High-resolution crystal structure of the leucine-rich repeat domain of the human tumour suppressor PP32A (ANP32A)

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Acidic leucine-rich nuclear phosphoprotein 32A (PP32A) is a tumour suppressor whose expression is altered in many cancers. It is an apoptotic enhancer that stimulates apoptosome-mediated caspase activation and also forms part of a complex involved in caspase-independent apoptosis (the SET complex). Crystals of a fragment of human PP32A corresponding to the leucine-rich repeat domain, a widespread motif suitable for protein–protein interactions, have been obtained. The structure has been refined to 1.56 Å resolution. This domain was previously solved at 2.4 and 2.69 Å resolution (PDB entries 2je0 and 2je1, respectively). The new high-resolution structure shows some differences from previous models: there is a small displacement in the turn connecting the first α -helix ($\alpha 1$) to the first β -strand ($\beta 1$), which slightly changes the position of $\alpha 1$ in the structure. The shift in the turn is observed in the context of a new crystal packing unrelated to those of previous structures.

1. Introduction

Acidic leucine-rich nuclear phosphoprotein 32A (PP32A) contains an N-terminal leucine-rich repeat (LRR) domain. PP32A acts as a tumour suppressor and its expression is altered in many tumours. It favours the activation of caspase 9 through the apoptosome (Pan *et al.*, 2009) and is also implicated in caspase-independent apoptosis since it is a member of the SET complex (Lieberman, 2010; Beresford *et al.*, 2001). The Set oncoprotein gives name to the SET complex, which translocates to the nucleus in response to reactive oxygen species and mitochondrial damage.

In addition, PP32A is involved in other activities such as modulating histone acetylation and transcription as it forms part of the INHAT (inhibitor of histone acetyltransferases) complex, where in association with the Set oncoprotein (Muto *et al.*, 2007) it inhibits the histone-acetyltransferase activity of p300/CBP (CREB-binding protein) and p300/CBP-associated factor by histone masking (Seo *et al.*, 2002). PP32A is also implicated in the inhibition of protein phosphatase 2A (Li *et al.*, 1996). PP32A also participates in other biochemical processes such as regulation of mRNA trafficking and stability by its association with HuR (Hu-antigen R), which increases the stability of mRNA binding to the 3'-untranslated region (UTR) region (Mazroui *et al.*, 2008)

PP32A has three differentiated regions: an N-terminal LRR domain and a central region which connects the LRR domain to a highly acidic C-terminal domain. The LRR domain has a curved shape that has been shown to be appropriate for building protein–protein interaction modules (Bella *et al.*, 2008). This domain is not only important because it might be responsible for many PP32A interactions, but also because

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Table 1

Macromolecule-production information.

Source organism	Human
DNA source	cDNA library
Forward primer	GCGCATATGGAGATGGGCGAGCGGATTC
Reverse primer	CGCCTCGAGTTAGTCATAGCCGTCGAGATA
Expression vector	pET-28a
Expression host	<i>E. coli</i> BL21 (DE3) CodonPlus RIPL
Complete amino-acid sequence of the construct produced	MGSSHHHHHSSGLEVLFGQPHMEMGRRIQLELR-NRTPFDVKELVDNSRSNEGKLEGLTDFEEL-EFLSTINVGLTSIANLPKLNKLLKLELSDNRV-SGGLEVLAEKCPNLTHNLNLSGNKIKDLSTIEP-LKKLENLKSLDLFNCVETNLNDYRENVFKLLP-QLTYLDGYD

several LRR domain crystal structures exhibit their ligand-binding domain in this concave surface.

Our high-resolution human PP32A LRR domain structure (PDB entry 4xos) shows some differences compared with previously reported structures (Huyton & Wolberger, 2007), including some modifications at the end of helix $\alpha 1$ and the loop connecting it to the $\beta 1$ strand. This change is involved in the generation of a totally different crystal packing. A chloride ion is located in the vicinity, interacting with residues towards the end of the $\alpha 1$ – $\beta 1$ turn and the initial $\beta 1$ residues.

2. Materials and methods

2.1. Macromolecule production

A fragment of the gene encoding human PP32A (protein residues 1–149) was cloned into a modified version of the pET-28a vector (Table 1). This construct was expressed in *Escherichia coli* BL21 (DE3) CodonPlus RIPL cells (Invitrogen) for 3 h at 303 K following induction with 1 mM IPTG when the cell density had reached a value of between 0.6 and 0.8. The cells were harvested, washed with PBS and stored at -80°C until use.

For purification, the cells were sonicated with a lysis buffer consisting of 50 mM Tris–HCl pH 7.5, 0.1% Triton X-100, 500 mM NaCl, 10% glycerol, 1 mM β -mercaptoethanol (BME). To remove cell debris, the lysate was centrifuged prior to loading onto a 5 ml chelating nickel HisTrap HP column (GE Healthcare). The column was washed with six column volumes of buffer A (50 mM Tris–HCl pH 7.5, 350 mM NaCl, 5% glycerol, 2 mM BME) and the protein was eluted using a gradient to buffer B (50 mM Tris–HCl pH 7.5, 350 mM NaCl, 5% glycerol, 500 mM imidazole, 2 mM BME). These buffers were selected from Thermofluor stability assay results (Boivin *et al.*, 2013; Reinhard *et al.*, 2013). Elution fractions were analyzed by SDS–PAGE and the fractions containing PP32 were treated overnight at 4°C with recombinant human rhinovirus 3C protease fused to glutathione *S*-transferase. After digestion, the sample was loaded onto a Sepharose column covalently linked to glutathione in order to remove the protease. The sample was concentrated and injected onto a Superdex 200 16/60 size-exclusion column (GE Healthcare) equilibrated with 20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM BME.

Table 2

Crystallization conditions.

Method	Sitting-drop vapour diffusion
Plate type	96-well plate
Temperature (K)	294
Protein concentration (μM)	300
Buffer composition of protein solution	20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM BME
Composition of reservoir solution	20% PEG 3000, 0.1 M HEPES pH 7.5, 0.2 M NaCl
Volume and ratio of drop	0.4 μl (1:1)
Volume of reservoir (μl)	0.4

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	ALBA synchrotron
Wavelength ($\text{\AA}$)	0.9795
Temperature (K)	100
Detector	Pilatus 6M
Crystal-to-detector distance (mm)	258.67
Rotation range per image ($^{\circ}$)	0.1
Total rotation range ($^{\circ}$)	120
Exposure time per image (s)	0.1
Space group	$P12_11$
a, b, c ($\text{\AA}$)	48.04, 50.88, 63.02
α, β, γ ($^{\circ}$)	90, 110.19, 90
Mosaicity ($^{\circ}$)	0.865
Resolution range ($\text{\AA}$)	45.09–1.56 (1.615–1.559)
Total No. of reflections	87304 (7888)
No. of unique reflections	36978 (3587)
Completeness† (%)	90.50 (85.63)
Multiplicity	2.3 (2.2)
$\langle I/\sigma(I) \rangle^{\ddagger}$	12.14 (1.77)
$R_{\text{meas}}^{\S}$	0.05
Overall B factor from Wilson plot ($\text{\AA}^2$)	17.07

† The crystal orientation and morphology did not allow complete data collection. $\ddagger$ The mean $\langle I/\sigma(I) \rangle$ in the outer shell is 2.0 at 1.6 $\text{\AA}$ resolution. $\S$ $R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ are the observed intensities, $\langle I(hkl) \rangle$ is the average intensity and $N(hkl)$ is the multiplicity of reflection hkl .

2.2. Crystallization

Plate-like crystals with approximate dimensions of $0.2 \times 0.6 \times 0.7$ mm were grown by mixing 0.4 μl protein solution at 300 μM and 0.4 μl reservoir solution (20% PEG 3000, 0.1 M HEPES pH 7.5, 0.2 M NaCl) at 294 K using the sitting-drop vapour-diffusion method (Table 2). Crystals appeared within one week.

2.3. Data collection and processing

One- to two-week-old crystals were flash-cryocooled with liquid nitrogen without the use of any additional cryoprotectant. Diffraction data were collected from the crystals at 100 K on the XALOC beamline at the ALBA synchrotron, Barcelona, Spain. A complete data set was collected to 1.56 $\text{\AA}$ resolution from a single crystal. Data were reduced using *MOSFLM* (Battye *et al.*, 2011) and *SCALA* (Evans, 2006) from *CCP4* (Winn *et al.*, 2011).

2.4. Structure solution and refinement

Phases were determined by molecular replacement with *Phaser* (McCoy *et al.*, 2007), using the structure of the LRR

domain from PP32 (PDB entry 2je0; Huyton & Wolberger, 2007) as a model. The solution, consisting of two molecules in the asymmetric unit, was first refined as a rigid body and then by restrained refinement using *phenix.refine* (Afonine *et al.*, 2012) and manual model building using *Coot* (Emsley *et al.*, 2010). We also performed a final refinement cycle with TLS (translation/libration/screw) refinement (Schomaker & Trueblood, 1968). *phenix.validate* was used for model validation (Adams *et al.*, 2010).

Interchain interactions were analyzed with *CONTACT/ACT* (Kabsch & Sander, 1983) as implemented in *CCP4* (Winn *et al.*, 2011). Only high-probability electrostatic interactions have been considered for comparison. Model images were generated with *Chimera* (Pettersen *et al.*, 2004).

3. Results and discussion

The crystals belonged to space group $P2_1$ (Table 3) and contained two molecules in the asymmetric unit, with a solvent content of 42%, which is around 20% lower than those of the two previously reported structures. The higher resolution structure improves the global validation metrics, and the side-chain outliers decrease from 9.1% in the 2.4 Å resolution model to 1.1% in our 1.56 Å resolution structure. The final model included 149 residues of PP32A and three further amino acids at the N-terminus resulting from the cloning and protease-cleavage strategies. The 1.56 Å resolution structure has been refined to a final R_{work} and R_{free} of 0.1706 and 0.2048, respectively (Table 4). No PP32A residues in the asymmetric unit were found in the Ramachandran disallowed regions. The better resolution of our crystal allowed us to identify different rotamers for residues Leu22, Asn59, Arg75, Lys99, Thr105, Thr126, Tyr144 and Tyr148 compared with the molecules in

Table 4

Structure refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	45.09–1.559
Completeness (%)	90.50 (85.63)
σ Cutoff	1.63
No. of reflections, working set	36969
No. of reflections, test set	1851
Final $R_{\text{work}}^{\dagger}$	0.1603 (0.2514)
Final $R_{\text{free}}^{\ddagger}$	0.1929 (0.2808)
Cruickshank DPI§	0.13
No. of non-H atoms	
Protein	2456
Ion	2
Ligand	12
Water	163
Total	2633
R.m.s. deviations	
Bonds (Å)	0.007
Angles (°)	1.07
Average B factors (Å ²)	
Overall	28.0
Protein	25.6
Ion	27.0
Ligand	33.2
Water	30.3
Ramachandran plot	
Favoured regions (%)	97
Additionally allowed (%)	3
Outliers (%)	0

$\dagger R_{\text{work}} = \sum_{hkl} |F_{\text{obs}}| - |F_{\text{calc}}| / \sum_{hkl} |F_{\text{obs}}|$, where F_{obs} and F_{calc} are the observed and calculated structure factors, respectively. $\ddagger R_{\text{free}}$ is the same as R_{work} but was calculated using 5% of the total reflections that were selected randomly and excluded during refinement. $\S$ Diffraction-component precision indicator (Cruickshank, 1999).

PDB entries 2je0 and 2je1. A double conformation has also been detected for Arg28.

Superimposition of the C^{α} atoms among monomers from PDB entries 4xos, 2je0 and 2je1 shows a root-mean-square deviation (r.m.s.d.) of around 0.5 Å, indicating high similarity to the previous structures deposited in the PDB (Huyton & Wolberger, 2007). The main differences in the C^{α} trace are located in the turn between helix $\alpha 1$ and strand $\beta 1$ at the N-terminus (Fig. 1a). This difference is maintained in all of the molecules in the asymmetric unit from previous structures. In particular, Arg12 adopts a totally different conformation (Fig. 1b) in both molecules in the asymmetric unit, establishing a double salt bridge with Asp73 from a neighbouring molecule. As a consequence, both Leu11 and Asn13 also occupy different positions in our model with respect to the previously reported structures. The C-terminal part of the $\alpha 1$ helix is also displaced. This N-terminal modified region therefore shows a certain degree of conformational flexibility when comparing the different LRR domain structures, and is in agreement with the higher temperature factors observed in the $\alpha 1$ – $\beta 1$ loop. This finding might be functionally relevant since residues Thr15 and Ser17 have been shown to undergo cell cycle-regulated phosphorylation (Dephoure *et al.*, 2008).

Our structure shows the binding of a chloride ion towards the end of the $\alpha 1$ – $\beta 1$ turn. We assigned the chloride taking into account the ions that were present during purification and crystallization. The chloride interacts with the N^{η} atom of Arg14, the main-chain N atoms of Lys20 and Glu21 and two water molecules (Fig. 2). This interacting region immediately

Figure 1

(a) Superimposition of a ribbon representation of PDB entries 4xos (magenta), 2je0 (cyan) and 2je1 (green) showing a shift of the $\alpha 1$ – $\beta 1$ turn and the chloride position in 4xos (grey). (b) Comparison of the Arg12 side-chain distribution in the shifted area of PDB entries 4xos, 2je0 and 2je1 using the same colour scheme.

Figure 2

Model inside a $F_o - F_c$ map (green) contoured at the 6σ level. The map was calculated in the absence of chloride and the two interacting water molecules. Interacting distances of the chloride ion (grey) with N atoms belonging to Arg14, Lys20 and Glu21 and with water molecules are shown.

precedes the first LRR motif of the protein (Fig. 1*a*) in the vicinity of the largest conformational change between the previously reported structures and our model. The chloride ion site would be occupied by the side chain of Glu21 from neighbouring molecules in the asymmetric units of both PDB entries 2je0 and 2je1, making the presence of this ion incompatible with the previously reported crystallographic space groups.

Despite belonging to different space groups, the structures with PDB codes 2je0 and 2je1 reveal related crystal packing (Supplementary Table S1). This degree of conservation is lost when compared with our PP32A structure since most of the contacts are not maintained. The new packing observed is related to the conformational change of the turn connecting the first α -helix to the first β -strand (Supplementary Table S1). PISA analysis (Krissinel & Henrick, 2007) revealed no change in the protein assembly, which remains as a monomer. This is in agreement with the behaviour of the PP32A LRR domain in size-exclusion chromatography. We can therefore conclude that the differences observed in crystal packing, the new conformation of the $\alpha 1$ – $\beta 1$ turn and the presence of the chloride ion do not have any influence on the oligomerization state of the LRR domain.

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