The coiled-coil domain of glycosomal membrane-associated *Leishmania donovani* PEX14: cloning, overexpression, purification and preliminary crystallographic analysis

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The glycosomal membrane-associated *Leishmania donovani* protein PEX14, which plays a crucial role in protein import from the cytosol to the glycosomal matrix, consists of three domains: an N-terminal domain where the signalling molecule binds, a transmembrane domain and an 84-residue coiled-coil domain (CC) that is responsible for oligomerization. CCs are versatile domains that participate in a variety of functions including supramolecular assembly, cellular signalling and transport. Recombinant PEX14 CC was cloned, overexpressed, affinity-purified with in-column thrombin cleavage and further purified by size-exclusion chromatography. Crystals that diffracted to 1.98 Å resolution were obtained from a condition consisting of 1.4 M sodium citrate tribasic dihydrate, 0.1 M HEPES buffer pH 7.5. The crystals belonged to the monoclinic space group C2, with unit-cell parameters $a = 143.98$, $b = 32.62$, $c = 95.62$ Å, $\beta = 94.68^\circ$. Structure determination and characterization are in progress.

1. Introduction

The kinetoplastid parasite *Leishmania* is the causative agent of a diverse spectrum of human diseases collectively known as leishmaniasis. Kinetoplastids possess several novel molecular, biochemical and structural features that are not present in mammalian cells (Sloof & Benne, 1993). The glycosomes, which are surrounded by a single phospholipid bilayer membrane and are related to the peroxisomes of higher eukaryotes and the glyoxysomes of plants (Michels & Hannaert, 1994; Opperdoes & Borst, 1977; Opperdoes & Michels, 1993), compartmentalize a multitude of indispensable metabolic and biosynthetic pathways that include glycolysis, purine salvage, ether-lipid biosynthesis and β -oxidation of fatty acids (Fairlamb, 1989; Michels *et al.*, 2000; van den Bosch *et al.*, 1992). The glycosomes and peroxisomes lack nucleic acid and protein translational machinery, and nuclear-encoded glycosomal/peroxisomal proteins are synthesized on cytosolic ribosomes and post-translationally imported to these microbodies utilizing topogenic signals termed peroxisomal targeting signals 1 and 2 (PTS1 and PTS2) located at the C-terminus and N-terminus, respectively (Purdue & Lazarow, 1994; Blattner *et al.*, 1992; Gould *et al.*, 1989; Lametschwandtner *et al.*, 1998; Swinkels *et al.*, 1991). Newly synthesized folded proteins (cargos) containing either the PTS1 or PTS2 signals, bound to cargo-laden receptors (PEX5 and PEX7; Brocard & Hartig, 2006; Lazarow, 2006), are trafficked to the glycosome surface and dock by binding with the membrane-associated protein peroxin-14 (PEX14). After binding of the cargo-laden receptor, PEX14 forms a large and transient or

dynamic membrane pore through which the cargos are transported into the glycosomal matrix, without any additional requirement for membrane potential or NTP-derived energy. The coiled-coil domain is believed to be involved in the formation of the PEX14 homomeric assembly and is essential for pore formation. In yeast and trypanosomes, knockdown of PEX14 using RNA interference causes the mis-targeting of PTS1 and PTS2 proteins to the cytosol and the disruption of peroxisome/glycosome biogenesis, leading to a lethal phenotype (Shimizu *et al.*, 1999; Kessler & Parsons, 2005). The glycosomal enzymes are thus novel attractive chemotherapeutic targets in kinetoplastids (Parsons *et al.*, 2001; Sommer & Wang, 1994).

A coiled coil, which consists of two or more α -helices that wrap around each other to form a superhelix, is a structural motif that is predominantly used as a platform for higher order assembly. This ubiquitous assembly, which accounts for ~3% of all coding sequences (Rackham *et al.*, 2010), is versatile and can undergo changes ranging from a few ångströms to nanometers to oligomer changes (Calladine *et al.*, 2013), and elucidation of the associated mechanisms is an active area of research (Srivastava *et al.*, 2013; Nayak *et al.*, 2016; Karade *et al.*, 2020).

In *L. donovani*, PEX14 includes an N-terminal signature motif containing a PEX5-binding motif (Purdue & Lazarow, 1994; Subramani *et al.*, 2000; Cyr *et al.*, 2008), a hydrophobic region and a coiled-coil (CC) motif (Albertini *et al.*, 1997; Will *et al.*, 1999; Brocard *et al.*, 1997; Jardim *et al.*, 2002; Cyr *et al.*, 2018). The 84-residue C-terminal domain (residues 244–327) is predicted to form a coiled-coil domain upon oligomerization by online coiled-coil identification software, *i.e.* COILS (Lupas *et al.*, 1991), Paircoil2 (McDonnell *et al.*, 2006), MARCOIL (Zimmermann *et al.*, 2018) and MultiCoil (Wolf *et al.*, 1997). Here, we report the cloning, overexpression, purification and crystallization of the coiled-coil domain of PEX14 (PEX14CC) with the aim of elucidating its functional mechanism.

2. Materials and methods

2.1. Cloning

The DNA encoding the coiled-coil domain was amplified by polymerase chain reaction (PCR). The PCR was comprised of *L. donovani* genomic DNA as a template, a gene-specific pair of primers (Table 1), *Taq* DNA polymerase, MgCl₂ and dNTPs. The PCR-amplified product was cloned into a T-overhang linear vector (pTZ57/R) and was subcloned into the bacterial cell expression vector pGEX-KG (Addgene, USA) between the BamHI and HindIII restriction sites. The final recombinant construct was confirmed by restriction double digestion followed by nucleotide sequencing (Sanger's method).

2.2. Overexpression

The recombinant expression plasmid was introduced into the *Escherichia coli* BL21 (DE3) pLysS expression host strain

Table 1

Macromolecule-production information.

Source of organism	<i>L. donovani</i>
DNA source	<i>L. donovani</i>
Forward primer	5'-GGATCCCCAGCCGCCCTCACTGAAGAA GTA-3'
Reverse primer	5'-AAGCTTTTCGCTGTGCTGTGTCGC CTC-3'
Cloning vector	pTZ57R/T
Expression vector	pGEX-KG
Expression host	<i>E. coli</i> BL21 (DE3) pLysS
Complete amino-acid sequence of the construct produced	PAALTEEVKRLQTELDEAKEALANERKKCA DLAVSAAKIRADKQQLSRANDRLTQQID GLKKDIEKLEREKSSAVGEATQTAE

for the production of intracellular PEX14 coiled-coil domain. The positive clone-containing colony was inoculated into Luria-Bertani (LB) medium supplemented with 100 µg ml⁻¹ ampicillin at 37°C and induced with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) on reaching an OD_{600 nm} of 0.6–0.8. After induction, the culture was grown for a further 10–12 h at 16°C. The cells were harvested by centrifugation and the pellet was resuspended in lysis buffer (50 mM Tris-HCl pH 8, 200 mM NaCl) in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) at 1 mM. The cells were lysed by sonication at 25% amplitude (cycles of 10 s on and 10 s off) for 30 min and the cell debris was removed by high-speed centrifugation for 30 min. All experiments were carried out at 4°C.

2.3. Purification

The cell-free lysate was passed through a GST-IGA column pre-equilibrated with equilibration buffer (50 mM Tris-HCl buffer pH 8, 200 mM NaCl) and washed with three column volumes of the buffer. 12 U of bovine thrombin per millilitre was added to the buffer and incubated for 10 h at 279 K. The GST-tag-cleaved PEX14CC construct was eluted in the same buffer and elution fractions were pooled before a second step of purification by size-exclusion chromatography on a HiLoad Superdex 16/600 S-200 column (GE Healthcare, USA) pre-equilibrated with the same buffer, using a decreased NaCl concentration (50 mM; Figs. 1a and 1b). The purity of the sample was checked at each step by 15% SDS-PAGE (Supplementary Fig. S1).

2.4. Crystallization and data collection

The initial crystal screening for the PEX14CC construct at 45 mg ml⁻¹ was performed at 288 K with a nanolitre micro-drop robotic system (Mosquito, TTP Labtech) using a 96-well sitting-drop tray and various commercially available crystallization screens (Crystal Screen, Crystal Screen 2, PEG/Ion, Index and the AmSO₄ Suite from Hampton Research and Qiagen). One of the conditions from Crystal Screen (condition D2) yielded microcrystals in 15 days. This condition was further optimized to produce separate crystals by altering the crystallization condition and the drop size ratio and volume, and by the use of additives; the best crystal was obtained when the condition was supplemented with 4% (v/v) 1,3-butanediol

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Temperature (K)	288
Protein concentration (mg ml ⁻¹)	45
Buffer composition of protein solution	50 mM Tris-HCl pH 8, 50 mM NaCl
Reservoir solution	1.4 M sodium citrate tribasic dihydrate, 0.1 M HEPES-Na pH 7.5, 4% 1,3-butanediol
Volume and ratio of drop	4 µl, 2:2
Volume of reservoir (µl)	500

Figure 1
Purification and SDS-PAGE analysis of recombinant PEX14CC. (a) SDS-PAGE analysis of recombinant PEX14CC after the purification step. Molecular-weight markers (lane 1; labelled in kDa) and purified recombinant PEX14CC protein (lane 2) are shown. The protein band on 15% SDS-PAGE shows a size of >10 kDa owing to low electrophoretic mobility of the protein. (b) Size-exclusion chromatography of PEX14CC on a HiLoad Superdex 200 16/600 column. The fraction at 106 ml corresponds to a monomer, while the fractions at 87 and 49 ml correspond to a tetramer and a higher order oligomer, respectively (Supplementary Fig. S1).

Table 3
Data collection and processing.

Diffraction source	XRD2, Elettra
Wavelength (Å)	0.9784
Temperature (K)	100
Detector	PILATUS 6M
Space group	C121
<i>a</i> , <i>b</i> , <i>c</i> (Å)	143.98, 32.62, 95.62
α , β , γ (°)	90, 94.68, 90
Oscillation range (°)	1.0
No. of images	380
Matthews coefficient (Å ³ Da ⁻¹)	2.08–3.01
Solvent content (%)	38–59
Resolution range (Å)	47.65–1.98 (2.02–1.98)
Total No. of reflections	171270 (11107)
No. of unique reflections	30666 (2067)
Multiplicity	5.6 (5.4)
$\langle I/\sigma(I) \rangle$	9.9 (2.0)
Mosaicity (°)	0.89
Completeness (%)	97.3 (92.9)
<i>R</i> _{merge} [†]	0.079 (0.647)

[†] $R_{\text{merge}}(I) = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $\langle I(hkl) \rangle$ is the average intensity of the *i* observations of reflection *hkl*.

and a 2 µl:2 µl drop was equilibrated against the reservoir solution (Table 2). For X-ray data collection, a single crystal was mounted on a cryo-loop using 20% glycerol as a cryo-protectant solution and flash-cooled directly in a nitrogen stream at 100 K. The diffraction data were collected using a PILATUS 6M detector on beamline XRD2 at the Elettra synchrotron, Italy. The data were indexed and integrated with *iMosflm* (Battye *et al.*, 2011), while scaling was performed with *AIMLESS* from the *CCP4* suite (Winn *et al.*, 2011) (Table 3).

3. Results and discussion

Recombinant PEX14CC from *L. donovani* was expressed in *E. coli* host cells as a soluble N-terminally GST-tagged protein. The protein was successfully purified and the GST tag was subsequently removed by treatment with thrombin followed by gel-filtration chromatography (Fig. 1a). The yield of the protein was 10 mg per litre of culture. In initial crystallization trials, we used a range of protein concentrations (5–25 mg ml⁻¹) and various crystallization screens with the sitting-drop and hanging-drop vapour-diffusion methods. A basal precipitation was observed in a few conditions in two weeks and the protein concentration was increased to 45 mg ml⁻¹, which yielded small crystals in a fortnight. Optimization of this crystal condition using 4% 1,3-butanediol from the Additive Screen (Hampton Research) produced crystals (Figs. 2a and 2b) that diffracted to a resolution of 1.98 Å (Fig. 3) on beamline XRD2 at Elettra, Trieste, Italy. 380 images of 1.0° oscillation were collected and were used in data processing using *iMosflm* (Battye *et al.*, 2011) and *AIMLESS* (Evans & Murshudov, 2013) from the *CCP4* suite of programs (Winn *et al.*, 2011). The data set has a signal-to-noise ratio of 9.9 (2.0 in the highest resolution shell) with a mosaicity of 0.89° (Table 3). Data-reduction analysis indicated that the crystal belonged to the monoclinic space group *C*2, with unit-cell parameters *a* = 143.98, *b* = 32.62, *c* = 95.62 Å, β = 94.68°. Matthews coefficient calculations (Matthews, 1968) suggested

the presence of 4–6 molecules in the asymmetric unit, with a solvent content of 38–59% and a V_M of between $2.08 \text{ \AA}^3 \text{ Da}^{-1}$ (four molecules) and $3.01 \text{ \AA}^3 \text{ Da}^{-1}$ (six molecules). Following data reduction, structure solution was initiated by molecular replacement calculations in *Phaser* (McCoy *et al.*, 2007) from the *CCP4* suite of programs using templates suggested by the *Phyre2* server (Kelley *et al.*, 2015).

However, none of the templates used (PDB entries 1wt6, 3swk, 4yto, 5jxc and 6a9p) produced unambiguous results, with the best solution so far being obtained using the human dystrophin myotonic kinase CC domain (PDB entry 1wt6; Garcia *et al.*, 2006) with a sequence identity of 25% (Supplementary Fig. S2), with a TFZ of ~ 9 and an LLG of ~ 300 for four protomers in the asymmetric unit, that appears to be acceptable upon preliminary visual examination. Initial rigid-body refinement of this structure solution resulted in fairly high R factors (R_{work} of $\sim 50\%$ and R_{free} of $\sim 52\%$). The solution of coiled-coil structures by molecular-replacement calculations is seldom straightforward and requires continuous efforts and machine time, although significantly advanced

(a)

(b)

Figure 2
Crystallization of recombinant PEX14CC protein. (a) Photograph of the crystallization well. (b) A crystal of PEX14CC. The approximate dimensions of the crystal are $1600 \times 400 \times 100 \text{ μm}$.

Figure 3

X-ray diffraction pattern of a PEX14CC crystal; the resolution shells are shown by concentric circles.

software has recently become available. Structure-solution efforts are also in progress using *AMPLE* (Bibby *et al.*, 2012) and *MrBUMP* (Keegan & Winn, 2008) in addition to *Phaser*. Secondary-structure prediction analysis suggests that 82 of the 84 residues adopt a single helical conformation, which adds another layer of complication. As there are no methionine residues (Table 1) in the structure, SeMet derivatization is also ruled out; heavy-atom soaking experiments are in progress.

4. Related literature

The following references are cited in the supporting information for this article: Gouet *et al.* (1999) and Thompson *et al.* (1994).

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