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Interaction of apurinic/apyrimidinic endonuclease 2 (Apn2) with Myh1 DNA glycosylase in fission yeast

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ABSTRACT

Oxidative DNA damage is repaired primarily by the base excision repair (BER) pathway in a process initiated by removal of base lesions or mismatched bases by DNA glycosylases. MutY homolog (MYH, MUTYH, or Myh1) is a DNA glycosylase which excises adenine paired with the oxidative lesion 8-oxo-7,8-dihydroguanine (8-oxoG, or G°), thus reducing G:C to T:A mutations. The resulting apurinic/apyrimidinic (AP) site is processed by an AP-endonuclease or a bifunctional glycosylase/lyase. We show here that the major *Schizosaccharomyces pombe* AP endonuclease, Apn2, binds to the inter-domain connector located between the N- and C-terminal domains of Myh1. This Myh1 inter-domain connector also interacts with the Hus1 subunit of the Rad9–Rad1–Hus1 checkpoint clamp. Mutagenesis studies indicate that Apn2 and Hus1 bind overlapping but different sequence motifs on Myh1. Mutation on I^{261} of Myh1 reduces its interaction with Hus1, but only slightly attenuates its interaction with Apn2. However, E^{262} of Myh1 is a key determinant for both Apn2 and Hus1 interactions. Like human APE1, Apn2 has 3′-phosphodiesterase activity. However, unlike hAPE1, Apn2 has a weak AP endonuclease activity which cleaves the AP sites generated by Myh1 glycosylase. Functionally, Apn2 stimulates Myh1 glycosylase activity and Apn2 phosphodiesterase activity is stimulated by Myh1. The cross stimulation of Myh1 and Apn2 enzymatic activities is dependent on their physical interaction. Thus, Myh1 and Apn2 constitute an initial BER complex.

1. Introduction

Reactive oxygen species and radiation can lead to DNA strand breaks and base lesions that must be repaired to maintain genomic stability, prevent carcinogenesis, and control aging [1]. Oxidative DNA lesions are repaired primarily by the base excision repair (BER) pathway [2]. A frequent and highly mutagenic oxidative lesion is 8-oxo-7,8-dihydroguanine (8-oxo-G or G°), which mispairs with adenine during DNA replication, leading to G:C to T:A mutations [3–5]. MutY homolog (MYH, MUTYH, or Myh1) is a DNA glycosylase which excises adenine from A/G° in the first step of BER pathway [3–5]. The resulting apurinic/apyrimidinic (AP) sites are generally processed by AP-endonucleases that catalyze a 5′ cleavage of the phosphodiester backbone, producing a 3′-OH [6,7]. Alternative pathways independent of AP-endonucleases have been identified [8,9]. The downstream BER enzymes then complete the repair process. These enzymes and basic steps of BER pathway are highly conserved among diverse organisms.

MYH/Myh1 repair is essential for genome stability because its deficiency leads to higher mutation rates in both mouse and fission yeast cells [10,11]. In addition, mutations in the human *MYH* (*hMYH*) gene are associated with colorectal cancer as in MYH-associated polyposis (MAP) [12–16]. Eukaryotic MYH enzymes contain unique motifs not found in prokaryotic MutY that mediate interactions with partner proteins involved in DNA replication, mismatch repair, and DNA damage response (reviewed in [3,17]). These interactions are critical to direct MYH repair to daughter DNA strands, to drive the repair pathway to completion, and to coordinate BER with DNA damage response. Cell cycle checkpoint provides surveillance mechanisms to activate the DNA damage

Abbreviations: 3′-dRP, 3′-α,β-unsaturated aldehyde; 8-oxoG or G°, 7,8-dihydro-8-oxoguanine; 9–1–1, Rad9–Rad1–Hus1; AP, apurinic/apyrimidinic; APE1 or Apn1, AP-endonuclease 1; Apn2, AP-endonuclease 2; BER, base excision repair; BSA, bovine serum albumin; DTT, dithiothreitol; FAM, fluorescein; GST, glutathione S-transferase; h, human; IDC, interdomain connector; k_{obs}, rate constants; MAP, MYH-associated polyposis; MBP, maltose binding protein; MYH, MUTYH, or Myh1, MutY homolog; Nth1, endonuclease III homolog; PCNA, proliferating cell nuclear antigen; *S. cerevisiae* or Sc, *Saccharomyces cerevisiae*; *S. pombe* or Sp, *Schizosaccharomyces pombe*; THF, tetrahydrofuran abasic site analog; UDG, uracil DNA glycosylase.

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response, thus preserving genomic integrity [18,19]. Checkpoint sensors Rad9, Rad1, and Hus1 form a heterotrimeric (9–1–1) complex [20,21] whose structure [22–24] is remarkably similar to that of proliferating cell nuclear antigen (PCNA) [25–27]. We recently showed that the interdomain connector (IDC) located between the N- and C-terminal domains of hMYH is uniquely oriented to interact with human AP endonuclease 1 (hAPE1) and the 9–1–1 complex [28]. We have shown that *Schizosaccharomyces pombe* Myh1 (SpMyh1) also interacts with the 9–1–1 complex and mutations in the IDC region of SpMyh1 cannot complement *myh1*Δ phenotypes [28–30].

AP-endonuclease is a multifunctional enzyme that participates in many aspects of DNA metabolism [31,32]. AP-endonuclease activity cleaves the phosphodiester bond 5′ to an AP site and its associated phosphodiesterase activity removes various forms of 3′-blocked lesions at DNA strand breaks to generate a 3′-OH DNA end [33,34]. Because AP sites are mutagenic and cytotoxic [17], they must be recognized by a downstream enzyme such as AP-endonuclease immediately after the action of a DNA glycosylase. A "passing-the-baton" model has been proposed for BER [35,36]. However, the underlying molecular mechanisms remain unclear. It has been proposed that the 9–1–1 complex may serve as a platform to coordinate BER at the sites of DNA damage because it interacts with nearly every enzyme in BER (reviewed in [37]). Human major repair AP-endonuclease, APE1, has been shown to interact with several DNA glycosylases [38–43]. MYH is the only glycosylase that can form a stable complex with APE1 [39]. In *S. pombe*, there are three AP endonucleases (Apn1, Apn2, and Uve1) [33,44]. However, none of these AP endonucleases has been shown to interact with DNA glycosylases.

It has been suggested that *S. pombe* Apn2 is the major AP endonuclease [33,44]. Because the AP endonuclease activity of SpApn2 is very weak, it has been suggested that the AP lyase activity of the bifunctional glycosylase SpNth1 (endonuclease III) provides the major incision at AP sites [33,45]. The 3′-α,β-unsaturated aldehyde (3′-dRP) produced by SpNth1 is then further processed by the phosphodiesterase activity of SpApn2 [33,45]. Here, we provide the first biochemical characterization of Apn2. We show that recombinant Apn2 expressed in bacteria has 3′-phosphodiesterase activity but processes a weak AP endonuclease activity which cleaves the AP sites generated by Myh1 glycosylase. SpApn2 interacts with the IDC region (residues 245–293) of Myh1 which is also a Hus1 binding site, however, Apn2 and Hus1 use overlapping but different sequence motifs. Myh1 and Apn2 cross stimulate each other's enzymatic activity. Thus, Myh1 and Apn2 can act synergistically as a physical unit to maintain genomic stability.

2. Materials and methods

2.1. Cloning of glutathione S-transferase (GST) tagged SpApn2

The full-length cDNA of SpApn2 encoding 523 residues was amplified by PCR from the plasmid pNBR110 [44] (kindly provided by S. Mitra, University of Texas Medical Branch) using Pfu DNA polymerase (Stratagene) with the appropriate primers (listed in Table S1). All oligonucleotides were purchased and purified by HPLC from IDT. The PCR products were digested with BamHI and XhoI and then cloned into pGEX4T-2 to express GST fusion protein. The plasmids were transformed into *Escherichia coli* DH5α cells (Invitrogen), and selected via ampicillin resistance. DNA sequencing revealed that the Apn2 clone in pGEX4T-2 vector contained Ser (AGT codon) at position 254. However, SpApn2 sequence in the gene bank (NCBI Reference Sequence: NP_595522.1) indicates Asn (AAT codon) at this position. Further analysis showed that the *Apn2* gene in the original template plasmid (pNBR110) [44] already contained the same AAT to AGT change. The GST-tagged Apn2^{S254} was expressed in Rosetta cells (Invitrogen).

2.2. Cloning, expression, and purification of His-tagged full-length Apn2

The plasmid pGEX4T-Apn2 containing the full-length cDNA of SpApn2^{S254} was digested with BamHI and XhoI and isolated cDNA fragment was ligated into BamHI/XhoI digested pET21a vector to obtain pET21a-SpApn2^{S254}. QuickChange site-directed mutagenesis (Strategene) using pET21a-SpApn2^{S254} plasmid as a template and primers listed in Table S1 was employed to obtain pET21a-SpApn2^{N254} plasmid. The pET21a-SpApn2^{N254} plasmid was then further used to construct pET21a-SpApn2$^{N254/S295}$ plasmid by similar QuickChange site-directed mutagenesis. Both mutations were verified by DNA sequencing.

SpApn2^{S254}, SpApn2^{N254}, and SpApn2$^{N254/S295}$ proteins were expressed in *E. coli* BW528 [*nfo-1*::kan Δ(*xth-pncA*)90] (kindly provided by Bernard Weiss) containing a lambda DE3 lysogen to avoid the contamination of *E. coli* AP endonucleases. The DE3 lysogenic stain was constructed according to the procedures described by Invitrogen. The cells were cultured in Luria-Bertani broth containing 100 μg/ml amplicilin at 37 °C. Protein expression was induced at an A_{590} of 0.6 by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.2 mM. After 16 h at 20 °C, the cells were harvested. The His-Apn2 proteins were first purified by Ni-NTA resin (Qiagen) under native conditions according to the manufacturer's protocol. The proteins from Ni column were diluted with buffer A (20 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol and 0.1 mM PMSF) and further purified by 1 ml SP column (GE Health) equilibrated with buffer A containing 0.05 M KCl. Upon washing with 12 ml of equilibration buffer, the column was eluted with 20 ml of buffer A containing a linear gradient of KCl (0.05–1 M). The fractions that contain the His-Apn2 proteins (confirmed by SDS-polyacrylamide gel analysis) were pooled, further purified by 1 ml Heparin column (GE Health) equilibrated with buffer A containing 0.05 M KCl. Upon washing with 12 ml of equilibration buffer, the column was eluted with 20 ml of buffer A containing a linear gradient of KCl (0.05–1 M). Because heparin chromatography only increased Apn2 purify slightly, SpApn2$^{N254/S295}$ protein was not further purified by Heparin column. The fractions that contain the His-Apn2 protein (confirmed by SDS-polyacrylamide gel analysis) were pooled, divided into small aliquots, and stored at −80 °C. The concentrations of His-Apn2 proteins were determined by the Bradford method.

2.3. Cloning, expression, and purification of His- and maltose binding protein (MBP)-tagged Apn2$^{1–303}$

The cDNA (encoding residues 1–303) of SpApn2^{S254} was amplified by PCR from the plasmid pGEX4T-Apn2 using Pfu DNA polymerase (Stratagene) with the appropriate primers (listed in Table S1). The PCR products were digested with BamHI and NotI, ligated into BamHI/NotI digested pLM303 vector which can express dual N-terminal His- and MBP-tagged proteins. The plasmid was transformed into *E. coli* DH5α cells (Invitrogen), and selected via kanamycin resistance.

To express the His-MBP-tagged SpApn2$^{1–303}$ (His-MBP-Apn2$^{1–303}$) protein, the plasmid was transformed into the *E. coli* Rosetta (Invitrogen) strain. The cells were cultured in Luria-Bertani broth containing 25 μg/ml kanamycin at 37 °C. Protein expression and purification procedures were similar to those described for His-Apn2. The fractions that contain the His-MBP-Apn2$^{1–303}$ protein (confirmed by SDS-polyacrylamide gel analysis) were