The role of the human Ape2 in the DNA repair

Péter Burkovics

Supervisor: Dr. Haracska Lajos

Hungarian Academy of Sciences, Biological Research Centre, Institute of Genetics

Szeged, 2006

Introduction

Cellular DNA is subject to attack by a variety of agents of exogenous and endogenous origins. Oxidative attack on DNA by free radical species happens continuously during normal cellular metabolism, generating abasic sites and DNA strand breaks with modified 3'-termini, such as 3'-phosphate or 3'-phosphoglycolate (3'-PG). Abasic (AP) site, which is one of the most common lesions that arise in cellular DNA, can also be formed due to the action of DNA glycosylases on modified bases or by spontaneous base hydrolysis. It has been estimated that as many as 10000 bases are lost spontaneously in a mammalian cell per day. DNA strand breaks with blocking 3'-termini can also arise due to the β-lyase activity of some DNA glycosylases. The non-coding AP-sites as well as modified 3'-DNA ends are inhibitory to synthesis by DNA polymerases (Pols), or if bypassed, are highly mutagenic; consequently, their repair is essential for retaining the stability of the genome.

AP sites and single strand DNA breaks with 3` modified termini are repaired mainly by the base excision repair (BER) system. In BER, class II AP endonucleases play a central role by incising the DNA 5' to AP sites to generate accessible 3'-OH termini prior to repair synthesis. In addition, several class II AP endonucleases are able to function in the removal of 3'-blocking termini thereby generating accessible 3' DNA ends for repair synthesis by DNA polymerases.

Class II AP endonucleases have been classified into two families, the exonuclease III (ExoIII) and endonuclease IV (EndoIV) families, based on their homology to the two *Escherichia coli* enzymes. In *E. coli* the main AP endonuclease is ExoIII representing 90% of the cellular AP endonuclease activity, while Endo IV accounts for 10% of the total activity (97). In *Saccharomyces cerevisiae,* the Apn1 and Apn2 proteins represent the EndoIV and the ExoIII family, respectively. Apn1 exhibits a strong AP-endonuclease activity and accounts for more than 90% of the total AP endonuclease activity in yeast cells. In contrast, the Apn2 protein has only a weak AP endonuclease activity but exhibits a strong 3'-exonuclease and 3'-phosphodiesterase activities. The functional consequences of the enzymatic activities

of Apn1 and Apn2 is indicated by that the *apn1Δ* strain displays a much higher level of sensitivity to the alkylating agent methyl-methanesulfonate (MMS) than the *apn2Δ* strain; in contrast, neither of the single mutant strains, only the *apn1Δapn2Δ* double mutant showed sensitivity to the oxidative agent H_2O_2 (98). These observations indicate that in *S. cerevisiae* Apn1 has a more prominent role in the repair of AP sites than the Apn2, whereas for the processing of 3' oxidatively damaged DNA termini Apn1 and Apn2 contribute equally. Contrary to these findings, in *Schizosaccharomyces pombe*, which is generally believed to be phylogenetically closer to mammalian cells than *S. cerevisiae*, Apn2 provides the major AP-endonuclease activity while the Apn1 serves only as a back-up activity.

In humans, EndoIV homologues are not known, but two ExoIII family proteins, Ape1 and Ape2, have been identified. Ape1 exhibits robust AP endonuclease activity, which accounts for more than 95% of the total cellular activity, and Ape1 is considered to be the major AP endonuclease in human cells. It also possesses 3'-phosphodiesterase and 3' exonuclease activities that are about two orders of magnitude less efficient than its AP endonuclease activity.

The role and the biochemical properties of Ape2 have not been ascertained yet. This is largely due to the difficulty in the purification of human Ape2 as its overexpression causes extreme cytotoxicity in *E. coli*. A partially purified sample of human Ape2 has been shown to exhibit a weak AP endonuclease activity. Its limited AP-endonuclease activity taken together with the fact that in human cells Ape1 accounts for more than 95% of the total AP endonuclease activity indicates that human Ape2, similarly to its *S. cerevisiae* homologue may play only a limited role as an AP endonuclease. Also, a novel role of Ape2 is suggested by recent studies that showed that APE2-null mice have a growth retardation phenotype which is accompanied by a G2/M arrest of proliferating lymphocytes, indicating that Ape2 is required for proper cell cycle progression. In addition, Ape2 has been shown to localize not only to the nucleus but to some extent to the mitochondria, in which it may help to maintain the function and integrity of mitochondrial DNA, even if threatened by the attack of reactive oxygen species produced during oxidative phosphorylation. These observations strongly suggest that the function of Ape1 and Ape2 proteins differs considerably in human cells.

To shed more light on the role of human Ape2, we have purified recombinant Ape2 from yeast, and characterized it biochemically. Here, we report novel enzymatic activities for human Ape2. We find that recombinant Ape2 exhibits only a weak AP-endonuclease activity; however, it shows strong 3'-5' exonuclease and 3'-phosphodiesterase activities. Also, we show that human Ape2 is able to remove preferentially mismatched and mutagenic paired 3'-nucleotides from DNA. Additionally, we demonstrate that PCNA stimulate the Ape2 3'-5' exonuclease activity. Based on these findings, we discuss a possible role for Ape2 in processing 3'-damaged termini or 3'-mismatched nucleotides.

Aims

In our study, our goal was the purification and *in vitro* characterization of the human Ape2 protein. In particular, our aim was to determine the enzymatic activities of the Ape2, and through these activities to gain insight into the biological functions of the Ape2 in DNA repair.

Results

The Ape2 cDNA was PCR amplified from a human cDNA library followed by cloning it in frame downstream of the GST gene in a yeast expression vector. We also made a similar construct expressing the Ape2D277A protein containing a point mutation at the D277 residue, which corresponds to a conserved metal binding site in the active centre of class II AP endonucleases. We expected this mutation to render Ape2 catalytically inactive. The resulting GST-Ape2 fusion proteins were purified from a protease-deficient *S. cerevisiae* strain on Glutathione-Sepharose beads.

To examine whether the purified recombinant Ape2 has an intrinsic AP-endonuclease activity, we assayed Ape2 using a 75-nt duplex DNA substrate, in which the 5'-^{32}P-end labelled strand contained a single abasic site at position 31 from the 5'-end. Incubation of Ape2 with the 75-nt DNA substrate generated a 30-nt labeled oligomer indicating that Ape2 like other class II AP-endonucleases incised the DNA 5' to the AP site. Using the Ape2 D277A mutant protein in the same assay we could not detect any incision of the DNA substrate confirming that the D277A mutation indeed rendered Ape2 catalytically inactive and, that the observed AP endonuclease activity is intrinsic to Ape2. However, Ape2 is a weak AP-endonuclease

indicated by that even at optimal NaCl concentration Ape2 incised only seven percent of the DNA substrate despite its two and half fold molar excess over DNA substrate.

When we examined the AP endonuclease activity of Ape2 in a buffer containing no NaCl, we noticed the gradual shortening of the 75-mer template that indicated an exonucleolytic digestion of the DNA from the 3' end. To determine whether the observed 3'-5' exonuclease activity was intrinsic to Ape2, or due to an exonuclease contamination of our protein sample, we assayed the wild type and active centre mutant Ape2 D277A proteins on a double-stranded DNA substrate containing a 5'-^{32}P-labeled strand with recessed 3' terminus. Incubation of increasing amount of Ape2 with this DNA substrate generated a series of smaller products, indicating that Ape2 exonucleotically digested the DNA from the 3' terminus. In contrast, the D277A active centre mutant Ape2 did not show any 3'-5' exonuclease activity demonstrating that the observed robust 3'-5' exonuclease activity was intrinsic to the Ape2 protein. Importantly, when the DNA substrate was in two-fold molar excess over Ape2, under optimal reaction conditions Ape2 hydrolyzed nearly hundred per cent of the exonuclease substrate and only seven per cent of the AP endonuclease substrate. Therefore Ape2 is dominantly a 3'-5' exonuclease.

Next we tested whether Ape2 is also able to remove 3'-blocking termini. For this we constructed a DNA substrate by annealing a 70-nt long oligonucleotide to a 35-nt oligomer containing a 3'-PG terminus. Enzymatic removal of the 3'-PG results in a 3'-hydroxyl terminus (3'-OH), and 3'-PG is released in the form of phosphoglycolic acid. The 3'-OH group confers lower electrophoretic mobility to the 35-nt oligomer on a denaturing polyacrylamide gel. Incubation of Ape2 with the PG-containing DNA substrate resulted in the appearance of a lower mobility band on the gel, which indicated that Ape2 had a 3'-phosphodiesterase activity. Unlike the wild type protein, the Ape2 D277A mutant did not exhibit such an activity. These results indicate that the Ape2 protein has a 3'-phosphodiesterase activity.

To characterize further Ape2 exonuclease, first we examined for its optimal reaction conditions. The exonuclease activity of Ape2 was strongly dependent upon metal ion. Ape2 was about five fold more active in the presence of manganese than of magnesium ion. Addition of salt inhibited the exonuclease activity, and at 100 mM NaCl concentration Ape2 exhibited only twenty percent of its exonuclease activity measured in buffer containing no salt, and in the presence of 200 mM salt almost no

exonuclease activity could be detected. The Ape2 showed optimal activity in the pH range of 6.0 to 8.0.

To determine the DNA substrate requirement for the exonuclease activity, we compared the exonuclease activity of Ape2 for its ability to hydrolyze single-stranded DNA, blunt-ended duplex DNA, partial DNA duplexes with either a protruding or a recessed 3'-terminus, or a single nucleotide gap containing heteroduplex DNA. Ape2 exhibited the strongest exonuclease activity on a recessed 3'-end containing partial DNA duplex and fairly strong activity on a single nucleotide gap containing heteroduplex DNA. In addition, Ape2 also exhibited exonuclease activity on blunt-ended substrate. However, Ape2 showed only residual activity on single stranded DNA and protruding 3'-terminus.

Short-patch base excision repair proceeds via a nick and a single nucleotide gap containing heteroduplex DNA. To examine whether Ape2 has any preferences for these DNA substrates, we compared the exonuclease activity of Ape2 on a nick and 1, 2, 3, or 4-nt long gap containing DNA duplexes. Our results revealed that Ape2 exonuclease had two-fold stronger activity on a single nucleotide gap containing substrate than on a nicked DNA, and increasing the gap size from 1-4 nucleotides did not make significant difference.

To examine the effect of base-base hydrogen bounding at the primer-template junction on the exonuclease activity of Ape2, we assayed Ape2 on various combinations of recessed DNA with 3'-mispairs as substrates. Quantitation revealed that Ape2 removed 3' mismatched nucleotides two to forty-fold more efficiently than those matched correctly. For example, Ape2 had a very limited activity on a 3' recessed DNA duplex containing a matched 3'A opposite from a template T, however it exhibited a strong nuclease activity on a DNA containing unmatched 3' A opposite from G, A, or C. From these experiments we conclude that Ape2 exonuclease has a significant preference for mispaired nucleotides at the recessed 3'-end of DNA.

Proliferating cell nuclear antigen (PCNA) is a highly conserved eukaryotic homotrimeric protein that assembles around the DNA to form a sliding clamp and acts as a processivity factor for the replicative DNA polymerase. PCNA interacts with a large variety of proteins involved in DNA replication, DNA repair, and cell cycle control. More recently, it has been shown that PCNA interacts with the TLS polymerases and yeast Apn2, and that Ape2 and PCNA are in the same protein complex in human cells. Based on these observation, we hypothesized, that Ape2 and

PCNA directly interact, and PCNA modifies the enzymatic activities of Ape2. We focused mainly how the PCNA modifies the 3'-5' exonuclease activity of the Ape2. Addition of the PCNA in our standard Ape2 exonuclease assay increased dramatically the 3'-5' exonuclease activity of the Ape2. To verify this result, we decided to determine the PCNA binding site of the Ape2. We found three putative PCNA binding motifs in the Ape2 sequence, which can be responsible for the Ape2 PCNA interaction. We have made point mutations in these PCNA binding motifs (Y396A F397A, F492A Y493, F511A F512A). In the standard exonuclease assay all mutant proteins showed the same activity as the wild type Ape2. After the addition of the PCNA to the exonuclease reaction only one mutant (Y396A F397A mutant) showed the same increased 3'-5' exonuclease activity as the wild type Ape2. The other two mutants (F492A Y493, F511A F512A) were defective in this stimulation. This result supports that the PCNA binding increases the 3'-5' exonuclease activity of the Ape2, and it suggests that the Ape2 has two PCNA binding motifs which work cooperatively.

Tutshimoto at all described that the amount of Ape2 increased in the replication fork after desoxy-uridine treatment. Therefore, we examined the 3'-5' degradation of recessed end heteroduplex DNA containing either thymine or desoxy-uracil on the 3' end. Ape2 had a very weak activity on the thymine containing substrate DNA, but it had a robust activity on the desoxy-uracil containing substrate DNA. To characterize this difference we determined the kinetic parameters of the 3'-5' exonuclease activity on the thymine and desoxy-uracil containing DNA substrate. The comparison of the V_{max}/Km values showed that the desoxy-uracil substrate is prefered to thymine substrate.

Endogenous oxidative DNA lesions are most abundant and inevitable as cells generate reactive oxygen species trough aerobic respiration. The occurrence of oxidized bases in DNA can result from either direct oxidation or misincorporation of oxidized deoxyribonucleosid monophosphate by DNA polymerases from the nucleotide pool. These oxidized bases are mutagenic and therefore must be removed by an efficient DNA repair processes. During the replication polδ bypasses the 8-oxoG lesion inefficiently and an adenine is preferentially incorporated opposite 8-oxoG. To achieve a lower mutation rate, the mispaired adenine must be eliminated. One possible way is that the Ape2 exonuclease removes this mutagen adenine. Therefore we tested how the Ape2 acts on the DNA substrates which contain on the

3’ end an A:8-oxoG and C:8-oxoG base pair. To see the difference between them we determined the kinetic parameters and the V_{max}/Km values. In our experiment the Ape2 cleaved the adenine 8.5 times better than it cleaved the cytosine opposite the 8-oxoG. This result indicates that the 3’-5’ exonuclease activity of Ape2 can provide an efficient way to decrease the mutation rate during the replication of 8-oxoG lesion containing DNA.

Conclusions

In this study, we have clarified the enzymatic activities of human Ape2 and give some suggestion for its functional importance. Ape2 is a multifunctional enzyme, which has only a weak AP endonuclease, a fairly strong 3’-phosphodiesterase, but a robust 3’-5’exonuclease activity. Importantly, we reveal that the Ape2 exonuclease is most active on 3’-recessed heteroduplex DNA and is able to remove mismatched nucleotides preferentially.

Previously, Ape2 has been purified only partially due to its insolubility and its cytotoxicity on the *E. coli* and it has been shown to possess a very weak AP-endonuclease activity. In agreement with that study, our attempt to purify active recombinant Ape2 from *E.coli* was not successful. However, we managed to overcome the problem by overexpressing Ape2 in fusion with GST in the yeast *S. cerevisiae*. To confirm that the observed 3’-5’ exonuclease, 3’-phosphodiesterase, and the extremely weak AP-endonuclease activities are intrinsic to Ape2, we also purified a mutant Ape2 protein, in which we altered the conserved Asp277 residue to Ala. This amino acid in Ape2 is equivalent to residues Asp328 in *S. cerevisiae* Apn2, and Asp283 in human Ape1, which have been shown to be involved in metal binding. As expected, the Ape2 D277A protein did not show activity in any of the enzymatic assays thereby confirming that the detected AP-endonuclease, 3’-phosphodiesterase, and 3’-exonuclease activities are intrinsic to Ape2.

Interaction with PCNA increases the 3’-5’ exonuclease activity, but not the AP endonuclease activity of Ape2. We determined the PCNA binding motifs of Ape2 by examining the effect of PCNA on the exonucleotic activity of pont mutant Ape2. We showed that Ape2 has two PCNA binding motifs, which work cooperatively. The strong dependence of the Ape2 3’-5’ exonuclease activity on the PCNA suggests, that the main function of the Ape2 in the cell is attached tightly to the PCNA.

It has recently been published that APE2-null mice show abnormalities in proliferating haemopoietic organs, such as dyshaematopoiesis, defect in lymphopoiesis, and delayed S-phase and G2/M-phase arrest. It has been suggested that the increased accumulation of AP sites in the absence of the AP endonuclease activity of Ape2 is the underlying cause of these phenotypes. However, our biochemical data show a very inefficient AP endonuclease activity of Ape2 arguing that this activity can not play a major role *in vivo*. In contrast, due to its significant 3'-phosphodiesterase activity, Ape2 might have a major function in the removal of 3'-PG termini formed in DNA by oxidative agents. This theory is also supported by our previous study, in which we provided genetic evidence for the involvement of *S cerevisiae* Apn2 in the repair of 3'-PG residues.

Another clue for the main function of Ape2 can be found in its strong 3'-5' exonuclease activity, in the functional dependence on the PCNA, and in its preference for removing mismatched nucleotides from the 3' primer end. These results indicate that Ape2 might have a role as a proofreader in processes where new DNA synthesis occurs such as DNA repair synthesis.

It has been recently described, that the amount of Ape2 protein in the replication foci increased after desoxy-uridin treatment. We analyzed the 3'-5' exonuclease activity of the Ape2 on uracil containing DNA substrate. The 3'-5' exonuclease activity of the Ape2 was 10 times more active on desoxy-uracil, than on the thymine containing DNA substrate. The desoxy-uracil incorporation is a base of an anticancer drug (5-fluorouracil). Because of the fact that Ape2 has a strong desoxy-uracil removing 3'-5' exonucleas activity, it can act as a suppressor of this anticancer drug. Based on our observation, we predict that the inhibition of Ape2 might increase the efficiency of this type of anticancer drugs.

Recently it has been demonstrated, that several class II AP endonucleases such as yeast Apn1 and human Ape1 are able to remove 3' end incorporated 8-oxoG. Above this, the DNA polymerase-δ (polδ) has a highly mutagenic activity on 8-oxoG containing DNA substrate, because polδ incorporates adenine opposite the 8-oxoG more than 90% frequency. Considering these studies, we examined how Ape2 removes the adenine or cytosine opposite the 8-oxoG from the 3' end. We showed that the 3'-5' exonuclease activity of the Ape2 was 8.5 times better on the A:8-oxoG DNA substrate than on the C:8-oxoG. In summary, our result is the first

demonstration, that Ape2 has an antimutagenic 3'-5' exonuclease activity, which can provide a higher fidelity for the replication of damaged DNA.