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Crystallization and preliminary crystallographic analysis of the chimeric protein LKB1–14-3-3 ζ

LKB1, a tumour suppressor, plays key roles in cell polarity, cell growth and energy metabolism. 14-3-3 proteins bind to LKB1 and suppress its functions. A chimera containing 14-3-3 ζ and its binding region in LKB1 was constructed and the chimeric protein LKB1–14-4-3 ζ was purified and crystallized. The crystal of LKB1–14-4-3 ζ diffracted to 2.9 Å resolution and belonged to space group R32, with unit-cell parameters $a = b = 130.262$, $c = 264.960$ Å. Structure determination and refinement are in progress.

1. Introduction

LKB1, a serine/threonine protein kinase, is mutated in patients with Peutz–Jeghers syndrome, an autosomal dominant genetic condition that results in a predisposition to a wide spectrum of benign and malignant tumours (Hemminki *et al.*, 1998; Jenne *et al.*, 1998). LKB1 functions as a tumour suppressor by controlling cell division, regulating cell polarity and coupling cell growth to energy metabolism (Jansen *et al.*, 2009; Spicer & Ashworth, 2004; Williams & Brenman, 2008). Restoring LKB1 expression in a number of tumour cell lines suppresses their growth by inducing G1 cell-cycle arrest (Tianen *et al.*, 1999).

LKB1 has been further investigated as a major upstream kinase of AMP-dependent protein kinase (AMPK), a master regulator of energy homeostasis and cell growth (Woods *et al.*, 2003). It directly phosphorylates AMPK within the kinase domain at Thr172 to activate AMPK (Hong *et al.*, 2003; Shaw *et al.*, 2004). In addition, it activates at least 12 other members of the AMPK-related protein kinase family through phosphorylation of a conserved threonine in the T-loop of the kinase domain (Alexander & Walker, 2011; Lizcano *et al.*, 2004).

LKB1 signalling is regulated by post-translational modification and subcellular translocation (Alessi *et al.*, 2006; Boudeau *et al.*, 2003; Tianen *et al.*, 2002). It is phosphorylated on multiple residues. There are nine phosphorylation sites on LKB1: Ser31, Ser325, Thr366, Ser428 and Ser431 are phosphorylated by many different upstream kinases, while Thr185, Thr189, Thr336 and Thr402 are autophosphorylation sites (Alessi *et al.*, 2006; Xie *et al.*, 2008). Phosphorylation at Thr336 and Ser428 has been shown to modulate the catalytic activity or subcellular localizations of this kinase (Sapkota *et al.*, 2002; Xie *et al.*, 2008).

The 14-3-3 family proteins, which include seven isoforms β , ϵ , η , γ , τ , ζ and σ in mammals, can bind to target proteins depending on phosphorylated serine or threonine residues mainly within the two 14-3-3 binding motifs, RSXpSXP (mode I) and RXXXpSXP (mode II), of the target proteins (where pS represents phosphoserine and X represents any residue; Fu *et al.*, 2000; Yaffe *et al.*, 1997). The function of 14-3-3 proteins can be generally classified into three main types: (i) changing the conformation of a binding partner, (ii) physically occluding sequence-specific or structural features of a binding partner and (iii) acting as a scaffold to anchor proteins to one another (Dougherty & Morrison, 2004).

A recent study demonstrated that 14-3-3 interacts with LKB1 by binding to phosphorylated Thr336 (Bai *et al.*, 2012). Through this interaction, 14-3-3 inhibits the binding of LKB1 to its substrates, such as AMPK, to suppress the kinase function of LKB1. At the cellular level, the effect of 14-3-3 on LKB1 alleviates G1 cell-cycle arrest and

apoptosis of HeLa cells induced by LKB1. However, the structural basis of the interaction between LKB1 and 14-3-3 has remained puzzling. Here, we report the purification, crystallization, diffraction data collection and preliminary crystallographic analysis of the chimeric protein LKB1–14-3-3 ζ .

2. Materials and methods

2.1. Cloning and expression

The DNA sequence of human 14-3-3 ζ was amplified from human cDNA and cloned into pET-28a with an N-terminal 6 $\times$ His tag. The DNA sequences of the GGSGGS linker and LKB1 fragment (amino acids 333–340) with a phosphomimetic mutation T336E were inserted into the 3' end of the coding sequence in the 14-3-3 ζ expression plasmid. The expression order of all elements in this fusion protein is His tag, 14-3-3 ζ , GGSGGS linker, LKB1 fragment. The recombinant plasmid was validated by sequencing.

The plasmid of 14-03-3 ζ -LKB1 was transformed into *Escherichia coli* BL21(DE3) Codon Plus competent cells. The cells were incubated in Terrific Broth medium with 30 $\mu\text{g ml}^{-1}$ kanamycin and 34 $\mu\text{g ml}^{-1}$ chloramphenicol at 310 K until the absorbance reached 1.0 at a wavelength of 600 nm. The temperature was then lowered to 293 K and isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.25 mM. The culture was subsequently incubated at 293 K for 18 h.

2.2. Protein purification

The following experiments were carried out at 277 K. The *E. coli* cells were collected at 6370g for 10 min and suspended in five times the volume of lysis buffer (20 mM HEPES pH 7.5, 500 mM NaCl, 20 mM imidazole, 5% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride). The cells were then broken by four passes through a high-pressure homogenizer (JNBio) at 130 MPa. The debris was removed by centrifugation for 40 min at 31 000g. The supernatant

was mixed with pre-equilibrated Ni Sepharose (GE Healthcare) for 1 h and the beads were then washed with lysis buffer without phenylmethylsulfonyl fluoride. The proteins were eluted with 300 mM imidazole in lysis buffer. The proteins were concentrated using a 10 kDa cutoff Amicon Ultra-15 (Millipore) and applied onto a Superdex 75 10/300 GL column (GE Healthcare) with 20 mM HEPES pH 7.5, 100 mM NaCl, 1 mM DTT. The purity of the protein was monitored by SDS-PAGE. Purified LKB1–14-3-3 ζ protein was concentrated to 10 mg ml $^{-1}$, aliquoted and stored at 193 K.

2.3. Crystallization and data collection

Crystallization trials were carried out at 289 K by the sitting-drop vapour-diffusion method. The 2 μl sitting drops consisted of 1 μl protein solution and 1 μl reservoir solution and were equilibrated against 100 μl reservoir solution. Crystals were optimized using sitting-drop and hanging-drop vapour diffusion.

Crystals were soaked in reservoir solution containing 25% glycerol and flash-cooled in liquid nitrogen. Diffraction data were collected from a single crystal on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF), People's Republic of China using 1 $^\circ$ oscillations with a crystal-to-detector distance of 350 mm. The diffraction data were processed using HKL-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

In order to purify and crystallize the complex of LKB1 and 14-3-3 ζ , residue Thr336 of LKB1 was mutated to glutamic acid to mimic the phosphorylated state; furthermore, the fragment of LKB1 (amino acids 333–340) containing this mutation was linked to 14-3-3 ζ by a GGSGGS linker. This chimera was purified by Ni-affinity chromatography and gel filtration. The protein eluted as a dimer in gel filtration and showed a single band on SDS-PAGE with a molecular weight close to the theoretical value of 30.3 kDa (Fig. 1).

Crystals of the chimeric protein LKB1–14-3-3 ζ appeared in two conditions: (i) 1.5 M Li $_2$ SO $_4$, 0.1 M bis-tris propane pH 7.0 and (ii) 1.2 M potassium/sodium tartrate, 0.1 M bis-tris propane pH 7.0. Further optimization was performed by changing the salt concentration and the pH of the buffer. After optimization, the best crystal was grown using a condition consisting of 1.4 M Li $_2$ SO $_4$, 0.1 M bis-tris

Figure 1
(a) Elution profiles of the chimeric protein LKB1–14-3-3 ζ from a Superdex 75 10/300 GL column and (b) SDS-PAGE of the eluted fractions. Lane M contains molecular-mass markers (labelled in kDa).

Figure 2
Crystals of the chimeric protein LKB1–14-3-3 ζ .

Figure 3
Diffraction pattern of a crystal of chimeric protein LKB1–14-3-3 ζ .

propane pH 7.0 (Fig. 2). A complete set of diffraction data was collected to 2.9 Å resolution (Fig. 3). We analyzed the data using the UCLA MBI Diffraction Anisotropy Server (Strong *et al.*, 2006) and found that the data were anisotropic. The crystal belonged to space group R32, with unit-cell parameters $a = b = 130.262$, $c = 264.960$ Å (Table 1). Assuming the presence of two molecules per asymmetric unit, the Matthews coefficient (V_M) was calculated to be $3.6 \text{ Å}^3 \text{ Da}^{-1}$, which corresponds to 65.6% solvent content (Matthews, 1968). We will try to solve the structure of this chimeric protein by molecular replacement using the structure of 14-3-3 ζ as the search model. Structure determination and refinement are in progress. The determination of the structure of the chimeric protein LKB1–14-3-3 ζ will lead to identification of the key residues involved in their association.

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Table 1

Data-collection statistics for the crystal of the chimeric protein LKB1–14-3-3 ζ .

Values in parentheses are for the outermost resolution shell.

Space group	R32
Unit-cell parameters (Å, °)	$a = b = 130.262$, $c = 264.960$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Wavelength (Å)	0.9793
Resolution (Å)	50.0–2.90 (2.95–2.90)
Total reflections	213600
Unique reflections	19507
Completeness (%)	100.0 (100.0)
Multiplicity	10.9 (11.3)
$R_{\text{merge}}^{\dagger}$	0.071 (0.922)
Mean $I/\sigma(I)$	43.0 (3.2)
Mosaicity (°)	0.300
Matthews coefficient ($\text{Å}^3 \text{ Da}^{-1}$)	3.6
Solvent content (%)	65.6

$$^{\dagger} R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}.$$

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