Acta Crystallographica Section F
Structural Biology Communications
ISSN 2053-230X

Crystallization and preliminary X-ray crystallographic study of human Hikeshi, a new nuclear transport receptor for Hsp70

Jinsue Song and Soo Jae Lee*

College of Pharmacy, Chungbuk National University, 48 Gaeshin-dong, Heungduk-gu, Cheongju, Chungbuk 361-763, Republic of Korea

Correspondence e-mail: sjlee@chungbuk.ac.kr

Received 3 September 2014
Accepted 1 November 2014

Hikeshi is a new nuclear transport receptor that plays an important role in the nuclear import of Hsp70 heat-shock proteins under thermal stress. Wild-type human Hikeshi and its Phe97Ala mutant were overproduced and purified using an *Escherichia coli* expression system. The purified proteins were crystallized using the hanging-drop vapour-diffusion technique. Wild-type crystals grew in space group $C222_1$, with unit-cell parameters a = 61.1, b = 137.8, c = 97.9 Å, α = 90.0, β = 90.0, γ = 90.0°. Phe97Ala mutant crystals were obtained in space group $P3_2$, with unit-cell parameters a = 85.7, b = 85.7, c = 69.1 Å, α = 90.0, β = 90.0, γ = 120.0°. These crystals diffracted to 1.8 and 2.5 Å resolution, respectively. This study is the first to yield structural insight into this highly unusual fourth import receptor after importins, NTF2 and TAP.

1. Introduction

During thermal stress, the level of molecular chaperones known as heat-shock proteins (Hsps) is increased to maintain the physiological homeostasis of the cell, which is an important requirement for cell survival (Wang *et al.*, 2004; Ellis & van der Vies, 1991; Mathew & Morimoto, 1998). Hikeshi, a nuclear transport receptor, plays an important role in the heat-shock-induced nuclear import of 70 kDa heat-shock proteins (Hsp70s) through interactions with proteins termed phenylalanine-glycine (FG)-repeat-containing nucleoporins (FG-Nups) in nuclear pore complexes (NPCs) (Kose *et al.*, 2012; Imamoto & Kose, 2012).

Under non-stress conditions, the well studied importin-β-family-mediated nucleocytoplasmic trafficking is down-regulated in response to stress, while Hikeshi-mediated nuclear import is up-regulated. Hikeshi-mediated transport is a unique intracellular pathway: it is modulated by a carrier unrelated to importin β and it uses the ATPase cycle of Hsp70s instead of the GTPase cycle of Ran. ATP-bound Hsp70 is directly recognized by Hikeshi for nuclear translocation, implying that carrier–cargo binding and release are regulated by the Hsp70 ATPase cycle (Kose *et al.*, 2012). Hikeshi is evolutionarily conserved from yeast to humans, with 70% sequence identity and 80% similarity (without insertions or deletions). A previous study revealed that Hikeshi interacts weakly, but significantly, with FG-Nups such as Nup62 and Nup153. Furthermore, Hikeshi binds directly to FG-Nups and translocates through NPCs, like all known nuclear transport carriers (Kose *et al.*, 2012). FG-Nups are important for mediating both the movement of cargo–carrier complexes through NPCs and for excluding other macromolecules from the central transport channel of NPCs (Stewart, 2007). FG-Nups have a specific feature: the highly unstructured FG sequence repeat, which is commonly FG, GLFG or F*x*FG (where *x* is usually a small residue such as Ser, Gly or Ala; Tran & Wente, 2006; Rout & Wente, 1994; Rout *et al.*, 2000). The Phe97 residue seems to serve an intervening role in recognizing an FG-repeat sequence. To identify the Hikeshi-mediated nuclear import pathway based on structural function, it is necessary to obtain a high-resolution atomic structure of Hikeshi. Here, we report the crystallization, diffraction data collection and preliminary X-ray diffraction data analysis of wild-type Hikeshi and a Phe97Ala mutant.

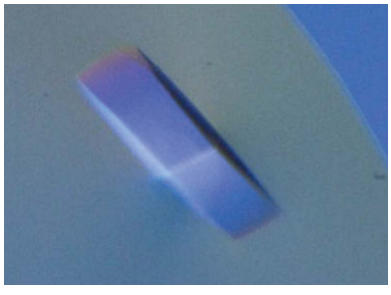

Table 1
Representative parameters from the crystallographic data measurement of wild-type Hikeshi and its Phe97Ala mutant.

Values in parentheses are for the outer shell.

	Wild type (native)	Phe97Ala (native)
Diffraction source	BL44XU, SPring-8	BL44XU, SPring-8
Crystal-to-detector distance (mm)	240	250
Exposure time per image (s)	2	1
Rotation range per image (°)	1	1
Total rotation range (°)	150	160
Wavelength (Å)	0.9192	0.9192
Space group	$C222_1$	$P3_2$
a, b, c (Å)	61.1, 137.8, 97.9	85.7, 85.7, 69.1
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 90.0, 120.0
Resolution range (Å)	39.9–1.88 (1.91–1.88)	50.0–2.50 (2.54–2.50)
Total No. of reflections	400933	77836
No. of unique reflections	33424	19994
Completeness (%)	99.1 (99.8)	99.8 (100)
Multiplicity	12.1 (12.2)	3.9 (3.9)
$\langle I/\sigma(I)\rangle$	66.0 (7.9)	10.2 (1.3)
$R_{\rm merge}$	0.076 (0.45)	0.072 (0.66)

2. Materials and methods

2.1. Cloning, overproduction and purification

The cDNA coding for full-length human Hikeshi (residues 1–197) cloned into the *Bam*HI and *Xho*I sites of the pGEX-6P-1 vector (a gift from Naoko Imamoto, RIKEN) was introduced into *Escherichia coli* strain BL21 (DE3) for protein overexpression. The transformants containing the target plasmid were grown in LB medium supplemented with 100 µg ml^{-1} ampicillin at 310 K until the culture reached an OD_{600} of 0.6 and were then induced with 1 m*M* IPTG for a further 16 h at 291 K. The cells were harvested by centrifugation at 6000*g* for 15 min at 277 K and resuspended in binding buffer (1× PBS, 5% glycerol, 10 m*M* β-mercaptoethanol) supplemented with 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF). The cells were sonicated on ice and the lysate was then collected by centrifugation at 18 000*g* for 40 min at 277 K.

The supernatant was loaded onto a gravity-flow column (Bio-Rad, USA) packed with 5 ml Glutathione Sepharose 4B resin (GE Healthcare) and the resin was extensively washed with binding buffer. Proteins were eluted from the resin with elution buffer (50 m*M* Tris–HCl pH 8.0, 10 m*M* reduced glutathione). The fusion protein was cleaved overnight with recombinant human rhinovirus (HRV 3C) protease (Doctor Protein, Republic of Korea) at 277 K and was desalted using an Amicon Ultra 5K concentrator (Millipore, USA). The sample was loaded onto a MonoQ HR 10/100 GL column (GE Healthcare) and the protein was eluted with a linear gradient (50–500 m*M*) of NaCl in binding buffer (50 m*M* Tris–HCl pH 8.0, 1 m*M* DTT). The protein was further purified by gel filtration using a HiLoad 16/60 Superdex 200 column (GE Healthcare) with buffer consisting of 20 m*M* Tris–HCl pH 8.0, 50 m*M* NaCl, 1 m*M* DTT at 277 K. The purified protein was concentrated to 10 mg ml^{-1} using an Amicon Ultra 5K. The Hikeshi Phe97Ala mutant was produced by PCR-based mutagenesis using a Muta-Direct Site Directed Mutagenesis Kit (iNtRON Biotechnology, Republic of Korea). The selenomethionine derivative of Hikeshi was expressed using *E. coli* BL21 (DE3) cells cultured in M9 minimal medium. Expression and purification procedures were performed as for native Hikeshi.

2.2. Crystallization and X-ray data collection

Initial conditions for crystallization were screened at 293 K by the hanging-drop vapour-diffusion technique using a PEG and salt screening kit from our laboratory, which was designed for the crystallization of protein–protein complexes. 50 m*M* DTT was added to the Hikeshi protein for crystallization. 1 µl protein solution was mixed with 1 µl reservoir solution and equilibrated against 400 µl reservoir solution. From optimization of the salt species, salt concentration and pH, we obtained a final crystallization condition for wild-type Hikeshi and the Phe97Ala mutant consisting of 1.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 7.4. The crystals were soaked briefly using reservoir solution supplemented with 30%(*w*/*v*) glycerol as a cryoprotectant and flash-cooled in liquid nitrogen. Data sets were collected using a Rayonix MX-225HE CCD detector supported by NSSRC on the BL44XU beamline at SPring-8 (Harima, Japan). The diffraction data were indexed, integrated, scaled and merged using *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Wild-type human Hikeshi and the Phe97Ala mutant were overexpressed and purified to homogeneity (approximately 95% purity) using Glutathione Sepharose 4B and Superdex 200 16/60 columns (Fig. 1). The protein was concentrated to 10 mg ml^{-1} for crystallization trials. After a further optimization screen of the salt species, salt concentration and pH, crystals diffracting to 1.8 Å resolution were obtained using a reservoir solution consisting of 1.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 7.4 (Fig. 2). The crystals of wild-type Hikeshi belonged to space group $C222_1$, with unit-cell parameters a = 61.1, b = 137.8, c = 97.9 Å (Table 1). The crystals of the Phe97Ala mutant diffracted to 2.5 Å resolution and belonged to space group $P3_2$, with unit-cell parameters a = 85.7, b = 85.7, c = 69.1 Å. The

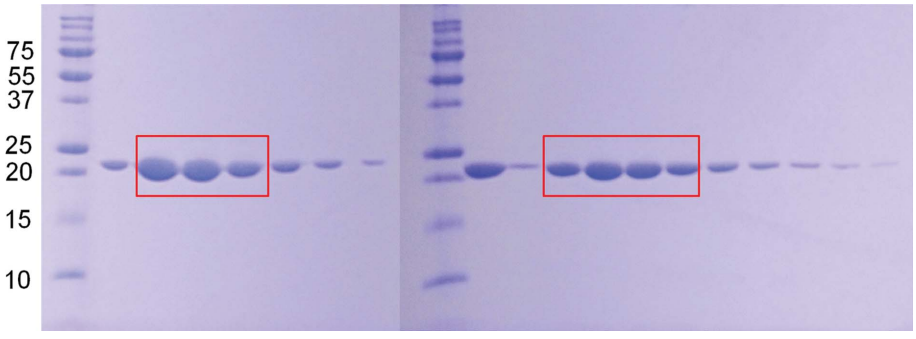

Figure 1
15% SDS–PAGE of purified Hikeshi proteins (left, wild type; right, Phe97Ala mutant), confirming the molecular weight of about 22 kDa. In each case the first lane contains molecular-weight standards (labelled in kDa) and the other lanes contain fractions from gel filtration. The protein samples in red boxes were used for crystallization.

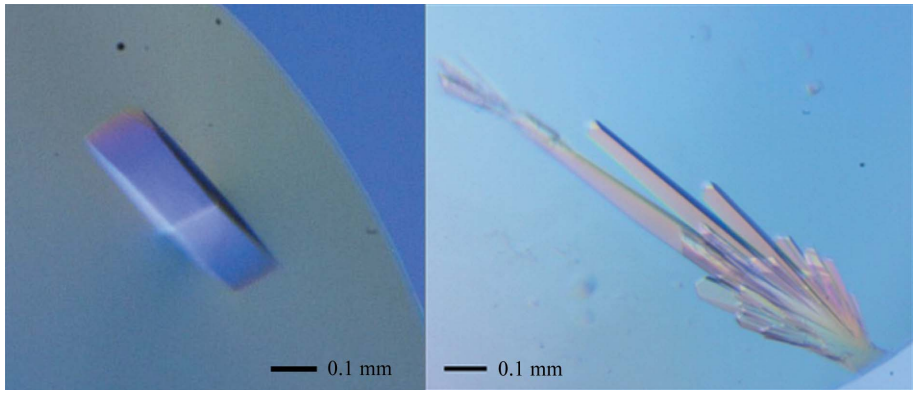

Figure 2
Crystals of wild-type Hikeshi (left) and the Phe97Ala mutant (right) grown in 1.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 7.4 at 293 K.

crystals of wild-type Hikeshi grew within one week in a plate shape with dimensions of about 0.25 × 0.3 × 0.08 mm at 293 K, while the Phe97Ala mutant crystals had dimensions of about 0.02 × 0.01 × 0.5 mm and a rod shape (Fig. 2). Crystals of the selenomethionine derivative of Hikeshi were grown under similar conditions. Preliminary crystallographic analysis gave calculated Matthews coefficients (V_M) of 2.38 and 2.24 Å^3 Da^{-1} with solvent contents of 48.3 and 45.05% for the wild type and the Phe97Ala mutant, respectively, assuming the presence of two molecules in the asymmetric unit (Matthews, 1968). The crystallographic parameters and diffraction data statistics are summarized in Table 1.

This research was supported by a research grant from Chungbuk National University in 2013. Synchrotron-radiation experiments were performed on BL44XU at SPring-8, Japan (2011B6500 and 2012A6500) and the Protein Beamline, Pohang Light Source, Republic of Korea.

References

Ellis, R. J. & van der Vies, S. M. (1991). *Annu. Rev. Biochem.* **60**, 321–347.
Imamoto, N. & Kose, S. (2012). *Nucleus*, **3**, 422–428.
Kose, S., Furuta, M. & Imamoto, N. (2012). *Cell*, **149**, 578–589.
Mathew, A. & Morimoto, R. I. (1998). *Ann. N. Y. Acad. Sci.* **851**, 99–111.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rout, M. P., Aitchison, J. D., Suprapto, A., Hjertaas, K., Zhao, Y. & Chait, B. T. (2000). *J. Cell Biol.* **148**, 635–651.
Rout, M. P. & Wente, S. R. (1994). *Trends Cell Biol.* **4**, 357–365.
Stewart, M. (2007). *Nature Rev. Mol. Cell Biol.* **8**, 195–208.
Tran, E. J. & Wente, S. R. (2006). *Cell*, **125**, 1041–1053.
Wang, W., Vinocur, B., Shoseyov, O. & Altman, A. (2004). *Trends Plant Sci.* **9**, 244–252.