Crystallization and preliminary X-ray crystallographic analysis of importin- α from *Neurospora crassa*

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Importin- α recognizes cargo proteins that contain classical nuclear localization sequences (NLS) and, in complex with importin- β , is able to translocate nuclear proteins through the nuclear pore complex. The filamentous fungus *Neurospora crassa* is a well studied organism that has been widely used as a model organism for fundamental aspects of eukaryotic biology, and is important for understanding the specific mechanisms of protein transport to the cell nucleus. In this work, the crystallization and preliminary X-ray diffraction analysis of importin- α from *N. crassa* (IMP α -Nc) complexed with a classical NLS peptide (SV40 NLS) are reported. IMP α -Nc-SV40 NLS crystals diffracted X-rays to 2.0 Å resolution and the structure was solved by molecular-replacement techniques, leading to a monomeric structure. The observation of the electron-density map indicated the presence of SV40 NLSs interacting at both the minor and major NLS-binding sites of the protein.

1. Introduction

The nuclear envelope present in eukaryotic cells allows the separation of the genetic material and the transcriptional machinery from the translational and metabolic processes in the cytoplasm. Nuclear proteins are synthesized in the cytoplasm and need to be transported into the nucleus through the nuclear pore complexes (NPCs). This translocation process usually occurs by active transport through factors termed karyopherins (importins and exportins; Görlich & Kutay, 1999). A pathway involved in this process is the 'classical' nuclear transport pathway which depends on an importin- α –importin- β heterodimer and requires a positively charged sequence within the cargo protein, known as a nuclear localization sequence (NLS; Lange *et al.*, 2007). Importin- α (IMP α) recognizes the cargo proteins that contain an NLS and importin- β (IMP β) is responsible for transport of the importin–cargo protein complex through the NPC.

Classical NLSs contain one (monopartite) or two (bipartite) clusters of positively charged amino acids, which are recognized by the IMP α . This protein receptor has two NLS-binding sites formed by conserved residues, known as the major and minor NLS-binding sites. The N- and C-terminal clusters of a bipartite NLS interact with the minor and major NLS-binding sites, while a monopartite NLS interacts only with the major binding site. The crystal structure of full-length IMP α showed that the major NLS-binding site is occupied by residues from its N-terminal region (IMP β -binding domain) that resembles an NLS (Kobe, 1999); thus, IMP α is auto-inhibited in the absence of IMP β . Therefore, N-terminally truncated IMP α has been used as a model for an IMP α –IMP β heterodimer in several crystal structures, since the truncated protein may simulate an IMP α –IMP β heterodimer (Rexach & Blobel, 1995; Conti & Kuriyan, 2000; Fontes *et al.*, 2000).

Crystal structures of IMP α from *Saccharomyces cerevisiae* (Conti *et al.*, 1998), *Oryza sativa* (Chang *et al.*, 2012), *Mus musculus* (Kobe, 1999) and *Homo sapiens* (Dias *et al.*, 2009) have been solved, showing they are built from repetitive sequences (armadillo repeats) and belong structurally to the class of proteins termed solenoid proteins (Kobe & Kajava, 2011), having sequence identities in the range 41–64%. The structural differences among IMP α from different organisms, although small, have fundamental importance for the specificity

in the transport of cargo proteins. The presence of different IMP α isoforms that are involved in the transport of different proteins highlights the importance of specificity in nuclear transport.

Neurospora crassa has been well studied in terms of classical and molecular genetics, and together with the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* has been much used as a model organism for fundamental aspects of eukaryotic biology. Nevertheless, compared with yeasts, *N. crassa* has developed greater morphological and developmental complexity as a consequence of its larger number of genes. Comprehension of the nuclear import mechanism in fungal cells could be an important tool for understanding their metabolism and growth (Stinnett *et al.*, 2007; Cupertino *et al.*, 2012), enabling the future applications of fungi in the development of specific drugs and treatments for infections and for applications in the biotechnological industry.

In order to obtain structural and functional information on the nuclear transport process in *N. crassa*, we recently cloned the cDNA encoding the N-terminally truncated importin- α from *N. crassa* (IMP α -Nc), produced the recombinant protein in *Escherichia coli* and performed biophysical experiments and bioinformatics studies (Takeda *et al.*, 2013). On the basis of the theoretical model proposed in this previous study and a sequence alignment among the IMP α s from different organisms, it was suggested that the presence of nonconserved amino acids relatively close to the NLS-binding region may be related to the binding specificity of the IMP α -Nc to cargo proteins (Takeda *et al.*, 2013).

In the present work, we report the crystallization experiments, X-ray diffraction data collection at 2 Å resolution and molecular-replacement solution of the N-terminally truncated importin- α from *N. crassa* bound to a classic NLS peptide (simian virus 40 large tumour antigen NLS peptide; SV40 NLS), aiming to obtain structural insights into the specific mechanisms of protein transport to the cell nucleus in *N. crassa*.

2. Materials and methods

2.1. Protein production and purification

The recombinant N-terminally truncated IMP α -Nc (75–549) was produced in LB medium as a hexa-His-fusion protein using the *E. coli* host strain Rosetta (DE3) pLysS (Novagen) as described previously (Takeda *et al.*, 2013). The protein was purified on a nickel-affinity column using an ÄKTAprime purification system (GE Healthcare) and eluted in a 0.15–3.0 M imidazole linear gradient, followed by

dialysis to remove imidazole and to change the buffer. Samples were concentrated to 12 mg ml⁻¹ and stored in a buffer composed of 20 mM Tris-HCl pH 8.0, 100 mM NaCl at -20°C. The purity was estimated to be 98% by SDS-PAGE.

2.2. Synthesis of the NLS peptide

The peptide corresponding to the simian virus 40 large tumour antigen NLS peptide (SV40 NLS; ¹²⁶PKKKRKV¹³²) was synthesized by Proteimax (Brazil) with purity higher than 95%. The peptide contains N- and C-terminal residues additional to the minimal identified NLSs to avoid artifactual binding at the termini (Fontes *et al.*, 2000).

2.3. Crystallization experiments

Crystallization experiments were performed using a protein concentration of 12 mg ml⁻¹ and a peptide:protein molar ratio of 8 for SV40 NLS peptide. Initial attempts to crystallize native and NLS-complexed IMP α -Nc were conducted with similar crystallization conditions used for IMP α from different organisms (Fontes *et al.*, 2000), but no crystal hits were obtained. The sparse-matrix method (Jancarik & Kim, 1991) was then used to screen crystallization conditions using several commercial kits, including Crystal Screen, Crystal Screen 2 and PEG/Ion from Hampton Research, The JSCG Suite from Qiagen and the PACT-HTS crystal screen from Jena Bioscience, using both native and NLS-complexed IMP α -Nc. The experiments were performed using sitting-drop vapour diffusion (McPherson, 1982), with MRC 2 Well Crystallization Plates (Swissci) and the Oryx4 automated crystallization system from Douglas Instruments.

No crystals were obtained for native IMP α -Nc, but protein crystals for IMP α -Nc with NLS SV40 peptide were found in two different conditions: (i) 0.2 mM sodium phosphate dibasic dihydrate, 20% (w/v) polyethylene glycol 3350 and (ii) 0.2 mM Bicine pH 8.5, 20% (w/v)

Figure 1
Crystal of the IMP α -Nc-SV40 NLS peptide complex.

Figure 2
Diffraction pattern image of the IMP α -Nc-SV40 NLS peptide complex.

Table 1

X-ray diffraction data-collection and processing statistics.

Values in parentheses are for the highest resolution shell. Data were processed using the *HKL-2000* suite (Otwinowski & Minor, 1997).

Unit-cell parameters (Å)	$a = 44.7, b = 63.6, c = 180.4$
Space group	$P2_12_12_1$
Resolution (Å)	40.0–2.00 (2.07–2.00)
Unique reflections	32551
Completeness (%)	91.4 (89.8)
$R_{\text{merge}}^{\dagger}$ (%)	9.8 (9.23)
Radiation source	D03B-MX1 station, LNLS
Data-collection temperature (°C)	–173
Average $I/\sigma(I)$	14.7 (4.7)
Multiplicity	10.4 (9.9)
Mosaicity	0.71
Matthews coefficient V_M (Å ³ Da ^{–1})	2.42
Molecules in the asymmetric unit	1
Solvent content (%)	49.1

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual measurement of the reflection with Miller indices hkl and $\langle I(hkl) \rangle$ is the mean intensity of that reflection. Calculated using reflections with $I > -3\sigma(I)$.

polyethylene glycol 6000 [conditions from the PEG/Ion crystallization kit (Hampton Research) and The JSCG Suite (Qiagen), respectively]. Despite the crystals from these two conditions showing a diffraction pattern when submitted to X-ray diffraction experiments, crystals from condition (ii) only diffracted to low resolution. After optimization of condition (i), the best crystals were obtained using reservoir solution (50 µl) consisting of 0.15 mM sodium phosphate dibasic dihydrate, 20% (w/v) polyethylene glycol 3350 at 277 K after approximately 1 week. The crystallization drops were composed of a mixture of 1 µl protein solution, 0.5 µl peptide solution and 0.5 µl reservoir solution. Fig. 1 shows a typical crystal obtained from this condition.

2.4. X-ray data collection and processing

X-ray diffraction data were collected from a single IMP α -Nc–SV40 NLS crystal at a wavelength of 1.58 Å using a synchrotron-radiation source (D03B-MX1 beamline, Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) and a MAR CCD image-plate detector (MAR Research). A crystal was mounted in a nylon loop and flash-cooled in a stream of nitrogen at –173°C using 10% (v/v) polyethylene glycol 400 as a cryoprotectant. The crystal-to-detector distance was 70 mm and an oscillation range of 1.5° was used, resulting in the collection of a total of 241 images (Fig. 2). The data were processed using the *HKL-2000* suite (Otwinowski & Minor, 1997).

3. Results and discussion

The data-collection statistics are shown in Table 1. The crystals belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 44.7, b = 63.6, c = 180.4$ Å. They are not isomorphous to the *M. musculus* importin- α –SV40 NLS complex (Fontes *et al.*, 2000) or to other *M. musculus* importin- α –monopartite NLS complexes (Takeda *et al.*, 2011). The diffraction images showed a pattern probably related to a major crystal surrounded by satellite crystals (Fig. 2); therefore, a wide-angle data set (360°) was collected in order to increase the completeness. The data set is 91.4% complete at 2.0 Å resolution, with an R_{merge} of 9.8%. Calculations based on the protein molecular weight indicate the presence of one molecule in the asymmetric unit. This corresponds to a Matthews coefficient (Matthews, 1968) V_M of 2.42 Å³ Da^{–1} with a calculated solvent content of 49.1%. These values are within the range for typical

protein crystals, assuming a value of 0.74 cm³ g^{–1} for the protein partial specific volume.

The crystal structure of IMP α -Nc–SV40 NLS was determined using molecular-replacement techniques implemented in *Phaser* (McCoy *et al.*, 2007) using as a model the coordinates of importin- α from *M. musculus* complexed to nucleoplasmin NLS (PDB entry 3ull, chain B; Marfori *et al.*, 2011) as the search model, which confirmed the presence of a monomer in the asymmetric unit. Inspection of the electron-density map indicated the presence of the SV40 NLS peptide at both minor and major NLS sites of the protein. The monomeric conformation obtained in the IMP α -Nc–SV40 NLS complex (this study) is in agreement with previous results for IMP α -Nc bound to NLS peptides (Takeda *et al.*, 2013) using analytical size-exclusion chromatography experiments, electrophoresis and dynamic light-scattering experiments. The monomeric conformation is a common characteristic of several N-terminally truncated importins- α from several organisms (Kobe, 1999; Dias *et al.*, 2009; Chang *et al.*, 2012). N-terminally truncated yeast importin- α is an exception, in which stabilization of the dimeric structure is achieved by the formation of an extensive dimeric interface which includes the linker-binding region of bipartite NLSs (Conti *et al.*, 1998).

In the present study, we also observed that it was impossible to crystallize N-terminally truncated IMP α -Nc without a ligand. This result is in agreement with our previous study (Takeda *et al.*, 2013), in which we demonstrated that N-terminally truncated unbound IMP α -Nc presented lower stability and has the tendency to form aggregates. This protein behaviour was also observed for N-terminally truncated importin- α from *Xenopus laevis* (Falces *et al.*, 2010). Indeed, attempts to crystallize importin- α from *M. musculus* without any ligand (Takeda, 2009; Mynott, 2009) were also unsuccessful.

Finally, despite the conservation of key residues among importins- α from different organisms (Takeda *et al.*, 2013), the presence of nonconserved amino acids close to the NLS-binding site may influence the binding of IMP α -Nc to specific cargo proteins. The crystal structure of IMP α -Nc may add important elements to the role of importin- α in the protein transport to the nucleus.

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References

- Chang, C.-W., Couñago, R. L. M., Williams, S. J., Bodén, M. & Kobe, B. (2012). *Plant Cell*, **24**, 5074–5088.
- Conti, E. & Kuriyan, J. (2000). *Fold. Des.* **8**, 329–338.
- Conti, E., Uy, M., Leighton, L., Blobel, G. & Kuriyan, J. (1998). *Cell*, **94**, 193–204.
- Cupertino, F. B., Freitas, F. Z., de Paula, R. M. & Bertolini, M. C. (2012). *PLoS One*, **7**, e44258.
- Dias, S. M., Wilson, K. F., Rojas, K. S., Ambrosio, A. L. & Cerione, R. A. (2009). *Nature Struct. Mol. Biol.* **16**, 930–937.
- Falces, J., Arregi, I., Konarev, P. V., Urbaneja, M. A., Svergun, D. I., Taneva, S. G. & Bañuelos, S. (2010). *Biochemistry*, **49**, 9756–9769.
- Fontes, M. R., Teh, T. & Kobe, B. (2000). *J. Mol. Biol.* **297**, 1183–1194.
- Görllich, D. & Kutay, U. (1999). *Annu. Rev. Cell Dev. Biol.* **15**, 607–660.
- Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
- Kobe, B. (1999). *Nature Struct. Biol.* **6**, 388–397.
- Kobe, B. & Kajava, A. V. (2011). *Curr. Opin. Struct. Biol.* **11**, 725–732.
- Lange, A., Mills, R. E., Lange, C. J., Stewart, M., Devine, S. E. & Corbett, A. H. (2007). *J. Biol. Chem.* **282**, 5101–5105.
- Marfori, M., Mynott, A., Ellis, J. J., Mehdi, A. M., Saunders, N. F., Curmi, P. M., Forwood, J. K., Bodén, M. & Kobe, B. (2011). *Biochim. Biophys. Acta*, **1813**, 1562–1577.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

- McCoy, A. J., Grosse-Kunstleve, R. W., Adams, P. D., Winn, M. D., Storoni, L. C. & Read, R. J. (2007). *J. Appl. Cryst.* **40**, 658–674.
- McPherson, A. (1982). *Preparation and Analysis of Protein Crystals*. New York: Wiley.
- Mynott, A. V. (2009). PhD thesis, University of New South Wales, Australia.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Rexach, M. & Blobel, G. (1995). *Cell*, **83**, 683–692.
- Stinnett, S. M., Espeso, E. A., Cobeño, I., Araújo-Bazán, L. & Calvo, A. M. (2007). *Mol. Microbiol.* **63**, 242–255.
- Takeda, A. A. S. (2009). PhD thesis, Universidade Estadual Paulista Júlio De Mesquita Filho – UNESP, Brazil.
- Takeda, A. A., de Barros, A. C., Chang, C.-W., Kobe, B. & Fontes, M. R. (2011). *J. Mol. Biol.* **412**, 226–234.
- Takeda, A. A. S., Freitas, F. Z., Magro, A. J., Bernardes, N. E., Fernandes, C. A. H., Gonçalves, R. D., Bertolini, M. C. & Fontes, M. R. M. (2013). *Protein Pept. Lett.* **20**, 8–16.