Cloning, recombinant production and crystallographic structure of Proliferating Cell Nuclear Antigen from radioresistant archaeon

Thermococcus gammatolerans

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Thermococcus gammatolerans is a strictly anaerobic, hyperthermophilic archaeon belongs to the order Thermococcales in the phylum Euryarchaeota. It was extracted from a hydrothermal vent from the Guaymas Basin (Gulf of California, Mexico). Different studies show that T. gammatolerans is one of the most radioresistant organisms known amongst the archaea. This makes it a unique model to study adaptations to the environment and to study DNA repair mechanisms in an organism able to tolerate harsh conditions. A key protein in these mechanisms is the Proliferation Cell Nuclear Antigen (PCNA). Its function is focused on their ability to slide along the DNA duplex and coordinating the activities of proteins mainly related to DNA edition and processing. Analysis of archaeal proteins have proven to be enormously fruitful because much of the information obtained from them can be extrapolated to eukaryotic systems, and PCNA is no exception. Here we report the cloning, recombinant expression and crystallographic structure of PCNA from T. gammatolerans (TgPCNA).

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1. Introduction

The mechanisms involved in DNA metabolism are essential to preserve genome integrity in every organism. In these mechanisms are present multiple highly ordered protein complexes such as one that catalyses a specific incision to remove 5′ flaps in double-stranded DNA substrates and those involving chromatin remodeling [1,2]. A key protein in these complexes is the Proliferation Cell Nuclear Antigen (PCNA) [3]. A protein evolutionarily highly conserved, which was initially identified as an autoantigen in patients with systemic lupus erythematosus [4]. Depending on
the life domain, sliding clamps can be organized, in solution and crystal structures, forming dimers or trimers. Whereas in bacteria it is assembled as a dimer (so-called β-clamp), in archaea and eukaryotes PCNA assembles into heterotrimeric protein structures. Crenarchaeota expressed typically as a homotrimer. While Thaumarchaeota contain only one gene encoding PCNA, which is amino acid (Ile/Leu/Met) and hydrothermal vent from the Guaymas Basin (Gulf of California, Thermococcales). Its complexity in terms of adaptation to extreme environments, exceptionally. Also, their three-dimensional structures have highlighted fruitful because much of the information obtained from them can be extrapolated from archaea to eukaryotes [26]. On the other hand, to date, only six crystallographic structures from archaeal PCNA have been deposited in the PDB.

All of the above highlights the importance of studying fundamental proteins such as PCNA in microorganism adapted to live in extreme conditions such as T. gammatolerans. A search in its genome, shows that T. gammatolerans only possesses a homolog gene PCNA, which is consistent with other Euryarchaeota [14,27]. Here we report the cloning, recombinant expression and crystallization of PCNA from T. gammatolerans (TgPCNA).

2. Materials and methods

2.1. Cloning of full-length TgPCNA

The open reading frame of 750 bp coding TgPCNA (accession code Tgam_1046) was PCR-amplified from genomic DNA from T. gammatolerans which was a generous gift from Dr. Patrick Forterre (Institut de Génétique et Microbiologie, Université Paris-Sud). The PCR primers were 5'-gtgctcagattt Cruciform TgACACCCTCTCTCTGTAAGGGAC-3' (forward) and 5'-gtgctcgGATTTTTCCTCTCCAAGGCGGAG-3' (reverse). These primers contained restriction sites for NdeI and BamHI restriction enzymes respectively (the recognition sequences are shown in bold). The purified PCR product was digested and ligated into a pcold-I, resulting in the recombinant vector pCold-I+ TgPCNA to overproduce the protein in fusion with an N-terminal hexahistidine tag. Bacterial strain for DNA propagation and cloning was DH5α. Positive clones were confirmed by automated DNA sequencing using local facilities (Institut de Génétique et Microbiologie, Université Paris-Sud). 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(GE Healthcare, Little Chalfont, United Kingdom) charged with Ni2+, previously and equilibrated with buffer A. TgPCNA was eluted with a 0–100 mM imidazole gradient in buffer B (50 mM Tris-HCl pH 7.5, 400 mM NaCl and 500 mM imidazole) using an AKTA Prime FPLC system (GE Healthcare, Little Chalfont, United Kingdom). Fractions corresponding to the largest peak were pooled and dialyzed against buffer C (50 mM Tris-HCl pH 7.5, 400 mM NaCl, 2 mM EDTA and 5 mM DTT), using a dialysis tubing cellulose membrane with molecular weight cut-off of 12,400 Da (Sigma-Aldrich, St Louis MO, USA) at 277 K for 12 h. Dialyzed TgPCNA was concentrated to 2 mL using an Amicon ultracentrifuge device with its mother liquor where water where exchanged by 20% of Gly-HCl pH 7.5, 400 mM NaCl and 500 mM imidazole) using an ÄKTA HiLoad 26/600 Superdex 200 pg gel filtration column (GE Healthcare, Little Chalfont, United Kingdom) previously equilibrated with buffer C and calibrated with molecular mass standards. The molecular weight markers were Aldolase (158,000 Da), Conalbumin (75,000 Da), Carbonic Anhydrase (29,000 Da) and Ribonuclease A (13,700 Da). Fractions with the highest protein concentration and purity were concentrated and analyzed by SDS-PAGE at 12%.

2.3. Protein crystallization

Protein concentration was measured by Bradford (Bio-Rad, Hercules, CA, USA) assay using bovine serum albumin dilutions as standards [30], densitometric analysis of proteins on SDS-PAGE and by absorbance at 280 nm. Initial protein crystallization screening was performed using a Mosquito LCP robot (TTP LabTech, Cambridge, United Kingdom) by sitting-drop vapor-diffusion method. The experiments were carried out using 96-well sitting drop iQ plate (TTP LabTech, Cambridge, United Kingdom) by sitting-drop vapor-diffusion method. The experiments were performed at 291 K.

3. Results and discussion

TgPCNA contains 249 amino acids residues and is annotated in the NCBI GenBank with the identification number ACS335481. An extensive search was conducted in the genome database from T. gammatolerans to identify duplicated PCNA genes as in the case of Thermococcus kodakaraensis [20]. Our search shows only one gene encoding a protein homologous to PCNA. To date, only six PCNAs of archaea have been deposited in the PDB as entry 5a6d, and a summary of data collection and refinement statistics are given in Table 1.

Crystallographic data and data-collection statistics of T. gammatolerans PCNA. Values in parentheses are for the highest-resolution shell.

| Beamsline | SSRL 14–1 |
|----------------|------------|
| Wavelength (Å) | 1.18 |
| Resolution (Å) | 49.86–2.8 (2.9–2.8) |
| Space Group | P3 |
| Temperature (K) | 100 |
| Detector | MARCCD |

| Unit-cell parameters (Å, °) | a = 92.669, b = 92.669, c = 63.632, α = β = 90, γ = 120 |
| Solvent content (%) | 56.13 |
| Unique reflections | 15,022 (1521) |
| I/σ(I) | 15.75 (2.64) |
| Average redundancy | 3.2 |
| Data completeness (%) | 99.69 (99.87) |
| Rmerge (%) | 0.0631 (0.5523) |
| Refinement | – |
| Rf (factor) (%) | 0.1883 |
| Rfree (%) | 0.2379 |
| R.m.s.d. bond distance (Å) | 0.011 |
| R.m.s.d. bond angle (°) | 1.51 |
| Average B factor (Å²) | 70.7 |
| Macromolecules | 70.7 |
| Ramachandran plot (%) | |
| Core | 93 |
| Disallowed | 0.82 |
| No. of protein atoms | 3898 |
| No. of solvent atoms | 0 |
| PDB code | 5a6d |

2.4. X-ray data collection and processing

Crystals were soaked in a cryoprotectant solution consisting of its mother liquor where water where exchanged by 20% of Glycerol, in order to maintained the remaining component contend, and mounted in a rayon cryo-loop and immediately flash-cooled in liquid nitrogen. Diffraction data were collected at the beamline 14–1 from the Stanford Synchrotron Radiation Light source, SSRL, Menlo Park, USA. Diffraction data were processed with XDS and scaled using XSCALAE [37]. According to the unit cell parameters indexed in XDS, space group and the molecular mass of each TgPCNA monomer, the packing of the crystal was analyzed using the Matthews Probability Calculator Server (http://www.rupweb.org/mattprob/default.html) and proposing 2 TgPCNA molecules in the asymmetric unit (AU). The structure was successfully determined by molecular replacement, in space group P 3 3 and with 2 copies in the AU. MR was performed with Phaser [38] and using the coordinates of PCNA1 from Thermococcus kodakaraensis tk0535 as a search model (PDB entry 3lx1 [20], with a sequence identity of 92% with TgPCNA). The TGPCNA coordinates were refined up to 2.8 Å resolution using a combination of REFMAC5 from the CCP4 suite [39], Phenix [40] and manual modeling using the molecular-graphics program Coot [41]. In all cases, due the low data parameter / diffraction data ratio, restrained Non-

Table 1

| Data-collection statistics. |
|-----------------------------|
| Beamline | SSRL 14–1 |
| Wavelength (Å) | 1.18 |
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| Unit-cell parameters (Å, °) | a = 92.669, b = 92.669, c = 63.632, α = β = 90, γ = 120 |
| Solvent content (%) | 56.13 |
| Unique reflections | 15,022 (1521) |
| I/σ(I) | 15.75 (2.64) |
| Average redundancy | 3.2 |
| Data completeness (%) | 99.69 (99.87) |
| Rmerge (%) | 0.0631 (0.5523) |
| Refinement | – |
| Rf (factor) (%) | 0.1883 |
| Rfree (%) | 0.2379 |
| R.m.s.d. bond distance (Å) | 0.011 |
| R.m.s.d. bond angle (°) | 1.51 |
| Average B factor (Å²) | 70.7 |
| Macromolecules | 70.7 |
| Ramachandran plot (%) | |
| Core | 93 |
| Disallowed | 0.82 |
| No. of protein atoms | 3898 |
| No. of solvent atoms | 0 |
| PDB code | 5a6d |

Crystalllographic Symmetries (NCS) were applied during the refinement process. Figures were generated using CCP4mg [42]. The atomic coordinates and structure factors have been deposited in the PDB as entry 5a6d, and a summary of data collection and refinement statistics are given in Table 1.

3. Results and discussion

Several E. coli strains were tested to over-express TgPCNA, and the best results were obtained with strain BL21 Star (DE3). The expression of recombinant TgPCNA did not show any significant adverse effects in the E. coli expression host. Approximately 50% of the heterologous induced TgPCNA was soluble in a lysis buffer. Histidine-tagged TgPCNA remain bound to the nickel column even after extensive washes. After first purification step, TgPCNA was
Fig. 1. Amino-acid sequence alignment of TgPCNA with other archaeal PCNA. Residues and structural elements present in PCNA are in blue boxes. (Tga: Thermococcus gammatolerans, GenBank accession number ACS33548.1; Tko: Thermococcus kodakarense, BAD84724.1 and BAD84771.1; Hvo: Haloferax volcanii, ADE03802.1; Pfu: Pyrococcus furiosus, BAA33020.2; Afu: Archaeoglobus fulgidus, AAB90899.1; Sso: Sulfolobus solfataricus, AAK40726.1, AAK41309.1 and AAK40734.1; Sto: Sulfolobus tokodaii, BAK54261.1, BAK54265.1 and BAK54417.1).
approximately 85% pure (Data not shown). However a second purification step applying the sample in a HiLoad 26/600 Superdex 200 pg gel filtration column was included (Fig. 2). This second purification step was used to increase TgPCNA purity before crystallization and determining the molecular weight. As a result we identified an anomalous migration in the recombinant expressed TgPCNA, which was confirmed by elution profile (Fig. 2a) and SDS-PAGE (Fig. 2b). By gel filtration, the molecular mass was approximately 52,000 Da, which is slightly larger than the theoretical mass but closer to the monomeric TgPCNA. Similar behaviors have been observed previously and it is speculated that may be due to the acidic nature of TgPCNA with a theoretical pI of 4.5 [36].

Protein droplets consisting of 1 μL PCNA (14 mg mL⁻¹ in 50 mM Tris-HCl pH 7.5, 400 mM NaCl, 2 mM EDTA and 5 mM DTT) were mixed with 1 μL precipitant solution. First, crystals were obtained by using the sitting-drop vapor-diffusion method. TgPCNA crystallized under different conditions, but best crystal growth was observed under condition No. 42 of Wizard Classic crystallization screen III (30% v/v PEG 4000, 200 mM Li₂SO₄ and 100 mM Tris-HCl pH 8.5) and under conditions previously described by Matsumiya [27]. Optimization matrices were designed and performed for both conditions. Optimization matrix for condition No. 42 evaluated how changes in PEG (10–35% w/v) and protein (10–16 mg mL⁻¹) concentration affect crystal growing, while matrix for Matsumiya condition evaluated changes in protein (10–16 mg mL⁻¹), ammonium sulfate (2.4–2.8 M) and MPD (5–10%) concentrations, as well as buffer pH (5.2–5.8). After 3 months crystals were collected, cryocooled and diffracted at the SSRL. The best data set was obtained from Matsumiya condition matrix, where prism-like crystal was obtained. The approximate dimensions of the crystal were 0.50 × 0.25 × 0.25 mm (Fig. 3). In brief, 1 μL of TgPCNA at 10 mg mL⁻¹ was mixed with 1 μL precipitant solution consisting in 2.8 M ammonium sulfate, 75% MPD and 100 mM citrate buffer pH 5.2.

The TgPCNA coordinates were refined up to 2.8 Å resolution using a combination of REFMAC5 from the CCP4 suite [39], Phenix [40] and manual modeling using the molecular-graphics program Coot [41] (Fig. 4a). In all cases, due the low data parameter/diffraction data ratio, restrained Non-Crystallographic Symmetries (NCS) were applied during the refinement process. Figures were generated using CCP4mg [42]. The atomic coordinates and structure factors have been deposited in the PDB as entry 5a6d, and a summary of data collection and refinement statistics are given in Table 1. The structure was then superposed with other PCNA, deposited at the PDB, to look for structural differences within them. The structures selected for this comparison belong to the organisms: T. kodakarensis (5dai); Pyrococcus furiosus (1iz5); Halofex volcanii (3ifv); Drosophila melanogaster (4hk1); Archaeoglobus fulgidus (1rxz); Sulfolobus sulfataricus (2ijx). The r.m.s.d. was: 5dai, 0.85; 3lx1, 0.88; 1iz5, 0.88; iz4, 1.25; 3ifv, 1.35; 4hk1, 1.38; 2ijx, 1.43; 5e0u, 1.47; 1rxz, 1.51; 1rwz, 1.51; 2ijx, 1.71 (Fig. 4b).

The superposition of coordinates from phylogenetically close and distant organisms yield to low rmsd values, between 0.88 Å for T. kodakarensis and 1.71 Å for S. sulfataricus. PCNAs respectively.
Unfortunately, there were no clear differences in the coordinates which allow us to speculate if TgPCNA is intrinsically resistant to the radiation and thus be a key factor for cell survival after irradiation.

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Appendix A. Transparency document

Transparency data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2016.08.004.

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