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Crystallization and preliminary X-ray crystallographic studies of transportin 1 in complex with nucleocytoplasmic shuttling and nuclear localization fragments

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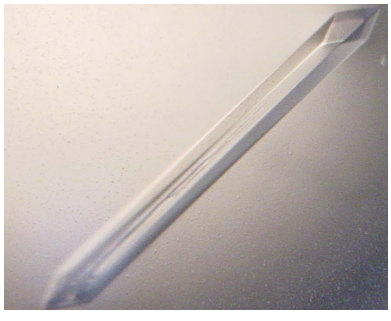
Nucleocytoplasmic transport of proteins with molar masses of larger than 60 000 is mediated by transport receptors. The transport receptor transportin1 (Trn1) transports various kinds of RNA-binding proteins such as JKTBP, hnRNP D and TAP. Trn1 was successfully cocrystallized with nucleocytoplasmic shuttling fragments of JKTBP and hnRNP D and a nuclear localization fragment of TAP. The crystal of the Trn1–JKTBP fragment complex belongs to space group $P2_12_12$, with unit-cell parameters a = 131.5, b =171.5, c = 68.2 Å. The crystals of Trn1 in complex with hnRNP D and TAP fragments are orthorhombic, space group $P2_12_12_1$, with unit-cell parameters a = 69.1, b = 119.1, c = 151.1 Å and a = 69.0, b = 119.1, c = 146.0 Å, respectively. The crystals diffracted to beyond 3.0, 3.2 and 2.4 Å resolution, respectively, using synchrotron radiation at SPring-8.

1. Introduction

In eukaryotic cells, the nucleocytoplasmic transport of macromolecules between the nucleus and the cytoplasm occurs through nuclear pore complexes (NPCs) by receptor-mediated diffusion (Görlich & Kutay, 1999; Nakielny & Dreyfuss, 1999; Fried & Kutay, 2003). Transport receptors target specific NPC proteins (nucleoporins) and a cognate nuclear localization and/or export sequence (NLS and/or NES) in transport substrates (cargoes). Subsequent translocation through the NPC occurs with RanGTP and the directionality of the translocation depends on the RanGTP concentration, which is high in the nucleus and low in the cytoplasm. Transportin 1 (Trn1) is a transport receptor that belongs to the importin β superfamily. Trn1 was first identified as the transport receptor for heterogeneous nuclear ribonucleoprotien (hnRNP) A1 containing an NLS called M9 (Siomi & Dreyfuss, 1995; Pollard *et al.*, 1996; Iijima *et al.*, 2006). Subsquently, a Trn1-mediated transport pathway has been found for various RNA-binding proteins such as hnRNP D (Suzuki *et al.*, 2005), the hnRNP D-like protein JKTBP (Kawamura *et al.*, 2002), HuR (Güttinger *et al.*, 2004), an mRNA-export factor TAP (Truant *et al.*, 1999), human papilloma virus type 16 E6 protein (Le Roux *et al.*, 2003), poly(A)-binding protein II (Calado *et al.*, 2000) and the ribosomal protein L23a (Jakel & Görlich, 1998).

Of the transport receptors that belong to the importin β superfamily, the best characterized is importin β (Conti *et al.*, 2006). Structural work on importin β in complex with the importin β-binding domain (IBB) of importin α (Cingolani *et al.*, 1999), cargoes of parathyroid hormone-related protein (PTHrP; Cingolani *et al.*, 2002) and sterol regulatory element-binding protein 2 (SREBP-2; Lee *et al.*, 2003) and RanGTP (Vetter *et al.*, 1999; Lee *et al.*, 2005) have shown that importin β is a superhelical molecule composed of 19 HEAT repeats with a flexible S-like conformation and that conformational changes induced by cargo binding and release plays a crucial role for transport. Importantly, the different regions of importin β are involved in binding to the different cargoes and importin α in distinct ways.

Similar to importin β, Trn1 is also a superhelical molecule, with 20 HEAT repeats (Chook & Blobel, 1999), but differs significantly from



importin β in the size and function of an acidic loop. The acidic loop that is inserted in HEAT repeat 8 of Trn1 may well be instrumental in RanGTP-mediated cargo dissociation (Chook *et al.*, 2002). Furthermore, Trn1 often targets nucleocytoplasmic shuttling fragments in cargoes that include both NLS and NES, which corresponds to NLS, whereas importin β targets nuclear localization fragments including only NLS. However, unlike importin β, nothing is known about the detailed structure of Trn1 in complex with cargoes, although a small-angle X-ray scattering study has shown that the M9-bound and M9-free forms of Trn1 are similar in gross shape but are more elongated than its RanGTP-bound form (Fukuhara *et al.*, 2004). The crystal structure analyses of Trn1–cargo complexes are therefore of great importance for better understanding of the cargo recognition and release by Trn1. Here, we report the crystallization and preliminary X-ray crystallographic analyses of Trn1 in complex with nucleocytoplasmic shuttling fragments of JKTBP and hnRNP D and a nuclear localization fragment of TAP.

2. Material and methods

2.1. Sample preparations

Human Trn1 (GenBank accession No. U72069) was expressed as a fusion protein with glutathione *S*-transferase (GST) in *Escherichia coli* BL21(DE3) cells and purified as described previously by Suzuki *et al.* (2005). The cells were collected and suspended in 20 m*M* HEPES pH 7.3, 110 m*M* potassium acetate, 10 m*M* DTT and 20% glycerol (buffer *A*). Cells were lysed by sonication on ice and centrifuged to remove cell debris. The supernatant was applied onto a glutathione Sepharose 4B (GS4B) column (Amersham Biosciences) and eluted with buffer *A* containing 20 m*M* glutathione pH 8.2. The eluate was treated with PreScission protease (Amersham Biosciences). After protease digestion, the N-terminus starts with residues Gly-Pro-Leu-Gly-Ser followed by the full-length sequence of Trn1 (GenBank accession No. U72069). The digest was purified by chromatography on HiTrap Q and Superdex-200 columns. The purified Trn1 was finally concentrated to 12 mg ml^{-1} in buffer *A* without glycerol using an Amicon Ultra-50 concentrator (Millipore).

Human TAP nuclear localization fragment (residues 61–102) was amplified in PCR with QuickClone HeLa cell cDNA (Clontech) as a template and cloned at a *Bam*HI/*Sal*I site of pGEX6P-1. The GST-fused TAP fragment was expressed in *E. coli* BL21(DE3) in the presence of 100 µ*M* IPTG at 303 K and purified by affinity chromatography. The eluate was treated with PreScission protease (Amersham Biosciences) and then purified on HiTrap SP and Superdex-75 columns. The TAP fragment was concentrated to 20 mg ml^{-1} in buffer *A* without glycerol using an Amicon Ultra-5.

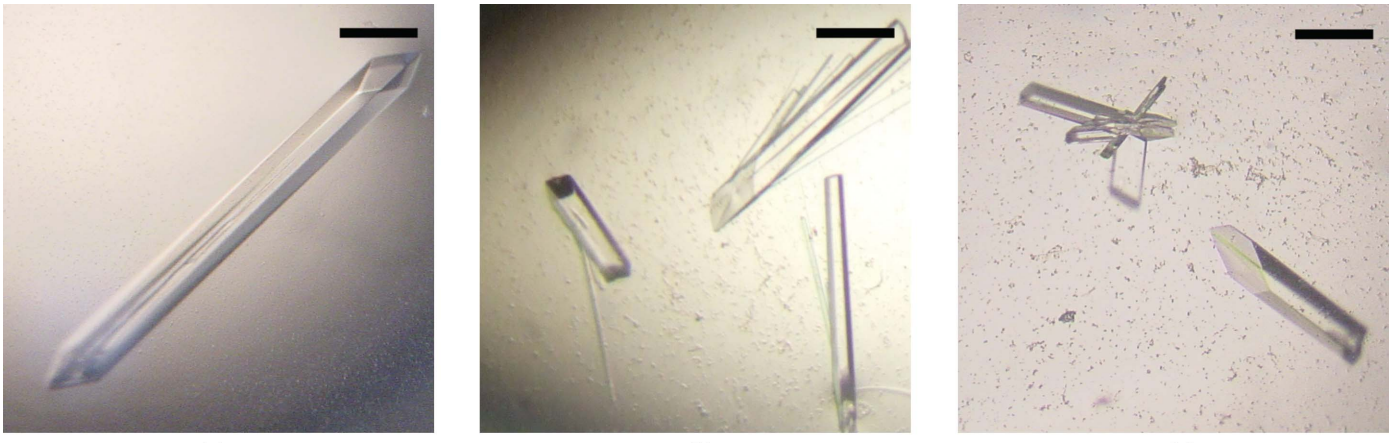
JKTBP nucleocytoplasmic shuttling fragment (residues 396–420) was chemically synthesized by the solid-phase method and purified by RP-HPLC. The fragment of hnRNP D (residues 332–355) was purchased from BEX Co. Ltd. These two peptide fragments were prepared at final concentration of 500 µ*M* in buffer *A* without glycerol.

2.2. Crystallization

Crystals of Trn1 in complex with the fragments of JKTBP, hnRNP D and TAP were prepared by cocrystallization. The Trn1–JKTBP fragment complex was prepared by mixing Trn1 at a final concentration of 5 mg ml^{-1} with a threefold molar excess of the JKTBP fragment and incubating on ice for more than 1 h. The complex was crystallized at 293 K by the sitting-drop vapour-diffusion method, equilibrating against 500 µl reservoir solution consisting of 100 m*M* Tris–HCl pH 8.4–8.8, 12–16%(*w*/*v*) PEG 20K, 100 m*M* NaH_2PO_4, and was grown by microseeding (Fig. 1*a*). Sitting drops (22 µl) were prepared by mixing 10 µl sample, 10 µl reservoir solution and 2 µl 150 m*M* phenol.

The Trn1–hnRNP D fragment complex was prepared by mixing Trn1 with a fivefold molar excess of the hnRNP D fragment at a final protein concentration of 5 mg ml^{-1}. The complex was crystallized at 293 K by the hanging-drop vapour-diffusion method, equilibrating against 200 µl reservoir solution consisting of 100 m*M* Tris–HCl pH 8.4–8.8, 20–24%(*w*/*v*) PEG 8K, 100 m*M* NaH_2PO_4 and 100 m*M* KCl (Fig. 1*b*). Hanging drops (4 µl) were prepared by mixing 2 µl sample and 2 µl reservoir solution.

The Trn1–TAP fragment complex was prepared by mixing Trn1 at a final concentration of 5 mg ml^{-1} with a threefold molar excess of the TAP fragment and incubating overnight at 293 K. The dissociation constant of Trn1 and the TAP fragment has been reported to be 18.2 n*M* (Chook *et al.*, 2002). A crystal of the complex was obtained at 293 K by the hanging-drop vapour-diffusion method, equilibrating against 200 µl reservoir solution consisting of 100 m*M* CAPSO pH 9.7–9.9, 14–18%(*w*/*v*) PEG 8K, 100 m*M* NaH_2PO_4 and 100 m*M* KCl (Fig. 1*c*). Hanging drops (4 µl) were prepared by mixing 2 µl sample and 2 µl reservoir solution.



(*a*) (*b*) (*c*)

Figure 1
Crystals of Trn1 in complex with the fragments of JKTBP (*a*), hnRNP D (*b*) and TAP (*c*). The scale bars represent 0.2 mm.

Table 1
Crystallographic data and data-collection statistics.

Values in parentheses are for the highest resolution shells (3.11–3.00 Å for Trn1–JKTBP, 3.31–3.20 Å for Trn1–hnRNP D and 2.49–2.40Å for Trn1–TAP).

	Trn1–JKTBP	Trn1–hnRNP D	Trn1–TAP
Wavelength (Å)	1.0000	1.0000	1.0000
No. of images	180	180	180
Oscillation per image (°)	1.0	1.0	1.0
Exposure time per image (s)	5.0	5.0	5.0
Space group	$P2_12_12$	$P2_12_12_1$	$P2_12_12_1$
Unit-cell parameters			
a (Å)	131.5	69.1	69.0
b (Å)	171.5	119.1	119.1
c (Å)	68.2	151.1	146.0
Resolution range (Å)	171.0–3.00	50–3.20	50–2.40
$R_{\rm merge}$† (%)	9.4 (38.1)	8.2 (54.1)	6.6 (56.1)
Average $I/\sigma(I)$	5.1 (1.1)	10.2 (1.7)	11.2 (2.0)
Multiplicity	4.8 (3.9)	5.7 (3.1)	6.0 (3.4)
No. of observations	149124	110635	283062
No. of unique reflections	31812	19610	47429
Data completeness (%)	98.5 (97.1)	91.9 (56.4)	98.4 (87.1)

† $R_{\rm merge} = \sum_h \sum_i I(h)_i - \langle I(h)\rangle|/\sum_h \sum_i I(h)_i$, where $I(h)$ is the observed intensity, $\langle I(h)\rangle$ is the mean intensity of a reflection h over all measurements of $I(h)$, $\sum_h$ is the sum over all measured reflections and $\sum_i$ is the sum over i measurements of a reflection.

2.3. Data collection and processing

For diffraction data collection at 100 K, crystals of the complexes with the JKTBP and TAP fragments were incubated in reservoir solution containing 20%(*v*/*v*) glycerol and that of the complex with the hnRNP D fragment was incubated in reservoir solution containing 15%(*v*/*v*) glycerol. All diffraction data were collected on the BL41XU beamline at SPring-8 (Hyogo, Japan) using an ADSC Quantum 315 CCD detector. The data from the Trn1–JKTBP fragment complex were processed with *PROCESS* (Pflugrath, 1999) and those from the Trn1–TAP fragment and Trn1–hnRNP D fragment complexes were processed with *HKL*-2000 (Otwinowski & Minor, 1997).

3. Results and discussion

Crystallographic data are given in Table 1. The crystal of the Trn1–JKTBP fragment complex belongs to space group $P2_12_12$, with unit-cell parameters $a = 131.5$, $b = 171.5$, $c = 68.2$ Å. The complexes of Trn1 with the hnRNP D and TAP fragments crystallized in space group $P2_12_12_1$, with unit-cell parameters $a = 69.1$, $b = 119.1$, $c = 151.1$ Å and $a = 69.0$, $b = 119.1$, $c = 146.0$ Å, respectively.

The presence of one Trn1 and one JKTBP fragment in the asymmetric unit gives a $V_{\rm M}$ value of 3.6 Å^3 Da^{-1} and a solvent content of 66.5%, which lie a little outside the range usually found for protein crystals (Matthews, 1968). The crystal of the Trn1–TAP fragment complex is fragile, indicating that the solvent content of the crystal is high. On the assumption that one Trn1 and one TAP fragment are present in the asymmetric unit, the $V_{\rm M}$ and the solvent content were estimated to be 2.8 Å^3 and 57%, respectively. The crystal of the Trn1–hnRNP D fragment complex was almost isomorphous with that of Trn1–TAP fragment complex. Table 1 lists the data-collection statistics. Structure determinations of these complexes by molecular replacement using the Trn1 structure in Trn1–RanGppNHp (PDB code 1qbk; Chook & Blobel, 1999) as a search model are in progress. Initial difference electron-density maps of the three crystals confirm the presence of the bound peptides.

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