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Crystallization and preliminary crystallographic analysis of the importin-β–SREBP-2 complex

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The nuclear-transport protein importin-β mediates the nuclear import of the transcription factor SREBP-2 without requiring adaptor proteins such as importin-α. An importin-β–SREBP-2 HLHZ domain complex was purified and crystallized. The crystals belong to space group $P2_12_12_1$ and show diffraction to at least 3.0 Å resolution. The unit-cell parameters are a = 101.0, b = 113.2, c = 240.0 Å. Structure determination using the MAD or SAD method is under way.

1. Introduction

Macromolecules that are transported into or out of the nucleus contain a nuclear-localization signal (NLS) or nuclear-export signal, respectively. The trafficking of macromolecules between the cytoplasm and nucleus through nuclear pore complexes (NPCs) is mediated by shuttling of transport factors or carrier molecules that bind their cargo on one side and release it on the other. An increasing number of signals and their carrier molecules have been identified in recent years and it has become clear that both import and export pathways are mediated by the importin-β superfamily. These events are regulated by the small GTPase Ran. Cargo binding and release is regulated by asymmetric distribution of the two nucleotide states of Ran, the so-called RanGTP gradient (Corbett & Silver, 1997; Gorlich & Kutay, 1999; Mattaj & Englmeier, 1998; Nakielny & Dreyfuss, 1999; Nigg, 1997). Importin-β is one of the best characterized transport factors in the importin-β superfamily and has been found to be unique within the receptor family because it recognizes nuclear proteins *via* adaptor molecules such as importin-α. In addition to the adaptor molecule-dependent pathway, importin-β also mediates the nuclear import of certain cargoes without requiring adaptor proteins. These cargo molecules include ribosomal proteins (Jakel & Gorlich, 1998), HIV Rev and Tat (Truant *et al.*, 1999), Rex protein of human T-cell leukaemia virus type 1p (Palmeri & Malim, 1999), GAL4 (Chan *et al.*, 1998), PTHrP precursor protein (Lam *et al.*, 1999), cyclin B1 (Moore *et al.*, 1999) and sterol regulatory element-binding protein 2 (SREBP-2; Nagoshi *et al.*, 1999; Nagoshi & Yoneda, 2001).

Studies on the protein-import mechanisms with no canonical NLS have extended our understanding of nuclear transport. Among these is the nuclear import of SREBP-2. SREBP-2 is a member of the SREBP family of transcription factors containing the basic helix–loop–helix leucine zipper (bHLH-Zip) motif (Brown & Goldstein, 1997). Unlike other bHLH-Zip transcription factors, SREBPs are synthesized as precursor molecules which are bound to the endoplasmic reticulum (ER) membrane and outer nuclear envelope in a hairpin orientation, with the N- and C-terminal regulatory segments projecting into the cytoplasm and the hydrophilic loop projecting into the lumen. The cytosolic N-terminal segment includes the bHLH-Zip domain, while the C-terminal regulatory segment interacts with a polytopic membrane protein, designated SREBP cleavage-activating protein (SCAP; Sakai *et al.*, 1997, 1998). When cells are deprived of cholesterol, SCAP escorts SREBPs to a post-ER component to reach the Golgi apparatus, where the site 1 protease (S1P) makes the first cut in the luminal loop, followed by a second cleavage at site 2 within the first membrane-spanning segment (Sakai *et al.*, 1998). This proteolytic processing liberates the transcriptionally active N-terminal fragment, designated the active form of the SREBP, from the membrane. The active form of the SREBP then enters the nucleus and activates a number of genes which control the synthesis and uptake of cholesterol and unsaturated fatty acids.

To date, one full-length importin-β crystal structure and four N-terminal half importin-β related structures have been reported. These structures include importin-β complexed with cargo molecules (Cingolani *et al.*, 1999, 2002), with RanGTP (Vetter *et al.*, 1999), with an FG-repeat protein (Bayliss *et al.*, 2000) or in a free form (Lee *et al.*, 2000).

In previous reports, a variety of *in vivo* and *in vitro* experiments showed that importin-β directly interacts with SREBP-2 and mediates import in a Ran-dependent manner (Nagoshi *et al.*, 1999; Nagoshi & Yoneda, 2001). In this

paper, the crystallization and preliminary crystallographic analysis of the importin-β–SREBP-2 HLHZ domain complex are reported. The structure of these complex proteins is helpful in understanding the nuclear-import mechanism of transcription factors.

2. Expression and purification

Full-length mouse importin-β, consisting of 876 residues, was cloned into the glutathione-*S*-transferase (GST) gene-fusion vector pGEX-2T. The expression of recombinant importin-β was induced in LB medium by 1 m*M* IPTG for 14 h at 293 K. Cells expressing fusion proteins were disrupted using ultrasonication on ice three times, each for 1 min. The fusion protein was purified from the lysates using glutathione-Sepharose (Pharmacia) affinity chromatography. After cleavage of the GST portion with thrombin, importin-β was further purified by MonoQ (Pharmacia) anion-exchange chromatography.

Human SREBP-2, including the helix–loop–helix leucine zipper domain (residues 343–460), was cloned into a GST gene-fusion vector, pGEX-6P-3. The expression of recombinant SREBP-2(343–460) was induced by 0.6 m*M* IPTG for 5 h at 303 K. Cells were disrupted using ultrasonication on ice three times, each for 1 min. Recombinant SREBP-2(343–460) was purified using glutathione-Sepharose affinity gels. After cleavage of the GST portion with precession protease, SREBP-2(343–460) was further purified by a Superdex 200 (Pharmacia) molecular-sieve gel-chromatography column. For the preparation of the importin-β–SREBP-2(343–460) complex, purified importin-β and SREBP-2(343–460) samples were mixed and incubated for 10 min and injected into a Superdex 200 (Pharmacia) molecular-sieve gel-chromatography column. For the complete separation of complex proteins from free importin-β proteins, a large amount of SREBP-2(343–460) was used. Two protein peaks, a peak from the complex of importin-β and SREBP-2(343–460) and a peak from residual SREBP-2(343–460), were confirmed by SDS–PAGE. Previously, we have reported that the active form of SREBP-2 exists as a stable dimer in solution and that the import-active complex is composed of a dimeric form of SREBP-2 and importin-β (Nagoshi & Yoneda, 2001). The final complex-sample buffer consisted of 20 m*M* HEPES–NaOH pH 7.3, 5 m*M* NaCl and 2 m*M* DTT. Both the preparation of SREBP-2(343–403) and the formation of the importin-β–SREBP-2(343–403) dimer complex were achieved using the same protocols as used for SREBP-2(343–460).

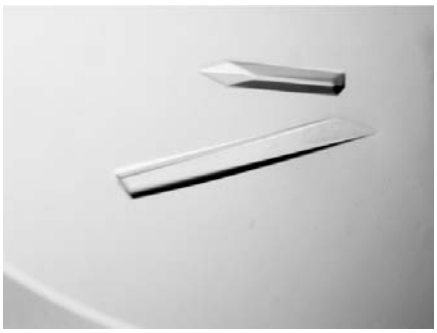

Figure 1
An example of crystals of the importin-β–SREBP-2(343–403) complex. The crystal dimensions are 0.5 × 0.08 × 0.08 mm. The crystal diffracted X-rays to at least 3.0 Å resolution.

3. Crystallization

Crystals of the complex of full-length mouse importin-β and human SREBP-2 (residues 343–460) were grown by the hanging-drop vapour-diffusion technique. Proteins were concentrated by centrifugation to a final concentration of 6 mg ml^{-1} in 20 m*M* HEPES buffer pH 7.3, 5 m*M* NaCl and 2 m*M* DTT as described above. The precipitant reservoir solution contained 50 m*M* MES buffer pH 5.8 with 2–3% PEG 8000, 20–30 m*M* $MgCl_2$ and 20% glycerol. The drop buffer (50 m*M* MES buffer pH 5.8 with 2–3% PEG 8000, 20–30 m*M* $MgCl_2$ and 10% glycerol) was prepared for mixing with an equal volume of protein buffers. Needle-shaped crystals appeared in a week at the edge of drops and grew for four weeks.

Crystals of the complex of full-length importin-β and SREBP-2 (residues 343–403) were grown by the hanging-drop vapour-diffusion technique. Proteins were dissolved in 20 m*M* HEPES buffer pH 7.3 with 2 m*M* DTT. 50 m*M* MES buffer pH 6.6 with 5–6% PEG 8000, 30 m*M* $SrCl_2$ and 20% glycerol was used as the reservoir solution. The precipitant solution contained 50 m*M* MES buffer pH 6.6 with 5–6% PEG 8000, 30 m*M* $SrCl_2$ and 10% glycerol. Equal volumes of 6 mg ml^{-1} protein solution and precipitant solution were mixed to make up a drop. Very small crystals appeared at 288 K within a week and crystals grew in rod shapes in four weeks to maximum dimensions of 0.6 × 0.08 × 0.08 mm (Fig. 1). The complex formation in crystal was confirmed by SDS–PAGE of dissolved crystals.

Table 1
Data-collection and processing statistics.

Crystal I and II are the experimental data for the importin-β–SREBP-2(343–460) complex and the importin-β–SREBP-2(343–403) complex, respectively. Values in parentheses are for the highest resolution shells.

	Crystal I	Crystal II
Space group	$C222_1$	$P2_12_12_1$
Unit-cell parameters		
a (Å)	123.7	101.3
b (Å)	189.0	113.4
c (Å)	112.9	240.1
Wavelength (Å)	0.9000	0.9000
Intensity data		
Resolution (Å)	41.7–3.5 (3.78–3.56)	40–3.0 (3.11–3.00)
Unique reflections	15811 (2503)	55607 (5450)
Redundancy	4.6 (4.6)	11.7 (4.3)
$I/\sigma(I)$	5.4 (2.4)	27.6 (2.9)
Completeness (%)	94.3 (92.6)	99.7 (99.5)
$R_{\rm merge}$	0.134 (0.333)	0.064 (0.300)

4. Data collection and analysis

All diffraction experiments were performed at the SPring-8 beamline BL44XU designed for biological macromolecular assemblies. The intensity data were acquired under cryogenic conditions (90 K) using a DIP6040 image-plate detector (Bruker) equipped with six 400 mm image plates. For the cryogenic diffraction experiment, glycerol was added to the crystallization buffer to 25%. Diffraction data were indexed, merged and scaled using *d*TREK* (Pflugrath, 1999) (crystal I in Table 1) and *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) (crystal II in Table 1). The importin-β–SREBP-2(343–460) complex crystal belongs to the orthorhombic space group $C222_1$, with unit-cell parameters a = 123.7, b = 189.0, c = 112.9 Å. This crystal diffracted to 3.5 Å (crystal 1 in Table 1). Because the importin-β–SREBP-2(343–460) complex crystals were severely stacked and diffraction was weak, we did not continue data collection. The value of the Matthews coefficient is 2.60 Å^3 Da^{-1} for one complex per asymmetric unit, corresponding to a solvent content of 52.8% (Matthews, 1968).

5. Conclusions

Diffraction experiments with the importin-β–SREBP-2(343–403) complex crystal were also performed at beamline BL44XU. The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 101.0, b = 113.2, c = 240.0 Å. The value of the Matthews coefficient is 3.0 Å^3 Da^{-1} for two complexes per asymmetric unit, corresponding to a solvent content of 58.7%. This crystal diffracted to a maximum of 3.0 Å and was suitable for structure determination

(crystal II in Table 1). Structure solution with the molecular-replacement method failed with model molecules using the importin-β structure (PDB code 1qgk) and the SREBP structure (PDB code 1amp). We are also preparing for expression of SeMet-substituted protein suitable for phase determination using the multiwavelength anomalous diffraction (MAD) or single-wavelength anomalous diffraction (SAD) method.

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