Requirement for XRCC4 and DNA Ligase IV in Alignment-based Gap Filling for Nonhomologous DNA End Joining in Vitro

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Abstract

In the nonhomologous end joining pathway of DNA double-strand break repair, the ligation step is catalyzed by a complex of XRCC4 and DNA ligase IV. Extracts of CHO-K1 cells are able to accurately rejoin a site-specific free radical-mediated double-strand break with partially complementary overhangs, by a mechanism involving alignment-based gap filling followed by ligation. Extracts of XR-1 cells, which lack XRCC4 and DNA ligase IV, carried out neither gap filling nor ligation. Supplementation of the extracts with recombinant XRCC4/ligase IV, but not with XRCC4 alone, restored gap filling and accurate end joining. The results imply that XRCC4 and ligase IV are essential for alignment-based gap filling, as well as for final ligation of the breaks.

Introduction

Under most conditions in mammalian cells, the majority of DNA DSBs are repaired by the nonhomologous end joining pathway, which joins DNA ends without reference to an intact homologous copy of the damaged sequence (1, 2). Although this repair pathway is somewhat error prone and can generate small deletions at the break site, in vitro end joining studies (3) suggest that even free radical-mediated DSBs with missing or damaged nucleotides are, in most cases, accurately rejoined by a mechanism involving Ku-dependent alignment of the broken ends, removal of damaged termini, fill-in of gaps in the aligned ends, and ligation (Fig. 1). The XR-1 cell line was isolated as a radiation-sensitive derivative of CHO-K1 cells, and XRCC4 was subsequently isolated as the factor that complements the extracts with recombinant XRCC4/ligase IV, but not with XRCC4 alone, restored gap filling and accurate end joining. The results imply that XRCC4 and ligase IV are essential for alignment-based gap filling, as well as for final ligation of the breaks.

Materials and Methods

Materials. The end-joining substrate is shown in Fig. 1. The 3'-PG-terminated substrate was constructed by ligation of 3'-PG oligomers into 5' overhangs of a plasmid substrate, as described previously (3). Extracts were prepared from suspension cultures of CHO-K1 and XR-1 cells by Dounce homogenization, as described previously (3). Aphidicolin (Sigma) was dissolved in DMSO at a concentration of 1.35 mg/ml and stored at −20°C. The complex of XRCC4 and histidine-tagged DNA ligase IV, or XRCC4 only, were prepared from baculovirus-infected insect cells, as described previously (5). In all cases, the proteins gave single bands on Coomassie Blue-stained polyacrylamide gels, with no evidence of proteolytic degradation, and the XRCC4, either alone or in complex with ligase IV, was an efficient substrate for phosphorylation by DNA-PK in vitro. Recombinant XRCC4/ligase IV complex was also purchased from Trevigen.

End-joining Reactions. Extracts (12.5 mg/ml protein) were dialyzed for 30 min against a mixture of 50 mM morpholinoethanesulfonate, NaOH (pH 7.5), 40 mM KCl, 10 mM MgCl₂, and 5 mM β-mercaptoethanol immediately before use. End-joining reactions contained 11 µl of extract, 1 mM ATP, 50 µM dNTP or deoxyribonucleoside 5'-triphosphate (ddNTP), 50 µg/ml BSA, and 10 ng of substrate in a total volume of 13 µl. Reactions were incubated for 6 h at 25°C and deproteinized by treatment with proteinase K followed by phenol extraction. DNA was cut with XbaI and XhoI and analyzed on sequencing gels as described previously (3).

Results

To examine end joining of free radical-mediated DSBs, we constructed a model DSB substrate consisting of a plasmid with partially cohesive PG-terminated 3' overhangs and a one-base gap in each strand (3). This substrate mimics the DSB that would result from free radical-mediated destruction of the two T nucleotides in the self-complementary sequence ACGT•ACGT. Accurate restoration of the original sequence would require PG removal, alignment of the self-complementary CG overhangs, fill-in of the one-base gaps with dTTP, and ligation (Fig. 1).

Extracts of CHO-K1 cells were able to repair this model free-radical-mediated DSB (Fig. 2), yielding a prominent repair product corresponding to accurate restoration of the original sequence, which was detected as a 43-base fragment after XhoI/BstXI cleavage (3). Previous studies (3) showed that this process is strictly dependent on Ku, suggesting that it reflects, at least to some degree, end-joining repair as it occurs in vivo. Additional ~23–24 base products were also detected, which may have resulted from resection-based end joining (1). They were apparently not head-to-head intermolecular end-joining products, because they were not present when DNA was cut with XhoI alone (data not shown). Curiously, supplementation of CHO-K1 extracts with XRCC4/ligase IV (Fig. 2, Lane 5) resulted in additional, as yet unidentified, end-joining products that appeared to be even longer than the accurate product.

By substituting ddTTP for dTTP in the end-joining reaction, it was possible to trap the filled-in but unligated intermediate, which is released as a 16-base fragment by XhoI cleavage (Fig. 2, Lane 4). Aphidicolin, at a concentration that should have been sufficient to completely inhibit the replicative polymerases α, δ, and ε (10), did not prevent either accurate end joining or gap filling (Fig. 2, Lanes 7–8). This result, combined with the efficient incorporation of ddTTP, suggests that gap filling is catalyzed by a member of the polX family, which includes polymerases β, λ, and μ (11).

References

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3 The abbreviations used are: DSB, double-strand break; DNA-PK, DNA-dependent protein kinase; PG, phosphoglycerate; dNTP, deoxyribonucleoside 5'-triphosphate.
between these data and a report that aphidicolin partially inhibits the generation of gap-filling-dependent end-joining products (12) remains to be resolved.

XR-1 cells are genetically deficient in XRCC4, and because DNA ligase IV is unstable in the absence of XRCC4, XR-1 extracts lack both XRCC4 and ligase IV (13). Thus, as expected, no end-joining products at all were detected when the same DSB substrate was incubated in XR-1 extracts (Fig. 3A, Lanes 1–2). To determine whether this end joining deficiency was caused by the absence of XRCC4/ligase IV or to some other property of the extracts, XR-1 extracts were supplemented with exogenous recombinant XRCC4/ligase IV. If XRCC4/ligase IV deficiency had blocked only the final ligation step, then a filled-in but unligated intermediate should have been formed in XR-1 extracts and should have been released by XhoI cleavage as a labeled 16-base fragment. The fact that no such fragment was detected suggests that XRCC4/ligase IV deficiency prevented not only ligation but gap-filling as well. To confirm this result, end-joining reactions in extracts from both cell lines were repeated with ddTTP substituted for dTTP. As expected, in XRCC4/ligase IV-supplemented XR-1 extracts, ddTTP (but not ddATP) trapped the filled-in but unligated intermediate, as shown by the presence of the predicted 16-base fragment and absence of the accurate repair product (Fig. 3A, Lanes 5, 6, and 8). However, no such filled-in intermediate was detected in unsupplemented XR-1 extracts (Fig. 3, Lane 2). These results strongly suggest that XRCC4/ligase IV is required for alignment-based gap filling.

Although DNA ligase IV is stable only in the presence of XRCC4 (13), XRCC4 is stable in the absence of ligase IV.4 To determine whether XRCC4 alone was sufficient to support alignment-based gap filling, XR-1 cells were supplemented with recombinant XRCC4. As shown in Fig. 3B, however, no gap filling was detected in XR-1 extracts supplemented with XRCC4 alone, implying that gap filling requires both XRCC4 and DNA ligase IV.

Discussion

Genetic data imply that DSB repair by nonhomologous end joining requires Ku, DNA-PKcs, XRCC4, DNA ligase IV (1, 2), and perhaps

4 S. M. Yannone, unpublished observations.
Artemis (14). XRCC4 exists in a tight complex with DNA ligase IV and stimulates its ligase activity (5–7), suggesting that this complex carries out the final ligation step in nonhomologous end joining.

Most naturally occurring DSBs, such as those resulting from free radicals, have missing, fragmented, or damaged nucleotides that will preclude repair by simple religation. Either these nucleotides must be replaced by a gap-filling mechanism, potentially allowing restoration of the original sequence, or the damaged ends must be trimmed to produce a ligatable substrate, resulting in a small deletion. At least in vitro, it appears that Ku-dependent end joining normally restores the original sequence by gap filling, as long as the ends have overhangs with residual complementarity, even as little as 2 bp (3). In the case of 3’ overhangs, such gap-filling requires prior alignment of the ends, presumably based on the annealing of the residual complementarity. X-ray crystallography of Ku bound to a DNA end shows that Ku forms a tightly fitting asymmetrical ring around DNA, suggesting that Ku could cradle two DNA ends and mold them into a continuous helix while, nevertheless, allowing access to the DNA termini on one side of the ring (15). In vitro end-joining studies (3, 16) imply that Ku is essential for alignment during gap filling but do not indicate whether Ku is sufficient for this function. The present results suggest that gap filling also requires the XRCC4/ligase IV complex, at least under conditions of the in vitro assay.

Recently, it was shown that DNA polymerase μ binds to XRCC4/ligase IV and stabilizes complexes between XRCC4/ligase IV and DNA, and that the combination of Ku, XRCC4/ligase IV, and polymerase μ could effect fill-in and ligation of a substrate with cohesive 4-base hydroxyl-terminated 3’ overhangs and a one-base gap in one strand (9). Although these studies did not explicitly address the question of whether XRCC4/ligase IV was required for gap filling, they provided further indirect evidence for such a requirement. They also suggested that the requirement for XRCC4/ligase IV in gap filling could be attributable, in part, to its role in recruiting polymerase μ to the aligned ends. XRCC4/ligase IV also probably helps to stabilize Ku-mediated DNA end-to-end association, as XRCC4/ligase IV itself promotes end-to-end association (17). All of these results, as well as the data presented above, suggest that both gap filling and ligation are carried out in the context of a repair complex that includes Ku, XRCC4, ligase IV, and polymerase μ. In neither experimental system, however, does DNA-PKcs appear to be required for these processes (3, 9). A key remaining question is how the putative complex of Ku, polymerase μ, and XRCC4/ligase IV sequentially affords access of the DNA termini to processing by polymerase μ and then by ligase IV, presumably even while Ku continues to bind to and align the two DNA ends.

References
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