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Research paper

EndoQ and EndoV work individually for damaged DNA base repair in *Pyrococcus furiosus*

Sonoko Ishino**, Naruto Makita, Miyako Shiraishi, Takeshi Yamagami, Yoshizumi Ishino*

Department of Bioscience and Biotechnology, Graduate School of Bioresource and Bioenvironmental Sciences, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka, Fukuoka 812-8581, Japan

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ABSTRACT

Base deamination is a typical form of DNA damage, and it must be repaired quickly to maintain the genome integrity of living organisms. Endonuclease Q (EndoQ), recently found in the hyperthermophilic archaea, is an enzyme that cleaves the phosphodiester bond 5′ from the damaged nucleotide in the DNA strand, and may primarily function to start the repair process for the damaged bases. Endonuclease V (EndoV) also hydrolyzes the second phosphodiester bond 3′ from the damaged nucleotide, although the hyperthermophilic archaeal EndoV is a strictly hypoxanthine-specific endonuclease. To understand the relationships of the EndoQ and EndoV functions in hyperthermophilic archaea, we analyzed their interactions in hypoxanthine repair. EndoQ and EndoV do not directly interact with each other in either the presence or absence of DNA. However, EndoQ and EndoV individually worked on deoxyinosine (dI)-containing DNA at each cleavage site. EndoQ has higher affinity to dI-containing DNA than EndoV, and cells produce higher amounts of EndoQ, as compared to EndoV. These data support the proposal that EndoQ primarily functions for, at least, dI-containing DNA.

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1. Introduction

DNA is constantly damaged by endogenous and environmental influences, even under physiological conditions in cells. Base modifications, including deamination, oxidation, alkylation, and hydrolysis, are important forms of damage because these modifications change the hydrogen bonding properties of the bases, thus altering the base pair specificity and leading to mutations during DNA replication [1,2]. Therefore, these damages must be repaired quickly to protect the genetic information on the genome [2,3]. Base deaminations, which are especially promoted by high temperature, ionizing radiation, aerobic respiration and nitrosative stress, convert adenine, guanine, and cytosine to hypoxanthine, xanthine, and uracil, respectively, and therefore, the repair of DNA containing deaminated bases should be highly efficient in hyperthermophilic organisms.

We analyzed uracil DNA glycosylase (UDG) [4] and AP endonuclease (APE) [5], as members of the base excision repair (BER) pathway in *Pyrococcus furiosus*. Both of the enzymes bind PCNA and may quickly perform hydrolysis of glycosidic bond and phosphodiester cleavage reactions successively [6]. Actually, the uracil glycosylase of UDG and the 3′-5′ exonuclease activities of APE are stimulated in the presence of PCNA *in vitro*. The BER pathway starts with a DNA glycosylase, and several enzymes belonging to the uracil DNA glycosylase (UDG) superfamily have been identified [7]. In contrast, the excision repair of damaged bases initiated by a single nick near the site of a DNA lesion is referred to as alternative excision repair (AER) [8]. The enzymes that introduce a nick on the 5′ side of the damaged base include UV damage endonuclease (UVDE) and ExoIII-type APE, and the repairs with these enzymes are especially classified in nucleotide incision repair (NIR). *Escherichia coli* VSR (very short patch repair) endonuclease is not included in NIR, although it recognizes a T/G mismatched base pair and creates a single nick on the 5′ side of the thymine. No homolog for UVDE and ExoIII has been found in the *Pyrococcus* genomes. On the other had, Endonuclease V, originally identified in *E. coli* as an endonuclease that nicks DNA containing a damaged base, and was subsequently proven to be a deoxyinosine (dI) 3′-endonuclease [9–11], and the EndoV-initiated pathway is very much apart from the above NIR pathways. EndoV homologs are conserved in Bacteria, Eukarya, and Archaea [12,13]. We characterized the EndoV

* Corresponding author.
** Corresponding author.
E-mail addresses: sonoko@agr.kyushu-u.ac.jp (S. Ishino), ishino@agr.kyushu-u.ac.jp (Y. Ishino).

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homolog from *P. furiosus* (PfuEndoV), and discovered its strict substrate specificity to hypoxanthine *in vitro* [14], same as earlier reported *Archaeoglobus fulgidus* EndoV (AfuEndoV) [15]. To elucidate the EndoV-mediated AER pathway in archaeal cells, the proteins related to the cleavage reaction of dI-containing DNA were screened, and the protein possessing the activity to cleave the phosphodiester bond 5′ from dI was identified. This novel endonuclease, designated as Endonuclease Q (EndoQ), is conserved only in *Thermococcales* and some methanogens in Archaea [16]. No homolog has been found in eukaryotic genomes, but a few bacteria, including *Bacillus subtilis,* possess homologs. The distribution pattern of the genes encoding the EndoQ homologs among the prokaryotic genomes is interesting.

The EndoQ-mediated repair may be classified into nucleotide incision repair (NIR). In the methanogenic archaeon, *Methanothermobacter thermoautotrophicus,* an ExoIII-like protein, Mth212, which shares the amino acid sequence identity with *E. coli* ExoIII (30%) and human ApeI (40%) [17], cleaves on the 5′-side of dU [18]. This archaeal ExoIII-dependent uridine repair procedure was biochemically demonstrated [19]. The repair pathways for the damaged bases in Archaea, with distribution of EndoQ, ExoIII, UDG, and APE were discussed in our previous report [16] and this work.

Both EndoV and EndoQ are specific endonucleases for DNA containing damaged bases, but the cleavage sites are opposite from the damaged nucleotide. Therefore, it is intriguing to determine how these two enzymes work at one damaged site, as the answer is important to understand the repair processes functioning with EndoV and EndoQ in the *Thermococcale* cells. In this study, we analyzed the interactions between EndoV and EndoQ, and how the dI-containing DNA is cleaved in the presence of the two endonucleases at the same time.

2. Materials and methods

2.1. Preparation of the recombinant PfuEndoQ and PfuEndoV proteins

The expressions of PfuEndoQ from pET21d-PF1551 and PfuEndoV from pET28a-PF0987 were performed in basically the same manner as previously described [14,16]. Briefly, PfuEndoQ and PfuEndoV were overproduced in *E. coli* BL21 CodonPlus (DE3)-RIL (Agilent) cells. The purification of PfuEndoQ was performed by heat treatment at 80 °C for 30 min, precipitation by ammonium sulfate, and sequential column chromatography steps using HiTrap Phenyl FF, HiTrap Heparin HP, and HiTrap SP HP (GE Healthcare). The purification of His-tagged PfuEndoV was performed by heat treatment at 80 °C for 30 min, and column chromatography using HisTrap HP and HiTrap SP HP (GE Healthcare). The protein concentrations were determined by measuring the absorbance at 280 nm, with theoretical extinction coefficients of 47,120 M^{-1} cm^{-1} and 7450 M^{-1} cm^{-1} for PfuEndoQ and PfuEndoV, respectively.

2.2. Co-immunoprecipitation experiments

P. furiosus cells were cultured in 300 ml of medium at 98 °C, as described previously [16], and were harvested at the late log phase ($A_{600} = 0.6$) by centrifugation (10 min, 5000 × g). The cells (1×10^{11} cells) were suspended and disrupted in 2.6 ml of lysis buffer (50 mM Tris–HCl, pH 8.0, 50 mM NaCl, 0.5 mM DTT, 0.1 mM EDTA, 0.1% Triton X-100, and 10% glycerol) by sonication, and the soluble extracts were obtained by centrifugation. A portion (20 μl) of rProtein A Sepharose Fast Flow (GE Healthcare) was washed three times with PBS-T (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, and 0.1% Tween 20), mixed with 10 μl of anti-TkoEndoQ [16] or anti-PfuEndoV antiserum [14], and incubated at room temperature for 1 h on a rotary shaker. Each mixture was washed twice with PBS-T, and then twice with 0.2 M triethanolamine, pH 8.0. The antibody was cross-linked to Protein A with dimethyl pimelimidate 2HCl (DMP, Thermo Scientific Pierce), according to the manufacturer's protocol. After equilibration of the antibody-conjugated Protein A resin with lysis buffer, an aliquot of the cell extracts (400 μl, 1.5×10^{10} cells) was added, and the mixture was incubated at room temperature for 1 h on the rotary shaker. The precipitates were washed thrice with lysis buffer, and the immunoprecipitated proteins were eluted from the beads with 40 μl of gel loading solution (50 mM Tris–HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol, 0.2 mg/ml bromophenol blue, and 2% SDS) at 98 °C for 3 min. The protein samples were subjected to SDS-12% PAGE, followed by western blot analysis. The proteins were detected with Immobilon Western reagents (Millipore) and an LAS-3000 mini image analyzer (Fujifilm). PfuEndoQ was cross-reacted with anti-TkoEndoQ antiserum, which was prepared by immunizing a rabbit with the recombinant EndoQ protein from *Thermococcus kodakarensis*.

2.3. In vitro pull-down assay

PfuEndoQ (10 μM) was incubated with His-tagged PfuEndoV (10 μM), in 120 μl of buffer A (20 mM potassium phosphate, pH 7.4, and 0.15 M NaCl) containing 20 mM imidazole, at 60 °C for 5 min. After the incubation, the protein solution was mixed with 60 μl of Ni-NTA agarose (QIAGEN) and 380 μl of assay buffer A containing 20 mM imidazole, and incubated at room temperature for 1 h. The resin was subsequently washed thrice with 300 μl of buffer A containing 20 mM imidazole. The bound proteins were eluted thrice with 60 μl of buffer A containing 0.5 M imidazole. The fractions were analyzed by SDS-12.5% PAGE, followed by staining with CBB. As a negative control, PfuEndoQ alone was subjected to the same procedures.

2.4. DNA substrates and cleavage assay

The dI-containing deoxyoligonucleotides (45-I25, 5′-dCGAACTGCCTGGAATCCTGACGACITGTAGCGAACGATCACCTCA), labeled by Cy5 at the 5′ terminus or FITC at the 3′ terminus, and the other deoxyoligonucleotides were obtained from Sigma Aldrich (Tokyo, Japan) and Hokkaido System Science (Sapporo, Japan). The labeled dI-containing dsDNA (I-dsDNA) was prepared by annealing 45-I25 and its complementary deoxyoligonucleotide (temp-45, 5′-dTGAGGTGATCGTTCGCTACATGTCGTCAGGATTCCAGGCAGTTCG), in 40 mM Tris-acetate, pH 7.8, and 0.5 mM magnesium acetate. The PfuEndoQ and PfuEndoV cleavage reactions were performed at 70 °C, in solutions containing 5 nM I-dsDNA, 50 mM Tris–HCl, pH 8.0, 1 mM DTT, 1 mM $MgCl_2$, 0.01% Tween 20, and 0.1 mg/ml BSA. The concentrations of the proteins and the reaction times are indicated for each reaction. The reactions were terminated with a double portion of stop solution (98% formamide and 10 mM EDTA). After an incubation at 98 °C for 5 min, the samples were immediately transferred to ice. The samples were separated by 8 M urea-12% PAGE in TBE buffer (89 mM Tris, 89 mM boric acid, and 2.5 mM EDTA, pH 8.3). The products were visualized with a Typhoon Trio + image analyzer (GE Healthcare).

2.5. Biochemical properties of the PfuEndoQ activity

The dependencies of the catalytic activity of PfuEndoQ on pH, reaction temperature, salt concentration, and divalent cations were measured. The basic reaction solution (10 nM 5′-Cy5-labeled I-dsDNA, 50 mM buffer, 1 mM DTT, 1 mM divalent cation, and 0.01% Tween 20) was modified as necessary. Cleaved products were

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