Crystallographic study of a novel DNA-binding domain of human HLTF involved in the template-switching pathway to avoid the replication arrest caused by DNA damage

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Received 3 February 2015

Accepted 24 March 2015

Edited by A. Nakagawa, Osaka University, Japan

Keywords: HIRAN domain; HLTF; template switching; DNA-binding domain.

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HLTF is a pivotal protein in the template-switching pathway that allows DNA synthesis to continue even in the presence of DNA damage by utilizing a newly synthesized undamaged strand as a template. HLTF has a novel DNA-binding domain termed HIRAN that has been recently found in various proteins, although its detailed function remains unclear. In this study, the HIRAN domain of human HLTF was successfully crystallized. The crystals belonged to space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 130.0$, $c = 150.1$ Å.

1. Introduction

A large amount of DNA damage occurs daily owing to internal and external factors. Damaged template DNA blocks accurate DNA synthesis by replicative DNA polymerase. Replication arrest leads to collapse of the replication fork and results in genomic instability, commonly causing various human diseases, including cancer, and aging. To avoid replication arrest and restart DNA synthesis even in the presence of DNA damage, cells employ DNA damage tolerance, also known as post-replication repair (reviewed by Friedberg *et al.*, 2014). DNA damage tolerance consists of two pathways to synthesize DNA: translesion and template-switched DNA syntheses. Translesion DNA synthesis (TLS) is transient DNA synthesis using a damaged template DNA by error-prone DNA polymerases specialized for DNA damage termed TLS polymerases. Template-switched DNA synthesis (template switching; TS), also known as the DNA damage-avoidance pathway, in which one newly synthesized strand is utilized as an undamaged template for DNA synthesis by replicative polymerases, is basically an error-free process.

DNA damage tolerance is regulated by post-translational modification of PCNA (proliferating cell nuclear antigen), which is an accessory protein of various DNA polymerases, to stimulate DNA synthesis. Monoubiquitination and Lys63-linked polyubiquitination of PCNA at Lys164 promotes the TLS and TS pathways, respectively (Hoege *et al.*, 2002). Monoubiquitination of PCNA is performed by the RAD6–RAD18 complex, the complex of an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase. In contrast, Lys63-linked polyubiquitination of PCNA at Lys164 is performed by a different E2–E3 complex, MMS2–UBC13 (E2) and RAD5 (E3). RAD5 (previously known as REV2) was initially iden-

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tified in budding yeast as a protein involved in DNA repair with DNA-dependent ATPase activity (Johnson *et al.*, 1992, 1994). Ten years later, it was shown that yeast RAD5 is an E3 ubiquitin ligase responsible for Lys63-linked polyubiquitination of PCNA (Hoege *et al.*, 2002). Interestingly, mammalian cells contain two RAD5 homologues, HLTf (helicase-like transcription factor) and SHPRH (SNF2, histone-linker, PHD and RING finger helicase), both of which are involved in the polyubiquitination of PCNA and the regulation of DNA damage tolerance (MacKay *et al.*, 2009; Motegi *et al.*, 2006, 2008; Unk *et al.*, 2006, 2008). HLTf and SHPRH have an SWI/SNF helicase domain involved in the fork-regression that is required for TS. The helicase domain is divided into N-terminal and C-terminal parts by an insertion of the RING domain involved in the polyubiquitination of PCNA (Fig. 1). Sequence analysis suggests that HLTf contains a novel domain referred to as HIRAN (HIP116 and RAD5 N-terminal) that is proposed to function as a DNA-binding domain and is found in various proteins involved in DNA metabolism (Iyer *et al.*, 2006), whereas SHPRH lacks the HIRAN domain. Bioinformatics studies have predicted that this domain is composed of 7–8 β -strands and a single helix, suggesting that it could potentially adopt a β -sandwich-like structure reminiscent of the cytochrome *f* or immunoglobulin folds (Iyer *et al.*, 2006). Nonetheless, the structural basis of and functional evidence for the HIRAN domain in DNA binding still remain unclear to date. To clarify the structure and function of the HIRAN domain of human HLTf and its implications for the mechanism of the TS pathway, we have attempted structure determination of the HIRAN domain of human HLTf. Human HLTf is composed of 1009 amino-acid residues with a molecular weight of 114 kDa. Based on bioinformatics such as domain-boundary prediction, secondary-structure prediction and disorder prediction, we designed the expression vector of the HIRAN domain of human HLTf including residues 58–174 (HLTf^{58–174}). In this study, we describe the crystallization of HLTf^{58–174} and the diffraction study of the crystal.

2. Materials and methods

2.1. Expression and purification of human HLTf^{58–174}

The cDNA encoding human HLTf^{58–174} was inserted into the BamHI–XhoI sites of the modified pET-28b vector. The plasmid encodes human HLTf^{58–174} with an N-terminal hexameric His tag (His-HLTf^{58–174}). His-HLTf^{58–174} was purified by the following procedure. The expression vector

Figure 1

Domain architecture of human HLTf. HLTf consists of HIRAN, SWI/SNF helicase and E3 RING domains. The helicase domain is divided into N-terminal and C-terminal parts by insertion of the RING domain.

Table 1

Macromolecule-production information.

The restriction sites of the primers are underlined.

Source organism	Human
Forward primer	CGCGGATCCGTTTATTGGAAGTTTGAGAGG
Reverse primer	GCGCTCGAGTCATTATGCAGGACCAATTAAAT-CC
Expression vector	pET-28b
Expression host	<i>E. coli</i> BL21(DE3)
Complete amino-acid sequence of the construct produced	MRGSHHHHHGSENLYFQGGSVLFGSLRGHVVL- RYYTGVVNNEMVALQRDPNNPYDKNAIKVNN- VNGNQVGHLLKELAGALAYIMDNKLAQIEGVV- PFGANNAFTMPLHMTFWGKEENRKAIVSDQLKK- HGFKLGA

was introduced into *Escherichia coli* strain BL21(DE3). The cells were grown at 310 K to a cell density of 0.5–0.8 at 660 nm in TB medium (4 l) and were cultured for a further 4.5 h after the addition of 0.1 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) per gram of cells, frozen using 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, 2 mM phenylmethylsulfonyl fluoride (PMSF) and 10 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 60 min at 277 K (48 400g). Subsequent purification was carried out at 277 K. The supernatant was applied onto Ni–NTA agarose resin (Qiagen) and the resin was washed with buffer I. His-HLTf^{58–174} was eluted with buffer containing 50–500 mM imidazole. The eluent was diluted with buffer II (50 mM HEPES–NaOH pH 7.4) and applied onto a HiTrap SP column (GE Healthcare) equilibrated with buffer II using an ÄKTApurifier plus chromatography system (GE Healthcare). The protein was eluted with a linear gradient from 50 mM to 1.0 M NaCl. The eluted protein was applied onto a HiLoad 16/60, Superdex 75 prep-grade column (GE Healthcare) equilibrated with buffer III (10 mM HEPES–NaOH pH 7.4, 100 mM NaCl) using an ÄKTApurifier chromatography system (GE Healthcare). The eluted protein was concentrated to 20 mg ml^{–1} using a Vivaspin (5 kDa NMWL) concentrator (Sartorius) and stored at 193 K until use. The purity of His-HLTf^{58–174} was confirmed by SDS–PAGE with Coomassie Brilliant Blue staining. Macromolecule-production information is summarized in Table 1.

2.2. Crystallization and diffraction study

Crystallization screening for His-HLTf^{58–174} was performed by the sitting-drop vapour-diffusion method using commercially available screening kits from Hampton Research (Crystal Screen and Crystal Screen 2) and Qiagen (The PACT, JSCG+ and PEGs Suites) at 293 K. The drops were prepared by mixing 0.5 μ l protein solution with 0.5 μ l reservoir solution. Prior to diffraction study, crystals were transferred into a buffer composed of 1.6 M magnesium sulfate, 0.1 M MES pH 6.5, 20% ethylene glycol for cryoprotection. Each crystal was

Table 2

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL-26B2, SPring-8
Wavelength (Å)	1.0000
Temperature (K)	100
Detector	Rayonix MX225
Crystal-to-detector distance (mm)	200
Rotation range per image (°)	1
Total rotation range (°)	180
Exposure time per image (s)	3
Space group	$P4_12_12$ or $P4_32_12$
a, b, c (Å)	130.0, 130.0, 150.1
α, β, γ (°)	90, 90, 90
Mosaicity (°)	0.159
Resolution range (Å)	50.0–3.30 (3.49–3.30)
Total No. of reflections	290257 (45322)
No. of unique reflections	20051 (3138)
Completeness (%)	99.6 (98.3)
Multiplicity	14.4 (14.4)
$\langle I/\sigma(I) \rangle$	17.0 (6.3)
R_{merge}	0.152 (0.508)

picked up in a nylon loop and cooled in liquid nitrogen, and was stored in liquid nitrogen using a Universal V1-Puck (Crystal Positioning Systems Inc.) until use. X-ray data collection was carried out on the Riken Structural Genomics Beamline BL-26B2 at SPring-8 (Sayo, Japan) using an MX225 CCD detector (Rayonix) and the SPACE (SPring-8 Precise Automatic Cryo-sample Exchanger) robotics controlled by the BSS (Beamline Scheduling Software) system. Diffraction data were integrated, scaled and averaged with XDS (Kabsch, 2010).

3. Results and discussion

Human HLTF^{58–174} was overexpressed as a His-tag-fused recombinant protein (His-HLTF^{58–174}) and purified to homogeneity using Ni-affinity, cation-exchange and size-exclusion chromatography. Single crystals of His-HLTF^{58–174} were obtained in various conditions including PEGs or salts in a few days. Eventually, single crystals grown with a reservoir solution consisting of 1.6 M magnesium sulfate, 0.1 M MES pH 6.5 at 293 K were used in the present X-ray diffraction study (Fig. 2). The diffraction study gave the crystallographic parameters for the crystal of His-HLTF^{58–174} given in Table 2. The crystal belonged to the tetragonal space group $P4_12_12$ or $P4_32_12$, with unit-cell parameters $a = b = 130.0$, $c = 150.1$ Å. The asymmetric unit was estimated to contain five to ten molecules with a V_M in the range from 3.86 to 1.93 Å³ Da^{−1}. Analysis of the self-rotation function did not show a clear peak corresponding to rotational symmetry in the asymmetric unit. A structural genomics group has deposited an NMR structure of the HIRAN domain (PDB entry 2I1i; Structural Genomics Consortium, unpublished work). However, structure determination by the molecular-replacement method

Figure 2
Crystals of His-HLTF^{58–174}.

using the NMR structure was not successful. Thus, the structure of the HIRAN domain may differ from the NMR structure. Structure determination using selenomethionine-labelled protein is now in progress.

Acknowledgements

We acknowledge the kind support of the beamline staff at SPring-8. This work was supported by grants from KAKENHI to AH and HH (23770125 and 25291017, respectively) and from the Protein 3000 Project and Targeted Protein Research Program (TPRP) to HH. The Takeda Science Foundation and The Naito Foundation also supported HH during the progress of this study.

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