

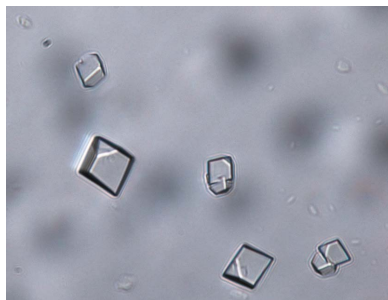
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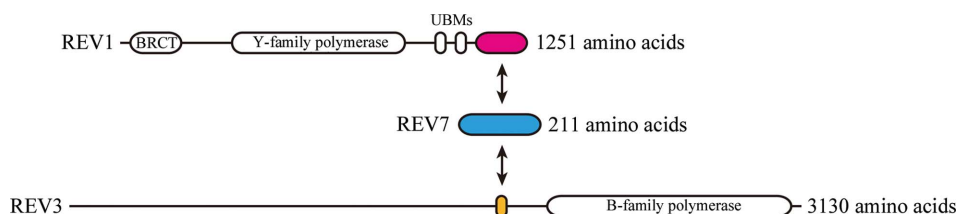
Crystallization and X-ray diffraction analysis of the ternary complex of the C-terminal domain of human REV1 in complex with REV7 bound to a REV3 fragment involved in translesion DNA synthesis

REV1, REV3 and REV7 are pivotal proteins in translesion DNA synthesis that allows DNA synthesis to continue even in the presence of DNA damage. REV1 and REV3 are error-prone DNA polymerases, while REV7 acts as an adaptor protein that links them together. A ternary complex of the C-terminal domain of human REV1 in complex with REV7 bound to a REV3 fragment has been crystallized. The crystals belonged to space group $P3_121$, with unit-cell parameters $a = b = 74.7$, $c = 124.5$ Å.

1. Introduction

Genomic DNA is constantly damaged by various factors, including endogenous and exogenous agents. The majority of DNA lesions stall the replicative DNA polymerases, DNA polymerase δ (Pol- δ) and Pol- ϵ , resulting in the arrest of DNA replication, which causes lethal effects such as genomic instability and cell death. Translesion DNA synthesis (TLS) is a mechanism that releases this replication blockage and allows DNA synthesis to proceed even in the presence of DNA damage. TLS is DNA synthesis using damaged DNA as a template and is performed by specialized error-prone DNA polymerases including REV1, Pol- η , Pol- κ , Pol- ι and Pol- ζ . It is generally considered that TLS includes two steps performed by at least two types of TLS polymerase, namely inserter and extender polymerases (reviewed in Moldovan *et al.*, 2007; Friedberg *et al.*, 2005). In the first step, an inserter polymerase (from the Y-family of DNA polymerases) such as REV1, Pol- η , Pol- κ or Pol- ι (Ohmori *et al.*, 2001) incorporates a nucleotide at the DNA lesion site where replicative polymerase has stalled. In the second step, the extender polymerase Pol- ζ then begins synthesis from this nucleotide. Pol- ζ is composed of two subunits: REV3 and REV7. REV3 is the catalytic subunit of Pol- ζ and is classed as a B-family DNA polymerase. Human REV3 is composed of 3130 amino-acid residues and has a molecular weight of 353 kDa. REV7 is a noncatalytic subunit of Pol- ζ . It has been reported that yeast Rev7 stimulates the polymerase activity of yeast Rev3 (Nelson *et al.*, 1996). Human REV7 is composed of 211 amino-acid residues with a molecular weight of 24 kDa and interacts with the central region of human REV3 (Fig. 1; Murakumo *et al.*, 2000). Recently, the crystal structure of human REV7 in complex with a human REV3 fragment has been reported, revealing the mechanism of the interaction between REV7 and REV3 and revealing that this interaction is indispensable for the cellular function of REV3 (Hara *et al.*, 2010). REV1 is a Y-family DNA polymerase which incorporates a single cytosine at the lesion site. Human REV1 is composed of 1251 amino-acid residues with a molecular weight of 138 kDa. In addition to its polymerase activity, REV1 interacts with other Y-family DNA polymerases (Pol- η , Pol- κ and Pol- ι) through its C-terminal domain (Fig. 1) (Murakumo *et al.*, 2001; Guo *et al.*, 2003; Ohashi *et al.*, 2004) and is therefore believed to function as a scaffold protein at sites of DNA damage. Furthermore, functional collaboration of REV1,

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Figure 1

Domain architecture and interactions of human REV1, REV7 and REV3. The C-terminal region of REV1, REV7 and the central region of REV3 involved in protein-protein interactions are shown in magenta, blue and yellow, respectively. Double-headed arrows indicate protein-protein interactions.

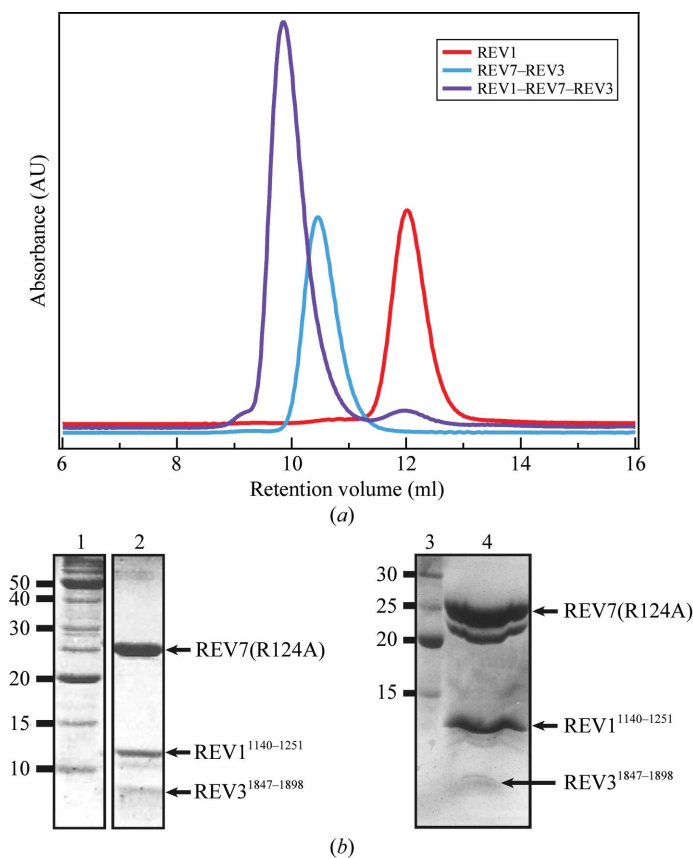
REV3 and REV7 in TLS has been reported (Okada *et al.*, 2005). We have recently reported the formation of the ternary complex of the C-terminal domain of human REV1, REV7 and a REV3 fragment, and our structural and functional studies have implied structural interplay between these three proteins in TLS (Hara *et al.*, 2010). We proposed that REV7 acts as an adaptor protein that functionally links REV1 and REV3. Recently, it has been suggested that exchange of Pol- δ and REV3, the catalytic subunit of Pol- ζ , occurs on accessory proteins bound to PCNA (Baranovskiy *et al.*, 2012).

To clarify the structural basis of communication between REV1, REV7 and REV3, we have attempted structure determination of the REV1-REV7-REV3 ternary complex. In this study, we describe the crystallization of the C-terminal domain of human REV1 in complex with human REV7 bound to a human REV3 fragment and the initial diffraction studies of the REV1-REV7-REV3 ternary complex.

2. Materials and results

2.1. Protein preparation

cDNA for the C-terminal domain of human REV1 (residues 1140–1251; REV1^{1140–1251}) was cloned in the pQE30 vector (Qiagen) at the *Bam*HI-*Sall* site. The plasmid encodes human REV1^{1140–1251} with an N-terminal hexameric His-tag. REV1^{1140–1251} was purified using the following procedure. The expression vector was introduced into *Escherichia coli* strain BL21 (DE3). The cells were grown at 310 K to a cell density of 0.5–0.7 at 660 nm and were grown for a further 3 h after the addition of 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG). The harvested cells were suspended in 2 ml buffer I (50 mM HEPES-NaOH pH 7.4, 500 mM NaCl, 40 mM imidazole) per gram of cells, cooled with liquid nitrogen and stored at 193 K. The cells were thawed in ice-water and lysed by the addition of 333 μ l buffer I containing 100 mM spermidine pH 7.4 and 4 mg ml⁻¹ lysozyme. The cells were incubated on ice for 30 min, heated in a 310 K water bath for 90 s and then incubated on ice for 30 min. The cell lysate was clarified by centrifugation for 30 min at 277 K. Subsequent purification was carried out at 277 K. The supernatant was applied onto Ni Sepharose 6 Fast Flow resin (GE Healthcare) equilibrated with buffer I. The resin was washed with buffer I and the His-tag-fused REV1^{1140–1251} was eluted with buffer I containing 200–300 mM imidazole. The eluate was diluted with buffer II (50 mM HEPES-NaOH pH 7.4) and applied onto a HiTrap Q HP column (GE Healthcare) equilibrated with buffer II using an ÄKTA chromatography system (GE Healthcare). The protein was eluted with a linear gradient from 0 to 600 mM NaCl. The eluted protein was applied onto a HiLoad 16/60 Superdex 75 prep-grade column (GE Healthcare) equilibrated with buffer III (5 mM HEPES-NaOH pH 7.4,


Figure 2

(a) Formation of the human REV1^{1140–1251}-REV7(R124A)-REV3^{1847–1898} ternary complex. SEC elution profiles (absorbance at 280 nm) of purified REV1^{1140–1251} (red line), the REV7(R124A)-REV3^{1847–1898} binary complex (blue line) and the REV1^{1140–1251}-REV7(R124A)-REV3^{1847–1898} ternary complex (purple line) are shown. (b) SDS-PAGE of the REV1^{1140–1251}-REV7(R124A)-REV3^{1847–1898} ternary complex. Lanes 1 and 2 in the left panel contain a molecular-weight marker (labelled in kDa) and the REV1^{1140–1251}-REV7(R124A)-REV3^{1847–1898} ternary complex obtained by SEC, respectively. Lanes 3 and 4 in the right panel contain a molecular-weight marker (labelled in kDa) and washed and dissolved crystals of the ternary complex, respectively. (c) Crystals of the REV1^{1140–1251}-REV7(R124A)-REV3^{1847–1898} ternary complex.

Table 1

Data-collection statistics of the crystal of the REV1^{1140–1251}–REV7(R124A)–REV3^{1847–1898} ternary complex.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.1000
Resolution range (Å)	20.0–3.0 (3.11–3.00)
Measured reflections	36414
Unique reflections	7829
Completeness (%)	93.6 (88.3)
Mean $I/\sigma(I)$	10.7 (2.1)
$R_{\text{merge}}^{\dagger}$	0.124 (0.386)

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

100 mM NaCl). The eluted protein was concentrated to 10 mg ml⁻¹ using an Amicon Ultra-15 3000 molecular-weight cutoff centrifugal filter unit (Millipore). The purity of REV1^{1140–1251} was confirmed by SDS–PAGE with Coomassie Brilliant Blue staining. Human REV7 with the R124A mutation [REV7(R124A)] in complex with a human REV3 fragment (residues 1847–1898; REV3^{1847–1898}) was prepared by the procedure reported previously (Hara *et al.*, 2009). To confirm the formation of the REV1^{1140–1251}–REV7(R124A)–REV3^{1847–1898} ternary complex, we performed a size-exclusion chromatography (SEC) analysis on a Superdex 75 10/300 GL column (GE Healthcare) equilibrated with buffer III. REV1^{1140–1251} and REV7(R124A)–REV3^{1847–1898} were mixed in a equimolar ratio (100 μM in buffer III). The SEC result clearly indicated that these three proteins form a ternary complex (Figs. 2*a* and 2*b*).

2.2. Crystallization

Prior to crystallization, the REV1–REV7–REV3 complex was prepared by mixing REV1^{1140–1251} and REV7(R124A)–REV3^{1847–1898} complex in an equimolar ratio [5 mg ml⁻¹ REV1^{1140–1251} and 10 mg ml⁻¹ REV7(R124A)–REV3^{1847–1898} in buffer III]. Initial crystallization screening for the ternary complex was performed by the sitting-drop vapour-diffusion method using a Hydra II Plus One liquid-handling system (Matrix). The drops were prepared by mixing 0.2 μl protein solution with 0.2 μl reservoir solution. Preliminary screening was performed using commercially available screening kits from Hampton Research, Emerald BioSystems and Qiagen. Small crystals were obtained using several conditions. These conditions were optimized using the hanging-drop vapour-diffusion method. Eventually, single crystals suitable for X-ray diffraction study were grown in one week with a reservoir solution consisting of 162 mM triammonium citrate, 18% (w/v) PEG 3350, 0.1 M TCEP–HCl (Fig. 2*c*).

SDS–PAGE showed that the crystals contained all three proteins, although a degradation product of REV7 also appeared (Fig. 2*b*).

A crystal was picked up in a nylon loop and cooled in liquid nitrogen prior to X-ray diffraction experiments. X-ray data collection was carried out on beamline BL-1A at the Photon Factory (PF), Tsukuba, Japan using a PILATUS 2M-F photon-counting detector (Dectris). Diffraction data were integrated, scaled and averaged with *HKL-2000* (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 1. The crystal belonged to the trigonal space group *P*3₂1, with unit-cell parameters $a = b = 74.7$, $c = 124.5$ Å. The asymmetric unit was estimated to contain one ternary complex ($V_M = 2.20$ Å³ Da⁻¹). Structure determination by the molecular-replacement method using the REV7(R124A)–REV3^{1847–1898} complex (PDB entries 3abd and 3abe; Hara *et al.*, 2010) as a search model is now in progress.

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