Incision at hypoxanthine residues in DNA by a mammalian homologue of the *Escherichia coli* antimutator enzyme endonuclease V

Ane Moe, Jeanette Ringvoll, Line M. Nordstrand, Lars Eide, Magnar Bjørås, Erling Seeberg, Torbjørn Rognes and Arne Klungland*

Centre for Molecular Biology and Neuroscience and Institute of Medical Microbiology, University of Oslo, Rikshospitalet, N-0027 Oslo, Norway

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**ABSTRACT**

Deamination of DNA bases can occur spontaneously, generating highly mutagenic lesions such as uracil and hypoxanthine. In *Escherichia coli* two enzymes initiate repair at hypoxanthine residues in DNA. The alkylbase DNA glycosylase, AlkA, initiates repair by removal of the damaged base, whereas endonuclease V, Endo V, hydrolyses the second phosphodiester bond 3' to the lesion. We have identified and characterised a mouse cDNA with striking homology to the *E.coli* *nfi* gene, which also has significant similarities to motifs required for catalytic activity of the UvrC endonuclease. The 37-kDa mouse enzyme (mEndo V) incises the DNA strand at the second phosphodiester bond 3' to hypoxanthine- and uracil-containing nucleotides. The activity of mEndo V is elevated on single-stranded DNA substrate *in vitro*. Expression of the mouse protein in a DNA repair-deficient *E.coli alkA nfi* strain suppresses its spontaneous mutator phenotype. We suggest that mEndo V initiates an alternative excision repair pathway for hypoxanthine removal. It thus appears that mEndo V has properties overlapping the function of alkylbase DNA glycosylase (Aag) in repair of deaminated adenine, which to some extent could explain the absence of phenotypic abnormalities associated with Aag knockout in mice.

**INTRODUCTION**

Cellular genomes are exposed to a wide range of toxic agents including ionising radiation, sunlight, chemical agents and intracellular reactive oxygen species (ROS). The molecular basis for the mutagenic and carcinogenic potency of these agents lies in their ability to alter the structure of individual DNA bases or the sugar–phosphate backbone (1). The most efficient way of correcting DNA damage is direct reversal of the specific lesion. This repair strategy has been identified for UV-induced pyrimidine dimers and alkylated bases in DNA, in both microorganisms and mammals (2–7). Excision repair is the other major pathway for correcting damaged or inappropriate DNA bases (8). Damaged base residues in DNA can be removed by one of two principally different mechanisms. Lesions generated endogenously by hydrolysis or exposure to ROS are corrected by base excision repair (BER), where release of the altered base in free form is mediated by a DNA glycosylase (9,10). Dipyrimidine adducts generated by exposure to UV light and other types of base damage that cause major helix distortions are generally processed by nucleotide excision repair (NER), in which the DNA chain is excised on each side of the lesion to release an oligonucleotide containing the damaged residue (8,11,12).

DNA is subjected to deamination at a physiologically significant rate. Hypoxanthine is the result of adenine deamination and can also be introduced by misincorporation of dIMP. Hypoxanthine pairs with cytosine during replication resulting in AT to GC transitions at deaminated adenes (13,14). Specific mechanisms for removal of deaminated bases have evolved. Several bacterial, archaean and eukaryotic organisms contain an evolutionary conserved enzyme that recognises deaminated bases in DNA. This 3′deoxyinosine endonuclease (Endo V), the product of the *nfi* gene in *Escherichia coli*, was first described by Gates and Linn and later extensively characterised by Yao and co-workers (15–17). Endo V was originally described as a nuclease cleaving near lesions that alters the secondary structure of DNA, and has subsequently been shown to have a broad substrate spectrum acting at AP sites (17), urea residues (17), base mismatches (18), flap DNA and pseudo Y structures (19) and loops and hairpins (19). The ability of Endo V to recognise all three deamination products in DNA, deoxyinosine, deoxyuridine and deoxyxanthosine, is not shared by any other known repair enzymes (20). Endo V incises the DNA at the second phosphodiester bond 3′ to the lesion, leaving a 3′ OH and a 5′ P termini. In *E.coli* there are three other known repair pathways initiated by cleavage of a phosphodiester bond (21). The UvrABC complex excises pyrimidine dimers and nucleotides containing bulky adducts (11,12). The MutSLH system removes regions containing mismatched bases (22), while the very short patch repair system removes
thymine resulting from deamination of 5-methylcytosine (23). In the Endo V-mediated repair pathway additional enzymes are required to excise the damaged base and complete the repair process. Kow recently suggested the action of a specific 5′–3′ exonuclease followed by polymerisation and ligation (24).

DNA repair enzymes acting on endogenous DNA lesions often show strong evolutionary conservation, both in sequence and function (25). Nfi homologues from Archaeoglobus fulgidus (20) and Thermotoga maritima (26) have recently been characterised. Here we describe the identification and characterisation of this gene from mouse and human cells. Endo V represents one of the most conserved DNA repair enzymes so far identified with 32% sequence identity between the mouse and E. coli orthologues. The mammalian Endo V homologue possesses DNA repair activities that are similar, but more limited, than those of E. coli Endo V. This antimutator enzyme initiates DNA repair by endonuclease cleavage at the second phosphodiester bond 3′ to the base lesion.

MATERIALS AND METHODS

Identification of mammalian expressed sequences with homology to E. coli Endo V (nfi)

A similarity search using the E. coli Endo V amino acid sequence as a query in a database of expressed sequence tags (ESTs) was performed. As a multiple sequence alignment was created using Clustal W (27). Candidate ESTs were identified and the murine clone (GenBank accession no. A1509431) ordered for further characterisation. The complete insert was sequenced without identification of the start codon. RML-Race (RNA Ligase Mediated Rapid Amplification of cDNA Ends; Ambion) was used in combination with 10 μg total RNA from normal mouse lung (Ambion) in order to clone the 5′ end of the cDNA. A full-length sequence carrying the complete ORF was then amplified with the sequence information from RML-Race and clone A1509431 by Qiagen One Step RT–PCR kit (Qiagen) using mRNA as template (primers; 5′-CTCA-AAGCCTCGAGGCGCTA-3′ and 5′-TGTGACTGTTGGTGAGGTCTC-3′).

Northern blot hybridisation

Northern blots containing samples from multiple mouse tissues purchased from Clontech (Mouse MTN™ blot; 7762-1) were probed for mni expression. Northern blot hybridisation was carried out using ExpressHyb solution (Clontech) as recommended by the manufacturer. Probes were labelled with [α-32P]dCTP (3000 Ci/mmol, Amersham) using the Rediprime DNA labelling system (Amersham).

Protein over-expression and purification

The 1017 bp mEndo V sequence was amplified and inserted into the pET-28b vector (Navagen), resulting in the expression vector pET-28b/mnfi. This was obtained by introducing NcoI and HindIII restriction sites at the start and stop, respectively, of the coding sequence using primers 5′-GGGCCCATGCTCACACGCGCGCT-3′ and 5′-CGGCCGCAAGCTTGGTGAGGTGACTGCTA-3′ in a PCR. The amplified fragment was ligated into the pET-28b vector resulting in the addition of a hexahistidine tag at the C-terminal end of the mEndo V protein. The plasmid carrying the mEndo V encoding gene was used to transform E. coli BL21 star (DE3) and positive colonies were inoculated in 500 ml of Luria–Bertani (LB) medium containing kanamycin (30 μg/ml). A negative control was generated by transforming the pET-28b vector into E. coli BL21 star (DE3). The cultures were incubated with shaking at 37°C until OD600 reached 0.4. IPTG was added to 1 mM and the cells were further grown for 4 h at 37°C. Cells were harvested by centrifugation at 4000 g for 6 min at 4°C, and resuspended in 10 ml of buffer A (50 mM Tris pH 8.0, 500 mM NaCl, 5 mM imidazole and 10 mM β-mercaptoethanol). Cells were disrupted by French press (800–1000 psig; Sinoamino) and the extract clarified by centrifugation at 17 000 g for 30 min at 4°C. A Ni2+-column, 6 mm in diameter, was packed with 1.0 ml of Ni2+-resin. The clarified extract containing histidine-tagged protein was loaded on the column and washed twice with 6× volume column, 4 and 15 mM imidazole, respectively, in buffer A. Bound protein was eluted with 500 mM imidazole dissolved in buffer A and collected in fractions of 0.5 ml. The fractions containing the protein of interest were desalted (50 mM Tris pH 8.0, 100 mM KCl and 20% glycerol) with a HiTrap column (Amersham). The protein was aliquoted into 10 μl fractions and stored at −20°C.

Enzyme assays

Single-stranded substrate preparation: a 24mer oligonucleotide containing a single hypoxanthine (5′-GGCGGATGACCC-hyp-GAGGCCCATC-3′) was 32P-labelled at the 5′ terminus with T4 polynucleotide kinase (MBI Fermentas) and [γ-32P]ATP (3000 Ci/mmol, Amersham). Double-stranded DNA substrate was produced by annealing a 49mer hypoxanthine containing oligonucleotide (5′-TAGACGGATGAAATTGGAGGATCGAAGTTGGATGAT-3′), 32P-labelled at the 5′ terminus, to a complementary strand containing a thymine (T) opposite to the hypoxanthine base (5′-ACTACCAAAATCCAACCTGTGCTCCTCAATAT-TCATCGGCTA-3′). All oligos include phosphorothioate linkages at the ultimate and penultimate 5′ and 3′ ends to reduce exonucleolytic attack. Activity of mEndo V on DNA containing uracil residues was assayed on a single-stranded uracil containing oligonucleotide (5′-GCTCATGCG-CAGXCGCCGTACTCG-3′, X = uracil). A reaction mixture (10 μl) containing reaction buffer (2 mM MgCl2, 50 mM Tris pH 7.5, 50 mM KCl, 5% glycerol, 1 mM DTT and 100 ng/μl BSA) and ~20 fmol 32P-labelled substrate was incubated at 37°C for 30 min with purified mouse Endo V. Reactions were terminated by addition of formamide loading mix. The sample was loaded onto a 6 or 20% 20% polyacrylamide gel containing 7 M urea and DNA molecules separated by electrophoresis. The gel was dried under vacuum and subjected to phororimaging for visualization.

Assay for mutation suppression in E. coli

The rate of spontaneous mutations was tested by growing cells on LB plates containing rifampicin (100 μg/ml). A wild type E.coli strain (TG:1) was compared with E.coli BK 2223 (alkA nfi in TG:1, this work). The open reading frame (ORF) of mEndo V gene was subcloned as described above and transformed into E.coli BK 2223 (BK2223/pET-28b/mnfi; alkA nfi mnfi+). For each experiment, an appropriate dilution
of the bacterial culture was grown on LB plates to estimate the number of colony forming units. Mutated bacteria were identified by seeding on rifampicin plates. The plates were incubated overnight at 37°C and the number of colonies counted.

**Site-specific mutagenesis**

Site-specific mutations were introduced in the mEndo V wild-type sequence by a PCR based ‘Quick change’ (Stratagene) method. Primers containing the mutation of interest were as follows: mutation S93P; primer A: 5'-GTGGGCTGAAGGCCCCTATGTGCAGGCTTCCGAGG-TCC-3', primer B: 5'-GGACCTTCGGAGCCAGGAGGTCGGTACATAGGGGCCTTCAGGCCAAC-3', and mutation Q133P; primer 1: 5'-GGGATGGAAACGGGGTGGTCTTACCCGCGCTTGAGTGCCTGGCACATAGGGGCCTTCAGGCCAAC-3', primer 2: 5'-GGTTGGGAGGCACCCCGAGAGCCGTTGACACCCGCGCTTCCATCC-3'. The PCR products were digested with DpnI and transformed into E.coli ER2566 by electroformation. Site-specific mutagenesis was verified by sequencing. The protocol described above was repeated for the construction of a double mutant with both amino acids changed. Prior to purification, the mutant sequences were transformed into E.coli BL21 star. Growth conditions and purification procedure were as described for the wild type protein.

**RESULTS**

Mouse and human proteins with extensive homology to E.coli Endo V

We have identified entries in the human and mouse EST database with striking sequence similarities to the E.coli nfi gene, termed hnfi and mnfi, respectively. The mouse clone was sequenced and the mRNA sequence was found to be completely identical to the GenBank sequence with accession number XM_203558. The corresponding protein has the interim name LOC277019 and accession number XP_203558. The ORF of mnfi encodes a putative polypeptide of 338 amino acids with a predicted molecular weight of 37.2 kDa. The amino acid sequence of mEndo V showed 32% sequence identity and 49% sequence similarity to E.coli Endo V (Fig. 1). The active site IGVAKK is conserved from E.coli to mouse and man. To our knowledge, a higher degree of conservation for a DNA repair function is seen only within the superfamily of uracil-DNA glycosylases (UDG/UNG) (28). The murine gene consists of nine exons spanning ~7.5 kb of chromosome 11. The sequenced 5' flanking region contains two putative TATA boxes, a CpG island and possible binding sites for several transcription factors and enhancer motifs [Bioinformatics and Molecular Analysis Section (BIMAS), NIH]. Domains conserved between the mammalian and bacterial enzyme include homology identified previously between E.coli Endo V and UvrC (Fig. 1B) (25).

Over-expression and purification of mEndo V

Several approaches were used to express and purify mEndo V. Expression and immediate purification of a C-terminal Histagged fusion protein was found to give highest yield and reproducible activity. A hexahistidine (His6) tag was attached to the C-terminal end of mEndo V by subcloning in the pET-28b vector. The protein was expressed at high levels following IPTG induction of E.coli BL21 star transformed with the plasmid carrying the mEndo V sequence, pET-28b/mnfi (Fig. 2, lane 2). The C-terminal histidine tag enabled purification by Ni²⁺ affinity chromatography. Elution from the column with 500 mM imidazole revealed a single major protein band with a molecular mass of ~37 kDa (Fig. 2, lane 3). The over-expressed and purified protein (lanes 2 and 3) also tested positively with a polyclonal antibody raised against a synthetic polypeptide of mEndo V (data not shown).

Expression patterns of mEndo V in mouse tissue

Expression patterns of mEndo V were investigated in several mouse tissues by northern blot hybridisation (Fig. 3). The labelled mEndo V cDNA probe consistently detected three different bands with the highest level in liver, and high levels in heart, kidney and testis. Several reports indicate high levels of DNA repair enzymes in these organs (29,30). The expected 1.8 kDa mRNA was detected in all tissues tested. Two larger transcripts, 6.4 and 3.6 kDa, were observed in all tissues examined and the nature of these are unknown, but are most probably unprocessed and partially processed mRNA. Northern analysis of brain mRNA indicates a low level of processed mRNA (Fig. 3). Positive entries for the mEndo V gene in the sequence database (NCBI, mouse EST database) includes lung, eyeball, brain, liver, testis, mammary and foetus, indicating a ubiquitous expression pattern. Similar results from the corresponding human EST database identified hEndo V expression in prostate, brain, skin, lung, kidney, eye, pancreas, spleen, placenta and uterus.

Endonuclease activity on DNA containing hypoxanthine or uracil

DNA containing hypoxanthine has been shown to be the main substrate for E.coli Endo V. We found significant endonuclease activity of such DNA also by the mEndo V enzyme (Fig. 4). The specific activity of the mouse homologue was approximately 50 times lower than for E.coli Endo V. From the data presented it is estimated that 2 fmol of E.coli Endo V and 100 fmol of mEndo V are required to cleave 1 fmol of a 32P-labelled single-stranded hypoxanthine-containing oligonucleotide in 30 min at 37°C. It should be noted that the background activity of non-specific nicking in DNA also seems to be conserved for the different Endo V orthologues (31). A possible role for this activity in vivo, e.g. in recombination, would explain its conservation. Higher activity was detected on single-stranded than on double-stranded substrate (Fig. 5) (16). Furthermore, the activity towards uracil is greatly reduced compared with hypoxanthine with the following substrate preference: ss DNA containing hypoxanthine > DNA ds containing hypoxanthine ~ ss DNA containing uracil. The mouse homologue is dependent on Mg²⁺ and no significant activity was observed towards uracil residues in double-stranded DNA, nor against 8-oxoguanine, C:C mismatches, AP-sites or 5' flap structures (data not shown).

Two proline residues that are conserved from E.coli to Schizosaccharomyces pombe are located within domains required for enzymatic activity (32). These two prolines are replaced with serine (S93) and glutamine (Q133) in the wild
type mouse protein. To test whether these changes could affect the enzymatic activity, we designed primers for site-specific mutations converting S93 and Q133 to prolines. Three mutant proteins were produced, the single mutants S93P and Q133P and the double mutant S93P/Q133P. Mutant proteins remained active. However, the activity was not increased relative to wild type (data not shown).

Suppression of the mutator phenotype of E.coli BK 2223 (alkA nfi) by mnfi

Nitrosative deamination by nitrous acid induces a moderate increase of mutations in an E.coli nfi mutant, whereas there is no increase in spontaneous mutations (33; data not shown). To block both pathways in the repair of hypoxanthine we constructed a double mutant of nfi and alkA, BK2223. AlkA also removes deamination products from DNA and previous experiments have suggested a role of AlkA in mutation avoidance following deamination (34,35). BK2223 (alkA nfi) showed a mild, but significant, mutator phenotype (Fig. 6). The mnfi cDNA, subcloned and introduced into BK2223, caused a complete reversion of the mutator phenotype. The antimutator effect was correlated with expression of mEndo V. However, it is unclear whether hypoxanthine or other unrecognized mEndo V substrates are the origin for the mutations.

DISCUSSION

Nature of mEndo V

Deamination of adenine to hypoxanthine induces AT to GC transitions in DNA (24). Here, we have described the cloning of a murine cDNA encoding an Endo V homologue that incises DNA at hypoxanthine residues. The enzyme is highly conserved from bacteria to mammals and, to our knowledge, uracil DNA glycosylase is the only other repair enzyme showing a similar degree of conservation. Aravind and
co-workers recently identified significant similarity between Endo V and certain motifs of the UvrC protein (Fig. 1B) (25). They further hypothesised potential structural elements and a requirement of two aspartates (D52 and D126 of the murine sequence) and a lysine (K155) for catalysis, and these residues are conserved in the mouse enzyme. Both UvrC and mEndo V represent damage-specific endonucleases cleaving near, but not next to, the DNA lesions. By analogy to UvrC, which only works efficiently together with UvrA and UvrB, mEndo V could also potentially be stimulated by other protein partners (36). Such stimulation is also observed for several other DNA repair enzymes (37).

Unexpectedly from the high degree of sequence conservation, mEndo V did not show a broad substrate specificity as has been described for E. coli Endo V. Similar data have been described by Liu and Roy for Endo V from A. fulgidus (38). They suggested that the ability of E. coli Endo V to recognize other DNA lesions was acquired later during the course of evolution. Huang and co-workers cloned and expressed a thermostable Endo V homologue in T. maritima and detected some activity on a uracil substrate (26). They propose a less important role of Endo V on these types of lesions compared with the much more active uracil glycosylase that is specific for uracil lesion, and out-compete Endo V for repair.

**Base excision repair versus alternative excision repair**

Several proteins required for removal of BER intermediates are essential for viability as judged by the embryonic lethality of gene-targeted mice (39). In contrast, no dramatic phenotypes have been produced by targeting DNA glycosylases that initiate the BER pathway by removing the damaged base (40–44). However, all these studies have been followed by discoveries of alternative DNA repair pathways for specific DNA lesions. For example, alternative repair of uracil residues in DNA is accomplished by the SMUG enzyme (43, 45), and a novel nuclear and mitochondrial glycosylase for thymine glycols (Tg) was revealed by disruption of the mouse Nih1 gene (44).
There are two major pathways for repair of deaminated bases in DNA: BER initiated by a damage-specific DNA glycosylase, and the AER initiated by Endo V (24). In the case of uracil, which is the most abundant deamination product, the two highly active and abundant glycosylases UDG and SMUG will account for almost all measurable activity for removal of uracil in DNA (43,45). Alkylpurine DNA glycosylases from mammalian cells (Aag/ANPG) efficiently excise hypoxanthine from DNA (34). As judged from in vitro assays of cell-free extracts from mice with a targeted deletion of Aag/ANPG, this is the major, or only, hypoxanthine DNA glycosylase (46,47). Nevertheless, Aag/ANPG null mice are viable with no apparent phenotypic abnormalities. For hypoxanthine removal, mEndo V could represent an important alternative repair pathway. Also, the Ogg1 mice lacking 8-oxoG DNA glycosylase activities appear to have alternative pathways for 8-oxoG repair (42,48).

Repair of deaminated purines is initiated by mEndo V which generates a nick in DNA, leaving the damaged hypoxanthine-containing nucleotide and an intact nucleotide penultimate and ultimate at the 3' side of the nick, respectively. The subsequent removal of the deaminated purine requires the excision of a small patch of DNA of at least 2 nt. Recent studies have identified 3'-5' mismatch-specific exonucleolytic activity of the apurinic/apyrimidinic endonuclease APE1 (49), or alternatively by the 3' flap-specific activity of Mus81 (50). Repair is completed by polymerisation and ligation. Repair synthesis is in red.

We suggest that mEndo V plays an important role in the repair of hypoxanthine in eukaryotic cells and is currently addressing this by constructing gene-targeted knockout mice lacking the mnf1 gene. By crossing mnf1 negative mice with Aag/ANPG null mice it should be possible to address the relative importance of mEndo V and/or Aag/ANPG for the repair of deaminated adenines in DNA.
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