Crystal structure of the Kelch domain of human NS1-binding protein at 1.98 Å resolution

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NS1-binding protein (NS1-BP), which belongs to the Kelch protein superfamily, was first identified as a novel human 70 kDa protein that interacts with NS1 of *Influenza A virus*. It is involved in many cell functions, including pre-mRNA splicing, the ERK signalling pathway, the aryl hydrocarbon receptor (AHR) pathway, F-actin organization and protein ubiquitylation. However, the structure of NS1-BP is still unknown, which may impede functional studies. Here, the structure of the C-terminal Kelch domain of NS1-BP (NS1-BP-C; residues 330–642) was determined at 1.98 Å resolution. The Kelch domain adopts a highly symmetric six-bladed β -propeller fold structure. Each blade of the β -propeller is composed of four antiparallel β -strands. Comparison of the Kelch-domain structures of NS1-BP and its homologues showed that the Gly–Gly pair in β -strand B and the hydrophobic Trp residue in β -strand D are highly conserved, while the B–C loops in blades 2 and 6 are variable. This structure of the Kelch domain of NS1-BP extends the understanding of NS1-BP.

1. Introduction

NS1-binding protein (NS1-BP), also known as IVNS1ABP, ARA3, KLHL39 or ND1, was first identified as a novel human 70 kDa protein that interacts with nonstructural protein 1 (NS1) of *Influenza A virus*. It belongs to the Kelch-protein superfamily and contains a BTB/POZ domain in its N-terminus, a BACK domain, and a Kelch domain in the C-terminus. It was reported that NS1-BP inhibits pre-mRNA splicing in HeLa cell nuclear extracts *in vitro* (Wolff *et al.*, 1998). It is concentrated in the nuclear speckles enriched with the spliceosome assembly factor SC35 and this localization is changed in *Influenza A virus*-infected cells (Wolff *et al.*, 1998). In addition, NS1-BP is involved in the alternative splicing of *Influenza A virus* M1 mRNA through interaction with heterogeneous nuclear ribonucleoproteins (hnRNPs; Tsai *et al.*, 2013). A previous report showed that NS1-BP regulates cell viability through inhibition of the NS1-mediated ERK activation which collaborates with CRKL (Miyazaki *et al.*, 2013). NS1-BP also increases the concentration of the functional aryl hydrocarbon receptor, a ligand-activated transcription factor that is involved in metabolic adaptation, dioxin toxicity and vascular development (Dunham *et al.*, 2006). Additionally, NS1-BP associates with MBP-1 and enhances its repressor activity on the c-Myc promoter to control c-Myc gene expression and cell proliferation. Moreover, NS1-BP plays a key role in the dynamic organization of the actin skeleton as a stabilizer of actin filaments by association with F-actin through Kelch repeats (Perconti *et al.*, 2007). Recent studies have demonstrated that NS1-BP

interacts with KLHL20 and functions as a negative regulator of Cul3-KLHL20 ubiquitin ligase to prevent ubiquitin-mediated proteolysis of PML and DAPK and suppresses colon cancer metastasis (Chen *et al.*, 2015). Although NS1-BP is important in mediating protein–protein interactions in these cellular functions, the structure of NS1-BP has not been determined.

The Kelch domain is important for NS1-BP to mediate protein–protein interactions. It contains five to seven repeated sequence motifs of a segment comprised of 44–56 residues (Adams *et al.*, 2000). In this paper, we determined the structure of the C-terminal Kelch domain of NS1-BP (NS1-BP-C; residues 330–642) at 1.98 Å resolution. Our data provide a structural basis for NS1-BP and extend the understanding of the structural features of the Kelch-repeat motif.

2. Materials and methods

2.1. Macromolecule production

The cDNA for human NS1-BP was obtained by reverse transcription of mRNA from HEK293 cells. The coding sequence for NS1-BP-C (residues 330–642) was cloned in pRSFDuet-1 expression vector (Novagen) with an N-terminal

His₆ tag. The constructed vector was then transformed into *Escherichia coli* strain BL21 (DE3) cells. The cells were cultured in Luria–Bertani (LB) medium with 100 mg l^{−1} kanamycin at 37°C to an OD₆₀₀ of 0.6–0.8. Expression of the target protein was induced with 0.5 mM IPTG at 16°C for 20 h. The bacterial cells were harvested by centrifugation at 4000 rev min^{−1} for 30 min. The cell pellet was resuspended in lysis buffer consisting of 20 mM HEPES pH 7.8, 200 mM NaCl, 30 mM imidazole, 5% glycerol. The cells were lysed on ice by ultrasonication and were then centrifuged at 16 000 rev min^{−1} for 30 min. The supernatant was mixed with pre-equilibrated nickel (Ni²⁺) beads (GE Healthcare) and incubated for 1 h at 4°C. The beads were collected and washed with lysis buffer. The protein was eluted with elution buffer consisting of 20 mM HEPES pH 7.8, 200 mM NaCl, 300 mM imidazole, 5% glycerol. The NS1-BP-C protein that eluted

Figure 1
Purification of NS1-BP-C using size-exclusion chromatography. (a) Elution profiles from gel-filtration chromatography of NS1-BP-C and BSA using a Superdex 200 10/300 GL (S200) column (GE Healthcare). (b) SDS–PAGE of purified NS1-BP-C after a S200 gel-filtration run. Lane *M* contains molecular-weight marker (labelled in kDa).

(a)

(b)

Figure 2
Photograph of NS1-BP-C crystals and an X-ray diffraction image. (a) Crystals grown under the optimal condition (0.2 M ammonium citrate tribasic pH 7.0, 20% PEG 3350) in a 24-well plate. (b) X-ray diffraction image of a selenomethione-labelled NS1-BP-C crystal.

Table 1
Macromolecule-production information.

Source organism	Human
DNA source	cDNA
Forward primer	GCGGATCCATGACTCCAAACTAAG
Reverse primer	GACTCGAGTTAAACTGGAAATCTTT
Cloning vector	pRSFDuet-1 vector
Expression vector	pRSFDuet-1 vector
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MGSSHHHHHSSQGSMTPKLSKLSFEMQQD ELIEKPMSPMQYARSGLTAEEMNGKLIA AGGYNREECLRTVECYNPHTDHWSEFLAP MRTPRARFQMAVLMGQLYVVGSGNGHSD DLSCGEMYDSNIDDWIPVELRTNRCNA GVCALNGKLYIVGSDPYGQKGLKNCNV FDPVTKLWTSAPLNIRRHQSAVCELGG YLYIIGGAESWNCLNTVERYNPENNTWT LIAPMNVARRGAGVAVLNGKLFVCGGFD GSHAISCVEMDPTRNEWKMMGMTSPR SNAGIATVGNTIYAVGGFDGNEFLNTVE VYNLESNEWSPYTKIFQF

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plates
Temperature (K)	289
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM HEPES pH 7.8, 200 mM NaCl, 5% glycerol
Composition of reservoir solution	0.2 M ammonium citrate tribasic pH 7.0, 20% PEG 3350
Volume and ratio of drop	2 µl; 1:1 ratio
Volume of reservoir (ml)	1

from the nickel column was further purified by size-exclusion chromatography on a Superdex 200 10/300 GL column (GE Healthcare) in a buffer consisting of 20 mM HEPES pH 7.8, 200 mM NaCl, 5% glycerol. The fractions containing pure protein were concentrated to 10 mg ml⁻¹ for crystallization screening. Selenomethionine-derivative protein was derived

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.	
Diffraction source	BL17U1, SSRF
Wavelength (Å)	0.9792
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	1.0
Total rotation range (°)	360
Exposure time per image (s)	0.5
Space group	<i>P</i> 3 ₂ 21
<i>a</i> , <i>b</i> , <i>c</i> (Å)	61.56, 61.56, 140.58
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.39–0.68
Resolution range (Å)	50.0–1.98 (2.0–1.98)
Total No. of reflections	459352 (37257)
No. of unique reflections	22017 (2037)
Completeness (%)	98.93 (93.58)
Multiplicity	20.9 (18.3)
$\langle I/\sigma(I) \rangle$	20.64 (7.74)
<i>R</i> _{meas}	0.0863 (0.431)
<i>CC</i> _{1/2}	0.999 (0.974)
Overall <i>B</i> factor from Wilson plot (Å ²)	34

using the same plasmid and *E. coli* strain, which was grown in M9 medium containing 50 µg ml⁻¹ selenomethionine. The induction conditions and purification procedure were similar to those for the native protein (Fig. 1). Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Preliminary crystallization experiments were performed using the hanging-drop vapour-diffusion method at 289 K. For initial screening, crystallization conditions were screened in 96-well crystal-growth plates using a Mosquito robot (TTP Labtech). After about 2 d, crystals were observed in several conditions from the Index kit (Hampton Research). The crystallization condition for these crystals was further

Figure 3
Overall structure of the Kelch domain of NS1-BP. (a) A ribbon diagram of the Kelch domain of human NS1-BP (residues 330–642) showing the six-bladed β -propeller structure. The blades of the β -propeller are numbered 1–6. The four β -strands in each blade are labelled A–D on blade 2. The N- and C-termini of the domain are labelled N and C, respectively. (b) Side view of the Kelch domain of human NS1-BP (residues 330–642).

optimized by altering the pH and the salt concentration. The best crystals were obtained using the conditions given in Table 2. Selenomethionine-labelled crystals were obtained under similar conditions as for the native crystal (Fig. 2*a*). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

To prevent radiation damage, the crystals were transferred into mother liquor containing 20% glycerol as a cryoprotectant for 10 s and were then flash-cooled in liquid nitrogen. A

sample diffraction image is shown in Fig. 2(*b*). Data sets were processed and scaled using *HKL-2000* (Otwinowski & Minor, 1997). Data-collection and processing information is shown in Table 3.

2.4. Structure solution and refinement

The initial phases for selenomethionine-derivatized NS1-BP-C were obtained by the single-wavelength anomalous dispersion (SAD) method. The Se atoms were located with *SHELXC/D* (Sheldrick, 2010). Phasing was carried out using

Figure 4

Comparison of the Kelch domains of NS1-BP and its homologues. (*a, b*) Superposition of the Kelch domain of NS1-BP (grey) on those of KEAP1 (PDB entry 1zgk; magenta) and KBTBD5 (PDB entry 4asc; cyan). The six Kelch repeats are numbered 1–6. The comparison highlights the differences in the *B*–*C* loop regions. (*c*) Structure-based sequence alignment of the second and the sixth Kelch-repeat motifs from the Kelch domains of NS1BP, KEAP1 and KBTBD5. Conserved regions are shown in blue boxes. Highly conserved residues are in white on a red background; moderately conserved residues are in red. Sequence alignment was performed using *ESPrpt* (Robert & Gouet, 2014).

Table 4

Structure solution and refinement.

Values in parentheses are for the outer shell.

Resolution range (Å)	26.65–1.98 (2.05–1.98)
Completeness (%)	98.93 (93.58)
No. of reflections, working set	22003 (2041)
No. of reflections, test set	1986 (189)
Final R_{cryst}	0.1888 (0.2321)
Final R_{free}	0.2326 (0.2960)
No. of non-H atoms	
Protein	2180
Water	134
Total	2314
R.m.s. deviations	
Bonds (Å)	0.006
Angles (°)	0.78
Average B factors (Å ²)	
Overall	40
Protein	40
Water	42
Ramachandran plot†	
Most favoured (%)	90.7
Allowed (%)	9.3

† Ramachandran plot statistics are based on PROCHECK (Laskowski *et al.*, 1993).

phenix.autosol and the initial models were built with *phenix.autobuild* (Terwilliger *et al.*, 2009). Refinement and model building were carried out iteratively using *phenix.refine* (Afonine *et al.*, 2012) and *Coot* (Emsley *et al.*, 2010). The data-collection and refinement statistics are shown in Table 4. Atomic coordinates and structure factors have been deposited in the Protein Data Bank (<http://www.pdb.org>) as entry 5yy8. All structural figures were prepared using *PyMOL* (<http://www.pymol.org>).

3. Results and discussion

The Kelch domain of NS1-BP is a highly symmetric six-bladed β -propeller (Fig. 3) and each blade is composed of four anti-parallel β -strands (*A–D*). The ‘top’ of the propeller contains the *A–B* and *C–D* loops, which connect strand *A* to strand *B* and strand *C* to strand *D*, respectively. The ‘bottom’ of the propeller contains the *B–C* loop and the interblade *D–A* loops. To identify the structural features of the Kelch domain of NS1-BP, we superposed it on two Kelch-repeat proteins, KEAP1 (Beamer *et al.*, 2005) and KBTBD5 (Canning *et al.*, 2013), that belong to the same subfamily. All three proteins display the typical β -propeller structure and the Kelch domain is similar in overall structure, with an overall root-mean-square deviation (r.m.s.d.) of about 1.68 Å using *PDBFold* (Krissinel & Henrick, 2004). In addition, several residues are conserved in these proteins, including a Gly–Gly pair in strand *B* and a hydrophobic tryptophan residue in strand *D*. Meanwhile, the greatest differences were found to be located in the blade 2 and 6 regions. NS1BP displays a short *B–C* loop in blades 2 and 6. In contrast, the *B–C* loop of blade 2 in KEAP1 and the *B–C* loops of blades 2 and 6 in KBTBD5 are more extended and arranged differently (Fig. 4). The KEAP1–Nrf2 complex has been reported and reveals that the properties of the substrate pocket are determined by the *D–A* loops as well as the variable *B–C* loops (Lo *et al.*, 2006). These *B–C* loop

features are expected to contribute to the substrate-binding specificity of Kelch-repeat proteins.

In summary, we determined the crystal structure of the Kelch domain of NS1-BP at 1.98 Å resolution. Sequential and structural comparison of NS1-BP-C with its homologues demonstrated that NS1-BP-C has the same conserved motifs and the same structural fold as its homologues. However, it possesses a different loop arrangement, including a short *B–C* loop in blades 2 and 6. These results will be helpful in future biochemical analyses of NS1-BP and extend the understanding of the structural features of the Kelch-repeat motif.

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