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The long N-terminus of the *C. elegans* DNA repair enzyme APN-1 targets the protein to the nucleus of a heterologous system

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ABSTRACT

We previously isolated from a *Caenorhabditis elegans* cDNA library, designed for two-hybrid screening, a gene encoding the DNA repair enzyme APN-1 using cross-specie complementation analysis of the *Saccharomyces cerevisiae apn1Δ apn2Δ tpp1Δ* triple mutant deficient in the ability to repair several types of DNA lesions including apurinic/apyrimidinic (AP) sites. We subsequently purified the APN-1 from this yeast mutant and demonstrated that it possesses four distinct DNA repair activities. However, following the re-annotation of the *C. elegans* genome we discovered that the functionally active APN-1 encoded by the cDNA from the library might lack 108 amino acid residues from the N-terminus. We therefore synthesized the entire *C. elegans apn-1* gene encoding the putative full-length APN-1 and created several N-terminal deletion mutants lacking either 63, 83 or 118 amino acid residues. The full-length APN-1, APN-1 (1–63Δ) and APN-1 (1–83Δ), but not APN-1 (1–118Δ) were stably expressed in the yeast triple mutant and cleaved the AP site substrate. However, only the full-length APN-1 rescued the yeast mutant from the genotoxicity caused by methyl methane sulfonate, a DNA damaging agent that creates AP sites in the genome. The full-length APN-1 was localized to the yeast nucleus, while APN-1 (1–63Δ) and APN-1 (1–83Δ) retained a cytoplasmic distribution. Our data suggest that the N-terminal region has no direct role in the DNA repair functions of APN-1 other than to target the protein to the nucleus and possibly to maintain its stability. Thus, the truncated APN-1, previously isolated from the two-hybrid library, ability to complement the yeast triple mutant depends on the engineered SV40 nuclear localization signal.

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1. Introduction

Caenorhabditis elegans APN-1 is a member of the endonuclease IV (Endo IV) family of DNA repair enzymes referred to as apurinic/apyrimidinic (AP) endonucleases/3′-diesterases and includes, for example, *Saccharomyces cerevisiae* Apn1 and *Escherichia coli* Endo IV (Daley et al., 2010). These proteins possess at least four enzymatic activities: (i) AP endonuclease that cleaves the DNA backbone 5′ to an AP site producing a 3′-hydroxyl group and a 5′-deoxyribose phosphate (Daley et al., 2010; Ramotar et al., 1991a), (ii) 3′-diesterase that removes a multitude of 3′-blocking groups such as 3′-phosphate present at DNA single strand breaks (Ramotar et al., 1991a; Karumbati et al., 2003), (iii) 3′- to 5′-exonuclease capable of removing a few nucleotides at nicked DNA to create a gap, (Karumbati et al., 2003; Ishchenko et al., 2005), and (iv) nucleotide incision repair activity that recognizes and removes certain oxidized bases by making an incision immediately 5′ to the damaged base to create a 3′-hydroxyl group (Gros et al., 2004; Ischenko and Saparbaev, 2002). The members of the Endo IV family perform a role in the base-excision DNA repair pathway to eliminate specific types of mutagenic DNA lesions that would otherwise compromise genomic integrity (Daley et al., 2010).

We have shown that RNAi knockdown of the *C. elegans apn-1* gene caused the animals to accumulate 5-fold higher levels of spontaneous mutations (Zakaria et al., 2010). The downregulation of the *apn-1* gene also sensitized *C. elegans* to DNA damaging agents such as methyl methane sulfonate (MMS), which indirectly creates AP site lesions as a result of the removal of alkylated bases by DNA glycosylases (Zakaria et al., 2010). Interestingly, these *apn-1* knockdown animals showed a delay in the progression of single cell embryo which has been ascribed to a defect in processing endogenous DNA lesions (Zakaria et al., 2010). It is unlikely that the endogenous lesions are due to the accumulation of AP sites or single strand breaks with blocked 3′-termini, as *C. elegans* embryos are proficient at processing these lesions due to the

Abbreviations: AP sites, apurinic/apyrimidinic sites; Endo IV, endonuclease IV; MMS, methyl methane sulfonate; NLS, nuclear localization signal; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate.

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conservation of another highly active AP endonuclease/3′-diesterase, EXO-3, that belongs to the EXO III family of apurinic/apyrimidinic (AP) endonucleases/3′-diesterases (Shatilla et al., 2005a; Shatilla and Ramotar, 2002). As such, we postulated that APN-1 might be required to perform a distinct repair function that is uncommon to EXO-3 (Zakaria et al., 2010).

Although the *C. elegans apn-1* gene was identified and the predicted protein shares high identity with the Endo IV family members, the APN-1 enzyme could not be purified and functionally characterized from *C. elegans* because the expression level of APN-1 is extremely low and the protein was highly susceptible to proteolysis (Shatilla and Ramotar, 2002). As such, we took advantage of the yeast heterologous model system to express and functionally characterized the *C. elegans* APN-1 (Ramotar and Demple, 1996; Ramotar et al., 1991b). This was achieved by the isolation of a plasmid, pGAL4-SV40(NLS)-apn-1, from a library containing *C. elegans* genes designed for two-hybrid screening in *S. cerevisiae* (Shatilla et al., 2005b). This plasmid rescued the DNA repair defects of a yeast mutant strain YW778 (*apn1*Δ *apn2*Δ *tpp1*Δ) lacking Apn1, as well as two additional enzymes Apn2 (a member of the EXO III family) and Tpp1 (possessing a 3′-phosphodiesterase activity) that are involved in processing similar DNA lesions as Apn1 (Shatilla et al., 2005b; Vance and Wilson, 2001a; Demple and Harrison, 1994). This yeast mutant YW778 exhibits exquisite sensitivities to various DNA damaging agents including MMS and hydrogen peroxide that produce AP sites and create strand breaks terminated with 3′-phosphate, respectively (Vance and Wilson, 2001b). The plasmid pGAL4-SV40(NLS)-apn-1 harbored the essential portion of the *apn-1* gene encoding a functional polypeptide that shared throughout its entire length 44 and 41% identity at the amino acid level with *E. coli* endo IV and *S. cerevisiae* Apn1, respectively (Shatilla et al., 2005b). Subsequent analysis of the re-annotated *C. elegans* database revealed that the cDNA harbored by the rescue plasmid was missing the N-terminal portion of the *apn-1* gene encoding residues 1 to 108. In fact, the *apn-1* gene expresses a longer transcript that has been substantiated by the detection of the N-terminal part of the transcript using real-time PCR (Zakaria et al., 2010). It is noteworthy that several other species including *Neurospora crassa, Aspergillus nidulans* and *Strongylocentrotus purpuratus* have Endo IV homologues that are predicted to carry significantly long N-terminal sequences (Daley et al., 2010). Thus, on the basis of the *C. elegans* database information and DNA sequence analysis, the complementing plasmid pGAL4-SV40(NLS)-apn-1 carried only the portion of the *apn-1* gene encoding residues 109 to 396 of the APN-1 protein.

We have established that the functional region of APN-1, which rescued the DNA repair defects of strain YW778, was located within residues 119 to 396 (Yang et al., 2012). This finding strongly suggests that the N-terminal portion of the full-length APN-1 is unlikely to play a role in DNA repair. As such, this finding incites the question regarding the purpose of the long N-terminus, that is, residues 1 to 118 of APN-1. Analysis of the missing portion of APN-1 from the plasmid pGAL4-SV40(NLS)-apn-1, that is, residues 1 to 108, for specific motifs revealed two segments that resemble nuclear localization signals (NLSs), suggesting that the putative extended N-terminus may serve to at least target the protein to the nucleus in *C. elegans*. We have shown that APN-1 (119–396) carrying the SV40 NLS can rescue the DNA repair defects of strain YW778 (Yang et al., 2012). However, this functional complementation does not occur if APN-1 (119–396) lacks the SV40 NLS (Yang et al., 2012). This observation strongly suggests that the N-terminal portion of APN-1 could be involved in targeting the protein to the nucleus. Herein, we provide evidence that the full-length APN-1 (1–396) can confer MMS resistance to the yeast mutant YW778, but not when it lacks the first 1–63 amino acid residues. This APN-1 (1–63Δ) variant retained full AP endonuclease activity, but lacked the ability to enter the nucleus and instead showed a cytoplasmic distribution as compared to the full-length APN-1.

2. Materials and methods

2.1. Yeast and bacterial strains

S. cerevisiae laboratory strain YW778 (*apn1*Δ*::HIS3 apn2*Δ*::KanMX4 tpp1*Δ*::MET15*) was generously provided by Dr. Tom Wilson (Ann Arbor, Michigan) and maintained on YPD agar and supplemented with adenine (20 μg/ml). *E. coli* laboratory strain DH5α (used for amplification of plasmids) was maintained on Luria Broth (LB) agar.

2.2. Plasmid construction

The full-length *C. elegans apn-1* cDNA gene encoding the entire open reading frame was synthesized and cloned into the vector pUC57 (Biobasic, Canada). This synthesized cDNA was fully sequenced and completely matched the sequence of the *C. elegans* genome. The full-length *apn-1* gene and three N-terminal deletions were amplified by PCR from the plasmid and cloned by gap-repair into the pYES2.0-GFP vector with the following primers: PYES-GFP-CeAPN1-FL-F1:5′-GCTG CTGGGATTACACATGGCATGGATGAACTATACAAAGAATTCATG GCTAAC AAAAAAGTAACA-3′; PYES-GFP-CeAPN1(Δ:1–63Nt)-F1:5′-GCTGCTGG GAT TACACATGGCATGGATGAACTATACAAAGAATTCGAAACATTAACT GAAGAAAA-3′; PYES-GFP-CeAPN1(Δ:1–83Nt)-F1:5′-GCTGCTGGGATT ACACATGGCATGGATGAACT ATACAAAGAATTCAACAAACCGAAAAAAAC AAG-3′; PYES-GFP-CeAPN1(Δ:1-118Nt)-F1: 5′- GCTGCTGGGATTACAC ATGGCATGGATGAACTATACAAAGAATTCATGTTGG GATTCCACGTGAG-3′; and PYES-GFP-CeAPN1-R1 5′-GGGACCTAGACTTCAGGTTGTCTA ACTCCTTCCTTTTCGGTTAGAGCGTTATCTTTTATCCATATTGT-3′ as previously described (Shatilla et al., 2005a; Aouida et al., 2004). In a similar manner, we used the pTW340 plasmid to express the full-length APN-1 and its two N-terminal deletions APN-1(1–63Δ) and APN-1(1–83Δ) as GST fusion proteins (Yang et al., 2012).

2.3. Extraction of proteins, SDS-PAGE and Western blot

These preparations and analyses were performed as previously described (Shatilla et al., 2005a). Briefly, cells were grown overnight in selective media lacking uracil, subcultured the next day and allowed to grow exponentially for an additional 4 h. Cells were harvested and stored at −80 °C overnight before preparation of the total protein extract using a bead beater. Total protein extracts were analyzed on SDS-PAGE followed by Western blot analysis using an anti-GFP monoclonal antibody (Sigma) at a dilution of 1:2000 and anti-mouse secondary antibodies at a dilution of 1:2000.

2.4. Preparation of oligonucleotide AP site substrate and AP endonuclease assay

A synthetic 42-base pair 5′-end [^{32}P]-labeled oligonucleotide with a uracil at position 21 d(GCTGCATGCCTGCAGGTCGA**U**TCTAGAG GATCCC GGGTACCT) and complementary strand containing G opposite to U d(CGACGTACGGACGTCCAGCT**G**AGATCTCCTAGGG CCCATGGA) was prepared as previously described (Shatilla and Ramotar, 2002; Masson and Ramotar, 1997). The uracil in the double-stranded DNA substrate was removed by uracil DNA glycosylase to create the resulting AP site (Shatilla and Ramotar, 2002; Masson and Ramotar, 1997).

The in vitro AP endonuclease assay was performed as previously described (Gelin et al., 2010). Briefly, 50 ng of the AP site substrate was incubated with the indicated amount of total protein extracts at 37 °C for 20 min in a final volume of 12.5 μl. Reactions were stopped with 5 μl formamide loading buffer (76% formamide, 0.3% bromophenol blue, 0.3% xylene cyanole, 10 mM EDTA), and heated at 65 °C for 3–5 min. The reaction product was separated on denaturing 10% polyacrylamide–7 M urea gel, exposed to a Fugi FLA-3000 Phosphor Screen and analyzed using Image Gauge V3.12 software. For K_M and k_{cat} determination of the full-length APN-1 and its two N-terminal deletions APN-1(1–

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