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Crystallization and preliminary X-ray diffraction analysis of human importin β -Snail zinc finger domain complex

Snail is a C2H2-type zinc finger transcriptional repressor that induces epithelial-mesenchymal transition by repression of E-cadherin expression levels during embryonic development and tumour progression. Snail is imported into the nucleus by importin β through direct binding with its four zinc finger domain. The complex between importin β and Snail four zinc finger domain was crystallized in order to understand the nuclear transport mechanism of Snail. The constituents of the complex were separately expressed and were then co-purified and crystallized by the hanging-drop vapour-diffusion method. The crystals belonged to space group *C*2, with unit-cell parameters $a = 228.2$, $b = 77.5$, $c = 72.0$ Å, $\beta = 100.9^\circ$ and diffracted to 2.5 Å resolution.

1. Introduction

Some epithelial cells that are non-motile can change their morphology to motile, fibroblast-like mesenchymal cells in a development process termed 'epithelial-mesenchymal transition' (EMT; Doble & Woodgett, 2007). This process is a normal embryonic development, but it is also fundamental to epithelial tumours with similar process mechanisms. One of the important changes in EMT progress is the down-regulation of E-cadherin, which is dependent on the overexpression of Snail. Snail is also related to various developmental processes, including neural differentiation, cell fate and left-right identity (Nieto, 2002; Peinado *et al.*, 2007).

Snail-family proteins are divided into Snail1, Snail2 and Snail3, which share evolutionarily conserved roles and structures in vertebrates. They are zinc finger transcription factors containing a SNAG domain in the N-terminal region and four to six highly conserved zinc finger domains in the C-terminal region. The repressor activity of Snail depends on the SNAG domain in cooperation with the zinc finger, and the zinc finger domain binds to DNA, recognizing specific DNA sequences or proteins (Peinado *et al.*, 2007).

In order to be activated, Snail must be imported into the nucleus by soluble transport factors. Although small molecules can pass through the nuclear pore complex (NPC) by passive diffusion, larger macromolecules require active transport systems facilitated by the interaction of transport factors (importins or exportins) with nuclear localization signals (NLS) or nuclear export signals (NES) of cargo proteins, respectively (Stewart, 2007). A recent study revealed that the zinc finger domain of Snail1 is recognized as an NLS by several importin family members, such as importin β , importin 7 and transportin (Mingot *et al.*, 2009). Importin α , an adaptor protein of importin β , inhibits nuclear import of Snail by competing against binding of importin β (Sekimoto *et al.*, 2011).

The full-length importin β structure has been determined with binding partners such as the importin β -binding (IBB) domain of importin α (Cingolani *et al.*, 1999), sterol regulatory element-binding protein 2 (SREBP-2; Lee *et al.*, 2003), RanGTP (Vetter *et al.*, 1999) and the N-terminal IBB of snurportin 1 (sIBB; Mitrousis *et al.*, 2008) by X-ray diffraction. However, prediction of the binding site of importin β with Snail protein is impossible owing to the flexibility of importin β . In this report, we describe the overexpression, purification, crystallization and preliminary X-ray diffraction analysis of the complex of importin β and Snail1 zinc finger domain.

2. Materials and methods

2.1. Cloning, expression and purification

Full-length human importin β (residues 1–876, molecular weight 97 170 Da, UniProt Q14974) was amplified by PCR with primers containing *Nde*I and *Xho*I restriction-enzyme sites and was subcloned into modified pET28-Tev vector containing a histidine tag and a *Tobacco etch virus* (TEV) protease cleavage site with sequence HHHHHHSSGENLYFQGH in the N-terminal region. The GST-fused zinc finger (ZF) domain of human Snail1 (residues 153–264, molecular weight 12 653 Da, UniProt O95863) was constructed with an amplified PCR fragment into the *Bam*HI and *Sal*I restriction-enzyme sites of the pGEX-4T-Tev vector, which expressed protein with a GST tag and a TEV protease cleavage site with sequence LVPDGSYDIPTTENLYFQGS. After TEV cleavage, non-native Gly and His (GH) residues and Gly and Ser (GS) residues remained before the importin β and the Snail ZF domain proteins, respectively. All constructs were confirmed by DNA sequence analysis (Solgent Co., Republic of Korea). The confirmed plasmids were transformed into *Escherichia coli* strain BL21(DE3) cells (Novagen). Importin β was precultured with fresh transformant for 5 h at 310 K in Luria-Bertani (LB) medium containing 50 $\mu\text{g ml}^{-1}$ kanamycin and was then inoculated into 2 $\times$ YT medium. Overproduction was induced by the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) for 16 h at 293 K. Snail ZF domain overproduction was induced with 0.5 mM IPTG and 1 mM ZnCl_2 at 298 K for 5 h. The cells were harvested by centrifugation at 6000 rev min $^{-1}$ for 10 min at 277 K and were stored at 193 K.

Importin β and Snail ZF domain cells were mixed and resuspended in buffer A [20 mM Tris-HCl pH 7.4, 300 mM NaCl, 1 mM dithiothreitol (DTT)] including 0.1 mM phenylmethanesulfonyl fluoride and were then lysed by sonication on ice. After centrifugation at 18 000 rev min $^{-1}$ for 40 min, the supernatant was purified with Phenyl Sepharose (GE Healthcare) and eluted with distilled water. The eluted sample was mixed with the same volume of buffer A and loaded onto Glutathione Sepharose 4 Fast Flow beads (GE Healthcare). The eluted sample was obtained with buffer A including 10 mM reduced glutathione and was incubated with TEV protease at 293 K overnight. To eliminate the GST tag, the sample was loaded onto Phenyl Sepharose again and eluted with distilled water. The complex was finally purified with a Superdex 200 26/60 (GE Healthcare) gel-filtration column which had been pre-equilibrated with 20 mM Tris-HCl pH 7.4, 1 mM DTT. The purified protein sample was concentrated to 30 mg ml $^{-1}$ with a centrifugal concentrator (Sartorius) and was stored at 277 K for crystallization. The protein concentration was determined by measuring the absorbance at 280 nm.

2.2. Crystallization and X-ray data collection

Crystallization of the importin β -Snail ZF domain complex was performed by the hanging-drop method using 2 μl drops consisting of equal volumes of protein solution and reservoir solution equilibrated against 400 μl reservoir solution at 293 K. The initial screening was performed using a PEG screen kit designed in our laboratory. A needle cluster-type crystal formed in 0.1 M Tris-HCl pH 7.5, 10% (w/v) PEG 8000, 20% (v/v) glycerol using a protein sample at 10 mg ml $^{-1}$; however, this was not reproducible. Therefore, we designed a pET28a-TEV-importin β vector for the elimination of 50 additional non-native N-terminal amino acids from the expression vector. The new subcloned importin β was co-purified with Snail ZF domain and was concentrated to 20 mg ml $^{-1}$. To stabilize the protein complex, 10% (v/v) glycerol was added directly to the concentrated protein

solution. The protein solution was then subjected to screening with a PEG screen kit as described previously (Song *et al.*, 2011). A needle cluster-type crystal appeared in a similar mother liquor to the initial crystallization condition within 2 d. The reservoir buffer consisted of 0.1 M bis-tris-HCl pH 6.9, 10% (w/v) PEG 8000, 0.5 M urea, 20% (v/v) glycerol. The protein composition of the crystal was confirmed by SDS-PAGE of dissolved crystals. A more concentrated protein sample (30 mg ml $^{-1}$) was suitable for improving the thickness of the crystals. The crystals grew to maximum dimensions (0.5 $\times$ 0.06 $\times$ 0.03 mm; Fig. 1) in a week. To obtain atomic resolution diffraction, we performed repeated detergent screenings under quite similar conditions. By adding *N*-nonyl β -D-glucoside to 0.5 CMC (critical micelle concentration), the reproducibility of single crystals was highly improved. Soaking in cryoprotectant for 10 min at 293 K was effective in improving the diffraction quality of the crystals. The final crystallization reservoir condition was refined to 0.1 M bis-tris-HCl pH 6.7, 11.5% (w/v) PEG 8000, 0.5 M urea, 15% (v/v) glycerol, 0.65 mM *N*-nonyl β -D-glucoside with 30 mg ml $^{-1}$ protein solution containing 20 mM Tris-HCl pH 7.4, 1 mM DTT, 7.5% glycerol. The final cryoprotectant solution consisted of 0.2 M bis-tris-HCl pH 6.9, 22% (w/v) PEG 8000, 1 M urea, 15% (v/v) glycerol, 10% (w/v) PEG 400 with 1 CMC of *N*-nonyl β -D-glucoside. Data sets were collected using a Rayonix MX-225HE CCD detector 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

Full-length importin β and Snail ZF domain were overexpressed in 2 $\times$ YT medium and co-purified. In the early stages, about 80% of the Snail ZF domain was lost during purification owing to the instability of the Snail ZF domain protein. Snail ZF domain was easily aggregated and stuck to Glutathione Sepharose, Phenyl Sepharose and Superdex 200 columns. Finally, we obtained 5 mg of importin β -Snail ZF domain complex from 9 l of importin β and 6 l of Snail ZF domain cell cultures. As the formation of the complex is disturbed by high salt concentrations, we used the final protein solution in the absence of salt for crystallization. If salt remained in the protein solution there was no crystal formation.

Early-stage crystals were formed in 0.1 M Tris-HCl pH 7.5, 10% (w/v) PEG 8000, 20% (v/v) glycerol with a protein solution at 10 mg ml $^{-1}$, but this was found to not be reproducible. After changing the importin β expression vector in order to remove extra

Figure 1
Crystals of the importin β -Snail zinc finger domain complex.

Figure 2
A diffraction image from the importin β -Snail zinc finger domain complex crystals.

residues and altering the concentration of the protein, a needle-type crystal was produced under similar conditions. To improve the crystal quality, additive and detergent screening was performed; however, this screening was not effective. The importin β -Snail zinc finger domain complex crystal only diffracted to 4.5 Å resolution and had a thickness of 0.02 mm. By concentrating the protein sample to 30 mg ml⁻¹, thicker crystals could be formed. Decreasing the glycerol content was also helpful in decreasing the crystal-growth rate. Under these altered conditions, single thick crystals were formed with the addition of sorbitol, inositol, D-galactose or *N*-nonyl β -D-glucoside from the additive and detergent screening. The presence of the zinc finger domain in the crystals was confirmed by an absorption-edge scan at the beginning of the diffraction experiment. We screened many crystals to improve the diffraction resolution. Finally, a suitable crystal was obtained using a reservoir buffer consisting of 0.1 M bis-tris-HCl pH 6.7, 11.5% (w/v) PEG 8000, 0.5 M urea, 15% (v/v) glycerol, 0.65 mM *N*-nonyl β -D-glucoside. X-ray data sets were collected on the BL44XU beamline at Spring-8, Harima, Japan and extended to 2.5 Å resolution (Fig. 2). Diffraction data were indexed in space group C2, with unit-cell parameters $a = 228.2$, $b = 77.5$,

Table 1

Summary of data-collection statistics.

Values in parentheses are for the outermost resolution shell.

Temperature (K)	100
Oscillation range (°)	1
Space group	C2
Wavelength (Å)	1.28226
Unit-cell parameters (Å, °)	$a = 228.2$, $b = 77.5$, $c = 72.0$, $\alpha = 90.0$, $\beta = 100.9$, $\gamma = 90.0$
Resolution range (Å)	2.51 (2.55–2.51)
Total reflections	168158
Unique reflections	41574 (2083)
Multiplicity	4.1 (4.0)
Completeness (%)	97.5 (99.2)
$R_{\text{merge}}^{\dagger}$ (%)	0.064 (0.415)
Mean $I/\sigma(I)$	34.7 (5.4)

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl) \rangle$ is the mean intensity of the reflection.

$c = 72.0$ Å, $\alpha = 90.0$, $\beta = 100.9$, $\gamma = 90.0^\circ$. One molecule is present in the asymmetric unit, with a solvent content of 60.0% (Matthews, 1968; Table 1). Structure determination is under way.

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