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Crystallization and preliminary X-ray crystallographic analysis of the human CKIP-1 pleckstrin homology domain

The casein kinase 2 interacting protein-1 (CKIP-1) is involved in many cellular functions, including apoptosis, signalling pathways, cell growth, cytoskeleton and bone formation. Its N-terminal pleckstrin homology (PH) domain is thought to play an important role in membrane localization and controls shuttling of CKIP-1 between the plasma membrane and nucleus. In this study, the human CKIP-1 PH domain was purified but problems were encountered with nucleic acid contamination. An S84D/S86D/S88D triple mutant designed to abolish nucleic acid binding was purified and successfully crystallized. Single crystals diffracted to 1.7 Å resolution and belonged to space group $P4_32_12$ with unit-cell parameters $a = 53.0$, $b = 53.0$, $c = 113.8$ Å, $\alpha = \beta = \gamma = 90.0^\circ$.

1. Introduction

The pleckstrin homology (PH) domain, composed of about 120 amino-acid residues, was first discovered and named by Haslam *et al.* (1993). There are reported to be ~252 examples of PH domains in the human proteome, where they are identified in proteins involved in cellular signalling, cytoskeletal organization and membrane trafficking (Lander *et al.*, 2001). PH domains are best known for their high affinity and specificity to the phosphoinositides (Lemmon, 2007), which are components of eukaryotic cell membranes. However, it has become evident that only a small proportion of PH domains share this high affinity for phosphoinositides and their functional properties are more diverse than previously thought (Lemmon, 2007, 2008). It has been suggested that PH domains instead adopt a 'dual key' strategy for efficient membrane targeting by interacting with both lipids and proteins on the target membrane (Godi *et al.*, 2004; Itoh & De Camilli, 2004).

The PH-domain-containing casein kinase 2 interacting protein-1 (CKIP-1) was originally identified as an interacting partner of the α -subunit of casein kinase 2 (CK2) (Bosc *et al.*, 2000). Subsequent studies have shown that CKIP-1 recruits a proportion of nuclear CK2 to the plasma membrane *via* its N-terminal PH domain, but is neither a substrate nor an enzyme regulator of CK2 kinase activity (Olsten *et al.*, 2004). CKIP-1 has been implicated in various functions to date, including regulation of muscle cell differentiation (Safi *et al.*, 2004), cytoskeleton reorganization (Canton *et al.*, 2005, 2006), cell apoptosis (Zhang *et al.*, 2005) and tumour cell growth (Lu *et al.*, 2008; Zhang *et al.*, 2007). It is reported to physiologically suppress bone formation in mice *via* augmented activity of the ubiquitin ligase Smurf1 (Lu *et al.*, 2008). CKIP-1 is also reported to be a candidate tumour suppressor because of its interaction with the serine/threonine kinase Akt and down-regulation of PI3K/Akt signalling and suppression of tumour growth *in vivo* (Tokuda *et al.*, 2007).

Human CKIP-1 is predominantly located in the plasma membrane, but can also be found in the nucleus in certain cell types and under certain nutritional conditions (Xi *et al.*, 2010). It is able to shuttle between the plasma membrane and nucleus in response to particular stimuli, although how this shuttling of CKIP-1 is regulated remains largely unclear. A recent study showed that, in contrast to full-length CKIP-1, the isolated CKIP-1 PH domain is predominantly located in the nucleus independent of the cell type (Xi *et al.*, 2010). The authors further showed that the N-terminal PH domain plays key roles in the localization of CKIP-1 on the plasma membrane and CKIP-1

Table 1

Data-collection statistics.

Values in parentheses are for the highest-resolution shell.

Space group	$P4_32_12$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 53.0$, $b = 53.0$, $c = 113.8$, $\alpha = \beta = \gamma = 90$
Number of molecules in the asymmetric unit	1
Wavelength ($\text{\AA}$)	1.005
Light source	SSRF-BL17U, U25
Detector	MX225
Temperature (K)	100
Resolution range ($\text{\AA}$)	50–1.70 (1.76–1.70)
Number of observed reflections	169305
Number of unique reflections	18387 (1810)
Completeness (%)	98.9 (100.0)
$\langle I/\sigma(I) \rangle$	26.8 (6.0)
Redundancy	9.2 (9.3)
$R_{\text{merge}}^\dagger$	0.061 (0.437)

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the mean intensity of the observations $I(hkl)_i$ of reflection hkl .

shuttling. Furthermore, shuttling of CKIP-1 was found to be inhibited as the result of a direct interaction between the PH domain and an auto-inhibition region mapped to a leucine zipper motif at the C-terminus of CKIP-1 (Fig. 1).

As a prelude towards studying the structural basis for regulation of CKIP-1 shuttling between the plasma membrane and nucleus, we have expressed, purified and crystallized its PH domain. In our study, we encountered low expression of the native CKIP-1 PH domain and severe nucleic acid contamination. An S84D/S86D/S88D triple mutant designed to abolish nucleic acid binding was purified and successfully crystallized, and a set of diffraction data was collected to 1.7 $\text{\AA}$ resolution for preliminary crystallographic analysis.

2. Materials and methods

2.1. Gene cloning

The gene encoding the human CKIP-1 PH domain (amino acids 22–136) was amplified by the polymerase chain reaction (PCR) from the full-length CKIP-1 gene (GenBank accession number NP_057358). The PCR product was purified and digested by *EcoRI* and *XhoI* before being inserted into the pET-28a vector. A PH S84D/S86D/S88D triple mutant (PH-3SD) was produced from the constructed pET-28a wild-type PH domain plasmid by site-directed mutagenesis using the Easy Mutagenesis System kit (Transgen, Beijing, China). The plasmids were verified by DNA sequencing and transformed into the *Escherichia coli* strain BL21(DE3).

2.2. Expression and purification

The transformed bacterial cells were cultured at 310 K, 200 rev min⁻¹ in LB medium supplemented with kanamycin to a final concentration 100 $\mu\text{g ml}^{-1}$. When the culture OD reached 0.6–0.8, we quickly reduced its temperature to ~ 289 K using an ice-water bath. IPTG (isopropyl β -D-1-thiogalactopyranoside) was added to the cooled culture to a final concentration of 0.5 mM for induction. After

Figure 1

Domain organization of human CKIP-1. PH, pleckstrin homology domain; LZ, leucine zipper; AIR, auto-inhibition region.

subsequent culturing for 18 h at 289 K, the cells were harvested by centrifugation (Beckman/Coulter Avanti-J26XP) at 5000 rev min⁻¹, 277 K for 20 min. The pellet was resuspended with cold lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol) and sonicated. The lysate was centrifuged at 277 K, 18 000 rev min⁻¹ for 40 min. The supernatant was then collected for recombinant protein purification.

The recombinant protein was purified with the Ni²⁺-NTA agarose affinity chromatography method. The supernatant was applied to the agarose pre-equilibrated with a buffer consisting of 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 10 mM imidazole. The unbound and loosely bound protein was washed with a buffer consisting of 20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole. The target protein was eluted with the elution buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 10% glycerol, 300 mM imidazole). Imidazole was removed from the eluted protein solution by dialysing against the dialysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol, 1 mM DTT) at 277 K overnight. The recombinant His-tagged PH-3SD protein was subsequently purified by size-exclusion chromatography (Superdex 200, GE Healthcare) for crystallization. The purity of the protein was estimated to be greater than 95% by SDS-PAGE.

2.3. Crystallization

The purified PH-3SD mutant protein, corresponding to amino acids 22–136 of human CKIP-1 with mutations at S84D/S86D/S88D and including an N-terminal His tag (with additional sequence MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSEF), was concentrated to 10 mg ml⁻¹ for crystal screening. Crystallization trials were performed using the sitting-drop vapour-diffusion method by mixing 1 μl protein solution with 1 μl reservoir solution at 291 K in 48-well plates. Initial screening was carried out using the following commercial screening kits: Crystal Screen, Crystal Screen 2 and Index

Figure 2

15% SDS-PAGE for evaluating the purity of the PH-3SD mutant prior to crystallization. Lane PH-3SD: PH domain mutant protein. Marker: Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific).

Figure 3
Optimal octahedral PH-3SD crystals grown in 0.2 M phosphate/citrate pH 5.6, 32% PEG 300. Crystals grew to size after approximately 2 weeks.

kits from Hampton Research, Wizard I, II, III and IV kits from Emerald BioSystems and the JCSG-*plus* kit from Molecular Dimensions. Crystals were discovered 1 week later from two different conditions of the JCSG-*plus* screening kit: 0.1 M phosphate–citrate pH 4.2, 40% PEG 300 (condition No. 30) and 0.17 M ammonium sulfate, 25.5% PEG 4000, 15% glycerol (condition No. 45). Crystals yielded from the second condition all diffracted poorly. Therefore, all subsequent optimization experiments were based on the first crystallization condition using the hanging-drop vapour-diffusion method. With a combination of varied precipitant concentration, buffer pH and protein concentration, the final optimized crystallization condition included 0.2 M phosphate/citrate pH 5.6, 32% PEG 300 (Hampton Research).

2.4. Crystallographic data collection and processing

X-ray diffraction data for the PH-3SD protein were collected at 100 K on beamline BL17U1 of the Shanghai Synchrotron Radiation

Facility (SSRF). Crystals were flash-cooled in a liquid-nitrogen stream and the reservoir solution with 10% (v/v) glycerol was used as cryoprotectant. Diffraction data were integrated, scaled and merged using the *HKL-2000* program (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 1.

3. Results and discussion

We had initially tried to express and crystallize the native PH domain of CKIP-1. However the expression in *E. coli* BL21(DE3) was very low and accompanied by severe nucleic acid contamination. We tried to remove the contamination by adding DNase and RNase enzymes, but the enzymes exhibited little effect. Furthermore, crystal screening experiments yielded no crystals for the purified native PH domain. After analysing the sequence of the PH domain, we identified a cluster of continuous basic amino-acid residues (amino acids 82–90, sequence: RKSRSRKK) in the CKIP-1 sequence. We hypothesized that the highly concentrated positive charge in this region may be the reason for the nucleic acid contamination. Thus, in order to neutralize the charge, we mutated the three serine residues in the cluster (Ser84, Ser86 and Ser88) to aspartic acid, such that the mutant protein bore the sequence RKDKDRDKK for residues 82–90. The recombinant mutant, named PH-3SD, exhibited no nucleic acid contamination when expressed in *E. coli* BL21(DE3) cells, in contrast to the native protein. Furthermore, the protein yields for the PH-3SD mutant were approximately fivefold higher than for the native PH domain.

After purification, the purity of the PH-3SD mutant was estimated to be greater than 95% by SDS-PAGE (Fig. 2). From crystal screening experiments, octahedral and thin plate-shaped crystals were observed from two conditions of the JCSG-*plus* screen: No. 30 and No. 45, respectively. The octahedral crystals exhibited better diffraction than the thin plate-shaped crystals when screened using our in-house X-ray system (Rigaku MicroMax-007 HF source and R-Axis HTC detector). Optimal octahedral crystals grew in approximately 2 weeks from a condition consisting of 0.2 M

Figure 4
A sample X-ray diffraction image collected from the PH-3SD mutant crystal. The enlarged view shows spots to 1.7 Å resolution.

phosphate–citrate pH 5.6, 32% PEG 300 (Hampton Research). A 1.7 Å resolution data set was collected from these optimal crystals using synchrotron radiation at the Shanghai Synchrotron Radiation Facility (SSRF). The crystal used for data collection and a sample X-ray diffraction image are shown in Figs. 3 and 4, respectively.

The PH-3SD mutant crystal used for data collection belongs to the space group $P4_32_12$ with unit-cell parameters $a = 53.0$, $b = 53.0$, $c = 113.8$ Å, $\alpha = \beta = \gamma = 90.0^\circ$. It is estimated that one PH domain molecule is present in the asymmetric unit with a Matthews coefficient of $2.67 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of approximately 54% (Matthews, 1968). Structure determination is currently underway by the molecular-replacement method using the structure of the PH domain of human AKT3 as a search model (27% sequence identity to the CKIP-1 PH domain, PDB entry 2x18; M. Vollmar, J. Wang, Y. Zhang, J. M. Elkins, N. Burgess-Brown, A. Chaikuad, A. C. W. Pike, F. Von Delft, C. Bountra, C. H. Arrowsmith, J. Weigelt, A. Edwards & S. Knapp, unpublished work).

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