Structure of the complex of phosphorylated liver kinase B1 and 14-3-3 ζ

Yongjian Lu, Sheng Ding, Ruiqing Zhou* and Jianyong Wu*

Department of Stomatology, Xin Hua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200092, People's Republic of China. *Correspondence e-mail: ruikingzhou@163.com, wjyxr@163.com

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The serine/threonine protein kinase liver kinase B1 (LKB1) is a tumour suppressor and plays important roles in development and metabolism. It phosphorylates AMPK and AMPK-related kinases to regulate multiple physiological processes. Mutations in LKB1 often occur in multiple cancers. LKB1 can be suppressed by 14-3-3 proteins in a phosphorylation-dependent manner. Previously, the structure of a 14-3-3 ζ –LKB1 fusion protein has been reported, revealing a phosphorylation-independent binding mode of LKB1 to 14-3-3 proteins. Here, the crystal structure of phosphorylated LKB1 peptide in complex with 14-3-3 ζ was solved, which provides a structural basis for the phosphorylation-dependent recognition of LKB1 by 14-3-3 proteins.

1. Introduction

Liver kinase B1 (LKB1, also named STK11) was first reported as its mutations frequently occur in Peutz–Jeghers syndrome (PJS) patients (Jenne *et al.*, 1998; Hemminki *et al.*, 1998). LKB1 is a serine/threonine kinase that controls multiple cellular processes such as cell growth, death and polarity (Jansen *et al.*, 2009; Alessi *et al.*, 2006; Yoo *et al.*, 2002). LKB1 can phosphorylate and activate AMPK and AMPK-related kinases, including MARK, NIAK, SAD and SIK (Hawley *et al.*, 2003; Woods *et al.*, 2003; Lizcano *et al.*, 2004). AMPK is an important metabolic sensor and response to cellular energy status (Hardie, 2014). AMPK is activated when the cellular AMP:ATP ratio increases. It promotes ATP-producing catabolic metabolism but suppresses biosynthetic metabolism *via* mTOR and other pathways, maintaining cellular energy homeostasis. LKB1 and MARK can act upstream of Hippo–YAP signalling, which controls organ size and tissue haemostasis during individual development (Mohseni *et al.*, 2014). NIAK and SAD kinases are activated by LKB1 to regulate axon initiation, growth and branching in cortical neurons (Barnes *et al.*, 2007; Courchet *et al.*, 2013). The LKB1–SIK axis is involved in lipid metabolism to regulate gluconeogenesis (Patel *et al.*, 2014; Choi *et al.*, 2015).

LKB1 is an important tumour suppressor. It can suppress tumour growth through regulating AMPK, p53, p73 and JNK, and tumour metastasis *via* MMP-2/9 and VEGF (Shackelford & Shaw, 2009; Gan & Li, 2014). Dysfunction of LKB1 often leads to tumorigenesis. Its loss-of-function mutations have been found in a variety of cancers such as breast, cervical, colorectal, lung and pancreatic cancers (Shackelford & Shaw, 2009; Alessi *et al.*, 2006; Gan & Li, 2014). Under physiological conditions, the activity and localization of LKB1 are regulated by its partners and post-translational modification (Gan & Li, 2014). The two most important regulators of LKB1 are the pseudokinase STRAD and the activator MO25 (Boudeau *et*

al., 2003; Baas *et al.*, 2003). The binding of STRAD and MO25 leads to the cytoplasmic localization and activation of LKB1 (Zeqiraj, Filippi, Deak *et al.*, 2009; Zeqiraj, Filippi, Goldie *et al.*, 2009). Many mutations of LKB1 found in PJS or tumour patients impair its binding to STRAD and MO25 (Zeqiraj, Filippi, Deak *et al.*, 2009). Several kinases, including ATM, PKA, PKC and RSK, have been reported to phosphorylate LKB1 at multiple sites, while their function needs to be further clarified (Sapkota *et al.*, 2001, 2002; Collins *et al.*, 2000; Xie *et al.*, 2006, 2008). LKB1 can also be prenylated at its C-terminal tail and targeted to the membrane compartment (Collins *et al.*, 2000).

14-3-3 proteins can modulate the functions of target proteins *via* changing their conformation and subcellular localization, and suppressing or promoting their association with other proteins (Dougherty & Morrison, 2004; Obsilova *et al.*, 2014). 14-3-3 proteins usually bind to consensus sequences with a phosphorylated serine or threonine in their partners, while phosphorylation-independent binding modes of 14-3-3 proteins have also been found (Obsil & Obsilova, 2011). Several studies have reported that 14-3-3 proteins are able to regulate the functions of LKB1 or its downstream kinases. For example, 14-3-3 binds the T-loop of SIK that is phosphorylated by LKB1 to activate SIK and change its cellular localization (Al-Hakim *et al.*, 2005). LKB1 phosphorylated at Ser334 by AKT associates with 14-3-3, preventing its interaction with STRAD and increasing its nuclear accumulation (Liu *et al.*, 2012). Upon serum starvation, LKB1 is autophosphorylated at Thr336, and this form of LKB1 also binds 14-3-3, inhibiting its association with the substrate AMPK (Bai *et al.*, 2012).

Previously, we reported the crystal structure of 14-3-3 ζ fused to an LKB1 fragment (amino acids 333–340, T336E; Ding *et al.*, 2015). The LKB1 fragment adopts two conformations to bind 14-3-3 ζ . One conformation of the LKB1 fragment resembles that of phosphorylated 14-3-3-binding partners, while the other is independent of phosphorylation. However, in this structure the phosphorylation-dependent binding of LKB1 to 14-3-3 ζ is not fully captured. Here, we solved the structure of 14-3-3 ζ in complex with a phosphorylated LKB1 peptide (pLKB1) in which Thr336 is phosphorylated, revealing a phosphorylation-dependent binding mode between LKB1 and 14-3-3.

2. Materials and methods

2.1. Cloning and protein expression

The DNA sequence for 14-3-3 ζ was cloned into pDEST17 vector with an N-terminal 6 $\times$ His tag. The expression plasmid was transformed into *Escherichia coli* BL21(DE3) CodonPlus competent cells. The cells were incubated in Terrific Broth medium with 30 $\mu\text{g ml}^{-1}$ ampicillin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol at 310 K until the OD₆₀₀ reached 1.0. The temperature was then changed to 293 K and the *E. coli* cells were incubated for a further 1 h. Protein expression was induced by isopropyl β -D-1-thiogalactopyranoside at a final concentration of 0.25 mM for 18 h.

2.2. Protein purification

The *E. coli* cells were collected by centrifugation at 6000g for 10 min and suspended in six times the volume of lysis buffer consisting of 20 mM HEPES–Na pH 7.5, 500 mM NaCl, 5% glycerol, 20 mM imidazole, 5 mM β -mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride; the cells were then broken by a high-pressure homogenizer at 80 MPa. Cell debris was removed by centrifugation for 40 min at 20 000g. The supernatant was mixed with 3 ml Ni Sepharose (GE Healthcare) for 1 h and the beads were then sequentially washed with ten times volume of lysis buffer containing 20 and 40 mM imidazole. The protein was eluted with five times the volume of lysis buffer plus 300 mM imidazole. The protein was concentrated and loaded onto a HiLoad 16/60 Superdex 200 pg column (GE Healthcare) pre-equilibrated with 20 mM HEPES–Na pH 7.5, 100 mM NaCl, 1 mM DTT.

2.3. Crystallization and data collection

5 mg ml^{−1} 14-3-3 ζ was mixed with synthesized pLKB1 peptide (331-RWRSMpTVVPYL-343, where pT represents phosphorylated threonine; Scilight-Peptide, Beijing, People's Republic of China) in a molar ratio of 1:5 for 1 h on ice. Crystallization was performed by the sitting-drop vapour-diffusion method. Initial crystal screening was performed with an ARI Crystal Gryphon instrument. Crystallization kits from Hampton Research and Molecular Dimensions were used for crystal screening. The 0.4 μl sitting drops consisted of 0.2 μl protein solution and 0.2 μl reservoir solution and were equilibrated against 60 μl reservoir solution. Crystals grew at 289 K. Crystal optimization was performed manually in a 48-well plate with 100 μl reservoir solution and 2 μl sitting drops.

The crystals were soaked in reservoir solution containing 20% glycerol and flash-cooled in liquid nitrogen. Diffraction data were collected on beamline BL17U1 at Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China from a single crystal using 1° oscillations and a crystal-to-detector distance of 350 mm. The diffraction data were processed using *HKL-2000* (Otwinowski & Minor, 1997).

2.4. Structure determination and refinement

The structure of the pLKB1–14-3-3 ζ complex was solved by molecular replacement with *Phaser-MR* in *PHENIX* (McCoy *et al.*, 2007; Adams *et al.*, 2010) using the structure of the 14-3-3 ζ –LKB1 fusion protein (PDB entry 4zdr; Ding *et al.*, 2015) as a search model. The model was completed using iterative cycles of manual building in *Coot* and refinement with *phenix.refine* in *PHENIX* (Afonine *et al.*, 2012; Emsley *et al.*, 2010). During refinement, a noncrystallographic symmetry restraint was applied and 10% of the reflections were selected for the calculation of R_{free} . Structural images were produced by *PyMOL* (<http://pymol.org/>).

2.5. Structure deposition

The coordinates and structure factors for the crystal structure of the LKB1–14-3-3 ζ complex have been deposited in the

(a)

(b)

Figure 1
(a) Crystals of the pLKB1–14-3-3 ζ complex. (b) A representative diffraction image from a crystal of the pLKB1–14-3-3 ζ complex.

Worldwide Protein Data Bank (wwPDB) under accession code 5wxn.

3. Results and discussion

3.1. Determination of the structure of the pLKB1–14-3-3 ζ complex

14-3-3 ζ was purified by affinity chromatography and gel filtration. The pLKB1 peptide was added to 14-3-3 ζ and initial crystallization screening was performed. Crystals appeared in several crystallization conditions: (i) Wizard III No. 5 (0.2 M ammonium chloride, 20% PEG 3350), (ii) PEG/Ion No. 38 (0.2 M ammonium tartrate, 20% PEG 3350), (iii) PEG/Ion 2 No. 11 (4% Tacsimate pH 5.0, 20% PEG 3350) and (iv) Crystal

Table 1

Data-collection and refinement statistics for the pLKB1–14-3-3 ζ complex.

Values in parentheses are for the highest resolution shell.

Data collection	
Wavelength (Å)	0.97915
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 72.77$, $b = 85.38$, $c = 112.74$
Resolution (Å)	50.0–2.93 (2.98–2.93)
Total No. of reflections	94121
No. of unique reflections	15953 (781)
R_{merge}	0.093 (0.937)
$\langle I/\sigma(I) \rangle$	22.07 (1.78)
Completeness (%)	100.0 (99.8)
Multiplicity	5.9 (5.9)
Refinement	
R_{work}	0.193
R_{free}	0.254
No. of atoms	
Total	3670
Protein	3652
Water	18
R.m.s.d.	
Bond lengths (Å)	0.009
Bond angles (°)	1.239
Ramachandran plot	
Favoured (%)	96.12
Allowed (%)	3.66
Outliers (%)	0.22
Average B factors (Å ²)	87

Screen 2 No. 40 (0.1 M Tris pH 8.5, 25% *tert*-butanol). After several rounds of optimization, the best crystals grew in a condition consisting of 0.15 M ammonium chloride, 18% PEG 3350 (Fig. 1a). One crystal diffracted to 2.93 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 72.77$, $b = 85.38$, $c = 112.74$ Å (Fig. 1b and Table 1). The multiplicity and completeness of the data set were 5.9 and 100%, respectively. The structure was determined by molecular replacement using the previously reported structure of the 14-3-3 ζ –LKB1 fusion protein (PDB entry 4zdr) as the search model (Ding *et al.*, 2015). The model was refined to a final R_{work} of 0.193 and R_{free} of 0.254. Details of the data-collection and refinement statistics are summarized in Table 1.

3.2. Crystal structure of the pLKB1–14-3-3 ζ complex

Two copies of the pLKB1–14-3-3 ζ complex are present in the asymmetric unit. In this structure 14-3-3 ζ forms a homodimer and each 14-3-3 ζ molecule binds one pLKB1 peptide just as in other 14-3-3–partner complexes, forming a heterotetramer (Fig. 2). The two copies of the pLKB1–14-3-3 ζ complex in the asymmetric unit have a similar conformation, with a root-mean-square deviation (r.m.s.d.) of 0.297 Å for C $^\alpha$ atoms. We chose one copy for structural analysis.

The pLKB1 peptide embeds into a groove surrounded by helices $\alpha 3$, $\alpha 5$, $\alpha 7$ and $\alpha 9$ of 14-3-3 ζ , which is the canonical partner-binding region (Obsil & Obsilova, 2011; Figs. 3a and 3b). The interface of the pLKB1–14-3-3 ζ complex is up to 802 Å² (PDBe PISA server). The phosphate group of phosphorylated Thr336 in pLKB1 coordinates residues Lys49 and Arg56 in helix $\alpha 3$ and residues Arg127 and Tyr128 in helix $\alpha 5$ of 14-3-3 ζ via ionic interactions and hydrogen bonds (Fig. 3b). The main chains of Ser334, Met335, Val337, Val338 and

Leu341 of pLKB1, as well as the side chain of Ser334, contact the side chains of Asn42 in helix α_3 , Lys120 in helix α_5 , Asn173 and Glu180 in helix α_7 , and Asn224 in helix α_9 of 14-3-3 ζ via hydrogen bonds. Hydrophobic packing also contributes to their association, especially in the C-terminal part of pLKB1 (Fig. 3c). The hydrophobic residues Met335, Val 337, Val338, Pro339, Tyr340 and Leu341 from pLKB1 pack with Val46 and Lys49 in helix α_3 , Phe117 in helix α_5 , Pro165, Ile166 and Leu172 in helix α_7 , and Ile217 and Leu220 in helix α_9 of 14-3-3 ζ , forming a hydrophobic core. Therefore, extensive ionic interactions, hydrogen bonds and hydrophobic interactions mediate pLKB1–14-3-3 ζ complex formation.

3.3. Comparison of the phosphorylation-dependent 14-3-3 binding mode of LKB1 with known modes

Previous studies have shown that there are three canonical phosphorylation-dependent modes of binding of 14-3-3 to its partners (Rittinger *et al.*, 1999; Yaffe *et al.*, 1997; Würtele *et al.*, 2003). They have distinct consensus sequences, R-X-X-pS/T-

X-P (mode I), R-X-X-X-pS/T-X-P (mode II) and pS/T-X(X)-COOH (mode III), where X is any type of residue and pS/T represents a phosphorylated serine or threonine. Although all of the pS/T residues in the three modes contact the same residues in the partner-binding groove of 14-3-3 and have a similar conformation, the other residues adopt different conformations. Compared with the consensus sequences of the three 14-3-3 binding modes, the sequence of the LKB1 peptide resembles that of mode I except that it has an extra Val338 in the +2 position (Fig. 4a). We compared the structure of the LKB1–14-3-3 complex with those of other 14-3-3–partner complexes (PDB entries 1qja, 1qjb and 1o9d; Rittinger *et al.*, 1999; Würtele *et al.*, 2003). We found that, consistent with the sequence similarity, the N-terminal part of the pLKB1 peptide adopts a similar conformation to 14-3-3 binding mode I (Figs. 4b–4e). However, owing to the presence of the extra Val338 in the +2 position, the LKB1 peptide has a relatively more outstretched conformation, contributing a larger interface with 14-3-3 (802 Å² for the pLKB1–14-3-3 ζ complex and 666 Å² for mode I; Rittinger *et al.*, 1999; Figs. 4b and 4c).

3.4. Comparison of phosphorylation-dependent and phosphorylation-independent binding modes of LKB1 to 14-3-3

Previously, we reported the crystal structure of the 14-3-3 ζ –LKB1 fusion protein (Ding *et al.*, 2015). In this structure, a phosphorylation-independent binding mode of LKB1 to 14-3-3 ζ was uncovered. Comparing the structure of the pLKB1–1493-3 ζ complex with that of the 1493-3 ζ –LKB1 fusion protein, we found that both of the LKB1 parts embed the partner-binding groove in 1493-3 ζ but possess different conformations (Fig. 5a). In the pLKB1–14-3-3 ζ complex pLKB1 adopts an outstretched conformation. The N-terminal and C-terminal parts of pLKB1 contact the polar residues and the hydrophobic groove of 14-3-3 ζ , respectively (Fig. 5b). The phosphorylated Thr336 of pLKB1 forms multiple interactions with 1493-3 ζ . However, in the 1493-3 ζ –LKB1 fusion protein LKB1 adopts a compact conformation, and nearly the whole LKB1 part interacts with the hydrophobic groove of 1493-3 ζ (Fig. 5c). Moreover, the phosphomimetic Glu336 of LKB1 in the 14-3-3 ζ –LKB1 fusion protein does not contact basic and

Figure 2
Overall structure of the pLKB1–14-3-3 ζ complex. The two 14-3-3 ζ molecules are coloured green and cyan. The two pLKB1 peptides are coloured purple and yellow. The $2mF_o - DF_c$ maps for the two pLKB1 peptides are contoured at 1.0σ and shown as a yellow mesh.

Figure 3
(a) The pLKB1 peptide binds to the partner-binding groove of 14-3-3 ζ . (b) The interaction of the pLKB1 peptide with 14-3-3 ζ via ionic interactions and hydrogen bonds. (c) The interaction of the pLKB1 peptide with 14-3-3 ζ via hydrophobic packing.

Figure 4
(a) Sequence alignment of the pLKB1 peptide and canonical 14-3-3 binding motifs. (b) Structure comparison of the pLKB1-14-3-3 ζ complex with 14-3-3-partner complexes with binding modes I (PDB entry 1qjb) (c), II (PDB entry 1qja) (d) and III (PDB entry 1o9d) (e).

Figure 5
(a) Structural comparison of the pLKB1-14-3-3 ζ complex and the 14-3-3 ζ -LKB1 fusion protein (PDB entry 4zdr). (b) Structure of the pLKB1-14-3-3 ζ complex. (c) Structure of the 14-3-3 ζ -LKB1 fusion protein.

polar residues in 1493-3 ζ , suggesting that the T336E mutation in LKB1 cannot fully mimic the phosphorylated Thr336 under physiological conditions.

In previous work and this work, we have solved crystal structures of the pLKB1-14-3-3 ζ complex and a 14-3-3 ζ -LKB1 fusion protein, uncovering phosphorylation-dependent and phosphorylation-independent binding features of LKB1 and 14-3-3. The two modes of binding of LKB1 to 14-3-3 may have diverse functions and regulate specific physiological processes, which should be revealed by further studies. Our

work will be of benefit to further functional studies of LKB1 regulation.

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