Crystal Structure of the Shrimp Proliferating Cell Nuclear Antigen: Structural Complementarity with WSSV DNA Polymerase PIP-Box

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Abstract

DNA replication requires processivity factors that allow replicative DNA polymerases to extend long stretches of DNA. Some DNA viruses encode their own replicative DNA polymerase, such as the white spot syndrome virus (WSSV) that infects decapod crustaceans but still require host replication accessory factors. We have determined by X-ray diffraction the three-dimensional structure of the Pacific white leg shrimp Litopenaeus vannamei Proliferating Cell Nuclear Antigen (LvPCNA). This protein is a member of the sliding clamp family of proteins, that binds DNA replication and DNA repair proteins through a motif called PIP-box (PCNA-Interacting Protein). The crystal structure of LvPCNA was refined to a resolution of 3 Å, and allowed us to determine the trimeric protein assembly and details of the interactions between PCNA and the DNA. To address the possible interaction between LvPCNA and the viral DNA polymerase, we docked a theoretical model of a PIP-box peptide from the WSSV DNA polymerase within LvPCNA crystal structure. The theoretical model depicts a feasible model of interaction between both proteins. The crystal structure of shrimp PCNA allows us to further understand the mechanisms of DNA replication processivity factors in non-model systems.

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Introduction

Proliferating Cell Nuclear Antigen (PCNA) is a member of the sliding clamp family of DNA-replication accessory proteins. Their functions are critical to processes such as cell cycle control, chromatin remodeling, gene expression, apoptosis, and DNA repair [1,2,3,4]. In most organisms PCNA is a homotrimer, in which its three subunits adopt a doughnut-shaped structure in a head-to-tail arrangement; this toroidal structure is extremely conserved in protozoa, humans, yeast and plants [5,6,7,8,9]. In bacteria, the PCNA homologue is called clamp, that is formed by a homodimeric assembly with a six-fold symmetry forming a toroidal structure similar to most PCNAs reported [10]. Only few organisms have a non-canonical homotrimeric structure as in the crenarchaeon Sulfolobus solfataricus and in the model plant Arabidopsis, where their PCNA are formed by heterotrimers [11,12].

The structure of PCNA is comprised by two α+β domains joined by an inter-domain connecting loop (IDCL) [7]. The PCNA molecule interacts with DNA by the inner face of the ring, which is composed by α-helices. Therefore, the arrangement of the α-helices in each monomer leads to a pseudo six-fold symmetry in the trimer comprised of 12 α-helices [13]. The inner face of the toroid has an array of basic residues positioned to provide favorable electrostatic interactions with the DNA-phosphate backbone. This structure allows PCNA to slide freely on DNA, once is assembled into DNA by the clamp loading complex [14].

In most cases, PCNA-interacting proteins contain a short sequence motif called PIP-box, which makes hydrophobic contacts with PCNA and has a consensus amino acid sequence QXXM/L/I/EX(F/Y)E/Y/F/Y [15]. However, there is also a novel PCNA-interacting motif (APIM) with an apparent consensus amino acid sequence MD(L/R)W(L/V/I)2(K/R) which is present in proteins involved in DNA repair and cell cycle control during genotoxic stress, the APIM motif was identified by bioinformatics analysis in about 200 nuclear proteins [16]. PCNA interacts with multiple protein partners and despite each PCNA binding protein has its specific contact site, most of them bind mainly through hydrophobic pocket formed by the IDCL, central loop and C-terminus in PCNA [17].

It is known that some viruses encode their own DNA polymerases and processivity factors as observed in T4 and...
Table 1. Data reduction and refinement statistics from *LvPCNA* structure.

| DATA SET                      | *LvPCNA*          |
|-------------------------------|-------------------|
| Space group                   | C2                |
| Unit-cell parameters (Å)      | *a* = 144.57, *b* = 83.38, *c* = 74.31, *β* = 117.6° |
| Data collection               |                  |
| Resolution range (Å)          | 40.0–3.0 (3.1–3.0) |
| Unique reflections            | 14802 (1128)      |
| *R*<sub>meas</sub>            | 0.094 (0.415)     |
| Completeness (%)              | 93.3 (97.0)       |
| *I*/σ(I)                      | 10.69 (3.57)      |
| Redundancy                    | 2.96 (3.10)       |
| Refinement statistics         |                  |
| *R*<sub>work</sub>/R<sub>free</sub>(%) | 0.2648/0.3108    |
| R.M.S.D. form ideal           |                  |
| Bond length (Å)               | 0.01              |
| Bond angles (°)               | 1.489             |
| Ramachandran plot, residues in |                  |
| Most favored regions          | 666 (87.32%)      |
| Additionally allowed regions  | 83 (10.83%)       |
| Outliers                      | 14 (1.85%)        |

Values in parenthesis represent the statistics at the highest resolution bin.

1°*R*<sub>meas</sub> is a redundancy-independent version of *R*<sub>symm</sub>.

\[ R_{\text{meas}} = \sum_{h} \frac{|I_h|}{g_{h,n}} \sum_{n} \left( \frac{I_h}{\bar{I}_h} - 1 \right)^2 \]

where \( I_h = 1/n_h \sum^{n_h} I_{h,n} \).

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Figure 1. Crystal structure of *Litopenaues vannamei* PCNA. The PCNA molecule is arranged as homotrimer and each monomer is shown in different color. The most important parts for protein-protein interaction of each monomer: Interdomain Connecting Loop (IDCL), Central Loop and C-terminal are labeled.

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Figure 2. Amino acid sequence alignment of PCNAs. The figure shows the high identity and similarity of *L. vannamei* PCNA with other species. Important domains for PCNA-protein interactions: Central Loop, Inter-domain Connector Loop (IDCL) and C-terminal are in colored boxes (blue, red and green respectively) and tagged.

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Figure 3. Electrostatic surface view of PCNA from shrimp *Litopenaeus vannamei* and human. The positively charged regions are colored in blue and negatively charged regions in red. The central channel is identified as a highly positive charged hole where the double-strand DNA can slide through it.

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RB69 bacteriophages or human viruses like herpes simplex and cytomegalovirus [18]. However, in some cases, pathogens like the Simian Virus 40 and bacteriophage T7 use proteins from their host as processivity factor for their genome replication [17,19].

The White Spot Syndrome Virus (WSSV) is a DNA virus that affects the shrimp aquaculture industry around the world [20,21,22]. It has been reported that this WSSV encodes its own DNA polymerase [23,24], and we have demonstrated that WSSV ORF514 encodes a bona fide DNA polymerase. In vitro, this polymerase had a low processivity, although the presence of a PIP-box in its sequence and the absence of putative processivity factors in the virus genome suggest that it utilizes a host processivity factor [24,25,26]. We have recently reported the cDNA sequence, recombinant overexpression, purification and crystallization of the shrimp *Litopenaeus vannamei* PCNA [25,27]. Moreover, others and ourselves have reported its gene expression during viral infection [25,28,29,30]. Herein we report the x-ray structure analysis of the first crustacean recombinant PCNA (*Lv*PCNA) and a model where PCNA interacts with viral DNA polymerase PIP-box as an approach toward structural understanding this feasible interaction.

**Materials and Methods**

**LvPCNA purification and protein crystallization**

Overexpression of recombinant *Lv*PCNA was carried using *E. coli* BL21 SI system and co-expression with chaperones was needed to obtain high yield of soluble recombinant protein. Metal affinity chromatography method was used for purification. Detailed description of overexpression, purification and *Lv*PCNA crystallization methods were previously reported [27].

Successful crystallization condition was: 300 mM CaCl$_2$.2H$_2$O, 100 mM sodium HEPES pH 7.5 and 30% v/v PEG 400. Thin hexagonal shaped crystals of approximately 0.1 x 0.6 mm were suitable for X-ray diffraction. The *Lv*PCNA crystal belonged to the C2 space group with unit-cell parameters $a = 144.6$ Å, $b = 83.4$ Å, $c = 74.3$ Å, $\beta = 117.6^\circ$ [27].

**X-ray data collection and crystallographic analysis**

Data collection from *Lv*PCNA crystals was carried on beam line X-4C of the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (BNL, Upton NY, USA), using a MarCCD 165 detector. The complete data covered 140° in 280 images, it was split and integrated independently using XDS and scaled together by XSCALE [31]. The phases were obtained by molecular replacement in PHASER [32] using an homology model of the *Lv*PCNA amino acid sequence (GenBank JN546075.1) as previously reported [25], based on the three-dimensional structure of human PCNA (PDB entry 1VYM) [33]. *Lv*PCNA refinement was carried out using the programs PHENIX [34]. Since the resolution was 3 Å, rigid body refinement and non-crystallographic symmetry between the monomers were imposed during refinement and manual rebuilding was done in COOT using $2F_o-F_c$ maps at 2 σ to adjust positions and rotamers [35]. The final structure was deposited in the Protein Data Bank with accession number 4CS5.

**Molecular docking of WSSV DNA polymerase PIP-box into *Lv*PCNA crystal structure**

In order to visualize if *Lv*PCNA could recognize WSSV DNA polymerase via its putative PIP-box, we performed a docking analysis using the software MOE 2102.10. The amino acid corresponding to the PIP-box from WSSV DNA polymerase was modeled by homology from residue 382 to 401, with the amino acid sequence ERAIGQHKILYYDIETTDKD. This template was selected by similarity with the sequence of a PIP-box peptide from Flap endonuclease 1 in complex with PCNA (PDB 1UL1) [36]. The final model for WSSV DNA polymerase PIP-box was refined from 25 intermediate models under the default parameters of the MOE homology modeling protocol using the CHARMM27 force field for energy minimization. The PIP-box binding site was defined from the resolved coordinates of *Lv*PCNA based on sequence identity on a multiple sequence and structural alignment of several PCNA crystallographic structures in complex...
with a PIP-box peptide or protein. A stochastic search of the best-fitted positions of the WSSV PIP-box peptide over the \( \text{LvPCNA} \) pocket was done using the MOE Dock platform under the Induced Fit protocol. Ligand placement was performed using the Alpha Triangle method and the London dG scoring function for at least 80,000 poses. From this output, 30 non-duplicate poses were retained for further refinement used to relax the poses by 500 iterations with the Force field scheme and the Affinity dG rescoring function under the CHARMM27 force field. Duplicates from the refinement process were removed and the best scoring 30 poses were retained for further analysis. The final file was used for elaboration of figures and diagrams using CCP4mg [37,38].

**Results and Discussion**

**Determination of the \( \text{LvPCNA} \) structure**

Electron density maps calculated from the molecular replacement initial model showed good coverage of the backbone and followed the alpha helical trace of the protein. \( \text{LvPCNA} \) had the cognate fold comprised by \( \beta-\alpha-\beta-\alpha-\beta-\beta-\beta \)-IDCL-\( \beta-\alpha-\beta-\alpha-\beta-\beta \)-topology with pseudo symmetry within each monomer. After several cycles of refinement in PHENIX and manual rebuilding in COOT, both \( R_{\text{work}} \) and \( R_{\text{free}} \) dropped, suggesting that the refinement strategy was correct. Final refinement values were \( R_{\text{work}} \) 0.2648 and \( R_{\text{free}} \) 0.3108 (Table 1).

To determine the quaternary structure of \( \text{LvPCNA} \) we run this purified protein at 1mg/ml into a Superdex 200 size-exclusion chromatography column and compared its elution profile with...
known molecular-mass standards. LvPCNA eluted in a complex of approximately 90 kDa, indicating that this protein assembles as a trimer in solution [27]. Accordingly to this previous result the molecular replacement found a trimer in the asymmetric unit. The backbone cartoon shows the canonical structure and although the IDCL (residues 117–133) had poor electron density, the density was conclusive to include the coordinates of those residues in the final model (Figure 1). LvPCNA amino acid sequence is highly conserved among species (Figure 2) and is structurally similar when compared with Drosophila PCNA [39], as it had a root mean square deviation (RSMD) of 0.5 Å for the α-carbon backbone. The central hole is highly positive charged as shown in Figure 3 and has a diameter of 30.5 Å, large enough to accommodate the double helical DNA and slide freely on it.

Construction of LvPCNA-WSSV PIP-box model

A peptide sequence containing the WSSV DNA polymerase PIP box was modeled and docked into the crystallographic structure of shrimp PCNA, which is its natural host. The docking of PIP-box peptide into the LvPCNA binding site was carried out at the cognate region but without constraints to a specific position within the pocket in a stochastic approach. It is remarkable that the docking algorithm led to seven similar poses for the PIP-box peptide into the pocket between the 30 best-scoring ones (Figure 4). This docking algorithm produces several solutions or poses of the peptide into pocket in a stochastic approach. It is remarkable that the docking algorithm led to seven similar poses for the PIP-box peptide into the pocket between the 30 best-scoring ones (Figure 4).

All these poses have an average RMSD of 2.2 Å for the α-carbon atoms of the entire peptides. The peptide corresponds to a region of 20 amino acids from residues 675 to 694 of the WSSV DNA polymerase ORF (GenBank NP_478036). The peptide adopts an extended structure with a single helical turn at the center of the consensus sequence QHKILYY, very similar to other PIP-box peptides. This cognate region but without constraints to a specific position within the pocket in a stochastic approach. It is remarkable that the docking algorithm led to seven similar poses for the PIP-box peptide into the pocket between the 30 best-scoring ones (Figure 4). The peptide adopts an extended structure with a single helical turn at the center of the consensus sequence QHKILYY, very similar to other PIP-box peptides. This cognate region but without constraints to a specific position within the pocket in a stochastic approach. It is remarkable that the docking algorithm led to seven similar poses for the PIP-box peptide into the pocket between the 30 best-scoring ones (Figure 4).

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The interactions between the PIP-box peptide and LvPCNA are shown schematically as a LigPlot diagram (Figure 5, panel A) [42]. The PIP-box peptide interacts with each PCNA monomer almost in the internal symmetry axis and almost perpendicular to the IDCL loop as shown in cartoon (Figure 5, panel B). However, this is a tight packing cavity as obtained by docking, where mostly hydrophobic interactions are leading the binding, the hydrophobic cavity is represented in a surface image where the PIP-box peptide is positioned and drawn as sticks (Figure 5, panel C). This pocket comes mainly from the IDCL (G127, P129, T131), central loop (S43, H44, V45, L47) and from C-terminus (F250, L251, A252, P253, I255) residues.

The interaction between peptide and PCNA is mainly hydrophobic, only the H7 and K8 residues from the peptide make polar contacts with PCNA residue A252 and S43, respectively (Figure 5, panel A) and some intra-molecular interactions were found within the PIP-box peptide. Main hydrophobic contacts are between PIP-box residues G5, Q6, Y11 and LvPCNA C-terminal domain L251, K254, P253, I255. The LvPCNA central loop residues M40, V45, H44 make hydrophobic contacts with I9, L10 of the PIP-box and only P129 LvPCNA IDCL residue makes hydrophobic contact with PIP-box Y12, the side chains of these residues are shown in Figure 5, panel D.

One feature observed during the docking process is that the algorithm produces several solutions or poses of the peptide into LvPCNA, and the internal peptide sequence Q6-HKILYYD-I14 has an RMSD smaller than 1 Å for those poses (Figure 4). This ensures that the computational docking is consistent and reliable, until further confirmation by X-ray crystallography studies of the complex LvPCNA with PIP-box peptide. To further envision the interaction between LvPCNA and WSSV DNA pol, a theoretical model of the polymerase was built around DNA (Figure 6) and a ring with the average radius of the PCNA was drawn for an estimation of the interaction and closeness of both proteins. In this model the PIP box of WSSV DNA pol is in a position that indicates that upon a conformation change it could interact with LvPCNA. Whether a conformational change occurs in WSSV DNA pol is necessary to produce the a tight interaction is something to be further explored.

Conclusions

The crystal structure of the LvPCNA has the expected trimeric ring shape, consistent with most of the eukaryotic PCNA reported. The results from docking suggest that WSSV polymerase has the capacity of binding the LvPCNA in the same way that most PCNA binding proteins do. This possible interaction is predicted as hydrophobic which has to be considered when proved experimentally to elect the correct method. Despite the experimental
phase of this interaction remains to be carried, it could lead to a future investigations toward generate an antiviral strategy that could prevent or disrupt this host-pathogen interaction, resulting in poor viral DNA replication and diminishing the pathogenicity of WSSV.  

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