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Crystallographic study of G178S mutant of human proliferating cell nuclear antigen

Proliferating cell nuclear antigen (PCNA) is an evolutionarily conserved protein that forms a ring-shaped homotrimer that functions as a sliding clamp for DNA replication. The *rev6-1* mutation of *Saccharomyces cerevisiae*, which inactivates both translesion DNA synthesis and damage-avoidance pathways while having little effect on normal cell growth, has a G178S substitution in the PCNA protein. Human PCNA protein carrying the G178S substitution was crystallized. Two crystal forms were obtained under similar conditions. Crystal forms I and II belong to space groups $P2_1$, with unit-cell parameters $a = 84.1$, $b = 130.2$, $c = 97.8$ Å, $\beta = 113.4^\circ$, and $P2_12_12_1$, with unit-cell parameters $a = 68.1$, $b = 100.2$, $c = 131.2$ Å, respectively. Structural analyses by molecular replacement are now in progress.

1. Introduction

Proliferating cell nuclear antigen (PCNA) was originally identified as a nuclear protein whose expression correlated with the proliferating stage of the cell (Miyachi *et al.*, 1978). It was rediscovered as a factor necessary for the replication of SV40 DNA *in vitro* and was shown to be an auxiliary protein for DNA polymerase δ (Prelich *et al.*, 1987). PCNA forms a ring-shaped homotrimer that encircles the DNA double helix and slides freely along the DNA axis, thereby providing the bound DNA polymerase with high processivity. It is now known that PCNA interacts with a large number of proteins with various functions such as replication, repair, cell cycling, chromatin assembly and sister-chromatid cohesion (Moldovan *et al.*, 2007). Thus, PCNA plays a biologically crucial role as a platform for various proteins to perform their respective functions. More recently, it has been recognized that PCNA undergoes various post-translational modifications under stress conditions. The most extensively studied example is ubiquitination by RAD6/RAD18 (E2/E3 ubiquitin-ligase) in DNA-damaged cells (Hoege *et al.*, 2002). Upon DNA damage, the RAD6/RAD18 complex monoubiquitinates PCNA at a defined residue (Lys164), some of which is further polyubiquitinated through Lys63 linkage of ubiquitin by RAD5/UBC13/MMS2. In yeast cells, monoubiquitination of PCNA at Lys164 stimulates translesion DNA synthesis by specialized DNA polymerases such as Pol η , Pol ζ and REV1 (Stelter & Ulrich, 2003) and polyubiquitination of PCNA is required for an error-free pathway of DNA-damage tolerance called the DNA damage-avoidance pathway, the detailed mechanism of which remains elusive.

A mutant of *Saccharomyces cerevisiae*, *rev6-1*, was found in a screen for mutants deficient for UV-induced reversion of the frame-shift mutation *his4-38* in *S. cerevisiae* (Lawrence *et al.*, 1985). The mutation abolishes both the translesion DNA-synthesis and damage-avoidance pathways, while having little effect on the growth rate. The *rev6-1* mutation was found to cause a substitution of Gly178 to serine (G178S) in the PCNA protein (Zhang *et al.*, 2006). The above phenotype of the *rev6-1* mutant suggests that the G178S mutant PCNA can support normal DNA synthesis by replicative DNA polymerases such as Pol δ , but cannot stimulate translesion DNA synthesis by Pol η , Pol ζ and REV1. The close location of Gly178 to Lys164 also suggested that the G178S substitution might prevent

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Figure 1

Sequence alignment of human and yeast PCNAs. Identical and homologous residues are highlighted by black and grey backgrounds, respectively.

ubiquitination at Lys164 by the RAD6/RAD18 complex. However, because Gly178 is located in the intersubunit β -sheet, it remains possible that the G178S substitution may affect the assembly of PCNA and consequently affect ubiquitination. To date, the structural and functional features of the PCNA G178S mutant have remained unclear at the molecular level. A sequence alignment of human and yeast PCNAs is shown in Fig. 1. Here, we describe structural studies of human PCNA (hPCNA) G178S mutant (hPCNA^{G178S}) as part of a wider functional analysis of the consequences of PCNA modification. In the present paper, the crystallization and X-ray crystallographic analysis of hPCNA^{G178S} are discussed.

2. Methods and results

2.1. Expression and purification of hPCNA^{G178S}

The G178S mutation was introduced into the expression vector of human PCNA, pT7-hPCNA (Fukuda *et al.*, 1995), using the Quik-Change mutagenesis kit (Stratagene Co.), resulting in pT7-hPCNA^{G178S}. The single mutation was verified by conventional sequence analysis (Applied Biosystems). hPCNA^{G178S} was expressed in *Escherichia coli* BL21 (DE3) harbouring the pT7-hPCNA^{G178S} expression vector. To date, various procedures for preparing recombinant hPCNA have been reported (Gulbis *et al.*, 1996; Zheleva *et al.*, 2000; Sakurai *et al.*, 2003; Bruning & Shamoo, 2004; Kontopidis *et al.*,

2005). However, we employed a simpler modified procedure to prepare hPCNA^{G178S} based on previous reports as follows. Expression was induced with 1 mM IPTG at OD₆₀₀ = 0.6 and growth continued for 12–16 h at 303 K. All subsequent procedures were performed at 277 K. Bacterial cells were harvested by centrifugation for 10 min at 5000g in a Beckman JLA8.1 rotor and the pellets were suspended in lysis buffer (25 mM Tris–HCl pH 8.5, 300 mM NaCl, 1 mM DTT, 1 mM PMSF, 1 mM EDTA, 0.1 mg ml⁻¹ lysozyme and 10% glycerol). Cells were lysed by sonication and cell lysates were clarified by centrifugation at 40 800g for 1 h. The supernatant was loaded onto three connected 5 ml HiTrapQ HP columns (GE Healthcare) and eluted using a linear NaCl concentration gradient (0.3–1.0 M). Fractions containing hPCNA^{G178S} were pooled, brought to a final ammonium sulfate concentration of 1.8 M and gently stirred for 1 h. The protein solution was clarified by centrifugation at 34 800g for 20 min. The supernatant was loaded onto a 5 ml HiTrap Phenyl HP column (GE Healthcare) equilibrated in 1.8 M ammonium sulfate, 1 mM EDTA, 10% glycerol and 25 mM Tris–HCl pH 8.0. hPCNA^{G178S} was eluted with a reverse linear gradient from 1.8 to 0 M ammonium sulfate. Fractions containing hPCNA^{G178S} were pooled and the protein solution was then concentrated and loaded onto a HiLoad 26/60 Superdex 200 column (GE Healthcare) equilibrated with 10 mM HEPES–NaOH pH 7.4 and 100 mM NaCl. The elution volume was slightly but significantly smaller than that of the wild-type protein. This might indicate some change in the molecular shape. Fractions containing hPCNA^{G178S} were collected and concentrated to 10 mg ml⁻¹ with Amicon Ultra 30 000 MWCO (Millipore) for crystallization. The homogeneity of the purified protein was checked by SDS–PAGE with CBB stain. The procedure described here is also applicable to the preparation of wild-type hPCNA.

2.2. Crystallization and X-ray data collection

Crystals of hPCNA^{G178S} were obtained using the conventional hanging-drop vapour-diffusion method. Typical hanging drops were prepared by mixing 2 μ l each of hPCNA^{G178S} solution and reservoir solution containing 100 mM bis-tris–HCl pH 5.42, 200 mM ammonium sulfate and 22% PEG 3350. Vapour diffusion was initiated against 200 μ l reservoir solution at 293 K. Rod-shaped crystals were obtained in a few weeks (Fig. 2). We could not obtain crystals of hPCNA^{G178S} using the crystallization conditions used for wild-type hPCNA (Kontopidis *et al.*, 2005), indicating that the mutation affects the physical properties of hPCNA. Prior to X-ray exposure, crystals were transferred to reservoir solution containing 20% ethylene glycol

Figure 2

Crystals of hPCNA^{G178S}.

Table 1Crystallographic data for form I and II crystals of hPCNA^{G178S}.

Values in parentheses are for the highest resolution shells (form I, 3.11–3.00 Å; form II, 2.90–2.80 Å).

	Form I	Form II
Space group	<i>P</i> 2 ₁	<i>P</i> 2 ₁ 2 ₁ 2 ₁
Unit-cell parameters		
<i>a</i> (Å)	84.1	68.1
<i>b</i> (Å)	130.2	100.2
<i>c</i> (Å)	97.8	131.2
β (°)	113.4	90
<i>V_M</i> (Å ³ Da ⁻¹)	2.85	2.49
Solvent content (%)	56.8	50.6
Wavelength (Å)	1.0000	1.0000
Resolution range (Å)	50.0–3.00	50.0–2.80
Measured reflections	130267	140786
Unique reflections	97831	22441
Completeness (%)	95.6 (83.6)	98.0 (93.0)
Mean <i>I</i> / σ (<i>I</i>)	12.8 (3.4)	13.4 (4.1)
<i>R</i> _{merge} [†] (%)	8.5 (44.3)	7.4 (34.4)

$$^{\dagger} R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl).$$

for cryoprotection and were flash-frozen in a N₂-gas stream at 100 K. Preliminary X-ray diffraction experiments revealed two crystal forms, I and II, that were obtained under similar conditions and were very similar in crystal shape. We sorted the crystals by preliminary X-ray experiments using UltraX or FR-D in-house generators with R-AXIS IV⁺⁺ imaging-plate detectors (Rigaku). X-ray diffraction data for crystal forms I and II were collected using an ADSC Quantum 315 CCD detector on beamline BL41XU at SPring-8 and an ADSC Quantum 210 CCD detector on beamline NW12A at Photon Factory Advanced Ring (PF-AR), respectively. All diffraction data were indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). The form II crystal used for data collection was obtained using reservoir conditions that included 10 mM cobalt dichloride. Data-collection statistics are given in Table 1. The form I and II crystals belong to the monoclinic space group *P*2₁, with unit-cell parameters *a* = 84.1, *b* = 130.2, *c* = 97.8 Å, β = 113.4°, and the orthorhombic space group *P*2₁2₁2₁, with unit-cell parameters *a* = 68.1, *b* = 100.2, *c* = 131.2 Å, respectively. To investigate noncrystallographic symmetry, self-rotation functions were calculated. Clear peaks appeared at κ = 120° for both diffraction data sets, suggesting that hPCNA^{G178S} forms a trimer in both crystals. Thus, the asymmetric unit is estimated to contain two trimers of hPCNA^{G178S} in the form I crystal, with a corresponding crystal volume per protein weight (*V_M*; Matthews, 1968) of 2.85 Å³ Da⁻¹ and a solvent content of 56.8%. In

contrast, the asymmetric unit is estimated to contain one trimer in the form II crystal, with a corresponding *V_M* value (Matthews, 1968) of 2.49 Å³ Da⁻¹ and a solvent content of 50.6%. Attempts to solve the structures of hPCNA^{G178S} in both forms are now in progress using molecular replacement.

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