Replication Protein A Confers Structure-specific Endonuclease Activities to the XPF-ERCC1 and XPG Subunits of Human DNA Repair Excision Nuclease*

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XPF-ERCC1 and XPG proteins are nucleases that are involved in human nucleotide excision repair. In this study, we characterized the structure-specific junction-cleaning activities of both nucleases using DNA substrates containing a bubble or loop structure. We found that the junction-cleaning activities of XPF-ERCC1 and XPG were greatly stimulated by human replication protein A (RPA), while heterologous single-stranded DNA-binding proteins could not substitute for human RPA. To test for specific interaction between RPA and XPF-ERCC1 as is known to occur between RPA and XPG, we employed a pull-down assay with immobilized “bubble” substrate. We found that the binding of XPF-ERCC1 complex to the bubble substrate was enhanced by RPA, suggesting a possible mechanism for RPA in the excision nuclease system, that is, the targeting of the nuclease substrates to their specific sites of action. Furthermore, the RPA-promoted junction cutting by XPF-ERCC1 and XPG nucleases was observed with “loop” substrates as well, raising the possibility that XPF-ERCC1, XPG, and RPA may function in removing loop structures from DNA, independent of the other subunits of the human excinuclease.

Nucleotide excision repair is a general repair system that plays an important role in maintaining genetic integrity (1, 2). This repair system removes damaged nucleotides from DNA by double incisions on both sides of the lesion in the damaged strand (3). Recently, the human and the highly homologous yeast nucleotide excision repair systems have been reconstituted from extensively purified proteins, which demonstrated that 14–15 polypeptides comprising replication protein A (RPA, (4) also known as HSSB or RF-A) and the general transcription factor, TFIIF, in addition to XP and ERCC proteins were essential for the dual incision step (4–6).

It was found that the two subunits of the excision nuclease had intrinsic endonuclease activities in the absence of the other components of the repair system. XPG was reported to possess a single-stranded DNA endonuclease activity (7, 8) and an exonuclease activity with 5′ to 3′ directionality (9). Similarly, it was found that XPF-ERCC1 had a single-strand-specific endonuclease activity and a weak activity on double-stranded UV-irradiated DNA, which was stimulated by RPA (10). Furthermore, it has been reported that the RAD1-RAD10 complex (11), the yeast counterpart of the human XPF-ERCC1 nuclease, and XPG (12) have structure-specific junction-cleaning activities with unique polarities. These results obtained with the model “undamaged” substrates suggested that XPF-ERCC1 and XPG were the 5′- and 3′-endonucleases of the dual incisions, respectively. A recent study using damaged DNA and specific antibodies supported this model (13).

Recently, it has been reported that RPA, which is absolutely required for dual incisions (4), specifically binds to XPA protein and XPG nuclease (14). These findings raised the possibility that RPA may play an important role in recognizing DNA lesions and then targeting the nuclease subunits of human excision nuclease system to their proper sites. Hence, we decided to investigate the effect of RPA on the nuclease subunits in the absence of other components of excision nuclease.

EXPERIMENTAL PROCEDURES

DNA Substrates—Structures of DNA substrates used in this study were schematically shown in Fig. 1. The sequence of the top strand of each substrate was identical: 5′-CTTTGAGAAGCCAGTAATGTATGCCGTGTGCCGC-3′. This top strand oligomer (90-mer) was 5′- or 3′-labeled with T4 polynucleotide kinase and [γ-32P]ATP or terminal deoxynucleotidyltransferase and [α-32P]dideoxy-ATP, respectively, and gel-purified. Hence, the size of the 3′-labeled top strand was 91-mer. After annealing with respective bottom strand oligomers, double-stranded DNAs with or without a bubble/loop structure were purified using non-denaturing 8% polyacrylamide gels. The sequences of bottom strands were: DS, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); B12, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); B30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); L30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); M30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); L30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); M30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); M30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); M30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); M30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer); M30, 5′-CCGGGATCCTGGATACTTACAGCCG-ATGGACACCGGGAATACTCCTCCATCCTCCGGAGCCTGAGGAGTATGG-3′ (90-mer). Mismatched bases in the bubble structures are underlined. Five pentamers of the labeled substrates per nuclelease reaction were used in all experiments in this study.

Proteins—The XPD cDNA tagged with 6× histidine using pRSET vector (Invitrogen) was overexpressed in Escherichia coli DR153 strain (15) and purified by a modified procedure of Jones and Wood (16). The DNA construct bearing the three-subunit human RPA was obtained from Dr. M. Wold (University of Iowa), and the recombinant RPA was expressed in E. coli and purified according to Henricksen et al. (17). Purification of XPF-ERCC1 complex was carried out as described previously (10). Protein concentrations were ~4 ng/μl (the heparin-agarose column fraction), ~10 ng/μl (the DEAE-column fraction), and 32 ng/μl (the XPA-affinity column fraction). XPG protein was overexpressed in a baculovirus/insect cell system using pVL1392-XPG/Baculogold viral DNA (Pharmingen) and SF21 cells and extensively purified by a modified procedure of O’Donovan et al. (5). Briefly, after separation on a phosphocellulose column, XPG-containing fractions identified by Western blotting were loaded onto an
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Affi-Gel blue (Bio-Rad) column. The column was extensively washed with buffer A (25 mM HEPES-KOH, pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) containing 150 mM KCl and subsequently with buffer A containing 1 M KCl. Final elution was achieved with a linear gradient of NaSCN (0.5–1.5 M) in buffer A. XPG fractions were dialyzed against storage buffer (25 mM HEPES-KOH, pH 7.9, 100 mM KCl, 12 mM MgCl2, 0.5 mM EDTA, 2 mM dithiothreitol, and 17% glycerol) and used in most of the experiments. For further purification, the elution fractions from the Affi-Gel blue column were dialyzed against buffer A containing 1 M (NH4)2SO4 and loaded onto a phenyl-Superose column. After extensive washing, XPG was eluted with a linear gradient of (NH4)2SO4 (0.6–0 M) in buffer A at a rate of dialysis against storage buffer. The purified XPG protein was active for complementing repair deficiency of UV135 (ERCC-5 XPA) and has no significant effect on the RPA-conferred junction-cutting activity.

**RESULTS AND DISCUSSION**

To study the effect of RPA on XPF-ERCC1 and XPG, we constructed substrates with 12- or 30-nt mismatched bases (bubble), or deleted nucleotides in the bottom strand (loop) (Fig. 1a). The bubbles were labeled 5' or 3' and characterized with respect to single-strandedness by S1 nuclease digestion. From the specific degradation by S1 nuclease, it is evident that the substrates used in our study contained no secondary structures in the bubble and loop regions (Fig. 1b). RPA, which has high affinity for single-stranded DNA, specifically bound to the 30-nt bubble or loop region (Fig. 1c) and promoted junction-cutting activity (Fig. 1d) from DNase I (Fig. 1d); it also bound to the 12-nt bubble and loop with less affinity (data not shown), in agreement with the report that a 30-nt-long single-stranded DNA is the minimum size for binding of RPA with high affinity (18).

XPF-ERCC1 is a single strand-specific endonuclease, which also nicks UV-irradiated supercoiled DNA in an RPA-stimulated reaction (10). Furthermore, it was found that the XPF-ERCC1 complex purified through the XPA-affinity column step contained a junction-cutting activity, which was not obvious in the purer fractions (the DEAE and the heparin-agarose fractions) (Fig. 2a). We reasoned that some other protein(s) present in the XPA-column fraction might have conferred the junction-cutting activity to XPF-ERCC1 detected with this fraction. Since RPA binds to these model substrates, we tested RPA for conferring the junction-cutting activity to the DEAE and the heparin-agarose fractions (Fig. 2b). In the absence of additional protein(s), the purer XPF-ERCC1 fractions have a weak single-stranded nuclease activity, which generates a ladder in the area of single strandedness (Fig. 2b, lanes 1, 4, and 8). The addition of RPA suppresses the nonspecific endonuclease activity while conferring a specific junction-cutting activity upon the nuclease enabling it to hydrolyze the strand which enters the bubble region in the 5’ to 3’ direction at the fourth phosphodiester bond before entering the junction (Fig. 2b, lanes 2, 3, 6, and 9). RPA also stimulated the junction-cutting activity of the XPA-column fraction (see below). In contrast, XPA, which is known to interact with XPF-ERCC1 (19), does not affect the single-stranded DNA endonuclease activity (Fig. 2b, lanes 5 and 10) and has no significant effect on the RPA-conferring junction-cutting activity of XPF-ERCC1 (Fig. 2b, lanes 7 and 11). Importantly, RPA failed to confer a junction-cutting activity to the S1 nuclease (Fig. 2c), indicating that the RPA-promoted junction-cutting activity is not caused by covering the bubble with RPA and leaving only the single-stranded junction open for a nonspecific single-stranded DNA endonuclease. The junction-cutting activity of XPF-ERCC1 was dependent on RPA concentration, and 40 ng of RPA per reaction...
lanes 1–5

nuclease.

d column fraction (lanes 1–3), the DEAE-column fraction (lane 4), b, effects of RPA and XPA on a junction-cutting activity of XPF-ERCC1. 5'-Labeled (lanes 1–7) or 3'-labeled (lanes 8–11) B30 substrates were preincubated on ice for 10 min with no protein (lanes 1, 4, and 8), 10 ng of RPA (lanes 3, 6, and 9), 20 ng of RPA (lanes 2), 10 ng of XPA (lanes 5 and 10), or 10 ng of XPA and 10 ng of RPA (lanes 7 and 11) prior to the incubation with 1 μl of the heparin-agarose column fraction (lanes 1–3) or the DEAE-column fraction (lanes 4–11) of XPF-ERCC1. In addition to the products caused by the XPF-ERCC1 junction-cutting activity, smaller fragments were reproducibly obtained with 5'-labeled substrates (lanes 2, 3, 6, and 7). These fragments might be by-products of XPF-ERCC1 junction cutting followed by 3' → 5' exonucleolytic degradation. However, it is not clear whether this exonuclease activity is intrinsic to XPF-ERCC1. Minor effects of XPA on the XPA-promoted junction-cutting activity (lanes 7 and 11) are due to experimental variability, because it has not been reproduced in repeated experiments. c, effects of RPA on the X1 nuclease activity. 5'-Labeled B30 substrate was preincubated on ice for 30 min with no protein (lane 1), the indicated amounts of RPA (lanes 2 and 3), or E. coli SSB (lanes 4 and 5), followed by the addition of S1 nuclease. d, titration of the RPA-conferred junction-cutting activity. Top, 3'-labeled B30 substrate was preincubated on ice for 10 min with the indicated amounts of RPA (lanes 1–5) or E. coli SSB (lane 6) prior to the incubation with 1 μl of the DEAE-column fraction of XPF-ERCC1. Bottom, quantitative analysis of the data shown in the top panel. e, DNA structure requirements for the RPA-conferred junction-cutting activity. 3'-Labeled B12 (lanes 1–3), B30 (lanes 4–6), L12 (lanes 7–9), or L30 (lanes 10–12) substrates were preincubated on ice for 10 min with the indicated amounts of RPA, followed by the addition of 1 μl of the XPA-affinity column fraction of XPF-ERCC1. M, size markers (x174/HinFI).

stimulated the activity about 64-fold (Fig. 2d). We wished to know the DNA structure requirements for the RPA-stimulated junction-cutting activity. As shown in Fig. 2e, the junction-cutting activity of the XPA-column fraction is greatly stimulated by RPA, and a smaller (12-nt) bubble structure is incised in the same way as the 30-nt bubble although with lower efficiency (lanes 1–3 compared with lanes 4–6). Interestingly, similar results were obtained with loop structures. Both the 12- and 30-nt loop substrates were incised in a manner similar to the bubble substrates (lanes 7–12).

Similar experiments were conducted with XPG nuclease, which is known to have a junction-cutting activity in a bubble substrate (12) and to interact with RPA (14). Fig. 3a shows the effects of RPA on the XPG junction-cutting activity with the 30-nt bubble and loop substrates. Under our experimental conditions, a very weak junction-cutting activity of XPG was observed with both substrates (~1% incision) (Fig. 3a, lanes 1 and 7), which is specific for the junction with entering the bubble/loop region in 3' to 5' direction. Addition of RPA stimulated the junction-cutting activity in a concentration-dependent manner (Fig. 3a, lanes 2–6 and 8–11), although the optimal RPA concentration and the major incision sites were different between the two substrates. The junction-cutting activity stimulated by RPA can be inhibited by anti-XPG antibodies (Fig. 3b). We failed to detect a significant XPG junction-cutting activity with smaller (12-nt) bubble and loop substrates under these conditions (data not shown).

These results, taken together, support a model of excision nuclease reaction where RPA may stabilize the unwound DNA region (presumably caused by TFIIH helicase activity) and recruit the two nucleases, XPF-ERCC1 and XPG, to their proper sites through physical interactions as is known to occur.
between RPA and XPG (14). In fact, consistent with this model, Lanes 1–3 contain RPA (0.2 μg), XPA (0.1 μg), and XPF-ERCC1 (1 μg), respectively. Immobilized B30 substrate was incubated with the indicated amounts of XPF-ERCC1 (lane 4), XPF-ERCC1 and RPA (lanes 5 and 6), XPF-ERCC1, RPA, and XPA (lane 7), RPA and XPA (lane 8), or RPA (lane 9). Immobilized DS substrate was incubated with 0.2 μg (lane 10) or 1.0 μg (lane 11) of RPA. The bound fractions were analyzed by Western blotting using a mixture of anti-p34 (RPA) (Onogene Science), anti-XPA, and anti-ERCC1 (13) antibodies.

In light of our results, which demonstrate stimulation of XPF-ERCC1 by RPA in the absence of XPA, we conducted a pull-down assay to test whether XPF-ERCC1 binding to the bubble substrate can be enhanced in the presence of RPA without the mediation of XPA (Fig. 4). The immobilized 30-nt bubble substrate was incubated with RPA, XPA, XPF-ERCC1, or a combination of these proteins. RPA bound to this substrate specifically and efficiently (lanes 5–8 compared with lanes 10 and 11), while XPF-ERCC1 bound to the bubble substrate weakly in the absence of RPA (lane 4). When XPF-ERCC1 was incubated with the substrate in the presence of RPA, more XPF-ERCC1 bound to the bubble substrate (lanes 5 and 6). In contrast, XPA did not affect the XPF-ERCC1 binding to this substrate (lane 7). Yeast RPA failed to facilitate XPF-ERCC1 binding to the bubble substrate (data not shown), consistent with the failure of yeast RPA to confer a junction-cutting activity to XPF-ERCC1. These results suggest that human RPA recruits XPF-ERCC1 onto the bubble substrate DNA, possibly either through direct interaction or by creating a unique DNA structure.

One reasonable question is why does the XPA-column fraction have a junction-cutting activity but the purer fractions do not? The contamination of RPA in the XPA-column fraction seems to be unlikely, because no trace of RPA (or XPA) could be detected by either silver staining or Western blotting with high sensitivity (data not shown, also see Fig. 4). A simple explanation is that XPF-ERCC1 has an intrinsic junction-cutting activity without RPA, which becomes nonspecific at low concentrations of the enzyme. Indeed, the diluted XPA-column fraction showed a nonspecific single-stranded DNA endonuclease activity (data not shown) rather than a junction-cutting activity (which could be restored by adding RPA).

Our results show that RPA plays an essential role in positioning the two nuclease subunits of the human excision nuclease system. Furthermore, these results show that, with loop structures of 12–30 nt in length, RPA, XPG, and XPF-ERCC1 are capable of removing the loop by incising the strand with extra nucleotides on both sides of the loops. The human MutL system is capable of eliminating mismatches and loops up to 4 nucleotides (24). However, it was reported that loops of 5, 8, and 16 nucleotides can be removed in a hMLH1-independent manner (25). Moreover, it has been found that the yeast Schizosaccharomyces pombe exonuclease I (26) and the Saccharomyces cerevisiae RT11 nuclease (27), which belong to the extended family of XPG/RAD2/Rad13 and FEN-1/Rad2 proteins, play roles in mismatch corrections. Thus, it is possible that XPG and XPF-ERCC1 proteins, in addition to their roles in damage excision repair, may participate in removing the looped-out DNA with a relatively large size and hence in genomic stability.

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