Characterization of the NTPR and BD1 interacting domains of the human PICH–BEND3 complex

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Chromosome integrity depends on DNA structure-specific processing complexes that resolve DNA entanglement between sister chromatids. If left unresolved, these entanglements can generate either chromatin bridging or ultrafine DNA bridging in the anaphase of mitosis. These bridge structures are defined by the presence of the PICH protein, which interacts with the BEND3 protein in mitosis. To obtain structural insights into PICH–BEND3 complex formation at the atomic level, their respective NTPR and BD1 domains were cloned, overexpressed and crystallized using 1.56 M ammonium sulfate as a precipitant at pH 7.0. The protein complex readily formed large hexagonal crystals belonging to space group $P6_122$, with unit-cell parameters $a = b = 47.28$, $c = 431.58$ Å and with one heterodimer in the asymmetric unit. A complete multiwavelength anomalous dispersion (MAD) data set extending to 2.2 Å resolution was collected from a selenomethionine-labelled crystal at the Swiss Light Source.

1. Introduction

Faithful chromosome segregation is essential for the prevention of genomic instability and aneuploidy, and ensures that daughter cells receive one copy of each chromosome (Chan & Hickson, 2011). The entanglement of sister chromatids can potentially occur during replication owing to the associated DNA topological changes. If these intertwined structures are not properly removed, DNA bridges can emerge during anaphase and the correct segregation of the chromosome will be compromised (Liu *et al.*, 2014). To aid the resolution of DNA entanglements between sister chromatids, several different protein complexes are recruited onto the DNA bridges. PICH (Polo-like kinase 1-interacting checkpoint helicase; also known as ERCC6L) co-localizes with BLM (Bloom syndrome helicase) and other DNA-repair proteins on ultrafine anaphase bridges (UFBs) in mitosis (Baumann *et al.*, 2007; Chan *et al.*, 2009; Wang *et al.*, 2008). Biochemical and biophysical characterization have shown that PICH is an ATP-dependent double-strand DNA translocase and is a member of the SNF2 family of ATPases (Biebricher *et al.*, 2013). PICH has an ability to coat UFBs along their entire length (Chan & Hickson, 2011; Biebricher *et al.*, 2013), promoting the association and co-localization of other DNA-repair complexes (Hutchins *et al.*, 2010). It has been proposed that PICH contributes to the resolution of entangled sister chromatids by being able to recognize and stabilize DNA under topological tension during anaphase (Biebricher *et al.*, 2013). Accordingly,

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the depletion of PICH produces an increase in the frequency of chromatin bridges (Hübner *et al.*, 2010; Kaulich *et al.*, 2012; Nielsen *et al.*, 2015).

The protein BEND3 (BEN domain-containing protein 3) was identified as a partner of PICH by co-immunoprecipitation experiments in nocodazole-arrested (prometaphase) cells (Pitchai *et al.*, unpublished work). Recent studies have shown that BEND3 is a heterochromatin-associated protein that is involved in transcriptional repression and that its over-expression produces extensive heterochromatinization (Sathyan *et al.*, 2011; Khan *et al.*, 2015; Saksouk *et al.*, 2014). BEND3 contains four BEN domains (Fig. 1*a*). This four- α -helix module has been proposed to mediate protein–DNA and protein–protein interactions in processes related to chromatin organization and transcription (Abhiman *et al.*, 2008; Dai *et al.*, 2013). The PICH translocase contains two TPR domains (Fig. 1*a*); these 34-amino-acid repeated sequence motifs are involved in protein–protein interactions (Zeytuni & Zarivach, 2012).

We speculate that PICH and BEND3 might act together to prevent chromatin-bridge formation during mitosis. To analyze this, we first confirmed that PICH and BEND3 interact directly *in vitro*. We then showed that the interaction of these two proteins is mediated by the amino-terminal TPR domain (NTPR) of PICH and the first BEN domain (BD1) of BEND3 (Pitchai *et al.*, unpublished work). Here, we report the

purification, crystallization and preliminary X-ray diffraction analysis of the complex of the two domains that mediates the interaction between PICH and BEND3.

2. Materials and methods

2.1. Construction of expression vectors

Codon-optimized sequences for human PICH (ERCC6L; UniProt Q2NKX8) and BEND3 (UniProt Q5T5X7) were obtained from the GeneArt gene-synthesis service (Thermo Fisher Scientific). In order to optimize the expression and solubility of the two interacting domains (Fig. 1*a*), we designed different constructs of the NTPR and BD1 domains. The corresponding DNA sequences were amplified by PCR from the synthetic genes. All plasmids created in this study are based on the pNIC28-Bsa4 vector (pCPR0011), which allows the use of the ligase-independent cloning method (Aslanidis & de Jong, 1990) and the expression of proteins with a His tag, a *Strep*-tag and a TEV protease site at the amino-terminus. After biochemical and biophysical analysis of the interactions (Pitchai *et al.*, unpublished work), we selected the following constructs for further crystallization experiments: NTPR (residues 2–72 from PICH; primers ERC6L-2FW, 5'-TACTTCCAATCCATGGAAGCAAGCCGTCGTTTTC-CGGAAG-3', and ERC6L-72RV, 5'-TATCCACCTTTAC-TGTTATTCTGCCAGTTCTTCCAGGGCTTC-3') and BD1

Figure 1

NTPR and BD1 domains. (a) Schematic representation of the domain architecture of PICH and BEND3 with the different domains indicated. PICH contains two TPR domains, a DEXH domain, a HELICc domain (HELIC) and a PICH family domain (PFD). BEND3 contains four different BEN domains. The interaction between the NTPR and BD1 domains, highlighted with red dotted boxes, is depicted by a black arrow. (b) SDS–PAGE gel showing the purified NTPR and BD1 domains (8.3 and 16.9 kDa, respectively). The first lane (*M*) contains molecular-weight protein markers (labelled in kDa). (c) ITC analysis of the NTPR–BD1 interaction. The dissociation constant (K_d), the ΔH and the stoichiometry of the interaction obtained from the binding isotherm are indicated. Each titration experiment was carried out in triplicate with different protein concentrations and temperatures.

Table 1
NTPR and BD1 production information.

Source organism	<i>Homo sapiens</i>
DNA source	Synthetic DNA (codon-optimized)
NTPR	ATGGAAGCAAGCCGTCGTTTCCGGAAGCAGAACGACTG- AGTCCGGAACAGGCAGCACATTATCTGCGTTATGTTA- AAGAAGCAAAAGAACCCACCAAAACGCGCATCTGGA- AGAAGCATTTAACTGTTTAACTCTGGCCAAAGACATC- TTTCCGAATGAAAAGTTCTGAGCCGCATCCAGAAAA- TTCAAGAAGCCCTGGAAGAACTGGCAGAA
BD1	ACCGAAATGGTTGCAAAATTTAGCCTCCGCTGAATAT- CAGCTGACCCGAGCAGAACTGAAACAAATTGTTGATC- AGAGCCTGAGCGTGGTGATCTGGCATGTCTGCTGCT- GGTTCAGCTGTTTCCGGAAGCTTTAGTGATGTTGAT- TTTAGCCGTGGTTAGCGCATGTGTTTTGTCAGCCA- AACGCAAACTGGAAGCCTGCATCTGCAGCTGATTCG- TAATTATGTGGAAGTTTATTACCGAGCGTTAAAGAT- ACCGCAGTTTGGCAGGCAGAAATGTCTGCCGAGCTGA- ATGATTTTTTTCAGCCGTTTTTGGGCACAGCGTGAAT- GGAAGATAGCCAGCCGAGCGGTGAGTTTGAAGCTTT- TTTGAAGCAGAACAGGTTGATCCGGGTCTTTCTGG- ATAACAAAGATCAAGAAGAAGCACTGAGCCTGGAT
Forward primer	
NTPR	TACTTCCAATCCATGGAAGCAAGCCGTCGTTTCCGGAAG
BD1	TACTTCCAATCCATGACCGAAATGGTTGCAAAATTTAG- CCTCC
Reverse primer	
NTPR	TATCCACCTTTACTGTTATTCTGCCAGTTCTTCCAGGGC- TTC
BD1	TATCCACCTTTACTGTTAATCCAGGCTCAGTGCTTCTTC- TTGATC
Cloning and expression	pNIC28-Bsa4 vector (pCPR0011)
vector	
Expression host	<i>E. coli</i> Rosetta
Complete amino-acid sequence of the construct produced (tags were removed for crystallization using TEV protease)	
NTPR	MHHHHHWSHPQFEKENLYFQSMESRRFPPEAEALSPEQ- AAHYLRVYKAEKATKNGDLEAFKLFNLAKDIFPNE- KVLRSRIQKIQEAELEAE
BD1	MHHHHHWSHPQFEKENLYFQSMEMVAKFPQPPPEYQLT- AAELKQIVDQSLSGDLACRLLLVQLFPPELFSVDVFSR- GCSACGFAAKRKLESLHLQLIRNYVEVYPSVKDTAV- WQAECLPQLNDFFSRFAQREMEDSQSPGQVASFEEA- EQVDPGHFLDNKDQEEALSLD

(residues 234–381 from BEND3; primers BEND3-234FW, 5'-TACTTCCAATCCATGACCGAAATGGTTGCAAAA-TTTCAGCCTCC-3', and BEND3-381RV, 5'-TATCCA-CCTTTACTGTTAATCCAGGCTCAGTGCTTCTTCTTG-ATC-3'). The protein sequences coded by these amplified DNAs and other relevant information are detailed in Table 1.

2.2. Protein expression and purification

Escherichia coli Rosetta competent cells (Novagen) were transformed with the different plasmids and small-scale expression assays were carried out to evaluate protein expression and solubility. Based on the results, we chose the best constructs for large-scale expression and purification (NTPR and BD1 DNA fragments). For expression of the domains, a single colony was inoculated into 5 ml LB containing 50 µg ml⁻¹ kanamycin and incubated overnight at 310 K at 200 rev min⁻¹. Thereafter, the bacterial culture was transferred to 1 l fresh LB containing 50 µg ml⁻¹ kanamycin and incubated at 310 K at 200 rev min⁻¹ until the OD₆₀₀ reached 0.8. Protein expression was induced by the addition of

1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and the cells were allowed to grow overnight at 291 K. The bacterial cells were harvested and were stored at 193 K until use.

The cell pellets were defrosted and resuspended in lysis buffer [50 mM sodium phosphate pH 7.5, 300 mM NaCl, one tablet of cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) per 50 ml, 1× BugBuster (Novagen), 50 U ml⁻¹ Benzonase, 10 mM imidazole, 10% glycerol, 0.5 mM TCEP]. After cell disruption using a French press, cell debris and insoluble particles were removed by centrifugation followed by sterile filtration (0.45 µm membrane pore size). The cleared lysates were sequentially purified by affinity chromatography on HisTrap HP (GE Healthcare) and Strep-Tactin Superflow (IBA Biosciences) columns, followed by reverse IMAC (HisTrap HP) after cleaving the N-terminal tags using His-tagged *Tobacco etch virus* (TEV) protease. The proteins were further purified by size-exclusion chromatography using a HiLoad 16/60 Superdex 75 pg column (GE Healthcare) equilibrated with 25 mM HEPES pH 8.5, 150 mM NaCl, 0.5 mM TCEP, 10% glycerol. Fractions containing the isolated NTPR and BD1 domains were pooled and concentrated to 7.6 and 5.9 mg ml⁻¹, respectively, using an Amicon Ultra-15 3K concentrator (Merck Millipore), flash-cooled in aliquots of 20 µl volume and stored at 193 K. The identity and integrity of the proteins were confirmed by mass spectrometry, CD spectroscopy and SDS-PAGE (Fig. 1b). Although the purified NTPR showed an anomalous migration on SDS gels in comparison with the molecular-weight markers (Fig. 1b; its apparent molecular weight appears to be lower than 6 kDa while it should be 8.3 kDa), mass-spectrometry experiments confirmed that it was not degraded. Following the above-mentioned protocol, roughly 2–3 mg pure NTPR protein and 0.5–1 mg pure BD1 protein could be obtained per litre of expression culture.

Since BD1 contains three methionine residues, selenomethionine-substituted BD1 was produced to obtain experimental phases for structure determination of the complex. We used the SelenoMethionine Medium Complete kit (Molecular Dimensions) for the preparation of labelled protein, following the manufacturer's instructions. Briefly, a single colony of *E. coli* Rosetta cells containing the BD1 expression plasmid was grown overnight in 50 ml minimal medium containing 50 µg ml⁻¹ kanamycin and L-methionine. The next day, 10 ml of this culture was inoculated into 1 l pre-heated (310 K) labelling medium (minimal medium containing L-selenomethionine and 50 µg ml⁻¹ kanamycin). Selenomethionine-substituted BD1 was expressed and purified in the same way as the native protein. Selenomethionine incorporation into the BD1 domain was verified by ESI-TOF intact protein analysis using mass spectrometry (micrOTOF-Q II, Bruker).

2.3. Isothermal titration calorimetry (ITC)

The formation of the complex between BD1 and NTPR was analyzed by ITC using a MicroCal iTC200 (GE Healthcare). Protein samples were dialyzed against ITC buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 0.5 mM TCEP). ITC data were

recorded on successive injections (2.5 μl) of BD1 (800 μM) into a cell containing NTPR (50 μM) at 298 K. The data were fitted into a hetero-association model using *NITPIC* (Keller *et al.*, 2012). The complex displayed a K_d of $4.3 \pm 0.6 \mu\text{M}$, a ΔH of $-0.798 \pm 0.05 \text{ kcal mol}^{-1}$ and a stoichiometry of 1:1 (Fig. 1c).

2.4. Crystallization

The formation of the NTPR–BD1 complex for crystallization assays was achieved by mixing the purified domains in a 1:3 molar ratio (BD1:NTPR) and incubating for 30 min at 277 K. Initial crystallization screening was carried out at 293 K by the sitting-drop vapour-diffusion method using commercially available screens (The JCSG+ Suite and The Protein Complex Suite from Qiagen, Crystal Screen HT from Hampton Research and Wizard Cryo 1 and 2 from Rigaku). Drops consisting of 100 nl reservoir solution and 100 nl protein complex solution (174 μM BD1 and 522 μM NTPR) were set up in 96-well plates (MRC 2 Drop, Swissci; 60 μl reservoir) using a Mosquito Crystal robot (TTP Labtech, Melbourn, England). The plates were stored and periodically imaged at 298 K using a Rock Imager 1000 automated imaging system and the data were managed using the *Rock Maker* software package (both from Formulatrix, Bedford, Massachusetts, USA). Several crystal hits were obtained after a few days of incubation and two conditions from The Protein

Complex Suite screen were chosen for optimization using a Dragonfly screen optimizer (TTP Labtech, Melbourn, England): condition G4 (2 M ammonium sulfate, 0.1 M Tris pH 8.0; Fig. 2c) and condition H4 (1.4 M sodium malonate; Fig. 2a). Verification of protein crystals was performed with the UV-sensitive camera equipped on the Rock Imager (Fig. 2). Crystals obtained from both conditions were further optimized by scaling up in 24-well hanging-drop VDXm plates (Hampton Research). Larger crystals (300 $\times$ 100 $\times$ 100 μm) were obtained within 2 d by mixing 1 μl protein complex and 1 μl crystallization solution (0.1 mM HEPES pH 7, 1.56 M ammonium sulfate; Fig. 2d). Selenomethionine-substituted crystals were obtained under the same final conditions (Figs. 2e and 2f). Analysis using SDS–PAGE and silver staining of the rinsed and dissolved crystals revealed the presence of both domains. Crystallization information is summarized in Table 2.

2.5. X-ray data collection and processing

Crystals were mounted on CryoLoops (Hampton Research) and flash-cooled in liquid nitrogen. Several cryoprotectants such as glycerol and 2-methyl-2,4-pentanediol (MPD) were tried at different concentrations before a suitable one was identified. For data collection under cryogenic conditions, crystals were briefly soaked in a universal cryosolution

Figure 2
NTPR–BD1 complex crystals. (a) Initial crystals obtained with The Protein Complex Suite screen condition H4 (native complex). (b) UV image of the previous crystals. (c) Initial crystals obtained with The Protein Complex Suite screen condition G4 (native complex). (d) Final optimized crystals (native complex). (e) Final optimized crystals (selenomethionine-labelled NTPR–BD1 complex). (f) CryoLoop containing selenomethionine-labelled crystals at the SLS synchrotron. The white grid represents the beam-focusing tool in raster mode and provides an idea of the size of the beam (each rectangle inside the grid is 50 $\times$ 10 μm) in comparison with the crystals.

Table 2
Crystallization of NTPR–BD1.

Method	Vapour diffusion
Plate type	96-well MRC 2 Drop plate (screening), 24-well VDXm plate (production)
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	2.9 (BD1), 4.3 (NTPR) (1:3 ratio; 171 μ M BD1 and 513 μ M NTPR)
Buffer composition of protein solution	25 mM HEPES pH 8.5, 150 mM NaCl, 0.5 mM TCEP, 10% glycerol
Composition of reservoir solution	0.1 mM HEPES pH 7, 1.56 M ammonium sulfate
Volume and ratio of drop	1:1 ratio protein:reservoir; 0.2 μ l (screening), 2 μ l (production)
Volume of reservoir	70 μ l (screening), 300 μ l (production)

consisting of mother liquor supplemented with 20% (w/v) glycerol.

Crystal testing and diffraction data collection were performed at 100 K on beamline X06SA at the Swiss Light Source (SLS), Paul Scherrer Institute, Villigen, Switzerland and at Max-lab, Lund, Sweden. X-ray images were recorded with a Pilatus 6M detector using the fine-slicing method (Dauter, 1999) with 0.1° oscillations at 100 K, a wavelength of 1.0 Å and a crystal-to-detector distance of 400 mm (see Table 2 for data-collection details and statistics). X-ray diffraction data sets were collected to resolutions of 2.2 Å from both native and selenomethionine-derivative protein crystals (Fig. 3). All data were integrated and scaled using *XDS* (Kabsch, 2010) and *SCALA* from the *CCP4* software suite (Evans, 2006; Winn *et al.*, 2011). To obtain phase information, three data sets, peak (0.9785 Å), inflection point (0.9787 Å) and remote (0.9709 Å), were collected at the Se *K* edge from the same selenomethionine-containing crystal (Table 3).

Figure 3
Diffraction pattern of an NTPR–BD1 crystal collected at the SLS (high-resolution reflections are at 2.2 Å).

Table 3
Data-collection and processing statistics for the NTPR–BD1 complex.

Values in parentheses are for the highest resolution shell.

	Selenomethionine MAD data set			
	Peak	Inflection	Remote	Native
Space group	<i>P</i> 6 ₁ 22			<i>P</i> 6 ₁ 22
No. of crystals	1			1
Diffraction source	X06SA, SLS			
Unit-cell parameters <i>a</i> , <i>b</i> , <i>c</i> (Å)	47.67, 47.67, 430.70			47.28, 47.28, 431.58
α , β , γ (°)	90, 90, 120			90, 90, 120
Wavelength (Å)	0.9785	0.9787	0.9709	1.000
<i>f</i> ', <i>f</i> '' (e)	−6.5, 4.1	−7.0, 3.8	−3.9, 3.8	
Temperature (K)	100	100	100	100
Detector	Pilatus 6M, Dectris			
Resolution range (Å)	50–2.5	50–2.5	50–2.5	50–2.2
<i>R</i> _{merge} † (%)	6.1 (22.6)	5.9 (26.4)	6.4 (37.6)	11.2 (68.1)
Crystal-to-detector distance (mm)	400			
Exposure time per image (s)	0.1			
Rotation range per image (°)	0.1			
Total rotation range (°)	360			
Total No. of reflections	186553	185896	182993	473961
No. of unique reflections	11290	11288	11357	15824
CC _{1/2} ‡	0.99	0.99	1.0	0.99
$\langle I/\sigma(I) \rangle$	29.9 (11)	30.4 (10)	27.8 (7.1)	16.8 (3.4)
Data completeness (%)	99.8 (98.4)	99.9 (99.3)	99.8 (98.7)	99.1 (94.3)
Multiplicity	16.6 (17.5)	16.5 (17.0)	16.1 (15.3)	30 (16.3)
Mosaicity (°)	0.16	0.17	0.18	0.15

† *R*_{merge} is defined according to *XDS* (Kabsch, 2010). ‡ CC_{1/2} is defined according to Diederichs & Karplus (2013).

3. Results and discussion

We expressed and purified the NTPR and BD1 domains, which have been identified as the interaction modules of the PICH and BEND3 proteins. Selenomethionine BD1 protein was also purified in order to solve the phase problem. Initial screening of crystallization conditions only yielded crystals when the NTPR–BD1 mixture was used; no crystals were obtained using the individual domains. Native and selenomethionine-derivative complexes exhibited the same behaviour in our crystallization assays and produced the same type of crystals (Fig. 2). The diffraction data also showed a resolution of 2.2–2.3 Å for both samples.

Based on the diffraction pattern (Fig. 3), the NTPR–BD1 crystals belonged to the hexagonal space group *P*6₁22 (No. 177), *P*6₁22 (No. 178) or *P*6₅22 (No. 179), with unit-cell parameters *a* = *b* = 47.28, *c* = 431.579 Å, α = β = 90, γ = 120°. To determine the packing of the NTPR–BD1 complex in the asymmetric unit of the crystal, we calculated the Matthews coefficient (Matthews, 1968). It yielded a *V*_M of 2.76 Å³ Da⁻¹, corresponding to a solvent content of 55.4%, with one NTPR–BD1 heterodimer in the asymmetric unit, in agreement with the ITC data. The structure is being solved by the three-wavelength MAD method using the selenomethionine data. We found the Se positions with *SHELX* (Sheldrick, 2008) using space group *P*6₁22. Currently, model building and refinement using *Coot* (Emsley *et al.*, 2010) and *PHENIX* (Adams *et al.*, 2010) are in progress. The structure of the NTPR–BD1 complex and its interpretation will provide the

first insights into the interaction between PICH and BEND3 and will provide further hints about their role in resolving the ultrafine DNA bridges.

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