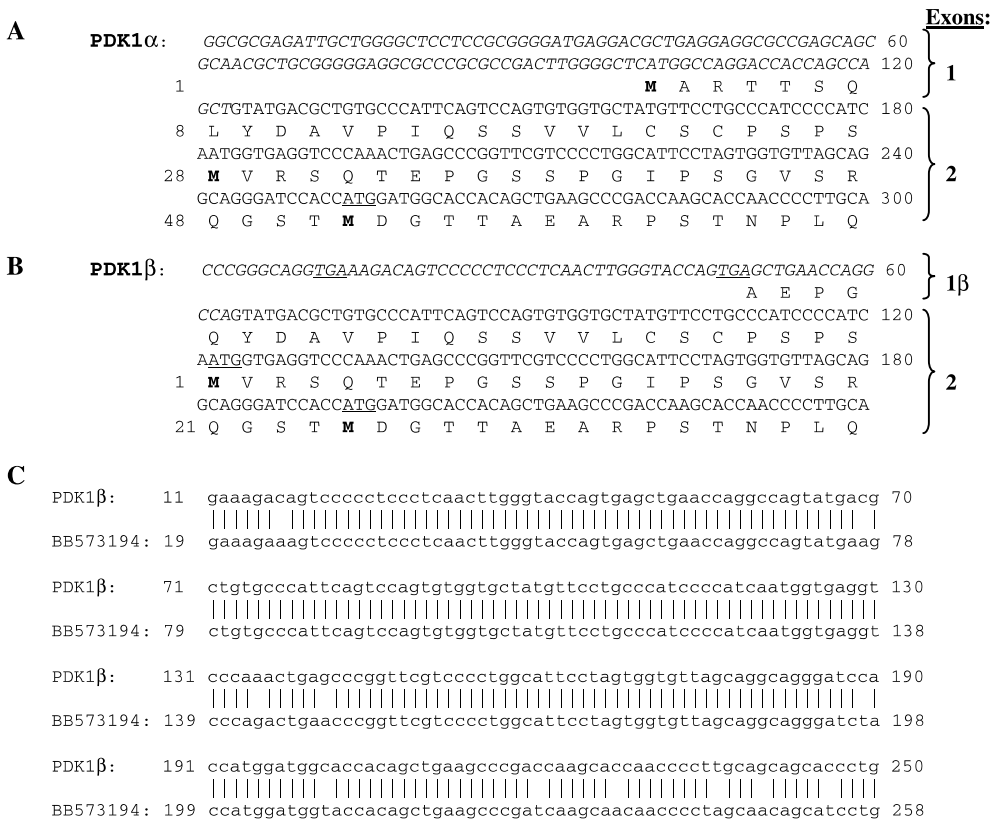

Fig. 1. Comparison of the 5′ cDNA sequences and the amino-terminus amino acid sequences of mPDK-1α (A) and mPDK-1β (B). The cDNA sequences which are not conserved in the two isoforms are italicized. The stop codons in mPDK-1β are underlined. The potential initiation sites in mPDK-1β are given in bold. (C) Alignment of the 5′ mPDK-1β cDNA sequence with EST clone BB573194.

ficed and tissues were collected and homogenized in lysis buffer A. The homogenates were centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatants were collected. Proteins in the supernatants were separated by 10% PAGE, transferred onto a nitrocellulose membrane, and examined by Western blot.

In vivo labeling, phosphoamino acid analysis, two-dimensional phosphopeptide mapping, and PDK-1 in vitro kinase assays. In vivo labeling, phosphoamino acid analysis, two-dimensional phosphopeptide mapping, and PDK-1 in vitro kinase activity assays were carried out as described previously [6,9].

Results

Cloning of PDK-1β

By Northern blot analysis, we recently detected two mRNA transcripts of PDK-1 (2 and 7 kb) in the mouse testis [6]. This finding led us to hypothesize that there may be tissue-specific isoforms of PDK-1. To test this hypothesis, we amplified cDNAs derived from mouse testis by 5′-RACE. We identified a cDNA identical to the mPDK-1α sequence recently isolated from mouse liver (Fig. 1A) [6], except at its extreme 5′-end (mPDK-1β, Fig. 1B). Unlike mPDK-1α, the mPDK-1β cDNA[1] contained two upstream in-frame stop codons, resulting in the selection of different start codon(s) from that of mPDK-1α cDNA. There are two potential start codons in the mouse testis PDK-1 cDNA, one is encoded by nucleotides 122–124 and another is encoded by nucleotides 194–196 (Fig. 1B). Sequence analysis of this region indicates that the sequence surrounding the second start codon better matched the consensus Kozak sequence (GCC(A/G)CCATGG) than the first start codon, with purines at both −3 and +1 positions [10] (see discussion below).

To obtain additional evidence confirming the presence of mPDK-1β mRNA transcript, we searched the mouse Expressed Sequence Tag database (dbEST) using the PDK-1β cDNA as probe. We identified a cDNA from testis (GenBank Accession No. BB573194) which almost completely matched the cDNA encoding PDK-1β (Fig. 1C). The presence of mPDK-1β cDNA in independent cDNA libraries further supports the presence of mPDK-1 isoforms in mouse tissues.

Mouse PDK 1α and PDK-1β are probably generated by alternative splicing

To test whether the mPDK-1 isoforms are generated by alternative splicing, we screened a mouse 129 genomic DNA library and isolated a 12 kb genomic DNA frag-

[1] The sequence reported in this paper has been deposited in the GenBank database (Accession No. AY062008).

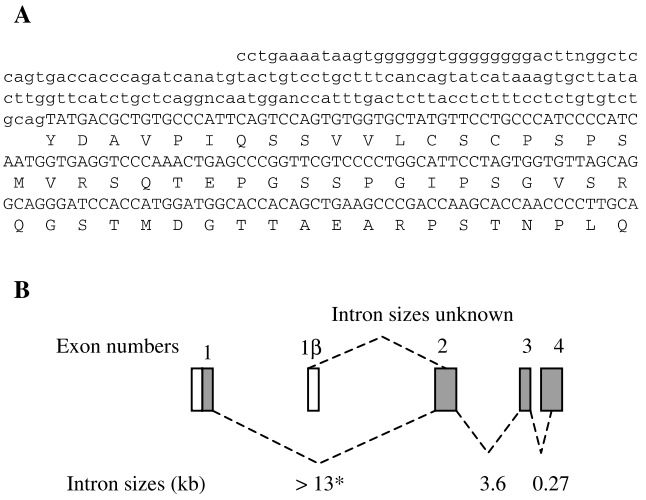

Fig. 2. mPDK-1 5′-genomic DNA sequence and its structural organization. (A) mPDK-1 genomic sequence flanking exon 2. The 5′-intron nucleotides flanking exon 2 are in lower case and the exon 2 nucleotides are in capital letters. (B) Potential genomic arrangement of mPDK-1 5′ exons to generate mPDK-1 alternative splicing isoforms. Coding regions are shaded. (*) The intron sizes are based on [11].

ment encoding mPDK-1. Sequencing analysis of the 5′-end of this genomic DNA revealed that it contained exons encoding the amino acid terminus of mPDK-1, including part of the kinase domain (Fig. 2A, and data not shown). However, exon 1, which is separated by an intron of 13 kb [11] and encodes the first eight amino acids of mouse PDK-1α (Fig. 2B), was absent from our isolated genomic DNA clone. Sequencing alignment of the PDK-1 cDNAs isolated from mouse liver and testis with the genomic DNA sequence indicated that the two cDNAs are different in sequences preceding exon 2, an exon which encodes amino acid residues 9–43 of mPDK-1α [6] (Fig. 2A). However, since our isolated genomic DNA clone was short at its 5′-end, we were unable to determine the size, the exact location, and the 3′ donor sequence of the exon that encodes the 5′-untranslated sequence of mPDK-1β, tentatively named exon 1β (Fig. 2B). Nevertheless, examination of the genomic sequence at the 5′-boundary of exon 2 indicated that the sequence fits with the donor–acceptor rule, with an AG pair located at 5′ of the exon 2 acceptor sequence (Fig. 2A) [12]. These findings suggest that the liver and testis mPDK-1 cDNAs were most likely caused by differential splicing of either exon 1 or exon 1β into exon 2 (Fig. 2B).

Expression of PDK-1 isoforms in cultured cells

To characterize the predicted protein products of mPDK-1 isoforms, we transfected CHO/IR cells with empty plasmid or plasmids encoding HA-tagged PDK-1 isoforms. Lysates from mPDK-1α- (Fig. 3A, lanes 2 and 5) and mPDK-1β- (Fig. 3A, lanes 3 and 6) transfected or mock-transfected (Fig. 3A, lanes 1 and 4) cells were

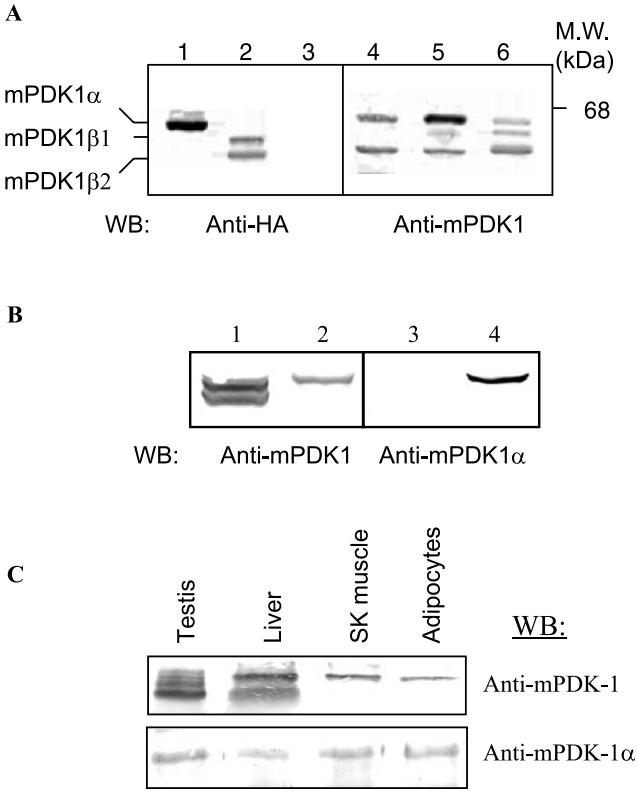

Fig. 3. Expression of mPDK-1 in CHO/IR cells. (A) Six μg of mammalian expression vectors containing mPDK-1α (lanes 2 and 5) or mPDK-1β (lanes 3 and 6) cDNAs were transfected into CHO/IR cells (in 60 mm plates) using LipofectAMINE (Life Technologies). As a control, 6 μg of empty pBEX plasmid was also transfected into the cells (lanes 1 and 4). Forty-eight h after transfection, cells were lysed in 200 μl SDS-sample buffer and 15 μl of whole cell lysates from each sample was separated by SDS–PAGE. Proteins on the gel were transferred onto nitrocellulose membrane and detected by Western blot with antibodies to the HA-tag (lanes 1–3) or to mPDK-1 (lanes 4–6). (B) The anti-mPDK-1α antibody recognizes overexpressed mPDK-1α but not mPDK-1β. Lysates from CHO/IR cells transiently expressing mPDK-1α (lanes 2 and 4) and mPDK-1β (lanes 1 and 3) were resolved by SDS–PAGE and transferred onto a nitrocellulose membrane. The expression of mPDK-1 isoforms was examined by Western bot using anti-mPDK-1 (lanes 1 and 2) or anti-mPDK-1α antibodies. (C) Expression of mPDK-1 isoforms in mouse testis. Mouse tissues were homogenized in lysis buffer and clarified by centrifugation. Cell lysates (300 μg protein) were incubated with a goat anti-mPDK-1 antibody. Immunoprecipitates were separated by SDS–PAGE and the expression of mPDK-1 isoforms was examined by Western blot using anti-mPDK-1 antibody (upper panel) or the anti-mPDK-1α-specific antibody (lower panel), respectively.

analyzed by immunoblotting with the antibody to the HA-tag (Fig. 3A, lanes 1–3) or with antibody raised against the C-terminus of mPDK-1 (Fig. 3A, lanes 4–6). In cells transfected with mPDK-1α cDNA, a protein of approximately 64 kDa was detected by the anti-HA antibody (mPDK-1α, Fig. 3A, lane 2). Transfection of CHO/IR cells with the mPDK-1β cDNA led to the expression of two proteins with molecular masses of approximately 61 and 58 kDa, respectively (mPDK-1β1

and mPDK-1β2, Fig. 3A, lane 3). As discussed above, the heterogeneity was probably due to the use of different initiation start codons during translation (Fig. 1A). Consistent with this notion, Western blot analysis using the anti-mPDK-1 polyclonal antibody revealed two endogenously expressed proteins in CHO/IR cells with similar molecular masses to those of mPDK-1α and mPDK-1β2 (Fig. 3A, lane 4).

Identification of PDK-1 isoforms in mouse testis

To further confirm the presence of PDK-1 isoforms, we examined PDK-1 expression in mouse testis and several insulin sensitive mouse tissues by Western blot using two different anti-PDK-1 antibodies. The anti-mPDK-1 antibody recognized both overexpressed mPDK-1α and mPDK-1β in CHO/IR cells (Fig. 3B, lanes 2 and 1), respectively. On the other hand, the anti-mPDK-1α-specific antibody, which was generated using the extreme amino-terminal 18 amino acid residues of mPDK-1α as an antigen, recognized only overexpressed mPDK-1α but not overexpressed mPDK-1β (Fig. 3B, lanes 4 and 3), respectively. Western blot of mouse tissues with the anti-mPDK-1 antibody revealed a single protein band with molecular weight of approximately 64 kDa in mouse liver, skeletal muscle, and adipocytes (Fig. 3C, upper panel). On the other hand, multiple protein bands were detected by this antibody in mouse testis (Fig. 3C, upper panel). Western blot using the anti-mPDK-1α-specific antibody revealed a single protein band in mouse testis that migrated to the same location as that of PDK-1 from other mouse tissues (Fig. 3C, lower panel). These results provide additional evidence for the presence of PDK-1 isoforms in mouse testis.

Expression of mPDK-1 isoforms in mouse brain

The PDK-1 homolog in *Caenorhabditis elegans* has recently been shown to play a role in regulating life span [13]. To test whether PDK-1 expression is affected by aging, we examined the expression levels of mPDK-1 isoforms in the brains of young (6 months) and aged (24 months) mice. We found that both mPDK-1 isoforms were expressed in the brains of young and aged mice (Fig. 4A, top panel). However, the expression level of mPDK-1β isoforms was notably increased in the brain from aged mice (Fig. 4A, top panel). This expression was specific and no difference in the expression levels of several other kinases involved in the insulin signaling pathway, such Akt, MAP kinase, and PKCζ was observed in both the liver and brain from the young and aged mice (Fig. 4A, second, third, and last panels).

To further characterize the expression of mPDK-1 isoforms in the brain, tissues from different functional regions of mouse brain, including hypothalamus, striatum, hippocampus, cortex, midbrain, forebrain, cerebellum, and olfactory bulb, were isolated from a 24-month-old mouse brain and the expression of mPDK-1 isoforms in the these regions was examined by Western blot of cell lysates using mPDK-1 and the mPDK-1α-specific antibodies. Fig. 4 shows the results of a representative experiment. Multiple protein bands with molecular weights corresponding to mPDK-1α and mPDK-1β were detected in different functional regions of mouse brain by the anti-mPDK-1 antibody (Fig. 4B, upper panel). On the other hand, only a single protein band with a molecular weight corresponding to that of mPDK-1α was detected by Western blot using the anti-mPDK-1α-specific antibody (Fig. 4B, lower panel). These results further confirm the presence of mPDK-1 isoforms in mouse brain.

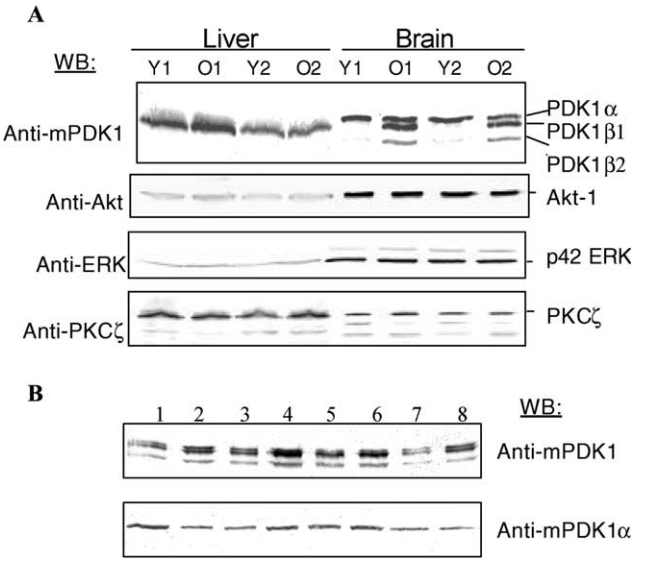

Fig. 4. Expression of mPDK-1 isoforms in mouse brain. (A) The expression of several kinases in the insulin signaling pathway in young (Y1 and Y2, 6 months) and old (O1 and O2, 24 months) mice. Mouse liver and brain homogenates (25 μg/lane) were separated by SDS–PAGE, transferred to nitrocellulose membranes, and analyzed by Western blot using antibodies to PDK-1 (top panel,), Akt (second panel), ERK (third panel), and PKCζ (bottom panel), respectively. (B) Expression of mPDK-1 isoforms in different functional regions of mouse brain. Mouse hypothalamus (lane 1), striatum (lane 2), hippocampus (lane 3), cortex (lane 4), midbrain (lane 5), forebrain (lane 6), cerebellum (lane 7), and olfactory bulb (lane 8) were isolated from a 24-month-old mouse and lysed in lysis buffer. Proteins in total cell lysates (8 μg/lane) were separated by SDS–PAGE and the expression of mPDK-1 isoforms in cell lysates was determined by Western blot using anti-mPDK-1 (upper panel) or anti-mPDK-1α (lower panel) antibodies.

mPDK-1 isoforms autophosphorylate at both serine and threonine residues

We previously found that mouse PDK-1α underwent significant autophosphorylation in vitro and in cells [6]. To further characterize the autophosphorylation of mouse PDK-1, we expressed HA-tagged mouse PDK-1α and PDK-1β in CHO/IR cells. mPDK-1 isoforms were

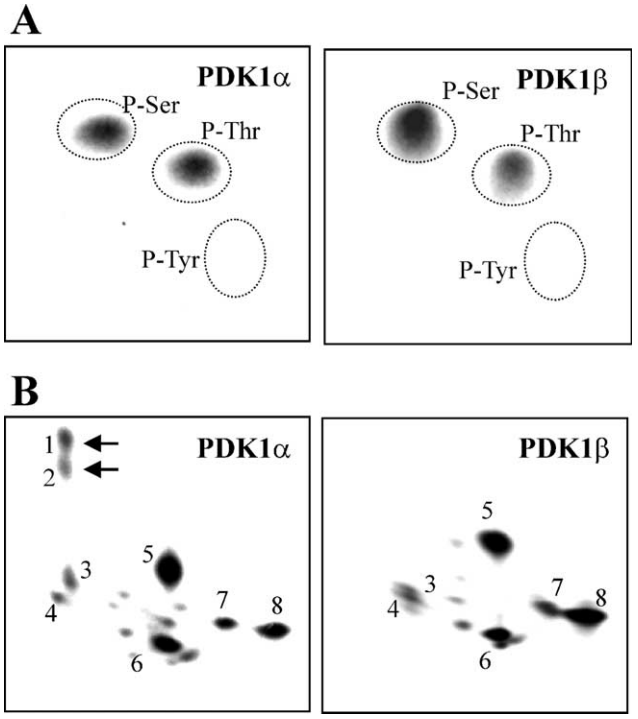

Fig. 5. Comparison of mPDK-1 isoform autophosphorylation. CHO/IR cells transiently expressing Myc-tagged mPDK-1α or mPDK-1β were serum starved for 1 h and lysed. mPDK-1 isoforms were immunoprecipitated using antibody to the Myc-tag. Bound mPDK-1 proteins were autophosphorylated in the presence of [^{32}P]γ-ATP, resolved by SDS–PAGE, transferred to a PVDF membrane, and analyzed by phosphoamino acid analysis (A) or by two-dimensional mapping studies (B). Phosphoamino acids or phosphopeptides were visualized by autoradiography

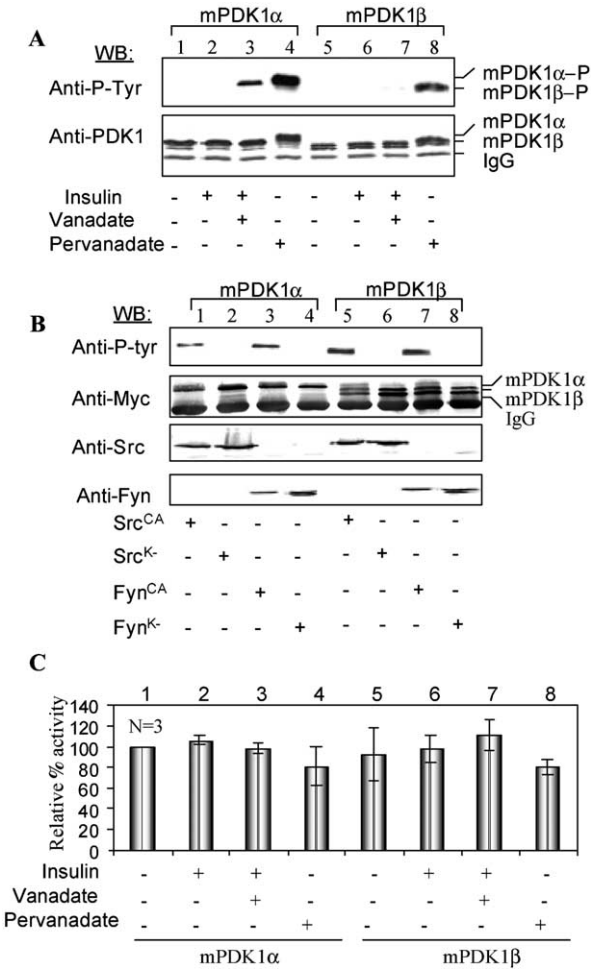

Fig. 6. Tyrosine phosphorylation of mPDK-1 isoforms. (A) CHO/IR cells overexpressing mPDK-1α (lanes 1–4) or mPDK-1β (lanes 5–8) grown in 60 mm plates were left untreated (lanes 1 and 5) or treated for 5 min with 10 nM insulin (lanes 2 and 6), 30 min with 1 mM vanadate followed by 5 min insulin treatment (lanes 3 and 7), or 15 min with 0.1 mM pervanadate (lanes 4 and 8). The HA-tagged mPDK-1 isoforms were immunoprecipitated with antibody to the tag and the tyrosine phosphorylation of the proteins was determined by Western blot using anti-phosphotyrosine antibody (upper panel). The expression of mPDK-1 isoforms was determined by Western blot with antibody to mPDK-1 (lower panel). (B) The effects of overexpressing constitutively active or dominant negative Src or Fyn on mPDK-1 tyrosine phosphorylation in cells. CHO/IR cells in 100 mm plates were transiently cotransfected with cDNAs encoding Myc-tagged mPDK-1 isoforms and constitutively active or kinase defective Src and Fyn as indicated. Thirty h post-transfection, cells were serum-starved for 1 h and lysed. mPDK-1 isoforms were immunoprecipitated with antibody to the Myc-tag and the tyrosine phosphorylation of mPDK-1 isoforms was detected by Western blot with anti-phosphotyrosine antibody (top panel). The expression of mPDK-1 isoforms, Src, or Fyn in these cells was detected by Western blot using antibodies to the Myc-tag (second panel), to Src (third panel), or to Fyn (bottom panel), respectively. (C) CHO/IR cells stably expressing HA-tagged mPDK-1α or mPDK-1β were treated as shown in (A). mPDK-1 isoforms were immunoprecipitated using an antibody to the HA-tag. In vitro kinase assays were carried out as described [6], using the peptide corresponding to the activation loop of Akt (KTFCGTPEYLAPEVRR) as a substrate. The relative mean percentage activity ± SE from three independent experiments is shown.

purified by immunoprecipitation and autophosphorylated in vitro in the presence of [^{32}P]γ-ATP. Phosphoamino acid analysis of the in vitro autophosphorylated PDK-1 isoforms showed that both mouse PDK-1α and PDK-1β were phosphorylated at serine and threonine residues, with a molar ratio of approximately 1:1 (Fig. 5A). Two-dimensional phosphopeptide mapping studies revealed no major differences in the autophosphorylation between these two isoforms, except that phosphopeptides 1 and 2 in mPDK-1α were absent in mPDK-1β (Fig. 5B, right panel). We also found that these two isoforms of mPDK-1 possess comparable kinase activities as determined by in vitro kinase assays, according to the protocol described recently [6] (Fig. 6C, lanes 5 and 6 vs. lanes 3 and 4). These findings suggest that the extreme amino terminal sequence is not essential for the kinase activity of the enzyme in vitro.

PDK-1 isoforms undergo differential tyrosine phosphorylation in response to pervanadate- or insulin treatment

hPDK-1 has recently been shown to undergo oxidative, nerve growth factor (NGF), and pervanadate-induced tyrosine phosphorylation [14–16]. To characterize

potential regulatory mechanisms of mPDK-1 isoforms, we investigated the tyrosine phosphorylation of these enzymes. CHO/IR cells transiently expressing either mPDK-1α or mPDK-1β were treated with or without insulin, insulin plus vanadate, or pervanadate and the tyrosine phosphorylation of mPDK-1 isoforms was examined by Western blot using an anti-phosphotyrosine antibody. While no detectable tyrosine phosphorylation of mPDK-1 isoforms was observed in untreated cells or cells treated with insulin alone (Fig. 6A, upper panel, lanes 1, 2, 5, and 6), treatment of cells with insulin in the presence of vanadate led to a significant tyrosine phosphorylation of mPDK-1α (Fig. 6A, top panel, lane 3). Under the same condition, no tyrosine phosphorylation of mPDK-1 was observed in the presence of vanadate alone (data not shown). Treatment of cells with pervanadate led to an even greater tyrosine phosphorylation of mPDK-1α than that treated with insulin and vanadate (Fig. 6A, lane 4 vs. 3). Under the same condition, pervanadate treatment also led to a significant tyrosine phosphorylation of mPDK-1β but to a lesser extent compared to that of mPDK-1α (Fig. 6A, lane 8 vs. 4). Our results showed that there are notable differences between insulin/vanadate and pervanadate-induced tyrosine phosphorylation of PDK-1 isoforms, suggesting that pervanadate is more effective than vanadate in inhibiting cellular protein tyrosine phosphatase. Consistent with this, it has been shown that vanadate competitively inhibits and pervanadate irreversibly inhibits protein tyrosine phosphatases [17]. It is interesting to note that the insulin- and vanadate-stimulated tyrosine phosphorylation of mPDK-1β was greatly decreased (Fig. 6A, lane 7 vs. lane 3), suggesting that these two isoforms may be differently regulated in cells.

Src and Fyn phosphorylate mPDK-1 isoforms to a similar extent in vivo

To characterize the mechanism accounting for the difference in insulin- and pervanadate-stimulated tyrosine phosphorylation of mPDK-1 isoforms, we investigated the tyrosine phosphorylation of mPDK-1 isoforms by Src tyrosine kinases, which has been shown to phosphorylate hPDK-1 at Tyr^{9}, Tyr^{373}, and/or Tyr^{376} in vitro [16]. Transient co-expression of a constitutively active form of Src with either mPDK-1α (Fig. 6B, top panel, lane 1) or mPDK-1β (Fig. 6B, top panel, lane 5) resulted in a marked increase in tyrosine phosphorylation of these isoforms. Under similar conditions, no tyrosine phosphorylation of mPDK-1 isoforms could be detected in cells co-expressing the kinase-inactive Src kinase (Fig. 6B, top panel, lanes 2 and 6). We also examined the tyrosine phosphorylation of mPDK-1 isoforms by another member of the Src tyrosine kinase family, Fyn. In contrast to the findings of Park et al. [16] who showed that overexpression of Fyn had no effect on human PDK-1 tyrosine phosphorylation, we found that co-expression of a constitutively active Fyn led to a significant tyrosine phosphorylation of both mPDK-1α and mPDK-1β (Fig. 6B, top panel, lanes 3 and 7). These findings suggest that mPDK-1 isoforms are potential in vivo targets of members of these Src tyrosine kinase family members. In addition, phosphorylation of Tyr^{9} in cells is mediated by a distinct kinase rather than Src or Fyn, since mPDK-1β, which does not contain the tyrosine residue corresponding to Tyr^{9} in mPDK-1α, was tyrosine phosphorylated to the same extent to that of mPDK-1α by either Src or Fyn.

To test whether tyrosine phosphorylation has an effect on the kinase activity of PDK-1 isoforms, we carried out in vitro kinase assays. We found that treatment of CHO/IR cells stably expressing mPDK-1α or mPDK-1β with insulin plus vanadate or pervanadate had no significant effect on the kinase activity of these PDK-1 isoforms (Fig. 6C). In addition, by Western blot using a phospho-specific antibody to Thr^{241} in the activation loop of human PDK-1, we found that treatment of cells with vanadate or pervanadate had no significant effect on the autophosphorylation of mPDK-1 in the activation loop (data not shown).

Discussion

Alternative RNA processing provides a common mechanism to generate diversity in the expression of complex transcription units which may allow for differences in cellular location, level of expression, response to extracellular stimuli, and function in the regulation of cell transformation, growth, differentiation, metabolism, and gene expression [18]. Examples of alternative splicing isoforms are found in a number of protein kinases including PKC and PKB. For PKC, alternatively spliced variants of the β and δ isoforms have been identified. PKCβI and PKCβII, which are generated by alternative splicing at the COOH-terminal V5 domain, have been shown to differ in their subcellular localization, substrate specificity, and ability to enhance insulin-stimulated GLUT4 translocation [19]. Three isoforms of PKB, PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3, have been identified and a splice variant of PKBγ, PKBγ1, has recently been described [20]. This splice isoform lacks the second regulatory phosphorylation site Ser^{473} in the C-terminal hydrophobic domain and differs from PKBγ in its responses to different stimuli, its specific activity, and its membrane translocation in response to insulin stimulation [20].

In the present study we report the cloning and characterization of a splice variant of mPDK-1, mPDK-1β. Some evidence supports the presence of this isoform in mouse tissues. First, mPDK-1β cDNAs can be found in independent clones (Fig. 1C), suggesting that the

mPDK-1β cDNA was not generated by a cloning artifact. Second, there are two mPDK-1 mRNA species in mouse testis [6], suggesting that they may encode distinct transcripts. Third, results from genomic DNA sequence analysis show that mRNAs encoding mPDK-1α and mPDK-1β differ in sequence proceeding exon 2, suggesting these isoforms are generated by alternative splicing between exon 1 (or 1β) and 2. Finally, by Western blot analysis using an mPDK-1α-specific antibody, we demonstrate that mPDK-1β, which differs from mPDK-1α at its extreme N-terminus, is specifically expressed in mouse testis and brain tissues.

The presence of PDK-1 splice variants that differ at their N-termini raises the possibility that these isoforms may be differentially regulated in cells. It has recently been shown that human PDK-1 is tyrosine phosphorylated in cells and the phosphorylation occurred at Tyr^{9}, Tyr^{373}, and Tyr^{376} [16]. mPDK-1α contains potential tyrosine phosphorylation sites corresponding to the tyrosine phosphorylation sites of human PDK-1 (Tyr^{9}, Tyr^{376}, and Tyr^{379}) and the protein is tyrosine phosphorylated in cells in response to the stimulation with pervanadate or insulin in the presence of vanadate (Fig. 6A). On the other hand, mPDK-1β, which does not contain the tyrosine residue corresponding to Tyr^{9} of human PDK-1 or mPDK-1α, was not efficiently phosphorylated in response to either insulin plus vanadate or pervanadate stimulation compared to mPDK-1α (Fig. 6A). These findings suggest that phosphorylation at Tyr^{9} may play an important role in the sequential phosphorylation of PDK-1 at other tyrosine phosphorylation sites. Consistent with this finding, mutation of Tyr^{9} in human PDK-1 decreased PDK-1 tyrosine phosphorylation [16]. However, the exact role of Tyr^{9} phosphorylation in cell signaling and the kinase(s) that phosphorylates Tyr^{9} remain unknown. It is interesting to note that overexpression of Src/Fyn in cells stimulated tyrosine phosphorylation of both mPDK-1α and mPDK-1β to a similar extent (Fig. 6B). These results suggest that Tyr^{9} in mPDK-1α may not be an in vivo Src/Fyn-mediated tyrosine phosphorylation site. These results are consistent with the findings that inhibition of v-Src reduced pervanadate-stimulated tyrosine phosphorylation at $Tyr^{373/376}$ but not Tyr^{9} in human PDK-1 [16]. Our results also suggest that treatment of cells with pervanadate or insulin plus vanadate may lead to activation of tyrosine kinases in addition to Src/Fyn.

In agreement with the earlier findings that PDK-1 undergoes vanadate- and pervanadate-stimulated tyrosine phosphorylation in cells [14–16], we found that treatment of cells with insulin plus vanadate or pervanadate induced significant tyrosine phosphorylation of mPDK-1 in CHO/IR cells. However, in vitro kinase assays showed that treatment of CHO/IR cells stably expressing mPDK-1α or mPDK-1β had little effect on the kinase activity of these isoforms. This result is in contradiction to the findings by Prasad et al. [15] or Park et al. [16] who showed that tyrosine phosphorylation stimulates the kinase activity of human PDK-1. The reason for this discrepancy is currently unknown but may be due to the differences in the types of cell studied or differences between transient or stable expression of PDK-1.

The specific expression of mPDK-1β in mouse testis suggests that this isoform may play a role in spermatogenesis, a process which consists of three phases: proliferation and differentiation of spermatogonia (the male germ line cells), meiotic division of spermatocytes, and development of post-haploid spermatids to sperm [21]. While the upstream signals regulating spermatogenesis remain largely unclear, the PI 3-kinase signaling pathway has been implicated in playing important roles in the regulation of this process [22,23]. Several PI 3-kinase downstream substrates such as PKCζ, PKB, and p70 S6 kinase are highly expressed in spermatogonia and inhibition of PKB and p70 S6 kinase has been shown to block stem cell factor (SCF)/*c-kit*-induced spermatogonial cell proliferation [22–24]. Since PDK-1 functions downstream of PI 3-kinase and upstream of PKB and p70 S6 kinase, the high level and specific expression of mPDK-1β in the testis suggests that this enzyme may play a potential role in regulating spermatogenesis. This hypothesis is consistent with a recent finding that the *Drosophila* gene *Dstpk61*, a functional homolog of mammalian *PDK-1*, generates cDNAs encoding multiple protein isoforms with variant N-termini [4]. In addition, these cDNAs contain transcriptional control elements which are found in transcripts under the male germline-specific transcriptional control [25]. Together, these findings suggest that specific expression of PDK-1 isoforms in the testis may play an important role in sex-specific events.

In the present study, we also found that the expression of PDK-1β is increased in the brain of aged mice. While the potential role of PDK-1 in brain activity remains to be established, there is a possibility that these isoforms may play a potential role in regulation of animal longevity. Recent studies in invertebrates have suggested that life span may be extended by a decrease in insulin signaling in the neurons. In *C. elegans*, the insulin-like pathway consists of proteins encoded by the genes *daf-2*, *pdk-1*, *akt-1*, *akt-2*, *daf-16*, and *daf-18* [26]. Loss-of-function mutations in the gene products of *daf-2* (insulin receptor homolog), *age-1* (PI 3-kinase homolog), *pdk-1* (mammalian PDK-1 homolog), and *akt* produce a state similar to that induced by deprivation of energy-supplying nutrients, a condition analogous to enhanced mammalian longevity induced by caloric restriction [13,27,28]. There is some evidence that in *C. elegans* the anti-aging action of the mutations is localized to neurons [28]. However, whether neuronal insulin/IGF-1 signaling plays a role in regulating the life span and aging process remains unclear. Although the

brain has been shown to possess an insulin-independent mechanism for glucose supply [29], it is interesting to note that most of the signaling molecules involved in insulin receptor signal transduction, including the insulin receptor [29], IRS-1 [30,31] and IRS-2 [32], Akt, PKCζ, and PDK-1 (this study), are highly expressed in the brain. These findings suggest a potential novel role(s) of insulin signaling in the central nervous system. Since mPDK-1β is specifically expressed in the brain of aged mice, characterization of the functional roles of this protein may improve our understanding of the physiological significance of insulin signaling in the brain and its potential involvement in the regulation of animal longevity and aging.

Acknowledgments

We thank Dr. C.X. Deng for providing the mouse genomic DNA library and Dr. S.A. Courtneidge for the cDNAs encoding constitutively active and kinase-inactive Src and Fyn. This study is supported by NIH Grants DK56166 (to F.L. and L.Q.D.), DK52543 (to R.S.), and PO3 AG13319 to the San Antonio Nathan Shock Aging Center at the University of Texas Health Science Center at San Antonio (to A.R.).

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