Structural Determinants for Cross-talk between Pyruvate Dehydrogenase Kinase 3 and Lipoyl Domain 2 of the Human Pyruvate Dehydrogenase Complex* 

Shih-Chia Tso‡§1, Masato Kato‡, Jacinta L. Chuang§, and David T. Chuang†*1

From the Departments of ‡Biochemistry and §Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390

Pyruvate dehydrogenase kinase isoforms (PDK1–4) are the molecular switch that down-regulates activity of the human pyruvate dehydrogenase complex through reversible phosphorylation. We showed previously that binding of the lipoyl domain 2 (L2) of the pyruvate dehydrogenase complex to PDK3 induces a “cross-tail” conformation in PDK3, resulting in an opening of the active site cleft and the stimulation of kinase activity. In the present study, we report that alanine substitutions of residues in the C-terminal region and the lipoyl-binding pocket of PDK3 similarly nullify L2 binding and L2-stimulated kinase activity. Alanine substitutions of residues Leu-140, Glu-170, and Glu-179 in L2 markedly reduce binding affinities of these L2 mutants for PDK3. Unlike wild-type L2, binding of these L2 mutants to PDK3 does not preferentially reduce the affinity of PDK3 for ADP over ATP. The inefficient removal of product inhibition associated with ADP accounts for the decreased stimulation of PDK3 activity by these L2 variants. Serial truncations of the PDK3 C-terminal tail region either impede or abolish the binding of wild-type L2 to the PDK3 mutants, resulting in the reduction or absence of L2-enhanced kinase activity. Alanine substitutions of residues Leu-27, Phe-32, Phe-35, and Phe-48 in the lipoyl-binding pocket of PDK3 similarly nullify L2 binding and L2-stimulated PDK3 activity. Our results indicate that the above residues in L2 and residues in the C-terminal region and the lipoyl-binding pocket of PDK3 are critical determinants for the cross-talk between L2 and PDK3, which up-regulates PDK3 activity.

The mammalian pyruvate dehydrogenase complex (PDC),2 which converts pyruvate to acetyl-CoA in the mitochondrion, is a 9 × 106-Da catalytic machine organized around dodecahedral transacetylase (E2p) core. To this E2p core, multiple copies of pyruvate dehydrogenase (E1p), dihydrolipoamide dehydrogenase (E3), E3-binding protein, pyruvate dehydrogenase kinase (PDK), and pyruvate dehydrogenase phosphatase are tethered through non-covalent interactions (1, 2). This mode of macromolecular organization is largely conserved in the family of mitochondrial α-ketoacid dehydrogenase complexes comprising PDC, the branched-chain α-ketoacid dehydrogenase (BCKD) complex, and the α-ketoglutarate dehydrogenase complex. The assembly of various enzyme components around the respective E2 scaffolds markedly facilitates catalysis and regulation of these macromolecular catalytic machines.

The human PDC is down-regulated by reversible phosphorylation through PDK (3–5). There are four PDK isoforms (PDK1–4) in mammalian and yeast mitochondria (6). Phosphorylation occurs at three serine residues (Ser-264, site 1; Ser-271, site 2; and Ser-203, site 3) in the E1p α subunit of PDC (7–9). Although phosphorylation of each site alone inactivates PDC, site 1 is most rapidly phosphorylated, whereas site 3 is the slowest among the three sites (7, 10). All four PDK isoforms phosphorylate site 1 and site 2, but only PDK1 modifies site 3 (10, 11). PDK isoforms exhibit tissue-specific expression. PDK1 is detected in heart, pancreatic islets, and skeletal muscles; PDK2 is expressed in all tissues; and PDK3 is present in testis, kidney, and brain, whereas PDK4 is abundant in heart, skeletal muscle, kidney, and pancreatic islets (12). The expression of PDK2 and PDK4 is induced in starvation and diabetes, which is reversed by insulin treatment (3, 13). Impairments in the insulin-induced down-regulation of PDK4 lead to the overexpression of PDK4 and shut off glucose oxidation in diabetic animals (4, 14). Therefore, PDK4 is a potential drug target for the treatment of type 2 diabetes.

The dimeric forms of PDKs are the biologically functional entities (10, 12, 15). PDK dimers are recruited to the PDC by preferentially binding to the inner lipoyl (L2) domain of the E2p chain (16). Binding of L2 to PDKs requires the covalently attached lipoyl group at the lysine 173 of L2 (17). Reduction or acetylation of the lipoyl group significantly increases the affinity of L2 for PDKs when compared with L2 containing an oxidized lipoyl group (14, 15). The activity of PDKs is stimulated upon binding to E2p, but the response of different isoforms to isolated L2 differs. PDK3 is robustly activated by E2p, and the bulk of this activation can be achieved using isolated L2 (14, 15). In contrast, PDK2 activity is not augmented by isolated L2; the activity of this PDK isoform is only stimulated through binding to E2p (15) or a di-domain (18) consisting of both the L2 and the E1p-binding domains. Individual isoforms exhibit different binding affinities for L2 in the order of PDK3 > PDK1 =...
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PDK2 > PDK4 (19). Recent studies have shown that the C-terminal tail regions of PDK3 and PDK2 are essential for the stimulation of kinase activity by isolated L2 and E2p, respectively (20, 21).

The crystal structure of PDK3 in complex with the L2 domain of human PDC has recently been determined (20). The structure shows that extensive surface areas in PDK3 are involved in binding L2; these include the hydrophobic lipoyl-binding pocket and the ionic patch in the N-terminal domain as well as the C-terminal tail of each PDK3 subunit. The C-terminal regions from both PDK3 subunits are in a “cross-tail” conformation such that the C-terminal tail from one PDK3 subunit constitutes an integral part of the lipoyl-binding pocket located in the N-terminal domain of the opposing subunit. A comparison with the L2-free PDK2-ADP (22) and BCKD kinase-ADP (23) structures discloses that the cross-tail configuration promotes conformational changes in the active site clefts of both PDK3 subunits, resulting in a largely disordered ATP lid in the ADP-bound form. Results from binding studies establish that the PDK3-L2 complex has lower affinity for ADP than ATP. It is therefore proposed that L2 stimulates PDK3 activity by disrupting the ATP lid, which otherwise traps ADP, to remove the feedback inhibition exerted by this product of the phosphoryl-transfer reaction. The binding of substrate analog dichloroacetate and other synthetic ligands to the N-terminal domain of PDK2 induces significant changes in the active site located in the C-terminal domain, resulting in the inhibition of kinase activity (24). Very recently, pyruvate along with ADP or ATP was shown to hinder the binding of PDK2 to a dimeric form of L2 and cause the association of the PDK2 dimer to a tetramer (25). The above findings, taken together, strongly support the view that the N-terminal domains play key roles in modulating kinase activities of PDK isoforms and the BCKD kinase (20, 23–25).

In the present study, we deciphered the interactions between PDK3 and L2 by alanine-scanning mutagenesis of residues at the interfaces between PDK3 and L2. The PDK3 or L2 mutants were characterized for binding affinities and L2-stimulated PDK3 activity. The results establish critical determinants in both L2 and PDK3 for interactions of L2 with the C-terminal tail and lipoyl-binding pocket of PDK3. This information provides a structural basis for understanding the mechanism for the L2-mediated up-regulation of PDK3 activity.

EXPERIMENTAL PROCEDURES

Vector Construction and Protein Expression—Recombinant human PDK3 with its N terminus fused to maltose-binding protein (MBP-PDK3) and N-terminally His6-tagged lipoylated L2 (residues 126–233 of human E2p) were expressed and purified as described previously (20). Mutations in MBP-PDK3 and L2 were introduced using the QuikChange site-directed mutagenesis system from Stratagene (La Jolla, CA). The PDK3 C-terminal end truncated mutants Δ (372–406), Δ (384–406), Δ (392–406), Δ (395–406), and Δ (398–406) were created by changing the codons for Phe372, Pro384, Pro392, Glu395, or Asp398, respectively, in PDK3 to a stop codon (TAA).

Kinase Activity Assay—PDK3 activity was measured at room temperature as the rate of [γ32P]phosphate incorporation into the E1p protein using [γ-32P]ATP as substrate. A typical reaction mixture (25 μl) contained 10 μg of E1p, 0.5 μM MBP-PDK3, and 0.1 mM [γ-32P]ATP (specific activity 500–1000 cpm/pmol ATP) in 50 mM MOPS buffer, pH 7.3, 20 mM potassium phosphate, 60 mM KCl, 5 mM dithiothreitol, 0.4 mM EDTA, and 2 mM MgCl2. The reaction was initiated by the addition of 2.5 μl of ATP solution and terminated after 4 min by adding 5 μl of 0.5 M EDTA. The aliquot (in 25 μl) was then blotted onto a 2.1-cm-diameter disk of Whatman P81 cation-exchange cellulose phosphate paper. After extensive washing with 0.7% phosphoric acid, protein-bound radioactivity was determined by liquid scintillation counting. An assay without PDK3 was used as a control to determine nonspecific incorporation of radioactivity.

Isothermal Titration Calorimetry (ITC)—The interaction of L2 or nucleotide with MBP-PDK3 was studied by ITC as described previously (20). All titrations were performed at 15 °C. For typical nucleotide binding measurements, MBP-PDK3 in the calorimeter cell was 15 μM, and nucleotide in the injection syringe was 75 μM, with 300-s spacing between injections. For L2 binding measurements, 50 μM MBP-PDK3 was loaded into the cell, and 300 μM L2 was in the syringe with 180-s spacing. Higher protein or nucleotide concentrations were used in case of weak binding to achieve accurate determination of binding parameters. For nucleotide binding experiments in the presence of L2, L2 was added to both the cell and the syringe to a final concentration of 50 μM. The concentrations of MBP-PDK3 and L2 were determined by using extinction coefficients ε278 nm of 114.8 and 11.2 mM-1 cm-1, respectively. The concentrations of ATP, ADP, and ATPγS were determined by using the same extinction coefficient of ε260 nm of 15.4 mM-1 cm-1.

Differential Scanning Calorimetry (DSC)—DSC measurements were carried out in a VP-capillary DSC system (MicroCal) in 50 mM potassium phosphate buffer, pH 7.5, containing 250 mM KCl. For each scan, 500 μM wild-type or mutant L2 protein was heated from 20 to 110 °C at a scan rate of 1 °C/min. Scans were corrected by subtraction of the reference scan, and protein concentrations were normalized to determine the melting temperature (Tm). All scans were performed in duplicate and averaged.

RESULTS

Binding Affinities of L2 Mutants for PDK3—The crystal structure of the PDK3-L2 complex we reported previously (20) shows that each subunit (I or II) of the homodimeric PDK3 binds one molecule of the L2 domain (L2 or L2′) from the E2p subunit of the human PDC (Fig. 1A). In Fig. 1A, the boxed area (in red) depicts interactions between L2 and the N-terminal domain from subunit I (in green) of PDK3. This interface comprises L2 residues (Leu140, Pro142, Glu162, Asp164, and Glu179) and helices α1, α3, and α13 from subunit I of PDK3 (Fig. 1B). Hydrophobic residue Leu140 was previously proposed to be critical for L2-stimulated PDK3 activity (14). Pro142 located on the loop region between β1 and β2 strands of L2 is likely to pack against hydrophobic residue Phe22 from PDK3. On the other hand, L2 residues Glu162, Asp164, and Glu179 may constitute a negatively charged ionic patch to interact with positively charged PDK3 residues Arg21, Lys-
374, and Arg-378 (Fig. 1B). A second region of interaction occurs between L2 residues and residues on the C-terminal tail from the subunit II of PDK3 (Fig. 1C, red box). Asp-172 and Arg-196 from L2 contact Arg-397 and Glu-395 of PDK3 subunit I, respectively, through ionic interactions (Fig. 1D). L2 residues Glu-170 and Thr-175 make hydrogen bonds to residues Ser-394 and Pro-392, respectively, from the C-terminal tail of PDK3 subunit II (Fig. 1D).

To establish the potential role of the above nine L2 residues in the cross-talk with PDK3, alanine substitutions for each of these L2 residues were carried out. Affinities of these L2 variants for PDK3 were determined by ITC. The PDK3 protein is largely insoluble; to increase its solubility, MBP is fused to PDK3 at the N terminus of the latter protein as described previously (20). Wild-type and mutant L2 proteins were titrated against the MBP-PDK3 fusion in a microcalorimeter as also described previously (20). Dissociation constants ($K_d$) and binding enthalpies ($\Delta H$) for these L2 mutants are shown in Table 1. The dissociation constants ($K_d$) and affinities for PDK3 of P142A and T175A are similar to those of the wild type (1.37 $\mu$M), although the $\Delta H$ values obtained with these two L2 variants are significantly reduced. The data suggest that Pro-142 is not essential for hydrophobic packing with Phe-22 from PDK3 and that the hydrogen bonding contributed by Thr-175 from L2 does not play an important role in PDK3 binding. Similarly, other L2 variants E162A, D164A, D172A, and R196A show only 2–3-fold increases in $K_d$ values over the wild type for binding to

| L2     | $K_d$ $\mu$M | $\Delta H$ kcal/mol |
|--------|---------------|----------------------|
| WT     | 1.37 $\pm$ 0.41 | $-7.95 \pm 1.58$ |
| L140A  | 9.76 $\pm$ 1.59 | $-8.18 \pm 0.50$ |
| P142A  | 3.35 $\pm$ 0.05 | $-3.98 \pm 0.31$ |
| E162A  | 2.92 $\pm$ 0.74 | $-6.82 \pm 0.70$ |
| D164A  | 2.10 $\pm$ 0.56 | $-6.38 \pm 0.48$ |
| E170A  | 2.01 $\pm$ 0.57 | $-6.84 \pm 0.09$ |
| D172A  | 2.60 $\pm$ 0.61 | $-3.88 \pm 0.35$ |
| T175A  | 1.65 $\pm$ 0.35 | $-5.70 \pm 0.30$ |
| E179A  | 12.25 $\pm$ 3.90 | $-3.56 \pm 0.61$ |
| R196A  | 2.83 $\pm$ 0.61 | $-6.62 \pm 0.87$ |
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The above results establish that reduced levels of stimulated PDK3 activity. The L140A, E170A, and E179A L2 variants exhibit markedly corresponding ligand concentrations. At 5 μM range stimulate PDK3 activity to near wild-type L2 levels at the wild-type L2 variants with binding affinities for PDK3 in the wild-type in the absence of L2. As expected, the P142A, D164A, and T175A 13.0-fold, respectively, over basal kinase activity measured in the absence of L2. The fold activation by L2 (ordinate) is defined as L2-stimulated PDK3 activity against basal PDK3 activity (one fold) measured in the absence—whether decreased protein stability is related to the reduced interactions with PDK3. The results indicate that ionic interactions conferred by Glu-170 from L2 (Fig. 1, B and D) are crucial for the high affinity binding of L2 to PDK3.

Effects of L2 Mutations on the Stimulation of PDK3 Activity—The ability of wild-type and mutant L2 to stimulate PDK3 activity was determined by measuring kinase activity in the absence and presence of three different concentrations (5, 50, and 100 μM) of the L2 ligand (Fig. 2). The wild-type L2 at 5, 50, and 100 μM concentrations stimulates PDK3 activity by 4.8-, 12.2-, and 13.0-fold, respectively, over basal kinase activity measured in the absence of L2. As expected, the P142A, D164A, and T175A L2 variants with binding affinities for PDK3 in the wild-type range stimulate PDK3 activity to near wild-type L2 levels at the corresponding ligand concentrations. At 5 μM concentration, the remaining six L2 mutants show lower levels of stimulated PDK3 activity than wild-type L2. Since this concentration is close to elevated Kd values of the L2 variants, the ability of these mutant L2 constructs to stimulate PDK3 activity correlates inversely with their Kd values for the kinase (Table 1). When the activator concentration is increased to 50 or 100 μM, only L140A, E170A, and E179A L2 variants exhibit markedly reduced levels of stimulated PDK3 activity. The Kd values of the latter three mutants are approximately 1 order of magnitude higher than that of the wild type (Table 1). Thus, the decreased fold activation indicates that the concentrations of these mutant ligands at 50 and 100 μM were non-saturating for the stimulation of PDK3 activity. The above results establish that Leu-140, Glu-170, and Glu-179 are critical determinants for L2 interactions with PDK3.

Effects of L2 Mutations on Protein Stability—To discern whether decreased protein stability is related to the reduced affinities of L2 variants for PDK3, melting temperatures (Tm) of the critical L2 mutants were measured by DSC. The L140A L2 mutant exhibits a Tm of 62.9 °C, which is markedly lower than that of the wild-type (69.1 °C) (Fig. 3). The result indicates that the L140A mutation significantly reduces the stability of the L2 protein. By comparison, the E170A and E179A substitutions produce Tm values of 70.5 and 70.9 °C, respectively, which are similar to that of the wild type. The data suggest that the latter two mutations have no significant effect on the stability of the L2 protein.

TABLE 2

Dissociation constants for nucleotide binding to MBP-PDK3 and MBP-PDK3 in complex with wild-type and mutant L2

For ITC measurements, nucleotide ATP or ADP (75 μM) was injected into the reaction cell housing 15 μM MBP-PDK3 in the presence of 50 μM wild-type or mutant L2. Standard deviations were calculated from three independent ITC measurements. WT, wild type.

| PDK-L2 complex | ATP Kd μM | ADP Kd μM |
|----------------|----------|----------|
| PDK3           | 0.16 ± 0.05 | 0.16 ± 0.05 |
| PDK3-L2(WT)    | 0.88 ± 0.21 | 1.50 ± 0.27 |
| PDK3-L2(L140A) | 0.35 ± 0.21 | 0.45 ± 0.14 |
| PDK3-L2(E170A) | 0.35 ± 0.15 | 0.50 ± 0.16 |
| PDK3-L2(E179A) | 0.14 ± 0.06 | 0.20 ± 0.02 |

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2). For E179A L2, the binding to PDK3 does not significantly alter affinity of the unbound PDK3 for either nucleotide. The results indicate that a tight binding of L2 to PDK3 conferred by residues Leu-140, Glu-170, and Glu-179 in the L2 domain is necessary for preferentially reduced affinity of PDK3 for ADP over ATP. As will be discussed below, the disparity in PDK3 affinities for the nucleotides is largely responsible for L2-stimulated PDK3 activity.

### Effects of the C-terminal Tail Truncations on L2-stimulated PDK3 Activity

To define the minimal length required for interactions with L2, the C-terminal tail region of PDK3 was serially truncated (Fig. 4A), and affinities of the truncated PDK3 mutants for wild-type L2 were measured. When titrated with wild-type L2, the ∆-(398–406) and ∆-(395–406) PDK3 mutants show smaller exothermic enthalpy changes (∆H) than the full-length PDK3 (Fig. 4B). The Kd values of ∆-(398–406) and ∆-(395–406) PDK3 mutants for L2 are 5.88 and 7.82 μM, respectively, when compared with 1.37 μM for the full-length PDK3 (Fig. 4C). Further deletions of the region proximal to the C-terminal end of PDK3, i.e. ∆-(392–406), ∆-(384–406), and ∆-(372–406), result in undetectable enthalpy changes (Fig. 4B) and failure to obtain Kd values for these mutants (Fig. 4C). The S394A PDK3 mutant located in the C-terminal tail region exhibits 4-fold lower affinity for L2 (Kd = 5.56 μM) than the wild-type kinase (Fig. 4C). Residue 394 in PDK3 is not conserved between human PDKs (Fig. 4A).

The substitution of Ser-394 with an arginine (as in PDK1 and PDK4) or a threonine (as in PDK2) does not significantly affect the affinity of PDK3 mutant for L2, with Kd values similar to that of wild-type PDK3 (Fig. 4C). When assayed in the absence and presence of L2, wild-type L2 stimulates kinase activity of the ∆-(398–406) and ∆-(395–406) PDK3 mutants by 6- and 2.8-fold, respectively, when compared with the full-length PDK3 at 12.2-fold (Fig. 4D). No L2-enhanced kinase activity was observed with the ∆-(392–406) PDK3 variant. No kinase activity was detected in the ∆-(384–406) and ∆-(372–406) mutants either in the absence or in the presence of wild-type L2 (Fig. 4D). The S394A mutation in the PDK3 C-terminal tail reduces the L2-mediated activation of kinase activity from 12.2-fold for the wild type to 6.3-fold for the variant (Fig. 4D). The S394R and S394T substitutions do not have significant effects on L2-stimulated PDK3 activity, with the latter exhibiting a slightly higher fold activation of basal activity than wild-type PDK3. The above results indicate that the entire C-terminal tail...
region of PDK3 is indispensable for high affinity L2 binding and L2-stimulated PDK3 activity. Moreover, residue Ser-394 in PDK3 is not a specific determinant that confers differential binding affinities for L2 between PDK isoforms.

**Critical Residues in the Lipoyl-binding Pocket for L2 Binding**—Fig. 5, A and B, shows interactions of the reduced dihydrolipoamide moiety from L2 with hydrophobic residues of α2 and α3 helices in the lipoyl-binding pocket of PDK3. Alanine-scanning mutagenesis of the PDK3 residues in the lipoyl-binding pocket was carried out to assess the roles of these residues in L2 binding and L2-mediated enhancement of PDK3 activity. When titrated with L2, the L27A, F35A, and F48A PDK3 variants show enthalpy changes that are too small to be detected for determining their dissociation constants (Fig. 5C). The L49A PDK3 mutant displays detectable enthalpy changes with a markedly higher $K_d$ value (7.77 μM) than wild-type PDK3 (1.37 μM). The yield of the F32A PDK3 mutant was too low to allow the measurement of affinity for L2 by ITC. The ability of L2 to stimulate the kinase activity of these PDK3 variants was also investigated. Wild-type L2 at 50 μM is able to activate the L49A PDK3 mutant with moderately reduced affinity for L2 to a level comparable with wild-type PDK3 (Fig. 5D). The basal activity of the L49A kinase variant in the absence of L2 increases by more than 2-fold relative to the wild-type kinase. It is of interest that the L27A PDK3 mutant that shows undetectable enthalpy signals when titrated with L2 is activated by more than 6-fold at 50 μM L2 concentration. The F32A, F35A, and F48A PDK3 mutants uniformly exhibit marginal basal and L2-stimulated kinase activities when compared with wild-type PDK3.

**DISCUSSION**

The binding of PDK to the E2p chain of the mammalian PDC leads to the stimulation of kinase activity by two accepted mechanisms. These include: 1) co-localization of the kinase with substrate E1p tethered to the E1p-binding domain of E2p and 2) the activation of kinase activity through conformational changes in the kinase active site (15, 16, 19). The ability of isolated L2 to efficiently stimulate PDK3 activity (15, 20) strongly suggests that...
L2-promoted conformational changes in the active site, rather than co-localization of the substrate, largely account for the enhanced kinase activity upon the recruitment of PDK3 to the E2p scaffold of PDC. The present study was undertaken to identify and characterize key determinants for the communication between PDK3 and L2, which up-regulates PDK3 activity. The study focuses on residues in L2 and the three L2-buried interfaces in PDK3: the N-terminal domain outside the lipoyl-binding pocket, the C-terminal tail, and the lipoyl-binding pocket.

It is of interest that among nine L2 residues for potential interactions with PDK3, based on the PDK3-L2 structure, only Leu-140, Glu-170, and Glu-179 are the critical determinants (Table 1 and Fig. 2). The significantly reduced binding affinity of L140A for PDK3 and the reduced fold activation of basal PDK3 activity confirms and extends the previous observation that the substitution of Leu-140 removed 80% of L2-activated PDK3 activity (14). Leu-140 is on the surface loop connecting β1 and β2 with the side chain pointing to inside of the L2 domain instead of the N-terminal domain of the PDK3 (Fig. 1B). Therefore, it is not apparent which residue(s) in PDK3 interacts with Leu-140 from L2. We speculated that Leu-140 might play a critical role in maintaining the native conformation of L2; a substitution of this residue might cause L2 to assume an altered conformation with reduced affinity for PDK3. This notion was confirmed by the significantly lower melting temperature of the L140A variant when compared with the wild-type L2, indicating the reduced stability of the mutant L2 protein (Fig. 3). Glu-179 in L2, which was also reported to be critical for L2-stimulated PDK3 activity (14), is located in a cluster of negatively charged residues facing positively charged residues Arg-21, Lys-374, and Arg-378 from PDK3 (Fig. 1B).

Moreover, Glu-179 is within the hydrogen-bonding distance to the main-chain amino group of Lys-374 in PDK3. We propose that in addition to the hydrogen bonding between Glu-179 (L2) and Lys-374 (PDK3), interactions between the two oppositely charged ionic patches are important to maintain the integrity of this interface area between L2 and PDK3. The E179A mutation does not significantly affect the melting temperature of the L2 protein (Fig. 3); therefore, residue Glu-179 apparently is not involved in maintaining the secondary structure integrity of L2.

It is noted that, unlike Glu-179, alanine substitutions of the two other residues Glu-162 and Asp-164 in the negatively charged patch from L2 have relatively insignificant effects on $K_d$ values for PDK3 and fold activation over basal PDK3 activity (Fig. 2 and Table 1). The data indicate that ionic patch interactions alone may not play a prominent role in maintaining this segment of the PDK3-L2 interfaces. In the PDK3-L2 structure, the side chain of Glu-170 from L2 is hydrogen-bonded to both the side chain and the main chain of Ser-394 on the C-terminal tail of PDK3 subunit II (Fig. 1D). This interaction is apparently important for the high affinity binding of L2 to PDK3, as indicated by the markedly increased $K_d$ value and the reduced fold activation of basal PDK3 activity with the E170A L2 mutant (Table 1 and Fig. 2). The very small enthalpy change ($\Delta H_f$) for this mutant may result from the loss of the critical hydrogen bonds between Glu-170 and Ser-394 from L2 and PDK3, respectively. Residue Glu-170 also does not participate in the secondary structure-forming activity of L2 since the E170A mutant shows a melting temperature similar to the wild-type (Fig. 3). Finally, residues Asp-172, Thr-175, and Arg-196 from L2, which are capable of forming ion pairs or hydrogen bonds with residues Arg-397, Pro-392, and Glu-395, respectively, on the PDK3 C-terminal tail (Fig. 1D), are apparently not critical determinants for PDK3-L2 interactions. This is indicated by the nearly wild-type $K_d$ values for the alanine mutants involving these residues (Table 1).

In the presence of 50 μM L140A, E170A, and E179A mutant L2 proteins, PDK3 shows similar $K_d$ values for nucleotides ATP and ADP (Table 2). The data strongly suggest that, unlike wild-type L2, these L2 variants are unable to remove the product inhibition imposed by ADP by preferentially reducing the affinity of PDK3 for ADP over ATP. This mechanism is likely responsible for the reduced fold activation of the basal PDK3 activity by these L2 mutants (Fig. 2). We have shown previously that the binding of wild-type L2 to PDK3 renders the ATP lid in the PDK3 active site more disordered in the presence of ADP than ATP, which promotes the accelerated release of product ADP (20). We have determined crystal structures of the PDK3-L2 (L140A), PDK3-L2 (E170A), and PDK3-L2 (E179A) complexes without the fused MBP moiety at 3.2, 2.5, and 2.5 Å resolution, respectively (data not shown). Interestingly, these structures are similar to the PDK3-L2 (wild-type) complex both in ADP-bound and in ATP-bound forms. The results suggest that the affinity of L2 for PDK3 is not the determining factor in the order-to-disorder transition of the ATP lid observed in the nucleotide-bound forms of PDK3 (20). The reduced affinity of the mutant L2 for PDK3 may indicate that a smaller fraction of the mutant ligand is bound to PDK3 than the wild-type L2. However, once the smaller number of mutant L2 domain molecules bind to PDK3, the ATP lid assumes the same conformations as those of PDK3 in complex with the nucleotide and wild-type L2. This mechanism is consistent with the reduced level of stimulated PDK3 activity by the mutant. It is conceivable that protein crystallization favors the bound form of PDK3 in the binding equilibrium since the unbound form of PDK3 in the absence of the fused MBP moiety tends to precipitate from the solution (20). These conditions result in the presence of a homogeneous population of PDK3 bound to the mutant L2 in the crystal lattice.

The serial truncations of the C-terminal tail on PDK3 lead to reduced or undetectable enthalpy changes when titrated with increasing concentrations of wild-type L2 (Fig. 4). In a separate study, nested deletions of the C-terminal region of PDK2 reduce the activation of basal kinase activity by a full-length E2p/E3BP construct (21). These findings establish that the C-terminal tails of PDK2 and PDK3 spanning 41 and 35 residues, respectively, are indispensable for L2 binding and L2-stimulated kinase activities. The C-terminal tail of one PDK3 subunit interacts with the bound L2 and becomes an integral part of the lipoyl-binding pocket in the other subunit (Fig. 5, A and B). The truncations of PDK3 subunit from the C-terminal end inward disrupt these interactions, resulting in the inability of PDK3 to bind L2. The loss of basal kinase activity with $\Delta$ (384–406) and $\Delta$ (372–406) PDK3 variants, but not the $\Delta$ (392–406), suggests that the C-terminal tail segment between residues 384 and 392 (Fig. 4A) is involved in maintaining the conformational integrity of the PDK3 homodimer. It is still
Cross-talk between PDK3 and L2 Domain

unclear what determinants confer differential affinities of PDK isoforms for L2. Residue Ser-394 in PDK3, which is hydrogen-bonded to Glu-170 in L2, is replaced by a threonine in PDK2 and an arginine in PDK1 and PDK4. However, substitution of Ser-394 in PDK3 with a threonine or an arginine does not appear to have significant effects on L2 binding (Fig. 4, C and D). This may be due to the fact that despite the loss of the otherwise accompanying side-chain/side-chain interactions, the hydrogen bonding between the side chain of Glu-170 from L2 and the main chain at the position 394 of PDK3 is unperturbed in these substitutions (Fig. 1D).

The requirement of the lipoyl moiety for the binding of PDK to the E2p was first described by the Roche group (16, 17). The crystal structure of the human PDK3-L2 complex shows that the lipoyl-binding pocket is formed by the loop region between helices α1 and α2 as well as helices α2, α3, and α8 (Fig. 5B). Hydrophobic residues Leu-27, Phe-35, Phe-48, and Leu-49 are invariant between PDK isoforms with Phe-32 replaced by a leucine in PDK4. The conservation of hydrophobic residues in the lipoyl-binding pocket is necessary for the tight binding of the lipoyl prosthetic group carried by L2. It is therefore not surprising that, except for residue Leu-49, alanine-scanning mutagenesis of the above invariant hydrophobic residues abrogates L2 binding to PDK3 and largely impedes L2-mediated stimulation of PDK3 activity (Fig. 4, C and D). Phe-32 from PDK3 is not in direct contact with the reduced lipoamide moiety, but the low yield of F32A indicates that this residue may be important for the folding and hydrophobic packing of the lipoyl-binding pocket. Residues Phe-35 and Phe-48 are critical because they flank the reduced dithiolane group of dihydrolipoamide in the PDK3-L2 structure. Residue Leu-49, on the other hand, is more than 5 Å away from the reduced lipoamide and may only have weak interactions with the aliphatic side chain. This conformation explains the moderately increased $K_d$ value of the mutant L49A PDK3 for L2 binding.

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