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Human Endonuclease V as a Repair Enzyme for DNA Deamination

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Abstract

The human endonuclease V gene is located in chromosome 17q25.3 and encodes a 282 amino acid protein that shares about 30% sequence identity with bacterial endonuclease V. This study reports biochemical properties of human endonuclease V with respect to repair of deaminated base lesions. Using soluble proteins fused to thioredoxin at the N-terminus, we determined repair activities of human endonuclease V on deoxyinosine (I)-, deoxyxanthosine (X)-, deoxyoxanosine (O)- and deoxyuridine (U)-containing DNA. Human endonuclease V is most active with deoxyinosine-containing DNA but with minor activity on deoxyxanthosine-containing DNA. Endonuclease activities on deoxyoxanosine and deoxyuridine were not detected. The endonuclease activity on deoxyinosine-containing DNA follows the order of single-stranded I > G/I > T/I > A/I > C/I. The preference of the catalytic activity correlates with the binding affinity of these deoxyinosine-containing DNAs. Mg²⁺ and to a much less extent, Mn²⁺, Ni²⁺, Co²⁺ can support the endonuclease activity. Introduction of human endonuclease V into *Escherichia coli* cells deficient in *nfi*, *mug* and *ung* genes caused three-fold reduction in mutation frequency. This is the first report of deaminated base repair activity for human endonuclease V. The relationship between the endonuclease activity and deaminated deoxyadenosine (deoxyinosine) repair is discussed.

Keywords

deamination; nitrosative stress; inosine; xanthosine; endonuclease; DNA repair

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Conflict of interest Statement

The authors declare that there are no conflicts of interest.

1. Introduction

Endonuclease V (endo V encoded by *nfi* gene) was initially discovered in *Escherichia coli* (*E. coli*) as a nuclease that acted on a variety of DNA damage [1, 2]. In the course of identifying hypoxanthine DNA repair activity, *E. coli* endo V was rediscovered as a deoxyinosine 3' endonuclease [3, 4]. Endo V in general hydrolyzes the second phosphodiester bond 3' to the aberrant site [3, 5–11]. Inosine (known as hypoxanthine as a base), xanthosine and oxanosine, and uridine are deamination products derived from adenosine, guanosine, and cytidine [12–15]. In addition to endonuclease activity on DNA containing deoxyinosine [5, 7, 9, 16], endo V was also found to be active on deoxyxanthosine [5, 17–19], deoxyuridine [2, 4, 5, 7, 20], and deoxyoxanosine [5, 6, 17]. Genetic analysis indicates that bacterial endo V is involved in repair of deoxyinosine, deoxyxanthosine, and *N*⁶-hydroxylaminopurine [21–23]. Homologs from endo V family proteins are diverse in substrate specificity. While bacterial endo V enzymes exhibit broad endonuclease activity towards different deaminated bases, endo V proteins from the archaeal species *Archaeoglobus fulgidus* and mouse seem to be active only on deoxyinosine-containing DNA [8, 9]. Endo V from *Salmonella typhimurium* appears to possess high affinity to deaminated bases as it is the only endo V enzyme tested that shows detectable binding to deoxyoxanosine-containing DNA [5, 6]. Interestingly, bacterial endo V enzymes also demonstrate endonuclease activity on mismatches [5, 7, 10, 24], small insertions/deletions (indels) [11], flap and pseudo-Y structures [11]. The mismatch and indel cleavage activity has been exploited for development of mutation identification or scanning methods [25–28].

The structure-function relationship has been extensively studied using endo V from the thermophilic bacterium *Thermotoga maritima* (Tma) as a model system. Sequence alignment has identified seven conserved motifs in endo V family proteins (Fig. 1A). D43 in motif II, E89 in motif III, and D110 in motif IV are identified as catalytic residues involved in coordination of a metal ion [17, 29]. Through a large-scale site-directed mutagenesis analysis, Y80, G83, L85 in motif III, G113, H116, R118 and G121 in motif IV, G136 and A138 in motif V, and S182 in motif VI were identified as residues that affect protein-DNA interactions [17]. The importance of Y80 in base recognition has been demonstrated by switching of base preference in mismatch cleavage by Y80A mutant [24]. The recently solved crystal structures of Tma endo V reveal that Y80 is situated in the PYIP wedge that occupies the space vacated by the flipped out hypoxanthine base [30].

Endo V family proteins are ubiquitous in bacteria, archaea, and eukaryotes (Fig. 1A). The human genome contains an endo V homolog located in chromosome 17q25.3. The biochemical and enzymatic properties of human endo V are not known. In this study, we report that human endo V is a deoxyinosine and deoxyxanthosine endonuclease. The single-stranded deoxyinosine endonuclease activity is 14-fold stronger than the single-stranded deoxyxanthosine endonuclease activity. Human endo V is active with Mg^{2+} , and to a much less extent with Mn^{2+} , Ni^{2+} and Co^{2+} as a metal cofactor. The mutation frequency in *E. coli* is suppressed by the presence of the wt human endo V, but not the active site D52A mutant. The biochemical and genetic analysis indicates that human endo V possesses deoxyinosine endonuclease activity that plays an important role for repair of deaminated purine damage *in vivo*.

2. Materials and Methods

2.1. Reagents, media and strains

All routine chemical reagents were purchased from Sigma Chemicals (St. Louis, MO), Fisher Scientific (Suwanee, GA), or VWR (Suwanee, GA). Restriction enzymes, *Taq* DNA

polymerase, Phusion high fidelity polymerase and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). BSA and dNTPs were purchased from Promega (Madison, WI). Anti-His (N-term) antibody and anti-rabbit IgG HRP-linked antibody were purchased from Cell Signaling Technology (Danvers, MA). The horseradish peroxidase substrate Opti-4CN and PVDF membrane for Western blot analysis were purchased from Bio-Rad (Hercules, CA). HiTrap chelating and Q columns were purchased from GE Healthcare (Piscataway, NJ). Oligodeoxyribonucleotides were ordered from Integrated DNA Technologies Inc. (Coralville, IA). LB medium was prepared according to standard recipes. Human endo V sonication buffer consisted of 50 mM Tris HCl (pH 7.4), 1 mM EDTA (pH 8.0), 2.5 mM DTT, 0.15 mM PMSF, 10% glycerol and 50 mM NaCl. GeneScan stop buffer consisted of 80% formamide (Amresco, Solon, OH), 50 mM EDTA (pH 8.0), and 1% blue dextran (Sigma Chemicals). TB buffer (1 ×) consisted of 89 mM Tris base and 89 mM boric acid. TE buffer consisted of 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA. *E. coli* host strain BL21(DE3)Δ3 [F^- , *ompT*, *hdsS_B*, ($r_B^- m_B^-$), *gal*, *dcm*, *sly*, (DE3), *nfi*, *ung*, *mug*] and JM109 [$e14^-$ (McrA $^-$) *endA1*, *recA1*, *gyrA96*, *thi-1*, *hdsR17*(r_k^- , m_k^+), *supE44*, *relA1Δ*(*lac-proAB*), [F^- , *traD36*, *proAB*, *lacFZΔM15*]] are from our laboratory collection. Plasmid pET28a-hnfi was constructed by PCR amplification of human cDNA (Fig. S1). *E. coli* wild type K-12 strain was obtained from *E. coli* Genetic Stock Center at Yale University (New Haven, CT).

2.2. Construction of *E. coli* BL21(DE3)Δ3 and Confirmation of the genotype

The strain *E. coli* BL21 (DE3) *mug⁻ ung⁻ nfi⁻* was constructed via transduction using the bacteriophage P1 *vir*. The individual mutant genes were obtained from the Keio Collection in the form of gene knockouts carrying a kanamycin gene cartridge [31]. A mutant gene was transduced into *E. coli* BL21(DE3) using a selection for kanamycin resistance. The resistance cartridge was then flipped out using *flp* sequences surrounding the kanamycin cartridge and *flp* recombinase supplied from the plasmid pCP20 [32]. Three rounds of transduction followed by kanamycin gene cartridge removal yielded the final strain.

The *E. coli* BL21(DE3)Δ3 strain was confirmed by PCR with wild type *E. coli* K-12 strain as positive control using the following primers: Ec.NFI.F, 5'- TAA AGT ACC CCA TGG GTG ATT ATG GAT CTC GCG TC-3' (The *NcoI* site is underlined); Ec.NFI.R, 5'- TAA AGG GTG GAT CCT AGG GCT GAT TTG CTG T-3' (The *BamHI* site is underlined); Ec.MUG.F, 5'-TGG GGT ACC CCA TGG GTT GAG GAT ATT TTG GCT CCA GGG-3' (The *NcoI* site is underlined); Ec.MUG.R, 5'-CCC GGA TCC TTA TCG CCC ACG CAC TAC CAG CGC CTG GTC-3' (The *BamHI* site is underlined); Ec.UNG.F, 5'-GGG AAT TCC ATA TGG CTA ACG AAT TAA CCT GGC ATG AC-3' (The *NdeI* site is underlined); Ec.UNG.R 5'-CCC AAG CTT CTC ACT CTC TGC CGG TAA TAC TGG-3' (The *HindIII* site is underlined). The PCR mixtures (50 μl) contained 40 ng of genomic DNA as template, 200 nM each primer pair, 50 μM each dNTP, 1 × *Taq* DNA polymerase buffer, and 1 unit of *Taq* DNA polymerase. The PCR procedure was composed of a pre-denaturation step at 95°C for 2 min, 30 cycles with each cycle consisting of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were electrophoresed on 1% agarose gel.

2.3. Plasmid construction, cloning, and expression of human endo V

The human endo V gene (*nfi*)-containing plasmid pET28a-hnfi and vector pET32a (Novagen) were digested with *NcoI* and *EcoRI*. The fragments containing the human endo V gene and digested plasmid pET32a, recovered from agarose gel, were purified with Gene Clean 2 Kit (MP Biomedicals). The ligation mixture (10 μl), containing 1 μl of 10 × ligation buffer (New England Biolabs), 400 units T4 DNA ligase (New England Biolabs), 100 ng of

vector and 300 ng of purified PCR products, was incubated at 4°C for overnight. The ligation mixture was transformed into *E. coli* strain JM109 competent cells prepared by a CaCl₂ method [33].

To express the N-terminal His-tagged human endo V gene, pET32a-hnfi was transformed into *E. coli* strain BL21(DE3)Δ3 by standard protocol [33]. An overnight *E. coli* culture of BL21(DE3)Δ3 containing pET32a-hnfi was diluted 100-fold into LB medium (1 liter) supplemented with 50 µg/ml ampicillin. The *E. coli* cells were grown at 37°C while being shaken at 250 rpm until the optical density at 600 nm reached about 0.6. IPTG was added to a final concentration of 0.5 mM. After growing at room temperature for an additional 16 h, the cells were collected by centrifugation at 4,000 rpm with JS-4.2 rotor in J6-MC centrifuge (Beckman Coulter) at 4°C and washed once with precooled sonication buffer.

To purify the human endo V protein fused to thioredoxin at the N-terminus, the cell paste from a 1 L culture was suspended in 10 ml of sonication buffer and sonicated at output 5 for 3 × 1 min with 5 min rest on ice between intervals in Branson Sonifier 450. The sonicated solution was clarified by centrifugation at 12,000 rpm with JA-17 rotor in Avanti J-25 centrifuge (Beckman Coulter) at 4°C for 20 min. The supernatant was transferred into a fresh tube and loaded into a 1 ml HiTrap chelating column. The bound protein in the column was eluted with a linear gradient of 15 column volumes of 0–1 M imidazole in chelating buffer A [20 mM Tris-HCl (pH 7.6), 10% glycerol and 50 mM NaCl] using a Bio-Rad BioLogic chromatographic system.

Fractions (200–400 mM imidazole) containing the human endo V protein as seen on 15% SDS-PAGE were pooled and dialyzed against HiTrap Q column buffer A [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% glycerol and 0.2 mM DTT] overnight at 4°C. The dialyzed sample was then loaded onto a 1 ml HiTrap Q column and eluted with a linear gradient of 15 column volumes of 0–1 M of NaCl in HiTrap Q buffer A. The putative human endo V protein was eluted at 200–400 mM NaCl. Fractions (200–400 mM NaCl) containing the human endo V protein as seen on 15% SDS-PAGE were pooled and dialyzed against HiTrap chelating column buffer A [20 mM Tris-HCl (pH 7.6), 10% glycerol and 50 mM NaCl] overnight at 4°C. The dialyzed sample was then loaded onto a 1 ml HiTrap chelating column and eluted with a linear gradient of 15 column volumes of 0–1 M imidazole in chelating buffer A. The human endo V protein was eluted at 200–400 mM imidazole. The homogeneity of the protein was examined by 15% SDS-PAGE analysis. The human endo V protein concentration was determined on SDS-PAGE using BSA as a standard.

2.4. Site-directed mutagenesis

An overlapping extension PCR procedure was used for construction of human endo V-D52A mutant [34]. The first round of PCR was carried out using pET32a-hnfi as template DNA with two pairs of primers, Hnfi.01F (5' TCA GGT ACC CAT ATG GCC CTG GAG GCG GCG GG 3', the *NdeI* site is underlined) and HVD52A.02R (5' TTC ACG AAG GAC AC GGC AAC GCC CCC GAC CCT CTG CA 3', the D52A site is underlined) pair & Hnfi.04R (5' TCA AAG CTT GAA TTC TCA ACA AAG TGC TGA GGA CTC TC 3', the *EcoRI* site is underlined) and HVD52A.03F (5' GGG TCG GGG GCG TT GCC GTG TCC TTC GTG AAA GGG GA 3', the D52A site is underlined) pair. The PCR mixtures (50 µl) contained 1 ng of pET32a-hnfi DNA as template, 200 nM each primer pair, 50 µM each dNTP, 1 × *Taq* DNA polymerase buffer, and 1 unit of *Taq* DNA polymerase. The PCR procedure was composed of a predenaturation step at 95°C for 2 min; 30 cycles with each cycle consisting of denaturation at 94°C for 15 s, annealing at 53°C for 30 s, and extension at 72°C for 1 min; and a final extension step at 72°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel and the expected PCR fragments were purified with Gene Clean 2 Kit (MP Biomedicals). This second run of PCR reaction mixture (100 µl),

which contained 3 μ l of each of the first run PCR fragment, 50 μ M each dNTP, 1 $\times$ Phusion high fidelity polymerase buffer, and 2 units of Phusion high fidelity polymerase, was initially carried out with a predenaturation step at 98°C for 2 min; five cycles with each cycle of denaturation at 94°C for 15 s and annealing and extension at 60°C for 4 min; and a final extension at 72°C for 5 min. Afterward, 100 nM outside primers (Hnfi.01F and Hnfi.04R) were added to the above PCR reaction mixture to continue the overlapping PCR reaction with the same condition for 25 cycles. The purified PCR products, digested with a pair of *NdeI* and *EcoRI* endonucleases, were ligated to cloning vector pET32a-hnfi treated with the same pair of restriction endonucleases. The recombinant plasmids containing the desired mutations were confirmed by DNA sequencing and transformed into *Escherichia coli* host strain BL21(DE3) Δ 3 for expression and protein purification.

2.5. Western blot analysis

Western blot analysis was carried out using an antibody raised against the N-terminal His-tag to confirm that the protein was overexpressed in *E. coli* cells. The protein samples were first separated on 15% SDS-PAGE, and then transferred onto a PVDF membrane by electroblotting at 100 V for 1 h using a Bio-Rad Mini Trans-Blot apparatus. The membrane was blocked with 1% low-fat milk. Anti-His (N-term) antibody (1 μ l) diluted in 5 ml of 1% BSA solution was added onto the membrane sealed in a plastic bag at room temperature for 1 h while shaking gently. After washing, anti-rabbit IgG HRP-linked antibody (1 μ l) diluted in 5 ml of 1% BSA solution was added onto the membrane sealed in a plastic bag at room temperature for 1 h with shaking gently. The color reaction was developed using Opti-4CN as a substrate.

2.6. Oligodeoxynucleotide substrates

The fluorescently labeled deoxyinosine- and deoxyuridine-containing substrates were prepared as described [7]. The sequences of the oligonucleotides are shown in Figure 2A. The oligodeoxyribonucleotides were dissolved in TE buffer at a final concentration of 10 μ M. The two complementary strands with the unlabeled strand in 1.2-fold molar excess were mixed, incubated at 85°C for 3 min, and allowed to form duplex DNA substrates at room temperature for more than 30 min. The fluorescently labeled deoxyxanthosine- and deoxyoxanosine-containing substrates were constructed as previously described [5, 35].

2.7. Human endonuclease V activity assay

DNA cleavage assays for human endo V were performed at 37°C for 60 min in a 10 μ l reaction mixture containing 10 nM oligonucleotide substrate, 100 nM human endo V, 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 1 mM EDTA, 5 mM MgCl₂, and 2% glycerol. Reactions were quenched by addition of an equal volume of GeneScan stop buffer. Samples were treated at 94°C for 3 min and 3.6 μ l of samples were loaded onto a 7 M urea-10% denaturing polyacrylamide gel. Electrophoresis was conducted at 1500 V for 1.5 h using an ABI 377 sequencer (Applied Biosystems). Cleavage products and remaining substrates were quantified using GeneScan analysis software.

2.8. Gel mobility shift assay

The binding reactions were performed at 37°C for 60 min in a 10- μ l volume containing 50 nM DNA substrate, 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 5 mM MgCl₂, and 2% glycerol, and 500 nM of human endo V protein. Samples were supplemented with 5 μ l of 50% glycerol and electrophoresed at 200 V on a 6% native polyacrylamide gel in 1 $\times$ TB buffer (89 mM Tris base and 89 mM boric acid) supplemented with 5 mM EDTA. The bound and free DNA species were analyzed using a Typhoon 9400 Imager (GE Healthcare)

with the following settings: photomultiplier tube at 600 V, excitation at 495 nm, and emission at 535 nm.

2.9. Spontaneous mutation frequency assay

A single colony was selected, inoculated into 4 ml liquid LB supplemented with 50 µg/ml ampicillin and grown at 30°C overnight [36]. IPTG was added to a final concentration of 0.5 mM. The culture continued to grow at 37°C for an additional 5 h. The cell culture was diluted and plated on LB plates with 50 µg/ml ampicillin. One ml cell culture was mixed with 3 ml of 0.7% soft agar and plated on LB plates with both 50 µg/ml ampicillin and 100 µg/ml rifampicin. The plates were incubated at 37°C for 24 h and cell numbers on amp^R and rif^R plates were counted, respectively. The mutation frequency was calculated as the results of rif^R colony number per 10⁹ amp^R colony number.

3. Results and Discussion

3.1. Expression and purification of human endonuclease V

Despite biochemical characterization of homologs from a variety of species, nothing is known about human endonuclease V. This is due to the difficulty of obtaining soluble and active human endo V protein from recombinant sources. Previously, we attempted to detect endo V activity from mammalian tissues without success, probably due to low activity in fractionated whole cell extracts/interference from nonspecific nucleases (L. Dong & W. Cao, unpublished data). In order to obtain soluble and active human endo V, we tried different constructs and induction conditions to express the *nfi* gene. Co-expression of human *nfi* gene with heat shock genes only yielded insoluble protein (H. Gao & W. Cao, unpublished data). Refolding of endo V from inclusion bodies did not generate active protein (H. Gao & W. Cao, unpublished data). Expression of *Schizosaccharomyces pombe* and *Arabidopsis thaliana nfi* genes met with similar difficulty (H. Feng & W. Cao, unpublished data). We then cloned human *nfi* into pET32a vector, in which the human *nfi* was fused downstream of the thioredoxin domain (Fig. S1). The entire sequence of the fused protein includes the *E. coli* thioredoxin gene, one S tag, two His tags and the full-length human endo V gene. The resulting plasmid pET32a-hnfi was then transformed into a special *E. coli* expression strain BL21(DE3)Δ3, in which the endogenous *E. coli nfi*, *mug* and *ung* were deleted. The deletion of these three genes prevents contamination of host endo V, MUG, and UNG proteins from interfering with assays of deaminated base repair activities. The genotype of the triple mutant strain was confirmed by PCR, which showed positive for the original strain but negative for the mutant strain (Fig. 1B). Human *nfi* gene was overexpressed in the mutant strain and purified by metal chelating and Q column chromatography (Fig. 1C). To confirm the soluble nature of the expressed protein, a Western blot analysis was performed on the whole cell extract, and supernatant after sonication and purified protein (Fig. 1D). Both the wt and the active site mutant D52A protein turned out positive, indicating that the expressed human endo V was soluble.

3.2. Deaminated base repair activity

Using the soluble human endo V protein purified from the mutant strain, we measured the repair activity towards all four deaminated bases in DNA, deoxyinosine (I), deoxyuridine (U), deoxyxanthosine (X) and deoxyoxanosine (O). Under the assay condition in which the enzyme was in excess ([E]:[S] = 10:1), human endo V showed strongest endonuclease activity towards deoxyinosine-containing DNA (Fig. 2B). The same activity was not detectable in the active site mutant D52A, indicating that the observed activity was intrinsic to human endo V (Fig. 2C). Human endo V was most active on single-stranded deoxyinosine-containing DNA (56%) followed by G/I (35%), T/I (24%), A/I (8%) and C/I (4%) (Fig. 2D). The fact both the human enzyme (with a thioredoxin domain) and the mouse

enzyme (without a thioredoxin domain) showed a similarly better activity on single-stranded deoxyinosine-containing DNA than double-stranded substrates is an indication that the thioredoxin domain had little effect on the repair specificity of the fusion protein. The enzyme also showed low level endonuclease activity on deoxyxanthosine-containing DNA (Fig. 2B), ranging from 4% for single-stranded X to 3% for G/X and T/X, 2% for A/X, and 1% for C/X (Fig. 2E). The activity on deoxyxanthosine was also intrinsic to human endo V since D52A did not show detectable activity (data not shown). On the other hand, human endo V did not show any endonuclease activity on either deoxyuridine- or deoxyoxanosine-containing DNA even under the assay condition in which the enzyme was in excess (Fig. 2B). Endo V enzymes from bacteria are also active on mismatch base pairs. Human endo V did not seem to contain these activities (data not shown). These data suggest that human endo V is primarily a deoxyinosine endonuclease with minor activity on deoxyxanthosine.

To better understand the kinetic differences of endonuclease activity on different deoxyinosine-containing DNA, a time-course analysis was performed (Fig. 3). The apparent rate constant was highest with single-stranded deoxyinosine-containing substrate (0.025 min^{-1}), followed by G/I (0.017 min^{-1}) and T/I (0.014 min^{-1}). The rate constants for A/I and C/I were not measured but should be significantly lower than the other three substrates. Since endo V requires divalent metal ion for its endonuclease activity, we examined the cleavage of T/I substrate using eleven metal ions (Fig. 4). Without a metal ion, human endo V showed no activity, indicating that a metal ion is essential for its catalytic activity (Fig. 4, EDTA). Human endo V was most active with Mg^{2+} as the metal co-factor. The activity with Mn^{2+} , Ni^{2+} and Co^{2+} was detectable but significantly less as compared with Mg^{2+} . To determine the binding affinity of human endo V to the deoxyinosine-containing DNA, we performed gel mobility shift analysis. As shown in Figure 5, human endo V was able to generate a shifted band in all five substrates. The intensities of the shifted bands roughly followed the order of its endonuclease activity with C/I being the weakest. These results suggest that the binding affinity of human endo V to deoxyinosine-containing base pairs correlates with its catalytic activity. In comparison, the human endo V-DNA complex is not as stable as bacterial endo V-DNA complexes [4, 5, 7, 29, 37].

3.3. Human endonuclease V as an antimutator

To assess whether of human endo V acts as an antimutator, we measured mutation frequency to rifampicin resistance [36]. The active site mutant D52A was used as a control. As shown in Figure 6, the mutation frequency was reduced three-fold in the presence of the wt human endo V. This result suggests that human endo V is an antimutator *in vivo*.

3.4. Biochemical properties of human endonuclease V

Human endonuclease V is an elusive repair enzyme that has escaped biochemical characterization for years. The successful production of soluble human endo V makes it possible to understand the biochemical properties of this important repair enzyme, which initiates so called Alternative Excision Repair pathway to remove deaminated base lesions [38]. The primary target of the endonuclease activity from human endo V appears to be deoxyinosine lesions with minor activity on deoxyxanthosine lesions. On the other hand, bacterial endo V enzymes have broader endonuclease activities on deaminated bases, including deoxyinosine, deoxyxanthosine, deoxyoxanosine and deoxyuridine [3–5, 16–18]. However, genetic and biochemical studies indicate that bacterial endo V primarily is involved in repair of deoxyinosine and deoxyxanthosine lesions [7, 22, 37, 39]. It is interesting that few amino acid substitutions influence the endonuclease activity on deoxyinosine and deoxyxanthosine of Tma endo V in an extensive site-directed mutagenesis analysis, indicating that the deoxyinosine and deoxyxanthosine endonuclease activity is well maintained within its structural fold [17].

The deoxyinosine endonuclease activity appears to be distinct in human endo V. Whereas bacterial endo V enzymes do not appear to be sensitive to the DNA base opposite to deoxyinosine [5, 7, 17], human endo V does. Besides the single-stranded deoxyinosine-containing DNA, the enzyme is most active on G/I followed by T/I > A/I > C/I (Figs. 2–3). Such a trend in activity in general is consistent with the stability of the inosine-containing base pairs in which the stability follows the order of C/I > A/I > T/I, G/I [40–42]. These results indicate that human endo V may significantly rely on the instability of the deoxyinosine-containing base pairs for recognition of the inosine base and subsequent endonucleolytic cleavage. The bacterial counterparts, on the other hand, may have evolved intrinsic exquisite ability to recognize inosine in any base pairs.

We now know that the human genome contains two repair activities for deoxyinosine, with human endo V being a primarily inosine-specific endonuclease and Alkyl Adenine Glycosylase (AAG) being a hypoxanthine DNA glycosylase. These two enzymes share some similarity in the repair of deaminated adenine base, in which they both are quite active towards T/I base pairs as well as G/I and A/I base pairs. The activity on C/I appears to be low for both enzymes (Fig. 2 and [35, 43–46]). The most significant contrast is that while human endo V is quite active on single-stranded deoxyinosine-containing DNA, human AAG has little activity on single-stranded deoxyinosine-containing DNA [35, 47]. The physiological significance of this difference is unknown; perhaps it allows human endo V, in coupling with other repair machinery, to repair single-stranded deoxyinosine lesions during DNA replication and transcription more effectively. This situation is similar to human NEIL1/NEIL2 versus OGG1 in which NEIL1/NEIL2 is active on single-stranded/bubbled 8-oxoguanine-containing DNA while OGG1 acts on 8-oxoguanine/C base pairs [48].

In summary, this work reveals the deamination repair activity of human endonuclease V. The biochemical activity detected in purified human endo V enzyme is consistent with its antimutator phenotype *in vivo*. Interestingly, an *nfi* mutant in fission yeast *Schizosaccharomyces pombe* shows a mutator phenotype and *nfi*^{-/-} mice demonstrate a cancer-prone phenotype [30]. These outcomes may underscore the importance of endonuclease V-mediated repair in prevention of cancer-prone mutations. More studies are needed to elucidate the nature of this rather unconventional alternative excision repair pathway.

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Highlights

- Human endonuclease V protein is produced in *nfi* deficient *E. coli* strain
- The primary activity in human endonuclease V is repair of deoxyinosine (I)
- The repair activity follows the order of single-stranded I > G/I > T/I > A/I > C/I

A

		D52A
Hsa	N-20-QARLKAH-19-RVGGVDVSF-33-APYVSGFLAFRE-19-PQVLLVDGNGVLHHRGFGVACHLG-5-PCVGVAKKLLQV-45-PLYISVGHR-16-RIPEPVROAD-42-C	
Mmu	N-20-QARLKAH-19-RVGGVDVSF-33-APYVSGFLAFRE-19-PQVLLVDGNGVLHHRGFGVACHLG-5-PCVGVAKKLLQV-45-PLYISVGHR-16-RIPEPVROAD-42-C	
Cel	N-435-QIRERLI-17-LVAGIDTSA-33-LPYIPQYLAVRE-19-PDVLCDGFGFHSRGCRCMACHVG-5-ASIGVAKNLTLLH-51-GVFVSAGYG-18-TTIEPIRAAD-18-C	
Ath	N-23-QDELKKK-25-YVGGVDMSE-33-VPYVPGFLAFRE-19-PQVLMVDGNGILHPRGFGVACHLG-5-PTIGVGKNNLHV-45-PIYVSVGHR-16-RVPEPIROAD-28-C	
Spo	N-16-QIELKGM-14-YVGLDISF-33-EDYVPGFLSFRE-12-IDILVDGNGVLHPRGFGVACHLG-5-PVVGVAKNYLHC-51-PVYVSIQNG-18-RVPEPIROAD-16-C	
Tma	N-19-QNELRKK-11-YVAGVDLSF-31-FPYIPGLLAFRE-14-PDVVFDGQGLAHPRKLGIAHMG-5-PTIGVAKSRLYG-33-PIFVSPGHL-18-RIPEPTRLAH-21-C	
Eco	N-11-QIELASS-13-LIAGADVGF-32-MPYIPGFLSFRE-14-PDLVFDGNGILHPRGFGVACHLG-5-PTIGVAKKRLCG-33-PLFIATGHR-17-RLPEPTRWAD-17-C	
Sty	N-09-QIELASS-13-FIGADVGF-32-MPYIPGFLSFRE-14-PDLVFDGNGILHPRGFGVACHLG-5-PTIGVAKKRLCG-33-PLFIATGHR-17-RLPEPTRWAD-17-C	
Ype	N-22-QQRASE-13-FIGADVGF-32-LPYIPGFLSFRE-14-PDLVFDGNGILHPRGFGVACHLG-5-PTIGVAKSRLYG-33-PIFVSPGHL-18-RIPEPTRLAH-21-C	
Sco	N-22-QDELRAR-13-RVTGVDVAY-32-FPYVPGLLAFRE-14-PGLVVCDCGYLAHPRRFLGASHLG-5-PTIGVAKNPTFF-33-PVFVSVGHR-17-RLPETTRRAD-14-C	
Bsu	N-20-QFNLRNR-13-TGAGVDLAY-34-VPYVSGFLAFRE-14-PDVVFDGNGILHPRGFGVACHLG-5-PTIGVAKSRLYG-33-PIFVSPGHL-18-RIPEPTRLAH-21-C	
Afu	N-12-QEEMSRS-13-YVGVDDQAF-34-FPYIPTFLMRE-14-RAAIMVDGSGIAHPRRCGLATYIA-5-PTVGTIKKRLFG-30-PIFISPGSY-17-KLPEPIRIAD-17-C	
Tac	N-127-QEYIARK-10-TVAADFVSF-27-FPYIPGFLAFRE-10-ADLLIDGNGILHPRFAGLATHAG-5-ASIGVAKHLTRS-22-MIVSPGNF-16-RYPWPLKLAH-17-C	
Fac	N-117-QEKIASM-10-NYAAIDVSF-27-FPYIPGFLAFRE-11-DALLIDANGILHPRRCGLATFAG-5-ATIGVAKSLMG-23-IVSPGNM-16-KYPAILKMHV-13-C	
Sso	N-13-QFLISK-10-KICGVDIAY-29-FPYIPGFLAFRE-12-CHLLVDGNGILHPRGFGVACHLG-5-PTIGVAKSRLYG-33-PIFVSPGHL-18-RIPEPTRLAH-21-C	
Pfu	N-10-QRRLSRK-12-RVGADVDSY-30-FPYIPTFFFLRE-12-PDVLVVEGHGKHAHPRRYGLASHIG-5-PTIGVAKRLRLG-12-AFVSVGNL-17-GYPKPLNIAD-12-C	

B

WT Strain		+	+	+
$\Delta 3$ (<i>nfi mug ung</i>)	+	+	+	+
<i>nfi</i> Primers	+	+		
<i>mug</i> Primers			+	+
<i>ung</i> Primers				+

M

C **D** **Figure 1. Expression of human endonuclease V**

A. Sequence alignment of endonuclease V. The amino acid residue selected for site-directed mutagenesis is highlighted and the resulting mutant is indicated above the arrows. Genbank accession numbers are shown after the species names. Hsa: *Homo sapiens*, BAC04765; Mmu: *Mus musculus*, XP_203558; Cel: *Caenorhabditis elegans*, 1731299; Ath: *Arabidopsis thaliana*, T10669; Spo: *Schizosaccharomyces pombe*, 1723511; Tma: *Thermotoga maritima*, NP_229661; Eco: *Escherichia coli*, NP_418426; Sty: *Salmonella typhimurium*, NP_463037; Ype: *Yersinia pestis*, NP_667835; Sco: *Streptomyces coelicolor*, CAB40676; Bsu: *Bacillus subtilis*, BSUB0019; Afu: *Archaeoglobus fulgidus*, NP_068968; Tac: *Thermoplasma acidophilum*, CAC11602; Fac: *Ferroplasma acidarmanus*, ZP_00001774; Sso: *Sulfolobus solfataricus*, NP_343804; Pfu: *Pyrococcus furiosus*, NP_578716. **B.** Confirmation of the genotype of BL21(DE3) $\Delta 3$ by PCR. WT strain: *E. coli* K-12 strain. **C.** SDS-PAGE analysis of the wt human endo V. Purified protein (~1 μ g) was electrophoresed on a 15% polyacrylamide gel containing 0.1% SDS. Protein bands were visualized by Coomassie staining. The molecular weight of the fused human endo V protein is 50.2 kD. **D.** Western blot analysis of the wt human endo V and mutant D52A proteins. Lanes 1 and 4: 10 μ l whole cell extract. Lanes 2 and 5: Supernatant collected after sonication (equivalent to 30 μ l cell culture). Lanes 3 and 6: purified protein (~0.5 μ g).

Figure 2. Cleavage of deaminated DNA by wt human endonuclease V and D52A mutant
A. Sequences of deoxyxanthosine (X)- and deoxyoxanosine (O)-, and deoxyinosine (I)- and deoxyuridine (U)-containing oligodeoxyribonucleotide substrates. FAM or TET: fluorophore. **B.** Cleavage activity of wt human endo V on I-, U-, X-, and O-containing substrates. Cleavage reactions were performed as described in Materials and Methods with 100 nM wt human endo V protein and 10 nM substrate. **C.** Cleavage activity of D52A mutant on I-containing substrates. Cleavage reactions were performed as described in Materials and Methods with 100 nM mutant D52A protein and 10 nM substrate. **D.**

Quantification of cleavage by wt human endo V on I-containing substrates. **E.**
Quantification of cleavage by wt human endo V on X-containing substrates.

Figure 3. Kinetic analysis of cleavage activity of wt human endonuclease V on single-stranded I, G/I and T/I substrates

A. GeneScan gel picture of cleavage activity of wt human endo V on T/I substrate. **B.** Time course analysis of cleavage activity. [S] = 10 nM, [E]:[S] = 10:1; (Δ) ss I; (□) G/I; (○) T/I. The apparent rate constants were determined by fitting the time-course data into a first-order rate equation using DeltaGraph (SPSS inc.).

Figure 4. Metal effects on T/I cleavage by the wt human endonuclease V

A. Cleavage activity of wt human endo V with different metals. Cleavage reactions were performed as described in Materials and Methods with 100 nM protein and 10 nM substrate with different metal ions (5 mM).

Figure 5. Binding analysis of wt human endo V on I-containing DNA substrates

A. Gel mobility shift analysis of binding of human endo V to I-containing substrate with 5 mM MgCl₂. Gel mobility shift assays were performed as described in Materials and Methods with 500 nM human endo V protein, 50 nM substrate and 5 mM MgCl₂. The first lane is the T/I substrate only used as a control. **B.** Quantification of binding affinity of human endo V to the T/I substrate.

Figure 6. Antimutator effect of human endo V in *E. coli* BL21(DE3) Δ 3 (*nfi*, *mug* and *ung*)
E. coli BL21(DE3) Δ 3 (*nfi*, *mug* and *ung*) cells transformed with pET32a-D52A (D52A) or pET-32a-hnfi (WT) were plated both in the presence and absence of rifampicin. Results are from three independent experiments.