Replication Protein A Stimulates Proliferating Cell Nuclear Antigen-Dependent Repair of Abasic Sites in DNA by Human Cell Extracts†

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ABSTRACT: Base excision repair (BER) pathway is the major cellular process for removal of endogenous base lesions and apurinic/apyrimidinic (AP) sites in DNA. There are two base excision repair subpathways in mammalian cells, characterized by the number of nucleotides synthesized into the excision patch. They are the "single-nucleotide" (one nucleotide incorporated) and the "long-patch" (several nucleotides incorporated) BER pathways. Proliferating cell nuclear antigen (PCNA) is known to be an essential factor in long-patch base excision repair. We have studied the role of replication protein A (RPA) in PCNA-dependent, long-patch BER of AP sites in human cell extracts. PCNA and RPA were separated from the other BER proteins by fractionation of human whole-cell extract on a phosphocellulose column. The protein fraction PC-FII (phosphocellulose fraction II), which does not contain RPA and PCNA but otherwise contains all core BER proteins required for PCNA-dependent BER (AP endonuclease, DNA polymerases δ, β and DNA ligase, and FEN1 endonuclease), had reduced ability to repair plasmid DNA containing AP sites. Purified PCNA or RPA, when added separately, could only partially restore the PC-FII repair activity of AP sites. However, additions of both proteins together greatly stimulated AP site repair by PC-FII. These results demonstrate a role for RPA in PCNA-dependent BER of AP sites.

Proliferating cell nuclear antigen (PCNA)[1] and replication protein A (RPA) are both involved in multiple DNA processes including DNA replication and repair (*1*−*4*). The role of these proteins in nucleotide excision repair is well documented (*5*−*8*). However, there is limited understanding of their function in base excision repair (BER). Abasic sites are thought to be repaired by two independent pathways. Short-patch BER involves single nucleotide replacement (*9*). In this pathway the AP sites are processed by the AP endonuclease HAP1 (Ape, Apex; *1*), which cleaves the phosphodiester bond immediately 5′ to the AP site, generating a DNA nick with 5′-sugar phosphate and 3′-OH ends (*10*). Removal of the 5′-terminal sugar phosphate residue results in a single-nucleotide gap that is then filled by a DNA polymerase and sealed by DNA ligase (*11*−*13*).

An alternative BER pathway involves AP endonuclease, DNA polymerase, DNA ligase, and the structure-specific endonuclease FEN1 (*14*−*16*). During this mode of repair, DNA polymerase first performs repair replication synthesis at the 5′ incised AP site. The displaced DNA flap containing 5′-sugar phosphate is then removed by FEN1, and the DNA nick is sealed by DNA ligase. PCNA was found to be an essential component for in vitro long-patch repair reactions reconstituted with pol δ and it has been suggested that it may play a role in the repair of the AP site by stimulating DNA polymerase δ (*14*, *15*). Further, it was shown that FEN1 interacts with PCNA and that the endonuclease activity of FEN1 is stimulated by PCNA (*16*−*19*). In contrast to the extensive understanding of the role of PCNA in the repair process, there is limited clarity about the role of RPA in this process. RPA was found to stimulate FEN1 activity with flap substrates containing long duplex regions (*20*) and also in an in vitro repair reaction reconstituted with pol ϵ, FEN1 and DNA ligase I (*21*). However, another study found that RPA was not involved in a PCNA-dependent repair reaction reconstituted with PCNA, HAP1, and partially purified fractions containing either pol δ or pol ϵ (*22*). Thus, the data on the role of RPA in long patch BER is unclear. We have developed an assay for PCNA-dependent repair and directly addressed the role of RPA in this reaction. We find that RPA stimulates the PCNA-dependent repair of AP sites in a crude protein fraction from human cells.

MATERIALS AND METHODS

Proteins, Antibodies, and Cells. Polyclonal antibodies raised against human FEN1 (human recombinant protein purified from bacterial cells) and the p125 subunit of DNA polymerase δ (recombinant protein provided by V. Podust) were purified by protein A−Sepharose chromatography.

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[1] Abbreviations: BER, base excision repair; AP sites, apurinic/apyrimidinic sites, abasic sites; AP-DNA, sites; DNA containing apurinic/apoyrimidinic; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; bp, base pair(s); FEN1, flap endonuclease; WCE, whole-cell extract; pol β or δ, DNA polymerase β or δ; NER, nucleotide excision repair; XP, xeroderma pigmentosum.

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Human RPA and PCNA were purified as previously described (*23*, *24*). RPA (p34) monoclonal antibodies were purified by protein A−Sepharose chromatography. Antibodies against the PCNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TL6 antibody recognizing DNA ligase I was a gift from A. Tomkinson. DNA pol β antibody was a generous gift from S. Wilson. Western blots were performed by standard procedure as recommended by the vendor (Novex, San Diego, CA). Protein concentrations were determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Normal human lymphoid cells AG9387 were obtained from the Human Genetic Mutant Cell Repository (Coriell Institute, Camden, NJ). Cells were grown in medium recommended by the supplier.

Preparation and Fractionation of Human Whole-Cell Extracts. All procedures were performed at 4 °C. whole-cell extracts (WCE) were prepared from 3−5 g of cells by the method of Manley et al. (*25*) and dialyzed overnight against buffer A (25 mM Hepes-KOH, pH 7.9, 2 mM DTT, 1 mM EDTA, 10% glycerol, 0.01% Nonidet P-40, and 0.1 mM phenylmethylsulfonyl fluoride) containing 0.15 M KCl. WCE protein (∼30 mg) was loaded onto a phosphocellulose column (3−5 mL bed volume) in buffer A containing 0.15 M KCl and washed with five column volumes of the same buffer. The flowthrough (∼15−20 mg of protein) was designated as phosphocellulose fraction I (PC-FI). Bound proteins were eluted with five column volumes of buffer A containing 1 M KCl (PC-FII). Fractions (0.5−1 mL) were collected and the peak protein fractions (∼10−15 mg of protein) were combined, concentrated on Centriprep 10 (Amicon), dialyzed against storage buffer (25 mM Hepes-KOH, pH 7.9, 0.1 M KCl, 12 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, and 17% glycerol), and stored at −80 °C.

Plasmid DNA Purification and Depurination. Plasmid DNA pUC19 was prepared by using a Qiagen plasmid DNA purification kit (Qiagen, Chatsworth, CA) followed by CsCl gradient centrifugation. DNA containing AP sites (AP-DNA) was prepared immediately prior to use by heating the plasmid DNA (0.5 mg/mL) in 30 mM sodium acetate (pH 5.0) at 70 °C for 8 min. The depurination was stopped by cooling on ice, and the pH was adjusted by addition of an equal volume of 0.1 M Hepes-KOH, pH 7.9. As determined by agarose electrophoresis, this treatment did not introduce any single- or double stranded breaks (data not shown).

DNA Repair Synthesis Assay. WCE or individual fractions were assayed as previously described (*26*). Standard 50 μL reactions contained 500 ng of plasmid DNA, 45 mM Hepes-KOH, pH 7.8, 70 mM KCl, 7.5 mM $MgCl_2$, 1 mM DTT, 0.4 mM EDTA, 2 mM ATP, 40 mM phosphocreatine (di-Tris salt, Sigma), 2.5 μg of creatine phosphokinase (type I, Sigma), 3.4% glycerol (Fluka), 20 μg of BSA, 50 μM each dCTP, dATP, and TTP, 5 μM dGTP, and 2 μCi of [α-^{32}P]-dGTP (3000 Ci/mmol, Amersham). The protein amounts of WCE and PC-FII used in the reactions were 100 and 50 μg, respectively, because during fractionation approximately 50% of the WCE protein loaded onto phosphocellulose column was recovered in PC-FII. For complementation reactions PC-FI, PCNA, or RPA was mixed with PC-FII and incubated on ice for 10 min, prior to the addition of substrate DNA. DNA repair synthesis reactions were carried out at 30 °C for the indicated time. After the reaction, plasmid DNA was purified from the reaction mixture by phenol deproteinization and ethanol precipitation, linearized with *Eco*RI, and separated by electrophoresis on 1% agarose gels followed by autoradiography. The amount of radioactivity was quantified with a PhosphoImager using ImageQuant software (Molecular Dynamics).

Construction of Closed Circular M13 DNA Containing a Single Uracil Residue. The synthetic DNA duplex corresponding to the sequence 5′-ATATACCGCGGCCGGCCG-ATCAAGCTTATT-3′ was cloned into the *Sma*I site of M13mp18 DNA. The recombinant single-stranded DNA, named M13In, was purified and the insert orientation was verified by sequencing. Double-stranded closed circular DNA containing a single uracil was constructed by priming 30 μg of single-stranded M13In DNA with a 6-fold molar excess of the oligonucleotide 5′-pATATACCGCGG**U**CGGCCGAT-CAAGCTTATT-3′ for the single-uracil construct or with 5′-pATATACCGCGG**C**CGGCCGATCAAGCTTATT-3′ for the undamaged control plasmid.

The 250 μL reaction mixture containing 50 mM Tris-HCl (pH 7.9), 50 mM NaCl, 7 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 100 μM ATP, 500 μM each dATP, dCTP, dGTP, and TTP, 40 units of T4 DNA polymerase, 35 Weiss units of T4 DNA ligase, and 100 μg of T4 gene 32 protein (all enzymes from Boehringer Mannheim) was incubated for 4 h at 37 °C. Closed circular DNA was isolated by CsCl/EtBr density gradient centrifugation, purified by butanol extraction, desalted and concentrated by centrifugation in a Centricon-10 microconcentrator (Amicon). DNA substrates were stored at −20 °C in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.

Restriction Analysis of in Vitro Repaired DNA. Single-uracil-containing or control DNA was incubated in the presence of 2 μCi of [α-^{32}P]dGTP (3000 Ci/mmol, Amersham) with WCE or PC-FII supplemented with PCNA and RPA at the same conditions as described for AP-DNA. After a 1 h reaction, DNA was purified from the reaction mixture by phenol−chloroform extraction and ethanol precipitation and treated with 20 units of the indicated restriction endonuclease for 3 h in a buffer supplied by the manufacturer. Reactions were evaporated in a Speedvac (Savant) and the pellet was dissolved in 20 μL of formamide-dye (0.1% xylene cyanol, 0.1% bromophenol blue, and 50% formamide), incubated for 5 min at 90 °C, and electrophoresed on a 20% polyacrylamide gel containing 7 M urea, 89 mM Tris−borate (pH 8.3), and 2 mM EDTA. The amount of radioactivity was quantified on a PhosphorImager (Molecular Dynamics).

RESULTS

Fractionation of BER Proteins by Phosphocellulose Column Chromatography. To examine the role of RPA in PCNA-dependent repair of AP sites in DNA, we have fractionated human whole-cell extract on a phosphocellulose column (Figure 1A) under conditions previously described for fractionation of RPA and PCNA from other proteins involved in nucleotide excision repair (NER) (*27*). WCE was loaded onto phosphocellulose columns and PC-FI was eluted with 0.15 M KCl. Another fraction, PC-FII, was eluted with 1 M KCl. BER proteins known to be involved in PCNA-dependent abasic site repair were identified in the phosphocellulose fractions by immunoblot analysis. PCNA and RPA were found exclusively in fraction PC-FI (Figure 1B), as

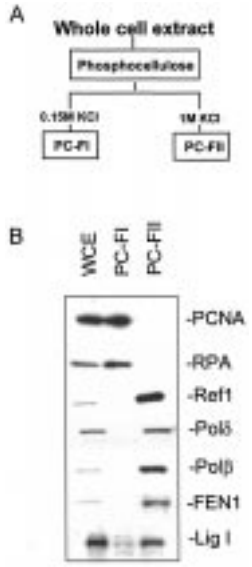

FIGURE 1: Fractionation of BER proteins on phosphocellulose column. (A) Whole-cell extract (WCE) fractionation scheme. (B) Immunoblot analysis of WCE and individual fractions with antibodies against the factors shown on the right. WCE (25 μg) from the normal human lymphoblastoid cell line AG9387 or 25 μg of protein from phosphocellulose chromatography fractions PC-FI and PC-FII were separated on SDS−PAGE, transferred onto a PVDF membrane, and immunoblotted.

also shown previously (*8*). All other proteins participating in PCNA-dependent BER were found in PC-FII. Endonuclease HAP1 (*1*), pol δ, pol β, and FEN-1 were found exclusively in fraction PC-FII (Figure 1B). DNA ligase I was mainly located in fraction PC-FII but at a lower level was also present in fraction PC-FI (Figure 1B). Thus, fraction PC-FII contains most of these proteins participating in PCNA-dependent BER, but it does not contain PCNA and RPA.

Repair of AP Sites in DNA Requires Both Phosphocellulose Fractions. The repair of AP sites was measured in the DNA repair incorporation assay developed by R. Wood et al. (*27*) and adopted for BER (*26*). Plasmid DNA containing AP sites or undamaged control DNA was incubated with WCE in the presence of all cofactors needed for DNA repair, including ^{32}P-labeled dGTP. After the reaction, the DNA was purified, linearized, and analyzed by agarose gel electrophoresis. The incorporation observed in WCE was highly dependent upon DNA damage (Figure 2). With prolonged incubation (more than 1 h), nonspecific incorporation gradually accumulated in the undamaged control DNA (data not shown).

To compare the capacity of AP-DNA repair in WCE and in the individual phosphocellulose chromatography fractions, we restricted the reaction time to 20 min. In addition, we used 500 ng/reaction plasmid DNA containing approximately 2 AP sites/molecule to provide a saturating amount of AP-DNA. Under these conditions the WCE efficiently supported repair incorporation of [^{32}P]dGMP into AP-DNA (Figure 3, lane 1). In contrast, there was no AP site repair activity in fraction PC-FI. Fraction PC-FII only supported limited repair incorporation (Figure 3, lanes 1−3). When the two fractions were combined the efficient repair of AP sites was restored, demonstrating that all necessary BER proteins were present in the two fractions and had not been lost or inactivated during chromatography (Figure 3, lanes 4−6).

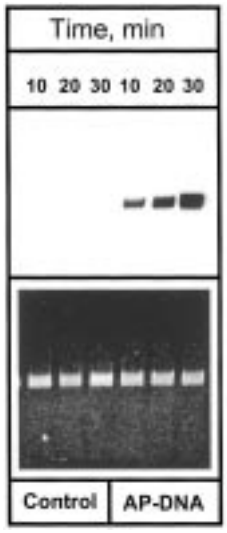

FIGURE 2: Repair of AP sites by human whole-cell extract. DNA repair synthesis assays in WCE (100 μg) were performed as described in the Materials and Methods section. The reactions contained 500 ng of plasmid DNA containing AP sites (AP-DNA) or undamaged DNA (control). DNA repair synthesis reactions were carried out at 32 °C for the indicated time periods. After the reaction, plasmid DNA was recovered, linearized with *Eco*RI, and subjected to electrophoresis on a 1% agarose gel. After electrophoresis the gels were stained with ethidium bromide and photographed (bottom panel) and then dried and subjected to autoradiography (top panel).

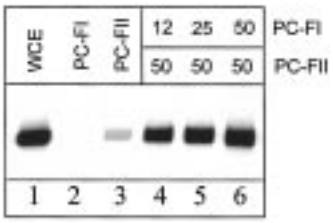

FIGURE 3: Reconstitution of AP site repair activity by mixing phosphocellulose fractions. Reactions were performed as described in the Materials and Methods section with 100 μg of WCE protein and 50 μg of PC-II protein. For complementation reactions,12.5, 25, or 50 μg of PC-FI was mixed with 50 μg of PC-FII and incubated on ice for 10 min prior to the addition of substrate DNA. Reactions were carried at 30 °C for an additional 20 min. After the reaction, plasmid DNA was recovered, linearized with *Eco*RI, and subjected to electrophoresis on a 1% agarose gel. The gel was dried and subjected to autoradiography.

Replacement of PC-FI by Purified PCNA and RPA. Fraction PC-FII is devoid of PCNA and RPA but otherwise contains the major proteins involved in PCNA-dependent BER. Our approach was to add purified proteins to this fraction and then assay for a stimulation of the repair of AP sites. Purified human PCNA and/or RPA were added to PC-FII in such a reconstitution experiment. PCNA, when added alone, slightly stimulates repair of AP sites by PC-FII (Figure 4, lanes 3 and 4). RPA alone has little effect on repair incorporation (Figure 4, lanes 5 and 6). The addition of both proteins stimulated repair synthesis (Figure 4, lanes 7 and 8). Increasing the amount of RPA and PCNA proteins led to further repair synthesis stimulation, indicating that these proteins are reaction-limiting components. Two hundred nanograms of each protein added to 50 μg of protein of the fraction PC-FII reconstituted repair synthesis to the WCE level (Figure 4, lane 8). The addition of the same amount of RPA and PCNA in a reaction containing undamaged DNA

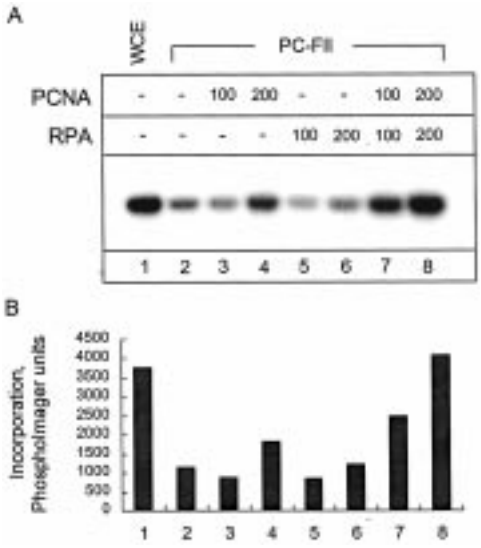

FIGURE 4: Replacement of PC-FI by purified human PCNA and RPA. (A) Reactions contained 500 ng of AP-DNA and 100 μg of WCE or 50 μg of PC-FII. When purified proteins were added (100 or 200 ng), reactions were preincubated on ice for 10 min before substrate DNA containing AP sites was added. All reactions were additionally incubated for 20 min at 30 °C and analyzed as described in the caption to Figure 2. (B) PhosphoImager quantification of the results presented in panel 3A.

only slightly stimulated background incorporation (data not shown). We thus conclude that RPA is involved in PCNA-dependent BER.

Damage Specificity of the PCNA-Dependent Reaction Supported by PC-FII. Experiments were conducted to ascertain that the PCNA-stimulated repair was specific to DNA damage rather than representing nonspecific DNA synthesis initiated at random nicks that might be introduced in DNA after incubation with fraction PC-FII. The repair was measured in the single-uracil-containing M13 DNA (Figure 5A) and control constructs after incubation with WCE, PC-FII, or PC-FII supplemented with PCNA and RPA. The DNA was purified from the reaction mixture, cleaved with restriction endonuclease, and analyzed by gel electrophoresis. The reaction with *Hin*dIII and *Eco*RI released a 59 bp fragment that contained a uracil residue in substrate DNA and an undamaged 22 bp fragment representing background incorporation into uracil-containing DNA. The quantitation of the gel shown in Figure 5B indicated that there was 8−10-fold more repair incorporation in the 59-mer *Hin*dIII fragment than in the 22-mer *Hin*dIII−*Eco*RI fragment of the uracil-containing plasmid incorporated by both WCE and PC-FII supplemented with PCNA and RPA (Figure 5B, lanes 3 and 5). There was no incorporation into the corresponding fragments generated from the undamaged control DNA after incubation in WCE or in PC-FII complemented with PCNA and RPA (Figure 5B, lanes 1 and 2). We thus conclude that the effect of RPA on PCNA-dependent repair is DNA damage-specific.

DISCUSSION

Repair of AP Sites in DNA by Human Whole-Cell Extract. The experiments presented here demonstrate for the first time that RPA and PCNA can act synergistically to stimulate long-patch BER. In contrast to our results, Stucki et al. (*22*) recently reported that BER reconstituted with PCNA, HAP1, and partially purified fractions containing either pol δ or pol ϵ showed no stimulation by RPA. It is unclear whether their repair assay was FEN1-independent. Since RPA probably exerts its effect on BER through the stimulation of FEN1

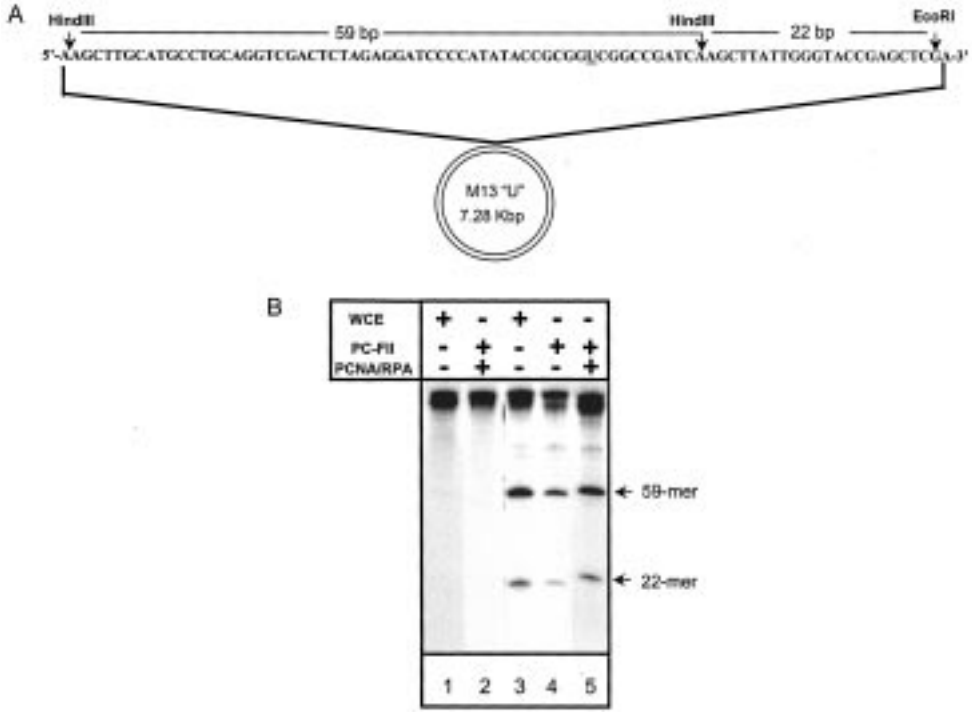

FIGURE 5: Restriction analysis of single-uracil-containing DNA repaired by whole-cell extract or PCNA- and RPA-complemented fraction PC-FII. Control (lanes 1 and 2) or single-uracil containing DNA (lanes 3−5) (100 ng) was incubated with 100 μg of whole-cell extract protein (lanes 1 and 3), 50 μg of PC-FII protein alone (lane 4) or 50 μg of PC-FII protein supplemented with 100 ng each of PCNA and RPA (lanes 2 and 5) under the conditions described in the Materials and Methods section. The DNA was recovered from the reaction mixture, digested with *Hin*dIII and *Eco*RI restriction enzymes, and analyzed by electrophoresis on a 20% polyacrylamide gel.

(*20*, *21*), this could explain the lack of stimulation by RPA observed by Stucki et al. (*22*). Alternatively, if RPA stimulates PCNA, HAP1, or pol δ/ϵ, it may only do so if the activity being stimulated is rate-limiting. In our experiments minimally fractionated extracts were used that contain all the known BER proteins (except the supplemented PCNA and RPA) and at ratios that closely mimic those found in whole-cell extracts. These are more physiological conditions compared with the experiments where repair reactions were reconstituted with partially purified proteins. The different conditions could account for the different results, but the mechanism of the RPA stimulation of certain BER reactions requires further investigation.

On the basis of the observation that RPA binds to single-stranded DNA and unwinds single-stranded regions by removal of secondary structure (*1*), it is reasonable to propose that an important function of RPA in PCNA-dependent BER is to stimulate single-stranded specific FEN1 endonuclease and/or increase pol δ/ϵ processivity. However, RPA may also have other functions. The biochemical data on the interaction of PCNA with DNA support the notion that PCNA can bind efficiently to closed circular DNA and form a ring around the DNA in the presence of the clamp loader replication factor C (RF-C), (reviewed in *3*). On the basis of these properties of PCNA and the observation that RPA and PCNA are both required for the long-patch BER process, we speculate that one of the functions of these proteins may be the loading of the other DNA repair proteins onto DNA provided by protein−protein interactions. The direct interaction between RPA and PCNA has been demonstrated by use of a PCNA affinity column (*28*). This physical interaction supports our finding of synergistic effect when both proteins were added to fraction PC-FII and supports the possibility that there is a functional interaction between these proteins as they participate in the repair reaction. In addition, both proteins interact with many other repair proteins. PCNA interacts with the DNA mismatch repair proteins MSH2 and MLH1 (*29*), nucleotide excision endonuclease XPG (*30*) and human DNA ligase I (*31*). Recently it was demonstrated that RPA interacts with uracil-DNA glycosylase (*32*). It also interacts with the XPA protein, which is involved in DNA damage recognition during NER (*33*, *34*) and stimulates endonucleases FEN1 (*19*, *21*), XPG, and XPF (*35*). Because so many different proteins interact with and are affected by PCNA and RPA, it would be reasonable to suggest that both proteins are involved in a coordination and catalysis of other DNA repair pathways in addition to long-patch BER pathway.

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