Supplementary data

Materials and methods

Preparation of the PDK3-L2 complex

The cDNA sequence coding the L2 domain (residues 126-233 of human E2p) was amplified from the E2p/E3BP plasmid kindly provided by Dr. Kirill Popov (University of Alabama at Birmingham) and cloned into the pTrcHisB vector (Invitrogen) as described previously (Chuang et al., 2002). The resulting plasmid was transformed into BL21 cells; the cells were grown in LB media at 37 °C. Prior to induction with 0.5 mM IPTG, 0.2 mM lipoic acid was supplemented in the medium to produce lipoylated His_6-tagged L2 (Liu et al., 1995), followed by culture for 15 hrs at 30 °C. Harvested cells were sonicated in a lysis buffer containing 100 mM potassium phosphate (pH 8.0), 500 mM NaCl, 0.5% Triton X-100, 1% Tween 20, 20 mM 2-mercaptoethanol, 1 mM benzamideine, 10% glycerol, 1 mM PMSF and 1 mg/ml lysozyme. The resulting cell lysate was centrifuged at 30,000 rpm for 30 min. The supernatant was extracted with Ni-NTA resin (Qiagen); the resin was washed in a column with buffer A containing 50 mM potassium phosphate (pH 7.5), 250 mM KCl, 20 mM 2-mercaptethanol, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride and 5% glycerol, followed by washing with buffer A containing 10 mM imidazole. Bound proteins were eluted with 250 mM imidazole in the buffer A. L2 domains were further purified with a Superdex 75 gel filtration column (Amersham) equilibrated with buffer A. Fractions containing L2 domains were combined, concentrated and stored at -80 °C.

DNA fragments encoding the entire mature sequence of human PDK3 (406 residues) were amplified by RT-PCR using RNA prepared from human skin fibroblast as template. The

DNA fragments were cloned into the pTrcHisB vector (Invitrogen), and the entire PDK3 sequence was confirmed by DNA sequencing. The resultant plasmid for expressing the N-terminally His_6-tagged PDK3 was transformed into BL21 cells containing the pGroESL plasmid. Expression of PDK3 was induced with 0.5 mM IPTG at 28 °C for overnight. Harvested cells were sonicated in the above lysis buffer. The cell lysate was centrifuged at 30000 rpm for 30 min. The supernatant was mixed with Ni-NTA resin, and bound proteins were eluted with buffer A containing 0.2% octaglucopyranoside and 250 mM imidazole. PDK3 was further purified on Superdex 200 gel filtration column (Amersham) equilibrated with buffer A containing 0.05% octaglucopyranoside. To the purified PDK3 solution, a 3-fold molar excess of lipoylated L2 was added to produce the PDK3-L2 complex. The complex was dialyzed overnight against 50 mM Hepes (pH 7.5), 50 mM KCl and 5%(v/v) glycerol. The PDK3-L2 preparation was concentrated to 15 mg/ml, and DTT was added to a 20 mM final concentration for crystallization.

Preparation of MBP-PDK3 fusion protein

To construct the plasmid expressing MBP-PDK3 fusion protein, the PDK3 gene was amplified by PCR using the plasmid for His_6-tagged wild-type PDK3 as a template. XbaI and HindIII restriction sites were introduced into the 5' and 3' end of DNA fragments, respectively. DNA fragments encoding the PDK3 sequence were subcloned into the XbaI/HindIII site of a pMALc vector (New England BioLabs), followed by transformation into BL21 cells containing the pGroESL plasmid for expressing chaperonins GroEL and GroES. The plasmid for C-terminally truncated MBP-PDK3 (Δ392-406) was created by replacing the codon for proline 392 (5'-CCC-3') for the wild-type PDK3 with a stop codon (5'-TAA-3') using the QuinkChange site-directed mutagenesis system (Stratagene). After cells were grown at 37 °C for 4 hrs, the

expression of MBP-PDK3 (wild-type or truncated protein) was induced with 0.5 mM IPTG at 28°C for overnight. Harvested cells were sonicated in the lysis buffer described above after treatment with lysozyme (1 mg/ml) for 30 min. After centrifugation at 30,000 rpm for 30 min, the supernatant was mixed with amylose resin (New England BioLabs), followed by extensive washing with buffer A. Bound proteins were eluted with buffer A containing 10 mM maltose. Eluted proteins were precipitated by ammonium sulfate (75% of saturation) and further purified on Superdex 200 FPLC gel-filtration column.

Other protein preparations

Human E1p protein was prepared as described previously (Korotchkina and Patel, 1995). The 60-meric E2p/E3BP core was expressed from the E2p/E3BP plasmid and purified as also described previously (Harris et al., 1997).

Structural determination

Crystals of the PDK3-L2 complex were produced at 20 °C by vapor diffusion using a reservoir solution containing 100 mM Na-citrate (pH 5.6), 50-80 mM $NaKH_2PO_4$ and 1M NaCl. Crystals appeared in one day and grew to a final size of ~ 600 x 600 x 500 μm^3 in 2 to 3 days. PDK3-L2 crystals were transferred into a stabilizing buffer (pH 5.6) containing 100 mM $NaKH_2PO_4$, 1.25 M NaCl, 30%(v/v) glycerol, and 100 mM Na-citrate for 3 to 4 hrs. For crystals of nucleotide-bound complexes, crystals were soaked in the stabilizing buffer containing 10 mM ADP or ATP and 10 mM $MgCl_2$. Crystals were flash-cooled after serial transfers into the stabilizing buffer containing 25%(v/v) glycerol with or without a nucleotide (ADP or ATP) and stored in liquid nitrogen. Diffraction data for the nucleotide-free complex (apo) were collected

with a rotating anode (RU-300, Rigaku) and an R-AXIS IV detector (Molecular Structure Corporation). Diffraction data for ADP- and ATP-bound complexes were collected with beamlines 19ID and 19BM in the Structural Biology Center at the Advanced Photon Source (Argonne, IL). The data were processed with MOSFLM (Leslie, 1992) or HKL2000 (Otwinowski and W. Minor, 1997). The crystals exhibit the symmetry of space group $P6_522$. Each asymmetric unit contains one PDK3 monomer bound to one L2 domain.

Structures of the PDK3-L2 complex were solved by molecular replacement using the program AMoRe (Navaza, 1994) with a model of PDK3, which was constructed by homology-modeling techniques using the program Swiss-Model (http://swissmodel.expasy.org/repository/sms.php?sptr_ac=Q15120) based on the published PDK2 structure (PDB code: 1JM6) (Steussy et al., 2001). After initial refinements of the PDK3 structure with REFMAC5 (Murshudov et al., 1997), the electron density map was improved using DM (Cowtan and Main, 1996). The NMR structure of L2 (PDB code: 1FYC) (Howard et al., 1998) was fitted in the improved density and re-modeled manually using the program O (Jones et al., 1991). During subsequent refinements, a lipoyl acid, ADP or ATP, a magnesium ion, potassium ions, and water molecules were gradually added. An oxidized dithiolane ring of the lipoyl group did not fit into the electron density map. In contrast, the reduced form of lipoamide (dihydrolipoamide) was successfully fitted in the map without any structural skewness (Figure 4, inset). In the final models, the N-terminal region of PDK3 (residues 1-11 for the ATP-bound form; residues 1-12 for both the apo- and the ADP-bound), the ATP lid (residues 311-319 for the ATP-bound; residues 307-319 and 322-323 for the apo- and ADP-bound, respectively), and the C-terminal region of L2 (residues 211-215) were disordered and not included. Data

processing and refinement statistics are summarized in Table I. Molecular graphics for structural representations were drawn by PyMOL (DeLano Scientific LLC.).

Kinase activity assays by E1p phosphorylation

The reaction mixture containing 1 μg of E1p protein, 0.2 μg of human MBP-PDK3 and increasing concentrations of lipoylated L2 (1-30 μM) or 40 nM lipoylated E2p/E3BP in 30 mM Hepes buffer (pH 7.35), 2 mM DTT, 1.5 mM $MgCl_2$, 0.2 mM EGTA were pre-incubated at room temperature for 10 min. The phosphorylation reaction was initiated by adding 0.4 mM [γ-^{32}P]ATP (specific activity 1.88 μCi/nmol ATP) to the above reaction mixture. After 2 min, the reaction was terminated by adding the reaction mixture at 1:3 dilution to a concentrated SDS-PAGE sample buffer comprising 50 mM Tris-HCl (pH 6.8), 5% (w/v) SDS, 50% (v/v) glycerol, 500 mM DTT, and 1% (w/v) bromophenol blue. The terminated reaction mixture was immediately placed in a 65 ºC water bath for 10 min prior to loading onto a 12% SDS-PAGE gel. After electrophoresis, gels were stained with Coomassie Blue R-250 and vacuum-dried. Quantitative analysis of ^{32}P incorporation to the E1p α subunit was carried out by scanning a storage phosphor screen with a Typhoon 9200 Variable Mode Imager (Molecular Dynamics) using ImageQuant software.

Binding measurements by isothermal titration calorimetry (ITC)

ITC measurements with the active preparation of PDK3 dimers were performed in a VP-ITC microcalorimeter from MicroCal (Nothampton, MA). Titrations were carried out in 50 mM potassium phosphate buffer (pH 6.3), 50 mM KCl, 10 mM $MgCl_2$, 20 mM β-mercaptoethanol at 15 °C. In a typical measurement for nucleotide binding, 36 injections (1 x 4 μl + 35 x 8 μl) of

ATP, ADP or ATPγS (75 μM) were made into 1.8 ml of the MBP-PDK3 fusion protein (15 μM, monomer) in the cell with 180 seconds between two consecutive injections, while the sample was stirred at 316 rpm. In experiments for studying the effect of the L2 domain on nucleotide binding, the L2 was added into both the cell and the injection syringe to the final concentration of 30 μM. For L2 binding measurements, the L2 concentration in the syringe was 537 μM, and the concentration of wild-type or a truncated PDK3 (Δ392-406) in the cell was 68.2 μM (monomers). The resulting ITC isotherms were analyzed by using the MicroCal ORIGIN software package, and the binding constant (K_A), the binding heat (ΔH), and apparent number of active binding site (N) were obtained. The concentrations of MBP-PDK3 and L2 were determined by using extinction coefficients ε_{278nm} of 114.8 $mM^{-1}cm^{-1}$, and 11.2 $mM^{-1}cm^{-1}$, respectively. The concentrations of ATP, ADP and ATPγS were determined by using the same extinction coefficient of ε_{260nm} of 15.4 $mM^{-1}cm^{-1}$.

Supplementary references

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