Reconstitution of Proliferating Cell Nuclear Antigen-dependent Repair of Apurinic/Apyrimidinic Sites with Purified Human Proteins*

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An apurinic/apyrimidinic (AP) site is one of the most abundant lesions spontaneously generated in living cells and is also a reaction intermediate in base excision repair. In higher eukaryotes, there are two alternative pathways for base excision repair: a DNA polymerase β-dependent pathway and a proliferating cell nuclear antigen (PCNA)-dependent pathway. Here we have reconstituted PCNA-dependent repair of AP sites with six purified human proteins: AP endonuclease, replication factor C, PCNA, flap endonuclease 1 (FEN1), DNA polymerase δ, and DNA ligase I. The length of nucleotides replaced during the repair reaction (patch size) was predominantly two nucleotides, although longer patches of up to seven nucleotides could be detected. Neither replication protein A nor Ku70/80 enhanced the repair activity in this system. Disruption of the PCNA-binding site of either FEN1 or DNA ligase I significantly reduced efficiency of AP site repair but did not affect repair patch size.

Base excision repair is the major mechanism that replaces bases having relatively small modifications, e.g. deoxuryracil, thymine glycol, or 8-oxo-guanine. In this process, the damaged bases are removed and replaced by specific DNA-N-glycosylases, producing apurinic/apyrimidinic (AP) sites as common intermediates. AP sites are also generated by spontaneous depurination/depyrimidination and by the action of many different DNA damaging agents. AP sites are one of the most abundant DNA lesions in living cells. Base excision repair further processes AP sites to complete repair. The primary mechanism for AP site repair includes the following reactions: 1) incision of the AP site to generate a nick with 3′-OH and 5′-deoxyribose phosphate (dRP) termini; 2) DNA synthesis at the incised site; 3) excision of the dRP group from the 5′ terminus; and 4) DNA ligation. Recent studies of in vitro repair systems derived from higher eukaryotes demonstrated that repair of AP sites could proceed by two alternative pathways: a DNA polymerase β (pol β)-dependent pathway and a proliferating cell nuclear antigen (PCNA)-dependent pathway (1, 2).

The pol β-dependent pathway requires three purified factors: AP endonuclease, pol β, and DNA ligase (3). To repair AP sites in this pathway, pol β catalyzes two reactions: DNA synthesis by a DNA polymerase activity and excision of the 5′-dRP group by a dRP lyase activity (4). Thus, the pol β-dependent pathway can efficiently repair unmodified natural AP sites, but cannot repair modified AP sites which are refractory to pol β-elimination catalyzed by pol β/dRP lyase. To permit repair of the modified AP sites, the flap endonuclease 1 (FEN1) removes the dRP group and its 3′-adjacent nucleotide(s) by hydrolysis (5, 6). Finally, DNA ligase (either ligase I or the ligase IIIα-XRCC1 complex in higher eukaryotes) seals the phosphodiester backbone to complete the repair reaction (3, 7, 8). Involvement of these enzymes in base excision repair in vivo is supported by studies with mutant mammalian cell lines. Pol β-deficient cells are hypersensitive to various alkylating agents, suggesting that the pol β-dependent pathway plays a major role in the repair of alkylated bases (9). Similarly, cell lines deficient in either DNA ligase I or DNA ligase III activity are also hypersensitive to DNA alkylating agents (see Ref. 10 for review). These observations indicate that DNA ligase I and DNA ligase III are not functionally redundant and presumably participate in different pathways that repair DNA lesions caused by alkylating agents.

The PCNA-dependent base excision repair system is an alternative to the pol β-dependent pathway. Immunocytological studies have demonstrated the formation of tight complexes of PCNA in nuclei following ionizing radiation, suggesting that the damage generated by such a treatment may be repaired at least in part by the PCNA-dependent pathway in vivo (11, 12). Recently, a consensus amino acid sequence for binding to PCNA was found in several proteins involved in base excision repair, such as FEN1, DNA ligase I, and a nuclear form of uracil DNA glycosylase (13, 14). These observations are consistent with the participation of PCNA in base excision repair in vivo. Since PCNA also interacts with both RF-C and pol δe, PCNA may act as a molecular adaptor coordinating the actions of the enzymes in this base excision repair pathway.

In order to test this hypothesis, it is a prerequisite to identify protein factors that are essential for the PCNA-dependent base excision repair pathway. An in vitro system for PCNA-dependent AP site repair was originally developed with three purified
proteins, AP endonuclease, PCNA, DNA polymerase δ (pol δ), and two protein fractions, BE-1B and BE-2 from Xenopus laevis oocytes (1). Subsequently, it was demonstrated that a purified protein, FEN1, could replace BE-2 (6). Biochemical properties of the BE-1B fraction suggested that replication factor C (RF-C) and at least one of the DNA ligases may be included in BE-1B. Here we have reconstituted this repair reaction with six purified human proteins: AP endonuclease, RF-C, PCNA, FEN1, pol δ, and DNA ligase I. We have also examined the effects of mutations in FEN1 and DNA ligase I which abolish the ability of these proteins to interact with PCNA on the repair reaction. In the accompanying paper, Pascucci et al. (15) also report reconstitution of AP site repair with purified components. Their data are essentially consistent with our results.

EXPERIMENTAL PROCEDURES

Protein Factors—Human AP endonuclease (also called Ref-1) and human FEN1 were overexpressed in bacteria as hexahistidine-tagged forms and purified by nickel-chelate column chromatography as described previously (16, 17). Untagged human PCNA was overexpressed in baculovirus-infected cells and purified as described previously (20). Human DNA ligase I was overexpressed in baculovirus-vectors which coded for each subunit of RF-C and purified as described previously (19). Human DNA ligase I was overexpressed in bacteria as hexahistidine-tagged recombinant proteins was examined as a function of time. Under the conditions used in this experiment, the repair reaction continued at a relatively linear rate for at least 2 h (data not shown). To examine intermediate products resulting from repair synthesis, we used a 5'-labeled DNA substrate (Fig. 2, lanes 1–8). With this substrate, repair synthesis was detected as elongation of the 5'-fragment incised at the AP site. Such products appeared when AP endonuclease, RF-C, PCNA, and pol δ were incubated together (lanes 6 and 7). Because of the circular nature of the DNA substrates, we assume that RF-C is required to load the clamp protein, PCNA, onto DNA. FEN1 was not essential for incorporation of one or two nucleotides (lane 6). However, the addition of FEN1 in the absence of DNA ligase I resulted in longer tracts of DNA synthesis (lane 7). This indicates that pol δ itself does not actively displace a downstream strand in the DNA synthesis from the incised AP site, but that it always pauses after a 1- or 2-nucleotide extension. We also observed a faint band resulting from 1-nucleotide incorporation in the absence of RF-C or PCNA, although this PCNA-independent DNA synthesis by pol δ did not appear to be efficient enough for AP site repair (lanes 3 and 4).

When all six proteins were added to the reaction, we still observed two products of incomplete repair, the incised fragment and the 1-nucleotide elongated fragment, in addition to the repaired product (Ref. 6; see below) may be a major rate-limiting step under the condition used in this experiment.

Excision of the AP site after incision by AP endonuclease was examined with a 3'-labeled DNA substrate which becomes reduced in length as a result of excision (Fig. 2, lanes 9–16). Although FEN1 is responsible for excision of AP sites in the PCNA-dependent pathway (6), FEN1 did not efficiently remove the 5'-terminal dRP group in the absence of loaded PCNA (lanes 10 and 11). Significant levels of AP site excision were

![FIGURE 1: STRUCTURE OF PRELABELED CIRCULAR DNA SUBSTRATES](image-url)
observed only when AP endonuclease, RF-C, PCNA, and FEN1 were incubated together (lanes 12 and 14). This result is consistent with our previous observation that FEN1 actively removes AP sites in the presence of both PCNA and RF-C-containing fractions (6). The major product of this excision reaction results from removal of the 5'-terminal dRP group and its 3'-adjacent nucleotide together, leaving a 2-nucleotide gap (6). Although it is now apparent that pol δ is not required for AP site excision (lane 12), addition of pol δ to the reaction facilitated longer excision in the absence of DNA ligase I (lane 14). Taking into account that the shortest excision reaction catalyzed by FEN1 generated a gap of 2 nucleotides, the ladder of intermediate products resulting from excision in the absence of DNA ligase I (lane 14) is essentially a mirror image of the ladder generated by DNA synthesis in the reaction with the same proteins (lane 7). This observation suggests that the DNA synthesis and excision steps are coordinated during the repair reaction. We detected faint bands of a fragment which was nearly 1 nucleotide longer than the 3' fragment incised by AP endonuclease (lanes 10, 11, and 13). This fragment seems to result from abortive ligation by DNA ligase I which has added AMP to the 5'-terminal dRP group but cannot complete the ligation reaction.

Replication protein A (RPA; also known as the human single-stranded DNA-binding protein, HSSB) is an essential factor for DNA replication and nucleotide excision repair (21, 26, 27). The involvement of RPA in these DNA transactions appears to be mediated by binding to single-stranded regions of DNA and specific protein-protein interactions (see Ref. 28 for review). With the exception of AP endonuclease, all the other protein factors that are employed by the PCNA-dependent pathway of AP site repair are also involved in DNA replication and/or nucleotide excision repair. Therefore, we tested whether RPA had any effect on AP site repair in our reconstituted system. Under these conditions, RPA did not have a significant effect on the repair reaction, although 100 ng of RPA moderately reduced the repair efficiency (data not shown).

The Ku70/80 heterodimer is a component of DNA-dependent protein kinase and is involved in nonhomologous recombination DNA repair and V(D)J recombination (29, 30). Since the Ku70/80 heterodimer binds not only to double-strand breaks but also single-strand breaks (31), we examined the effect of this protein complex on AP site repair. As with RPA, the Ku70/80 complex did not affect the repair efficiency (data not shown). Thus, we concluded that neither RPA nor Ku70/80 are essential for AP site repair.

**Patch Size of AP Site Repair in the PCNA-dependent Pathway**—Since FEN1 does not release a free dRP molecule but an oligonucleotide composed of the dRP group and its 3'-adjacent nucleotide(s), the PCNA-dependent pathway should replace at least two nucleotides during AP site repair. This is in stark contrast to the single nucleotide replacement by the pol β-dependent pathway for repair of unmodified natural AP sites (32). As shown in Fig. 2, repair synthesis catalyzed by pol δ proceeded up to 9 nucleotides in the presence of FEN1 (lane 7), whereas pol δ paused after incorporation of one or 2 nucleotides in the absence of FEN1 (lane 6). To determine directly the length of nucleotides replaced during the repair reaction (patch size), we conducted the repair reaction in the presence of α-thio-dNTPs, and subjected the repaired product to exonuclease III digestion (see Fig. 3A for schematic procedures). Since the 3'-5' exonuclease III activity cannot proceed beyond a phosphorothioate group, the size of the repaired DNA remaining after this enzymatic digestion will indicate the 3' end of the repair patch. We observed that the major product generated by exonuclease III digestion was 2 nucleotides longer than the AP site-incised DNA, although products which were longer by up to 7 nucleotides were detected as minor bands (Fig. 3B). Therefore, the reaction reconstituted with the six human proteins completed AP site repair predominantly by replacing two nucleotides.

**Effects of FEN1 and DNA Ligase I Mutations Abolishing Interaction with PCNA**—PCNA physically interacts with several proteins involved in DNA replication, repair, and cell cycle control. A consensus motif for binding to PCNA, QXX(I/L/M)XX(F/Y), has been found in p21Cip1/Waf1, FEN1, XPG, DNA ligase I, the large subunit of RF-C, a nuclear form of uracil DNA glycosylase, DNA-(cytosine 5) methyltransferase and many other proteins (13, 14, 33). This motif interacts with one of three hydrophobic pockets on the outer surface of the ring-shaped PCNA homotrimer (34). Substitution of the adjacent phenylalanine residues with arginine residues within the PCNA-binding motif of FEN1 (F343A/F344A) and DNA ligase I (F8A/F9A) results in the loss of physical interaction with PCNA but does not significantly affect the intrinsic catalytic activities.
of these proteins (17, 33). To examine if the binding to PCNA is essential for the participation of these proteins in the repair reaction, we compared the repair activity of wild-type FEN1 and DNA ligase I to the activity of their mutant forms in which the PCNA binding motif was disrupted. In a previous study, inactivation of the PCNA-binding site of FEN1 (F343A/F344A) slightly reduced AP site repair, but this effect was overcome by the addition of an excess amount of mutant FEN1 (17). A similar result was observed in the reconstitution experiments in which mutant FEN1 replaced the wild-type protein (Fig. 4, lanes 1 and 2). To examine the repair patch size in these reactions, fully repaired products were recovered from the polyacrylamide gel so that unligated intermediates would not interfere with the patch size assay. Since the repair patch produced in the reaction containing the FEN1 mutant was still predominantly 2 nucleotides in length (Fig. 4, lane 6), we concluded that the interaction of FEN1 with PCNA does not determine the size of the repair patch.

Finally, we compared the ability of the wild-type DNA ligase I and its mutant, which was deficient in the binding to PCNA, to function in the reconstituted repair reactions (Fig. 5). Ten ng of DNA ligase I supported repair of more than 80% of the AP sites regardless of whether it was the wild-type or the F8A/F9A mutant protein. At lower enzyme concentrations, however, the reactions with the mutant form of DNA ligase I generated a significantly lower amount of the repaired product than the reaction with the wild-type enzyme. More than 80% of the AP sites were still repaired in the presence of 1 ng of the wild-type DNA ligase I protein, whereas the reaction with the same amount of the mutant protein repaired less than 60% of the AP sites regardless of whether it was the wild-type or the F8A/F9A mutant protein.

Fig. 3. Patch size of AP site repair. A, schematic diagram of the patch size assay. After the repair reaction in the presence of α-thio-dNTPs, the 5'-labeled DNA substrate was restricted with PstI and HaeIII, and digested with exonuclease III, as described under “Experimental Procedures.” Exonuclease III can digest DNA from 3'-recessed end or blunt ends as generated by PstI. This nuclease can neither hydrolyze phosphorothioate diesters nor bypass them (24). α-Thio-nucleotides incorporated during the repair reaction are designated as S, B, patch size of PCNA-dependent AP site repair. The 5’-labeled circular DNA containing a synthetic AP site was repaired for 2 h by the six purified human factors. As indicated above each lane (+), the reactions contained either the normal four dNTPs (final 20 μM each) or the four α-thio-dNTPs (final 40 μM each). Following the repair reaction, the DNA samples in lanes 2 and 4 were digested with exonuclease III. Positions of the bands corresponding to the products containing a repair patch of 1–7 nucleotides are indicated in the margin.

Fig. 4. Patch size of the products repaired with a FEN1 mutant deficient in the interaction with PCNA. Repair reactions were conducted as in Fig. 3 with α-thio-dNTPs except that 10 ng of either the wild-type FEN1 (lane 1) or the F343A/F344A mutant (lane 2) were employed in addition to the five other factors. After incubation of the repair reactions for 2 h, the DNA samples were digested with PstI and HaeIII, and resolved by electrophoresis in a 7 M urea-containing polyacrylamide gel (lanes 1 and 2). Completely repaired products were recovered from the gel, annealed with pBS single-stranded DNA, and analyzed for their patch size (lanes 3–6) as described in the legend to Fig. 3. The samples loaded in lanes 3 and 4 were recovered from lane 1; the samples loaded in lanes 5 and 6 were recovered from lane 2.

Fig. 5. Titration of DNA ligase I in AP site repair. Indicated amounts of either the wild-type DNA ligase I (open circles) or the F8A/F9A mutant (closed circles) were employed for AP site repair assay in addition to other five protein factors as described in the legend to Fig. 2. The recovered DNA was digested with PvuII and AP endonuclease and analyzed in an 8 M urea-containing 6% polyacrylamide gel electrophoresis. The percentage of repaired DNA was calculated after scanning the gel with the Fuji BAS1000 PhosphorImaging System.
that pol efficiently perform the repair synthesis (1). It is noteworthy that a PCNA-deficient mutant is required for repair of methylation damage (39). These results suggest that the two PCNA-dependent DNA polymerases may be used for base excision repair of different types of damage in yeast. However, the conclusions of studies on base excision pathways in yeast are probably not applicable to mammalian cells, since there are clear differences in the damage-response mechanisms between single-cell organisms and multicellular organisms. For example, pol β-dependent base excision repair plays a major role in the repair of DNA damage caused by alkylating agents in mammalian cells but not in yeast cells. In addition, Klungland and Lindahl (5) and Dianov et al. (40) reported that pol β was also involved in the long patch pathway of base excision repair carried out by mammalian cell extracts. Although our results clearly indicate that pol β is not an essential factor for the PCNA-dependent repair pathway, coexistence of pol β may modify the repair reaction. Analysis of in vitro repair will be necessary for a precise assessment of the functional role of each DNA polymerase in mammalian base excision repair.

Reconstitution of PCNA-dependent AP Site Repair

RPA and Ku70/80 did not have any significant effects on the reconstituted system of AP site repair described here. Recently, Stucki et al. (37) also demonstrated that RPA did not stimulate but rather inhibited in vitro AP site repair catalyzed by PCNA and a fraction containing pol e, RF-C, and DNA ligase I. On the contrary, DeMott et al. (41) reported that the repair reaction of a preincised AP site catalyzed by pol e, FEN1, and DNA ligase I was stimulated by RPA. In this case, RPA seems to unwind a 5′-incised AP site and its adjacent nucleotides, leading to formation of a stable flap structure either directly or indirectly after strand-displacing DNA synthesis by pol e. The formation of a stable flap structure can stimulate excision of the AP site and its adjacent nucleotide(s) by FEN1 which has reduced excision activity at the incised AP site in the absence of PCNA and RF-C (6). In addition, unwinding of the incised AP site may increase the DNA synthesis activity of pol e. We suspect that these effects of RPA may be negligible if PCNA and RF-C are present in the AP site repair reaction. Nevertheless, our studies do not exclude the involvement of RPA in base excision repair in vivo. In addition, it is reported that the N-terminal region of uracil DNA glycosylase interacts with the C-terminal region of the 34-kDa subunit of RPA (42), suggesting possible modification of uracil DNA repair by RPA.

The experiment shown in Fig. 3 demonstrates that a majority of successfully repaired products in the PCNA-dependent reaction results from replacement of two nucleotides. This corresponds to the smallest patch size that is allowed by the excision mechanism of the AP site by FEN1, which generates a gap at least two nucleotides. The reconstituted system used in this study was able to elongate and excise up to nine nucleotides in the absence of DNA ligase I (Fig. 2, lanes 7 and 14). One possible mechanism which may serve for this limited patch size is that DNA ligase I may suppress the processivity of pol δ or the extensive excision by FEN1, although such activities of DNA ligase I have not been reported yet. Another possibility is that DNA synthesis at the incised AP site by pol δ may be carried with a significantly low processivity, which allows DNA ligase I to seal the strand after minimal DNA synthesis. The observation of a ladder resulting from 1 to 9-nucleotide elongation rather than the accumulation of the longest product
repair patch generated by the PCNA-dependent pathway is a flap structure that can be processed by FEN1 regardless of the efficiency of the repair reaction as previously observed (17). Inactivation of the PCNA-binding site of DNA ligase I also appears to serve as a molecular adaptor for DNA replication and repair by encircling DNA and bringing respective proteins together to the site of the reaction. Since one PCNA homotrimer can cleave a low processivity enzyme in this reaction.

Through interactions with many protein factors, PCNA appears to serve as a molecular adaptor for DNA replication and repair by encircling DNA and bringing respective proteins together to the site of the reaction. Since one PCNA homotrimer molecule has three binding sites and many proteins have been found to carry the consensus binding motif, it is possible that ternary complexes with different proteins are assembled on the PCNA ring. At the present time, the mechanism by which PCNA complexes with different proteins are assembled on DNA lesions in vivo remains unclear. The reconstituted AP site repair reaction described here provides a defined system for analysis of coordinated molecular interactions in base excision repair.

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