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Partial reconstitution of human interstrand cross-link repair in vitro: characterization of the roles of RPA and PCNA

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Abstract

The repair of DNA interstrand cross-links (ICLs) remains largely ill-defined in higher eukaryotic cells. Previously, we have developed assays that can be used to monitor the early stages of processing of ICLs in vitro. Here, we have used P11 phosphocellulose chromatography to fractionate HeLa nuclear extracts and have subsequently reconstituted these assays with the resulting fractions. RPA and PCNA were found in a single fraction, and were the only factors in this fraction required for the reconstitution of these assays. The roles of RPA and PCNA in the formation of incisions at ICLs and in the subsequent DNA synthesis step were assessed. RPA was found to be essential for both stages of ICL processing indicating that it is required for lesion recognition and/or for the subsequent endonucleolytic processing. PCNA is required for the DNA synthesis stage and although it is not critical for the incision stage of the reaction it does enhance this step presumably by a stimulation of lesion recognition by MutSβ. These findings define novel roles for RPA and PCNA in the processing of ICLs in mammalian cells.

Keywords: Interstrand cross-link repair; Psoralen; RPA; PCNA

As effective anticancer drugs, interstrand cross-linking agents are widely used in cancer chemotherapy. However, the mechanisms of repair of DNA ICLs in higher eukaryotic cells remain largely unresolved. ICLs are highly deleterious DNA lesions that interfere with many aspects of DNA metabolism including replication and transcription. In *Escherichia coli*, two mechanisms for the removal of interstrand cross-links (ICLs) have been described. A recombination-dependent mechanism was first identified by Cole and co-workers [1,2] and is the major pathway of repair of this lesion, while a recombination-independent pathway is a minor but highly mutagenic mechanism [3,4]. In both pathways nucleotide excision repair (NER) is required for the initial incisions that occur on either side of the ICL resulting in the formation of a single-stranded gap. In the major pathway the gap is repaired by a RecA-mediated recombination process using a homologous template as donor, while in the minor pathway the gap is filled by translesion synthesis requiring DNA polymerase II. In both pathways the remaining lesion is removed by NER. In *Saccharomyces cerevisiae*, the error-free repair of ICLs, as has been found in *E. coli*, appears to require components of both the nucleotide excision repair (*RAD3* group) and recombinational repair (*RAD52* group) pathways [5–7]. The role of the *RAD52* group was postulated to be required for the repair of double-strand breaks that were observed as intermediates during repair of psoralen or nitrogen mustard induced ICLs [8,9]. A more recent epistasis analysis has indicated that *SNM1*, *RAD51*, and *REV3* are elements of three distinct repair pathways each of which contributes to the removal of ICLs [10]. Mutants defective in all three of these genes have virtually no capacity to remove ICLs, indicating that there are no more than these three pathways for removal of these lesions in *S. cerevisiae*.

Similar to *E. coli*, mammalian cells exhibit both major and minor pathways of ICL repair. The minor pathway appears analogous to the *E. coli* pathway in

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that it requires NER and presumably a translesion polymerase, but not components of homologous recombination such as XRCC2 or XRCC3 [11,12]. This pathway has also proved to be highly mutagenic in response to psoralen adducts. Unlike in *E. coli* and perhaps yeast, the major recombination-dependent pathway does not require an intact NER pathway, since most mutants in this pathway exhibit only slight sensitivity to bifunctional alkylating agents [13,14]. Additionally, mutants defective in XPB or XPG have been shown not to be defective in the unhooking step of repair of nitrogen mustard ICLs in vivo [15], and results from our laboratory have shown that XPA, XPC, and XPG are not required for processing psoralen ICLs in vitro [16,17]. Although most NER mutants are not highly sensitive to ICL-inducing agents, mutations in either ERCC1 or XPF lead to extreme sensitivity to these agents [18]. Consistent with this cellular sensitivity, mutants in ERCC1 or XPF were found deficient in the unhooking step of ICL repair in vivo [15], and Ercc1 and Xpf are required for the initial processing of ICLs in vitro [16,17,19].

Recent findings from our laboratory have also shown that the mismatch repair (MMR) complex MutSβ is also required for the initial processing of psoralen ICLs in vitro [17]. Analogous to its role in MMR of binding to insertion/deletion loops, MutSβ was shown to recognize psoralen ICLs with high affinity. In addition, the binding of psoralen ICLs by MutSβ was strongly enhanced by PCNA. This latter finding is consistent with previous results showing that PCNA stimulates the recognition of mismatched DNA by both MutSα and MutSβ [20–23]. The demonstration that MutSβ has a distinct role in ICL processing indicates that this complex in cooperation with Ercc1–Xpf constitutes a novel excision pathway for the removal of these lesions. Here, we have initiated the fractionation and reconstitution of ICL processing in vitro, and have characterized the role of two additional proteins in this pathway, replication protein A (RPA) and proliferating cell nuclear antigen (PCNA). RPA is shown to be essential for the introduction of excisions at psoralen ICLs, while PCNA is shown to be a stimulatory factor for this process.

Materials and methods

Reagents. HeLa S3 cells for extract preparation were purchased from the National Cell Culture Center (Minneapolis, MN). Purified human RPA was generously provided by Z.-Q. Pan, and p21 was provided by J.W. Harper. Human PCNA was expressed from the PT7-hPCNA plasmid in *E. coli* BL21(DE3) and purified essentially as described [17].

Substrate preparation. Plasmids and psoralen interstrand cross-linked substrate for in vitro repair assays were prepared as previously described [16]. Briefly, two complementary oligonucleotides 5′-GC TCTCGTCTGTACACCGAAG and 5′-GCTCTTCGGTGTACA GACGAG were synthesized and phosphorylated. One hundred micrograms of this annealed duplex oligo was added to 4,5′,8-trimethylpsoralen at 5 μg/ml in 10 mM Tris (pH 7.5), 0.5 mM EDTA, and 25 mM NaCl. The sample was irradiated with 365 nm UVA light (10 min at 9 mW/cm^2) to effect formation of the ICL. The cross-linked oligonucleotide was purified by denaturing PAGE and inserted into a plasmid (pBSII-Nco) as described [16]. After ligation, covalently closed cross-linked template (CLT) plasmid was purified by CsCl–ethidium bromide gradient centrifugation.

In vitro repair assays. HeLa whole-cell extracts were prepared by the method of Manley et al. [24]. CRS assays were performed as previously described [16]. Briefly, 50 μl reactions contained 30 ng of CLT or CT, 100 ng of DT, 45 mM Hepes–KOH, pH 7.8, 75 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dATP, dGTP, and dTTP, and 8 μM dCTP, 2 μCi [^{32}P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, ~3% glycerol, 18 μg bovine serum albumin, and cell extracts or chromatography fractions as indicated. The reactions were incubated for 3 h at 22 °C and subsequently processed for analysis by agarose gel electrophoresis as described [16].

Incision assays were performed as described above for the CRS assay with the exception that the extracted DNAs were digested with *Bss*HII and examined by 6% PAGE [17]. Klenow fragment-dependent incorporation assays were performed as described above for the CRS assay with the following modifications. During the initial incubation with extract nucleotides were omitted from the reaction. For reactions performed in the absence of PCNA, after 2.5 h of incubation at 22 °C, 20 μM each of dATP, dGTP, and dTTP, 8 μM dCTP, 2 μCi [^{32}P]dCTP (3000 Ci/mmol), and 5 U Klenow fragment (−exo) (New England Biolabs) were added, and the incubation was continued at 30 °C for 30 min. For reactions performed in the presence of PCNA, after the incubation at 22 °C, DNAs were extracted and resuspended in 20 L polymerase I reaction buffer containing 20 μM each of dATP, dGTP, and dTTP, 8 μM dCTP, 2 μCi [^{32}P]dCTP (3000 Ci/mmol), and 1 U Klenow fragment (−exo). After the reactions were incubated for 30 min at 30 °C, the plasmids were recovered and subsequently analyzed by agarose gel electrophoresis.

Fractionation of HeLa nuclear extracts. HeLa nuclear extracts were prepared as described previously [25] and dialyzed against buffer A [25 mM Hepes–KOH (pH 7.8), 1 mM EDTA, 10% glycerol, 0.01% NP-40, 1 mM DTT, and 100 mM KCl]. After centrifugation at 10,000 rpm for 10 min at 4 °C, 20 ml of supernatant (5.7 mg protein/ml) was loaded onto a P11 phosphocellulose column, and 40 ml of flowthrough was collected and referred to as fraction FI (1.3 mg/ml). Proteins bound to the column were eluted sequentially with buffer A containing 0.4, 0.6, and 1.0 M KCl to yield 20 ml of fraction FII (2.3 mg/ml), 10 ml of fraction FIII (0.82 mg/ml), and 10 ml of fraction FIV (0.45 mg/ml), respectively. Each fraction was dialyzed against buffer B [25 mM Hepes–KOH (pH 7.9), 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 17% glycerol, and 2 mM DTT], aliquoted, and stored at −80 °C. For reconstitution assays, 4 μl HeLa nuclear extract or 8 μl FI, 4 μl FII, 2 μl FIII, and 2 μl FIV were added to each reaction as indicated.

Results

Partial reconstitution of ICL processing in vitro

To investigate the processing of ICLs in mammalian cells, we have developed an in vitro assay (referred to as CRS) wherein a single psoralen ICL located at a defined site within a plasmid induces DNA synthesis on both damaged and undamaged plasmids in cell-free extracts

[16]. The substrates for this assay are shown in Fig. 1A. This assay has been shown to be dependent upon Ercc1, Xpf, PCNA, RPA, and MutSβ, but not upon other proteins involved in xeroderma pigmentosum [16,17,19]. We have further demonstrated that this in vitro processing results in the uncoupling of the ICL ultimately resulting in the formation of double-strand breaks, and that this incision process is dependent upon Ercc1–Xpf and MutSβ [17]. To identify additional factors involved in processing ICLs, we fractionated HeLa cell nuclear extract using P11 phosphocellulose column chromatography into four fractions by step elution (Fig. 1B), and used these fractions to reconstitute both the CRS (Fig. 1C) and incision assays (Fig. 1D). The hallmarks of the incision assay are specific bands migrating at positions of 113 and 86 nucleotides [17]. In the presence of all four fractions activity was observed in both the CRS (Fig. 1C, lanes 1 and 2) and incision assays (Fig. 1D, lane 1), however, fraction FIV was not required for activity in either assay (Fig. 1C, lanes 3 and 4; Fig. 1D, lane 2). In the absence of either fractions FI or FII ICL-induced DNA synthesis was not observed (Fig. 1C, lanes 5, 6, 9, and 10; Fig. 1D, lanes 4 and 5). Interestingly, in the absence of fraction FIII,

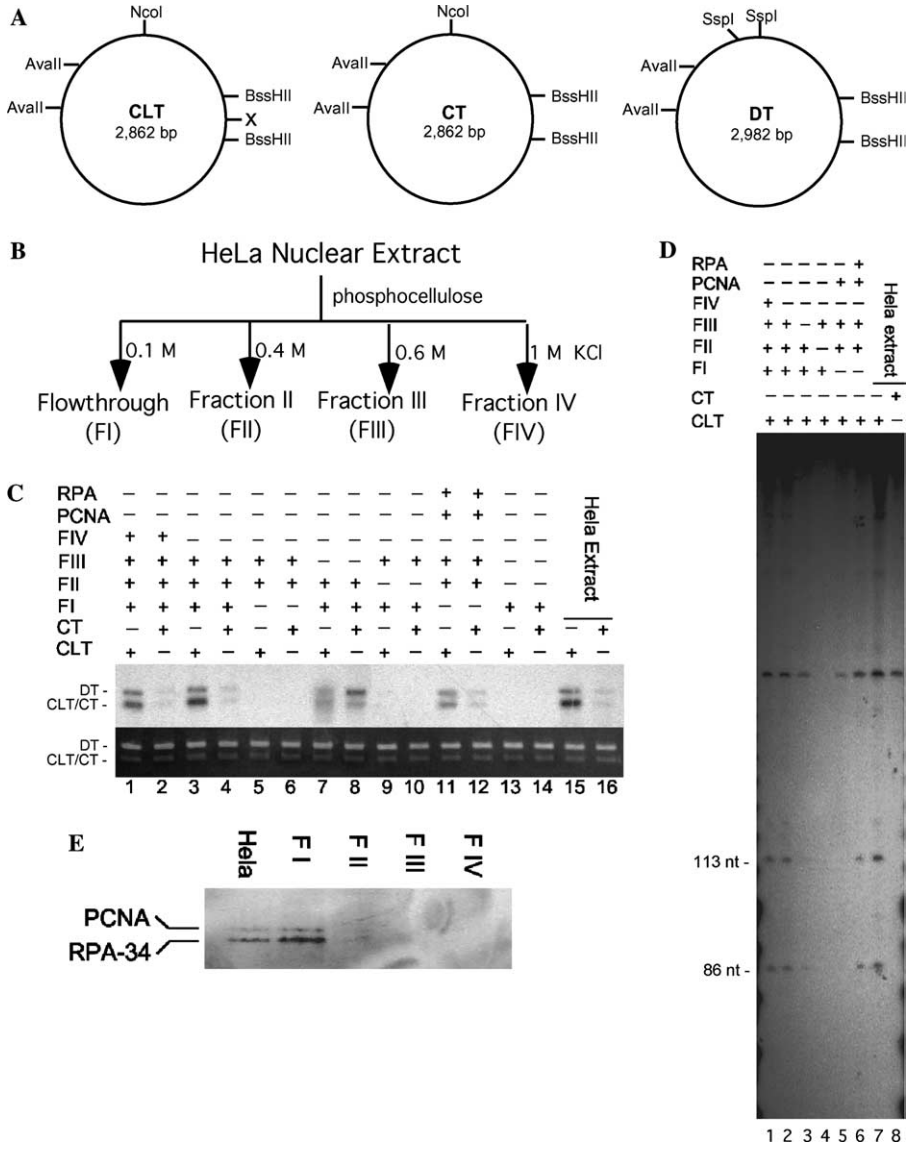

Fig. 1. Fractionation and reconstitution of ICL repair activities. (A) The CLT (for cross-linked template) plasmid contains a single psoralen cross-link at the site identified by an "X." The CT (for control template) plasmid is identical to the CLT except for the presence of the cross-link. The DT (for donor template) plasmid is identical to the CT plasmid except for the presence of a short segment containing two *Ssp*I sites that replace the *Nco*I site. (B) Fractionation of HeLa nuclear extracts by P11 phosphocellulose chromatography. (C) Reconstitution of ICL repair processing as indicated by the CRS assay. Final concentrations of added RPA and PCNA were 2 and 1.6 ng/ml, respectively. (D) Reconstitution of ICL repair processing as indicated by the incision assay. Concentrations of added RPA and PCNA were as indicated in (C). (E) RPA and PCNA elute with fraction FI. Immunoblot of P11 phosphocellulose fractions probed with anti-RPA-34 and anti-PCNA antibodies.

background DNA synthesis was very high as indicated by the incorporation levels observed in the control plasmids (Fig. 1C, lanes 7 and 8); however, only low activity was observed in the incision assay (Fig. 1D, lane 3). Thus, fractions FI, FII, and FIII are all required for full activity of ICL processing in vitro.

As previously described by others [26], we found that two replication/repair factors RPA and PCNA reside in fraction FI (Fig. 1E). We have demonstrated previously that both of these factors are required for the CRS assay [27]. As shown (Fig. 1C, lanes 11 and 12; Fig. 1D, lane 6), RPA and PCNA were able to replace fraction FI in both the CRS and incision assays indicating that these are the only required factors in this fraction. As determined by immunoblotting, the heterodimers Ercc1–Xpf and MutSβ were located in fraction FII, but were not able to substitute for this fraction in the CRS assay indicating that an additional factor(s) is present in this fraction and in fraction FIII (results not shown).

Requirement for RPA in the early stages of ICL processing

RPA has been shown to be required in the excision and DNA synthesis step of both NER and MMR [28–32]. We, therefore, examined the role of RPA in ICL processing. We first examined the requirement of RPA in the incision assay which requires both incision activity and gap filling DNA synthesis. To carry out this experiment, we used the reconstituted system containing fractions FII and FIII, and PCNA with increasing amounts of RPA. As expected, RPA was essential in this assay (Figs. 2A and B). In addition, the single strand binding protein from *E. coli* (SSB) could not replace RPA (Fig. 2A, lane 6) in this assay, suggesting that functions of RPA in addition to single strand binding, such as protection of the gapped intermediate, are required for ICL processing.

Our previous findings have shown that psoralen ICLs are uncoupled by incisions that occur in one strand on either side of the cross-link in vitro [17]. These initial incisions are then processed into a much larger gap presumably by an exonuclease, since the incorporation into the CLT can extend for at least a kilobase or more [16]. This gapped intermediate is then refilled by endogenous polymerases (see below). To examine the role of RPA in the early excision process, we performed the initial reaction in the absence of dNTPs and then used the Klenow fragment of polymerase I to replace the endogenous polymerases. This assay renders the DNA synthesis or gap filling stage of the reaction independent of RPA and thus allows a determination of the involvement of this protein in the introduction of incisions. As shown (Fig. 2C, lanes 3 and 4, and 8 and 10), in the presence of RPA, but not in its absence, extensive incorporation is observed in both the CLT and DT suggesting that RPA is required for the formation of the

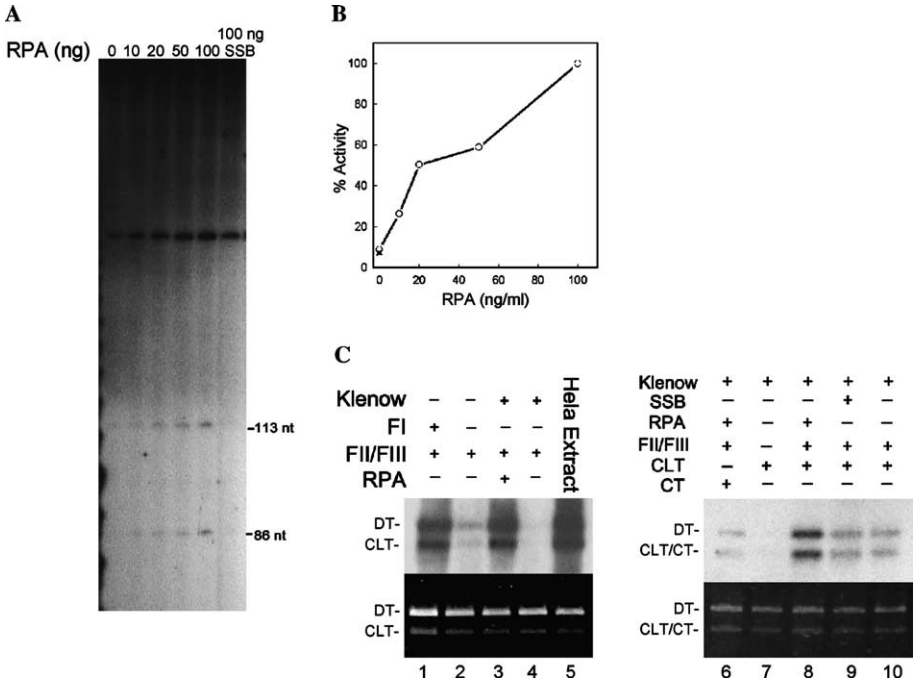

Fig. 2. RPA is required for the incision stage of ICL processing. (A) Dependence of the incision assay on RPA. Assays were conducted with fractions FII and FIII, and the indicated amounts of RPA and SSB. (B) Quantitation of the results shown in (A). "x" indicates the level of activity in the reaction supplemented with SSB. (C) Klenow fragment-dependent assays showing that RPA is required for the formation of the gapped intermediate in ICL processing. Final concentrations of added RPA and SSB were 2 ng/ml.

gapped intermediate. The Klenow fragment-mediated incorporation was dependent upon the presence of the ICL (Fig. 2C, compare lanes 6 and 8), and could not be substituted for by the addition of *E. coli* SSB (Fig. 2C, lane 9).

The role of PCNA in ICL processing

As described previously [27], PCNA is required for the CRS assay, and we have shown that it stimulates the binding of MutSβ to psoralen ICLs by electrophoretic mobility shift assays [17]. These findings suggest that PCNA, like RPA, may have a role in both the incision step as well as the subsequent DNA synthesis stage of ICL processing. To examine this question, we first showed that p21 (CIP/WAF), a known inhibitor of PCNA [33–35], strongly suppressed activity in the CRS assay (Figs. 3A and D). This dependence upon PCNA suggests that the endogenous polymerase(s) mediating incorporation into the CLT is either polymerase δ and/or ε. Both of these polymerases are sensitive to aphidicolin, and as shown (Fig. 3B) activity in the CRS assay is highly inhibited by this drug. Thus, we conclude that PCNA is required for the DNA synthesis stage of the CRS assay.

To determine if PCNA is also involved in the incision step of ICL processing, we again used a Klenow fragment-dependent assay. In this case, we incubated the CLT with HeLa extract in the absence of nucleotides in order to accumulate the gapped DNA intermediate. The DNA was subsequently extracted and recovered for incubation with the Klenow fragment in the presence of [^{32}P]dCTP and dNTPs. As shown (Fig. 3C), efficient incorporation into the CLT in this assay was dependent upon the cell extract, ICLs, and the Klenow fragment, but was not dependent upon the endogenous DNA synthesis machinery. This conclusion is indicated by the findings that 10 mg/ml of aphidicolin almost completely inhibited the CRS assay (Fig. 3B), but had little or no effect on the Klenow fragment-dependent assay (Fig. 3C, lanes 2 and 3), which should be unaffected by this drug. We also examined the effect of p21 on the Klenow fragment-dependent assay and in this case observed an approximate 50% inhibition (Fig. 3C, lane 4), suggesting that PCNA may have a role in the introduction of incisions at ICLs. To further assess this role, we used

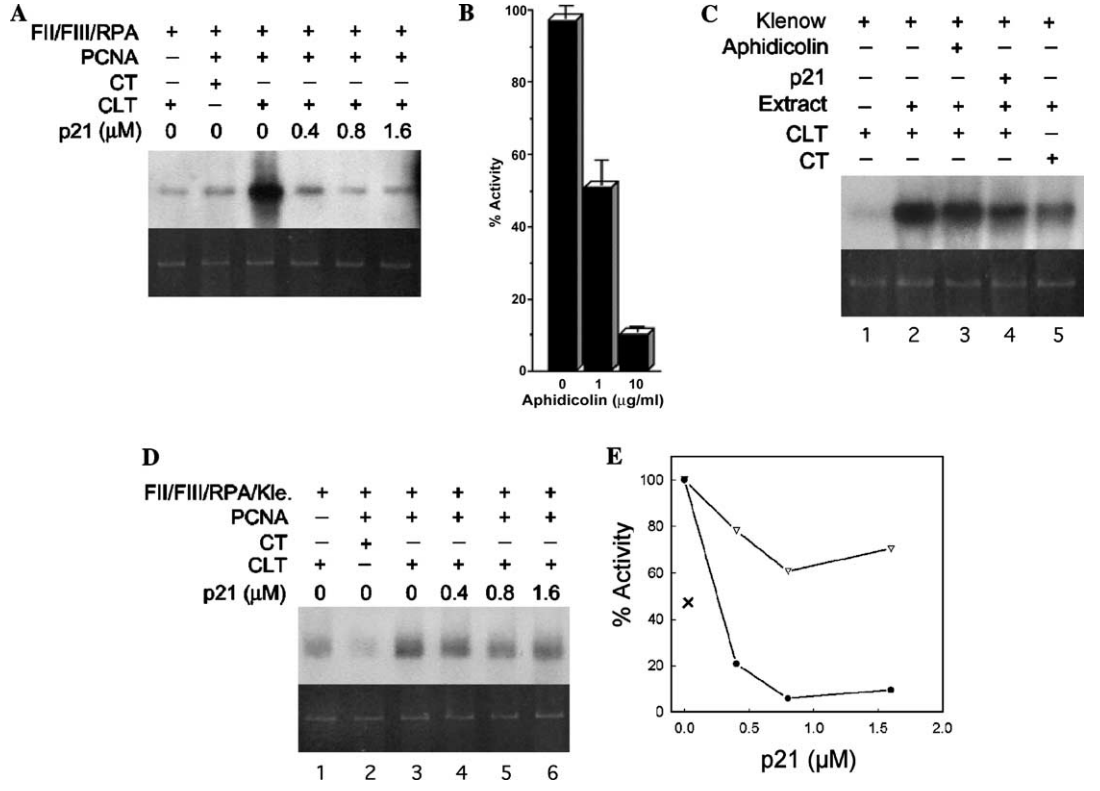

Fig. 3. PCNA stimulates but is not essential for the incision stage of ICL processing. (A) ICL-induced incorporation in the CLT is inhibited by p21 in reconstituted fractions. Final concentration of added PCNA was 0.1 mM. (B) Aphidicolin inhibits cross-link stimulated incorporation in the CLT. Aphidicolin was used at a concentration of 10 mg/ml. Standard deviations of the mean are indicated. (C) p21 partially inhibits ICL-induced incorporation as determined in the Klenow fragment-dependent assay in HeLa extracts. The final concentration of p21 was 1.4 mM. (D) PCNA stimulates formation of the gapped intermediate as determined by the Klenow fragment-dependent assay using reconstituted fractions. The final concentration of PCNA was 0.1 mM. (E) Quantitation of results shown in (A) and (D). "x" indicates the level of activity in the absence of PCNA (D, lane 1).

fractions FII and FIII in the Klenow fragment-dependent assay to determine the effect of PCNA on forming the gapped intermediate. Again we observed about a twofold increase in activity due to the addition of PCNA (Figs. 3D and E). p21 was also inhibitory in this assay, but did not quite reduce activity to the levels observed in the absence of PCNA (Figs. 3D and E). Thus, we conclude that PCNA stimulates the incision step of psoralen ICL processing in vitro by approximately twofold under these conditions.

Discussion

In our previous studies using a plasmid substrate with a site specific psoralen ICL, we have shown that the Ercc1–Xpf and MutSβ are required for incisions that lead to uncoupling or unhooking of the cross-link in vitro [16,17,19]. MutSα was unable to substitute for MutSβ in this reaction. Our findings thus indicate that Ercc1–Xpf and MutSβ cooperate in a novel pathway of ICL processing. Here, we have initiated the process of identifying additional components of this pathway by fractionation and reconstitution of these in vitro assays. These experiments have demonstrated that in addition to RPA, PCNA, Ercc1–Xpf, and MutSβ there are additional factors required to reconstitute the early stages of ICL repair processing. In addition, this fractionation has allowed us to characterize the role of RPA and PCNA in this mechanism.

PCNA has been shown to interact with Msh6 and Msh3 and to thereby stimulate the binding of MutSα and MutSβ to mismatched DNA [20–23]. We have shown previously that PCNA also stimulates the binding of MutSβ to psoralen ICLs [17] and our results, reported here, confirm those earlier findings by demonstrating

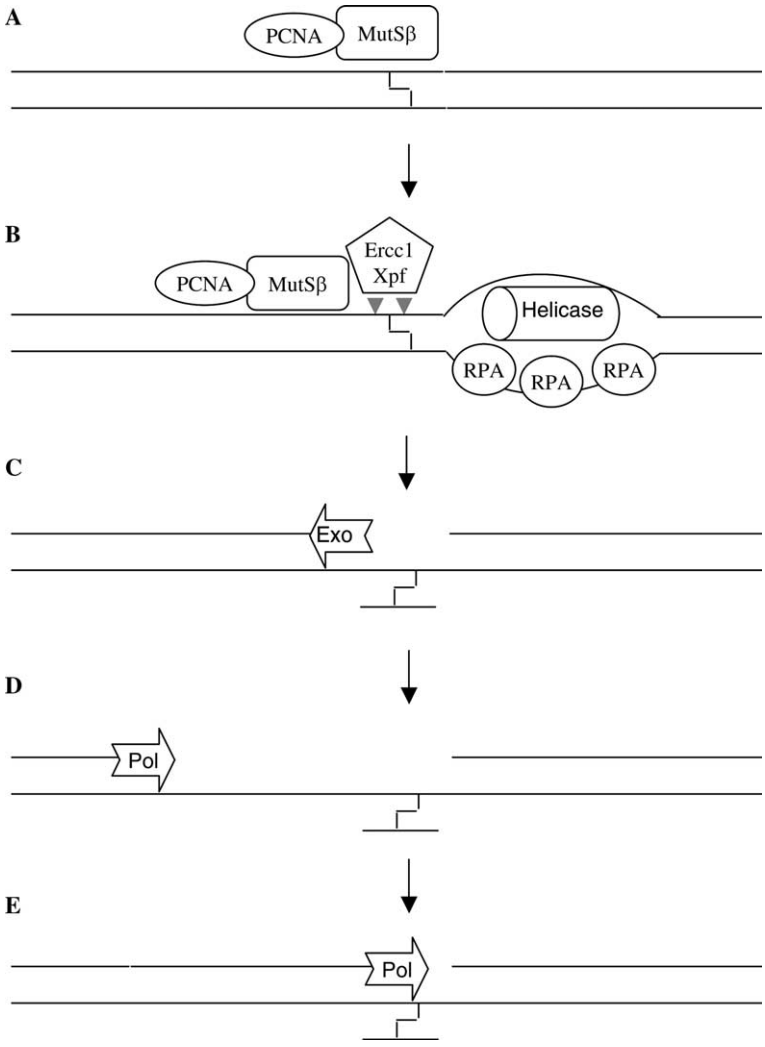

Fig. 4. Model depicting the processing of ICLs in vitro. (A) The binding of MutSβ to ICLs is stimulated by PCNA, (B) a DNA helicase in cooperation with RPA creates a bubble or open region proximate to the ICL. Ercc1–Xpf is recruited to the site and forms incisions on either side of the lesion as shown in (C). (D) An exonuclease resects the incised region leading to a larger gap that is subsequently filled in by a DNA polymerase. (E) The polymerase stalls precisely opposite to the remaining monoadduct.

that PCNA also stimulates the introduction of incisions at ICLs. PCNA is not essential for this process, since incisions were produced even in its absence. Both p21 and aphidicolin efficiently inhibited ICL-induced DNA synthesis. However, these agents have different effects on the introduction of incisions during the processing of ICLs due to their distinct mechanisms of inhibition. Aphidicolin suppresses the growth of eukaryotic cells by inhibiting DNA polymerase α, δ, and ε via competition for dNTP-specific binding sites on these enzymes [36,37]. Our results showed that aphidicolin does not inhibit the formation of the gapped intermediate that occurs during the processing of ICLs, indicating that these polymerases are not involved in the early stages of processing of ICLs. However, p21 was found to inhibit the incisions stimulated by PCNA nearly to the level observed in its absence. p21 has been shown to directly interact with a PCNA carboxyl-terminal domain and it shares a PCNA binding motif with Msh3 [23]. Thus, the inhibition of incision at ICLs by p21 may result from the competitive blocking of MutSβ binding sites on PCNA. p21 also inhibits PCNA-dependent DNA synthesis in DNA replication [34], the gap filling step of NER [38,39], as well as ICL-induced DNA synthesis [28], which is also observed in the reconstituted system described here (Fig. 3A).

RPA is a heterodimeric, multifunctional protein complex that plays an essential role in cellular DNA metabolism due to its involvement in DNA replication, recombination, and DNA repair [40,41]. During NER, it interacts with XPA to form a stable lesion recognition factor and to facilitate the formation of a pre-incision complex at sites of damage [28–31,42]. RPA is also required for the gap filling stage of NER [27]. In MMR, RPA also plays critical roles in both the excision and resynthesis stages of this pathway [32]. Our findings show that RPA is also required for the excision stage of ICL processing in mammalian cell extracts. The exact role of RPA in ICL processing is not clear, but a number of possibilities exist. As observed in NER, RPA may contribute to lesion recognition, however, since XPA is not required for ICL processing the mechanism of this recognition would differ from that observed in NER [30,31]. A second possibility is that RPA may facilitate unwinding of the duplex at the site of an ICL by a DNA helicase and also stabilize the resulting open or bubble structure. Recently, Kuraoka et al. [43] have shown that Ercc1–Xpf can incise on either side of a psoralen ICL when it is located near the junction of duplex and single-stranded DNA. Such a structure could be mimicked by the formation of a bubble or open helix proximate to an ICL through the action of a DNA helicase. There is precedence for RPA cooperating with a DNA helicase, since it has been shown to interact with SV40 large T antigen and to unwind the viral origin of DNA replication [44–46]. Also, RPA has been shown to stimulate the activity of a DNA helicase isolated from human cells [47]. A putative model encompassing these ideas and including the activities of factors that we have previously identified as required for ICL processing in mammalian cell extracts is shown in Fig. 4.

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