PCNA is involved in the EndoQ-mediated DNA repair process in Thermococcales

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To maintain genome integrity for transfer to their offspring, and to maintain order in cellular processes, all living organisms have DNA repair systems. Besides the well-conserved DNA repair machineries, organisms thriving in extreme environments are expected to have developed efficient repair systems. We recently discovered a novel endonuclease, which cleaves the 5′ side of deoxyinosine, from the hyperthermophilic archaeon, Pyrococcus furiosus. The novel endonuclease, designated as Endonuclease Q (EndoQ), recognizes uracil, abasic site and xanthine, as well as hypoxanthine, and cuts the phosphodiester bond at their 5′ sides. To understand the functional process involving EndoQ, we searched for interacting partners of EndoQ and identified Proliferating Cell Nuclear Angigen (PCNA). The EndoQ activity was clearly enhanced by addition of PCNA in vitro. The physical interaction between the two proteins through a PIP-motif of EndoQ and the toroidal structure of PCNA are critical for the stimulation of the endonuclease activity. These findings provide us a clue to elucidate a unique DNA repair system in Archaea.
same biochemical properties. Furthermore, it is of note that a homolog is found in a few bacteria, but so far not in any eukaryotic organism.

Biochemical characterization of EndoQ showed that it is involved in the damaged DNA base repair system. However, there is no evidence for how EndoQ functions in this process. Although another hypoxanthine specific endonuclease, Endonuclease V (EndoV), is considered to function in removing deaminated adenine in *P. furiosus*, as well as in *E. coli* and other prokaryotes, our *in vitro* analyses predicted that EndoQ and EndoV are not involved in the same repair pathway, but rather work independently. Furthermore, *Pfu*EndoQ is expected to act more effectively on hypoxanthine-containing DNA than EndoV from *P. furiosus* cells.

To address the question of how EndoQ works in the repair of damaged bases in DNA in the Thermococcales, we have been searching for its interaction partners. Proliferating cell nuclear antigen (PCNA) plays an essential role in DNA transactions, including replication, repair, recombination, and cell cycle control. PCNA is a ring-shaped trimeric complex. The central hole of the PCNA ring encircles double-stranded DNA to provide a scaffold to many proteins that acts on DNA, and it is called the clamp molecule. The β-clamp (identified as the β subunit of DNA polymerase III) in Bacteria has same functions as PCNA. Proteins interacting with PCNA possess a consensus sequence motif called PIP (PCNA-interacting protein) box (Qxxhxaxa; x, any amino acid; h, hydrophobic residues; a, aromatic residues). A similar motif to PIP box is also conserved as a β-clamp binding sequence in *Bacteria*. In this study, we found a PIP box-like motif at the C-terminal region of EndoQ. With respect to proteins that are involved in the early steps of the BER pathway from Archaea, previous studies showed that PCNA interacts with UDG and APE and enhances the glycosylase activity of UDG and the 3′–5′ exonuclease activity of APE in *P. furiosus*. It has also been shown that UDG from *Sulfolobus solfataricus* and from *Pyrobaculum aerophilum* interact with their PCNA. Hence, the PIP box-like motif in the EndoQ protein implies the possibility that PCNA is involved in EndoQ function. Here we report the physical and functional association of PCNA with EndoQ in *in vitro* and propose a repair pathway in the Thermococcales.

Results

EndoQ homologs have a PIP-box motif at the C-terminus. An alignment of the amino acid sequence showed that most EndoQ homologs from Archaea, except for the Methanomicrobiales, have PIP box-like motifs at their C-terminal region (Fig. 1). Thus we assumed that EndoQ proteins would interact with PCNA through the motifs. It is also of note that the endoQ gene is present in Bacteria, such as *Bacillus subtilis* and *Disulfuvivrio sp.*, although EndoQ is mainly conserved in Archaea. It is yet to be determined if these endoQ genes are expressed in the bacterial cells and have a function to cleave the DNA at the damaged site. However, the consensus sequences of the β-clamp binding motifs were found in the C-terminal region of the putative sequences of the bacterial EndoQ homologs. It will, therefore, be interesting to investigate if the physical and functional interactions between EndoQ and the clamp molecules from Bacteria, even though PCNA and β-clamp are thought to have evolved independently (see Supplementary Fig. S1).

Preparation of TkoEndoQ and TkoPCNA1 proteins. To investigate the interaction between EndoQ and PCNA from *T. kodakarensis*, we prepared the mutant EndoQ with truncation of the PIP-box-like sequence and mutant PCNA with point mutations at the interface of the protomers for disruption of the ring structure. We deleted the amino acids from position 409 to 421 for TkoEndoQ, and designated it TkoEndoQΔPIP. It is known that the D143A/D147A mutant of PfuPCNA cannot form a stable ring structure in solution, and therefore, the corresponding E143A/D147A mutations were made in TkoPCNA1. *T. kodakarensis* has two PCNAs, and PCNA1, but not PCNA2, is essential for cell viability. Recombinant proteins expressed in *E. coli*, i.e., TkoEndoQWT (MW: 48080.3), TkoEndoQΔPIP (MW: 46491.5), TkoPCNA1WT (MW: 28239.4) and TkoPCNA1Δ143A/D147A (MW: 28137.4) were purified to near homogeneity (Fig. 2). To confirm the disruption of the ring structure of TkoPCNA1Δ143A/D147A in solution, purified TkoPCNA1s were subjected to gel filtration analysis (see Supplementary Fig. S2). Each protein eluted as a single peak, but the elution positions were different. The molecular weight estimation of TkoPCNA1Δ143A/D147A was 37.3 k, while TkoPCNA1WT was 99.1 k from the elution profiles. It is already known that PCNA molecules are eluted slightly earlier than the calculated molecular weights. This result suggests that TkoPCNA1Δ143A/D147A exists as a monomer in solution even at a high concentration (160 μM). Maintenance of the structural conformation of TkoEndoQ after deletion of the C-terminal PIP region was supported by the comparison of the CD spectra from TkoEndoQWT and TkoEndoQΔPIP. Two spectra that were almost superimposed were obtained from the two proteins (see Supplementary Fig. S3). Further experiments were performed using these purified proteins.

Physical interaction between TkoEndoQ and TkoPCNA1. To investigate whether TkoEndoQ physically binds TkoPCNA1, surface plasmon resonance (SPR) analysis was performed using the purified proteins. As shown in Fig. 3, TkoEndoQ showed the positive sensorgram against the immobilized TkoPCNA1, and the responses increased in a protein concentration-dependent manner. The *Kd* value for the interaction between the two proteins was 55 nM, which was calculated from the sensorgrams of seven different concentrations of TkoEndoQ. On the other hand, TkoEndoQΔPIP did not show any response with TkoPCNA1 even at a high concentration up to 800 nM. These results clearly indicated that the PIP-box located in the C-terminus of TkoEndoQ is essential for its interactions with TkoPCNA1. In this experiment, TkoPCNA1 was fixed on a sensorchip at less than 2 μM, in which TkoPCNA1WT exists as a monomer in solution as we showed previously. Therefore, TkoEndoQ should binds to the monomeric form of TkoPCNA1 as observed in many other PCNA binding proteins.

Stimulation of endonuclease activity of TkoEndoQ by TkoPCNA1. To gain an information of how the physical interaction between EndoQ and PCNA contribute to DNA repair and the genome integrity, a cleavage...
## Putative PIP boxes in EndoQ homologs in Archaea

Table 1. Putative PIP boxes in EndoQ homologs in Archaea. PIP box consensus sequence is shown on the top of the panel: Q, a glutamine residue (magenta background); x, any amino acid; h, hydrophobic residues (L, I or M; cyan background); A, aromatic residues (F, Y or W; yellow background). The residues consistent with the PIP box consensus sequence are in the same color. Tko, *Thermococcus kodakarenisis* KOD1 (BAD85076); Ton, *Thermococcus onnurineus* NA1 (ACJ15906); Pfu, *Pyrococcus furiosus* DSM 3638 (AAL81675); Pab, *Pyrococcus abyssi* GE5 (CAH95947); Mac, *Methanosarcina acetivorans* C2A (AAM04083); Mma, *Methanosarcina mazei* Go1 (AAM31501); Mba, *Methanosarcina barkeri* str. Fusaro (AAM31501); Mja, *Methanocaldococcus jannaschii* DSM 2661 (AAB98023); Mig, *Methanotorrhis igneus* Kol 5 (AEE79206); Mae, *Methanocaldococcus jannaschii* Nankai-3 (ABR56895); Mmp, *Methanocaldococcus maripaludis* C5 (ABO388578); Mvo, *Methanocaldococcus voltae* A3 (ADI37103); Mth, *Methanothermobacter thermautotrophicus* str. Delta H (AAD85873); Sma, *Methanobrevibacter smithii* ATCC 35601 (ABQ73334); Mka, *Methanopyrus kandlerii* AV19 (AAM01639); Nar, *Nanoarchaeota archaeon* SCGC AAA011-222 (WP_039268906); Aar, *Aenigmarchaeota archaeon* JGI 0000106-F11 (WP_042665925).

The sequences were aligned by ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). PIP boxes of DNA polymerase B and DP2 from *P. furiosus* are shown on the bottom. Positions of the motifs are indicated by the amino acid number on the left (start) and right (end) of the sequences.

| PIP-box consensus sequence | Q | x | x | h | x | A | A |
|----------------------------|---|---|---|---|---|---|---|
| Tko            | R | S | I | T | E | F | L | 416 |
| Ton            | T | S | L | M | K | F | L | 419 |
| Pfu            | R | T | L | L | Q | Y | I | 418 |
| Pab            | L | N | L | M | E | F | L | 416 |
| Mac            | K | S | L | F | D | F | L | 416 |
| Mma            | K | S | L | F | D | F | L | 416 |
| Mba            | Q | S | L | F | D | F | L | 416 |
| Mja            | E | V | T | L | D | R | W | L | 783 |
| Mig            | N | T | L | D | A | W | L | 390 |
| Mae            | K | T | T | L | D | K | W | I | 422 |
| Mmp            | K | L | T | D | S | W | V | 392 |
| Mvo            | K | L | T | E | N | W | M | 392 |
| Mth            | A | E | T | L | D | A | Y | F | 385 |
| Msi            | L | T | T | L | D | N | F | 395 |
| Mka            | Q | R | T | L | D | E | L | I | 414 |
| Nar            | Q | K | K | L | G | E | F | 401 |
| Aar            | Q | K | N | L | D | Y | K | 416 |
| Pfu            | Q | V | G | L | T | S | W | L | 770 |
| Pfu            | V | I | S | L | D | D | F | F | 1260 |

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assay using TkoEndoQ and TkoPCNA1 was conducted. Using an assay condition, in which TkoEndoQWT exhibited 9% cleavage on one deoxyinosine (dI)-containing DNA, the rate of the cleavage was increased in a TkoPCNA1 concentration-dependent manner (Fig. 4a, lanes 2 to 5). When TkoPCNA1WT was added at 180, 600 and 1800 nM (60, 200 and 600 nM; as a trimer) to the reaction, the rate of the cleavage was increased to 11%, 24% and 41%, respectively. Conversely, when TkoEndoQΔPIP or the monomeric mutant of TkoPCNA1E143A/D147A was used, this stimulation was not detected. Notably, the TkoEndoQΔPIP mutant showed 6–7% cleavage either with or without TkoPCNA1WT (Fig. 4a, lanes 6 to 9). The TkoPCNA1E143A/D147A mutations did not affect the cleavage activity of the TkoEndoQWT (Fig. 4a, lanes 11 to 13). These results support our observation that the TkoPCNA1 stimulated endonuclease activity of EndoQ depending on the presence of the PIP box-like motif, and the ring structure of the PCNA is important for this function. The SPR experiment shown above supports that TkoEndoQ specifically binds to the monomeric form of TkoPCNA1. However, the ring structure TkoPCNA1 is necessary to stimulate the endonuclease activity of TkoEndoQ as shown here, although one EndoQ molecule on one PCNA...
Figure 2. Preparation of the recombinant proteins. The protein marker (NEB, P7703, lane 1) and 1 μg of each purified protein (TkoEndoQWT, lane 2; TkoEndoQΔPIP, lane 3; TkoPCNA1WT, lane 4; and TkoPCNA1E143A/D147A, lane 5) were subjected to SDS-12% PAGE followed by Coomassie brilliant blue (CBB) staining. The sizes of the marker are shown on the left of the panel.

Figure 3. Physical interaction between TkoEndoQ proteins and TkoPCNA1. Surface plasmon resonance (SPR) analysis was conducted using a Biacore system. TkoEndoQWT at various concentrations (12, 25, 50, 75, 100, 400 and 800; solid lines) and TkoEndoQΔPIP (800 nM, a dotted line) were injected on the chip immobilized with TkoPCNA1WT for 120 s in 10 mM HEPES, pH 7.4, 0.005% Tween20 and 0.4 M NaCl. The sensorgrams from TkoEndoQWT were fitted to the 1:1 reaction model to calculate $K_D$. $K_D = 55$ nM.
ring is enough and is possible to access the reaction site on DNA. Generally, PCNA binding proteins can bind a PCNA protomer, but needs a ring-structured PCNA to encircle DNA for their functional interactions. Interaction of EndoQ and PCNA is conserved in the Thermococcales. To confirm that the EndoQ-PCNA interaction is conserved in the Thermococcales, purified PfuEndoQ and PfuPCNA were used for the interaction/stimulation analyses (see Supplementary Fig. S4). PfuPCNA clearly stimulated the endonuclease activity of PfuEndoQ on the dI-containing DNA by 6–7 fold (see Supplementary Fig. S5). Because the purified TkoEndoQ protein has more non-specific binding property to DNA and proteins as compared with PfuEndoQ, a higher salt concentration (0.4 M NaCl) was required for its manipulation in vitro. In addition, the endonuclease activity of TkoEndoQ showed more salt-resistance than PfuEndoQ. From these differences, the cleavage assay for PfuEndoQ was performed under reduced concentration of NaCl (0.18 M). To confirm that the EndoQ and PCNA were in the same complex in the cells, an immunoprecipitation experiment was performed using extracts from exponentially growing P. furiosus cells and antibodies raised against TkoEndoQ and PfuPCNA (a cross-reactivity of PfuEndoQ against the anti-TkoEndoQ antibody was confirmed before this IP experiment). PfuEndoQ and PfuPCNA co-precipitated with anti-TkoEndoQ or anti-PfuPCNA antibody, respectively (see Supplementary Fig. S6).

Discussion
We presented here that EndoQs from T. kodakarensis and P. furiosus interact with PCNA, and therefore, EndoQ may be involved in the replication-associated repair pathway at the replication fork, as proposed previously for P. furiosus UDGs. It was also reported that APE of P. furiosus interacts with its cognate PCNA both in vivo and in vitro. Furthermore, an efficient BER process, in which UDG and APE are bound simultaneously to the same PCNA trimer, and an efficient progress of the repair process including the sequential cleavages of the glycosyl...
and dUTPase were proposed as a complex named ‘uracilosome’ for the efficient escape from uracil under hyper-substrate preference of PolD is the opposite, supports this prediction. We have also confirmed that the PolB of in vitro binding sites are not in the terminus, but the internal part of the proteins, and shorter versions of the PIP-box, C-terminus is also typical among the PCNA-binding proteins. In the case of UDG and APE in P. furiosus, respectively, and these are typical consensus sequences of the PIP-box. The location of these sequences at the very C-terminal regions. The predicted PIP-boxes are QRSITEFL in Thermococcales addition to these molecules, we propose here that EndoQ is a member of the ‘uracilosome’ in the Thermococcales and likely in other archaea harboring its homologs. Uracil is produced by the frequently occurring deamination of cytosine, especially at high temperatures, and therefore, it is possible that the hyperthermophilic archaea acquired the efficient prevention system to alleviate mutations by cytosine deamination.

We showed here that EndoQs also interact with PCNA likely through the PIP-box-like motif in their C-terminal regions. The predicted PIP-boxes are QRSITEFL in T. kodakarensis and QRTLIIQY1 in P. furiosus, respectively, and these are typical consensus sequences of the PIP-box. The location of these sequences at the very C-terminus is also typical among the PCNA-binding proteins. In the case of UDG and APE in P. furiosus, PCNA binding sites are not in the terminus, but the internal part of the proteins, and shorter versions of the PIP-box, AKTLE in UDG31 and T1AG14 in APE, were proposed as well as for PolB. We also have a shorter version of the PIP box, QKSF, in its internal site. The apparent Kᵦ values, calculated from the SPR analysis were 55 nM for TkoEndoQ and TkoPCNA1. These results suggest that EndoQ has stronger affinity to PCNA as compared with UDG and APE. The apparent Kᵦ values for PfuUDG and PfuAPE with PfuPCNA are 220 nM and 1 µM, respectively15,34. In consideration with a very close relationship between P. furiosus and T. kodakarensis, EndoQ may mainly work for uracil and other damaged bases in the Thermococcales cell.

The Thermococcales have one family B DNA polymerase (PolB) and one family D DNA polymerase (PolD), which are supposed to be replicative DNA polymerases. However, genetic analyses showed that the polB gene can be disrupted in T. kodakarensis genome and it may mainly work for repair processes. Our previous in vitro study showing that PolB prefers gap-filling type substrates to primer-extension type substrates, while the substrate preference of PolD is the opposite, supports this prediction. We have also confirmed that the PolB of P. furiosus has strand displacement activity in vitro (Kimizu et al., unpublished result). Taken together with these results, strand displacement DNA synthesis by PolB, cleavage of the resultant flapped DNA by Fen1 endonuclease, and nick-sealing by DNA ligase will occur after incision by EndoQ, as in the case of the BER pathway. PCNA will have an important role to provide a scaffold for EndoQ, PolB, Fen1 and Lig to work on DNA efficiently for their sequential tasks (Fig. 5). Further analyses will elucidate this prediction of the damaged base repair process. The presence of the gene in some bacteria is not surprising, as the methanogens tend to grow in association with bacteria in many environments including the soil and mammalian guts, and this important gene can be acquired through horizontal gene transfer. We are currently investigating the function of the EndoQ homologs in both the mesophilic and hyperthermophilic methanogens to help shed more light on the evolution and distribution of this very fascinating DNA repair enzyme.

In conclusion, we presented here the physical and functional interactions between EndoQ and PCNA. EndoQ is probably required for the efficient repair of damaged bases in hyperthermophilic archaea and evolved in the archael and bacterial domains to form a repairosome with PCNA.

Methods

Clones and proteins. The genes encoding the TkoEndoQ and PfuEndoQ with their C-terminal truncation (deletion of residues 409–421 and 411–424, respectively), designated ΔPIP, and TkoPCNA1 with mutations at E143A and D147A were generated by site-specific mutagenesis. We designed the TkoPCNA1 mutant that does not form a stable ring structure in solution based on the previous works17,20. The PCR reaction mixtures (25 µl) contained 25 ng pET-TK088 plasmid for TkoEndoQ, pET-PF1551 for PfuEndoQ, or pET-TK0535 for TkoPCNA1, 1 × PCR buffer for KOD-Plus-Neo DNA polymerase (TOYOBO, Osaka, Japan), 1.5 mM MgSO₄, 0.2 mM of each dNTP, 0.3 M primers (TkoEndoQ, 5’-ACGGTTGAGGAAAAGCCCAAG TGAAGGAGCTGGAAGTCAATGTTAC TGTCAACAGTGCTAACTGCTGCAACATG, 5’-AGGAATCCGTTATGCTCTTCATGCCTTCAGGTGGCTTTTCTCTTAAACTGCTT; PfuEndoQ, 5’-CGAGTTGGCCAAAGCTAAATGAGGA-CCCTGCTTTCAATTATATT and 5’-AAATATTTGAGGAGCTTCTCCATCTTAGTTCTGGCGCAAATC TG, stop codons are underlined) (TkoPCNA1E143A/D147A 5’-GTGAGGTTCTCAAGGCGCGCATAAAGGGCGGTCTCCCTGTCGAC and 5’-CTGACGAGGAAACGGCCCTTATGCGGCGGTCTGAGAACCCTAC), and 0.5 unit KOD-Plus-Neo DNA polymerase (TOYOBO, Osaka, Japan). The mixtures were heated at 95 °C for 30 s and then subjected to thermal cycling (14 cycles of 95 °C for 10 s, 55 °C for 30 s, and 68 °C for 5 min). The PCR products were digested with DpnI (NEB) at 37 °C for 1 h, and transformed into E. coli JM109 cells. Each full insert was sequenced to verify the targeted mutation. The expression and purification of TkoEndoQWT, TkoEndoQΔPIP, and TkoPCNA1 were performed as described previously. TkoPCNA1E143A/D147A was also purified basically as same as the TkoPCNA1WT.
Figure 5. Schematic representation of the model of PCNA-dependent process of EndoQ-mediated repair. PCNA-bound EndoQ recognizes a damaged base in DNA and makes an incision on its 5' side. PolB on the same PCNA synthesizes a new DNA strand coupled with the 5'-3' strand displacement of the forward strand. Fen1 cuts off the resulting flap and DNA ligase seals the nick.

however, we used a 5 ml HiTrap Heparin HP column (GE Healthcare) and a 1 ml MonoQ 5/50 column (GE Healthcare). Then protein concentrations were calculated by measuring the absorbance at 280 nm. The theoretical molar extinction coefficients of TkoEndoQ WT, TkoEndoQ ΔPIP, TkoPCNA1 WT, TkoPCNA1 E143A/D147A, PfuEndoQ WT, PfuEndoQ ΔPIP, and PfuPCNA WT were calculated as 48610, 48610, 5960, 5960, 47120, 45630, and 7450 M⁻¹ cm⁻¹, respectively.

Surface Plasmon Resonance (SPR) analysis. A Biacore J (GE healthcare) system was used to study the physical interaction between TkoEndoQ and TkoPCNA1. Purified recombinant TkoPCNA1 were bound to CMS sensor chip according to the manufacturer's recommendation. To measure the kinetic parameters, various concentrations of TkoEndoQ (12, 25, 50, 75, 100, 400 and 800 nM) were applied to the immobilized TkoPCNA1. All experiments were conducted at 25°C in a buffer containing 10 mM HEPES, pH 7.4, 0.4 M NaCl and 0.005% Tween20. Regenerations at the end of each cycle were achieved by injections of 2 M NaCl. The equilibrium dissociation constants (K_d) were determined from the association and dissociation curves of the sensorgrams, using the BIAevaluation program (GE healthcare).
DNA substrates and cleavage assay. The deoxyinosine (dl)-containing oligonucleotide (45-125, 5′-dGGAACCTGCTGGAATCTCTGACACGACGATTGACGAAAGATCACCGA), labeled by Cy5 at the 5′ terminus and its complementary oligonucleotide (45R, 5′-dTGAAGGTCATGCTGATGTACATGCGTGAGATTCC- AGGCAGTTGC) were obtained from Sigma Aldrich (Tokyo, Japan). Double-stranded DNA was prepared by annealing 45-125 and 45R in TAM buffer (40 mM Tris-acetate, pH 7.8 and 0.5 mM Mg(CH3COO)2). The cleavage reactions were performed at 75 °C for 10 min in a 20 μl reaction mixture, containing 50 mM Tris-HCl, pH8.0, 1 mM DTT, 1 mM MgCl2, 0.01% Tween20, 0.4 M NaCl, 5 nM DNA substrate, 10 nM TkoEndoQ and various concentrations of TkoPCNA1 (0.1, 0.8, 1.6, and 1.8 μM, as a monomer). Reactions were terminated with 40 μl of stop solution (98% deionized formamide, 10 mM EDTA and 0.1% OrangeG). After an incubation at 95 °C for 5 min, the samples were immediately placed on ice. The samples were separated by 8 M urea-12% PAGE in TBE buffer (89 mM Tris-borate and 2.5 mM EDTA). The gel image was visualized and the resulting bands were quantified with a Typhoon image analyzer (GE healthcare).

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Author Contributions
S.I., M.S., I.C. and Y.I. designed experiments. M.S., K.Y., T.Y. and S.I. performed experiments. Y.I., S.I., M.S. and I.C. prepared the manuscript.

Additional Information
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