Requirement for XLF/Cernunnos in alignment-based gap filling by DNA polymerases \( \lambda \) and \( \mu \) for nonhomologous end joining in human whole-cell extracts

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ABSTRACT

XLF/Cernunnos is a core protein of the nonhomologous end-joining pathway of DNA double-strand break repair. To better define the role of Cernunnos in end joining, whole-cell extracts were prepared from Cernunnos-deficient human cells. These extracts effected little joining of DNA ends with cohesive 5’ or 3’ overhangs, and no joining at all of partially complementary 3’ overhangs that required gap filling prior to ligation. Assays in which gap-filled but unligated intermediates were trapped using dideoxynucleotides revealed that there was no gap filling on aligned DSB ends in the Cernunnos-deficient extracts. Recombinant Cernunnos protein restored gap filling and end joining of partially complementary overhangs, and stimulated joining of cohesive ends more than twentyfold. XLF-dependent gap filling was nearly eliminated by immunodepletion of DNA polymerase \( \lambda \), but was restored by addition of either polymerase \( \lambda \) or polymerase \( \mu \). Thus, Cernunnos is essential for gap filling by either polymerase during nonhomologous end joining, suggesting that it plays a major role in aligning the two DNA ends in the repair complex.

INTRODUCTION

XLF/Cernunnos is a recently discovered core protein of the nonhomologous end-joining pathway of DNA double-strand break (DSB) repair (1,2). In humans, deficiency in Cernunnos confers immunodeficiency and microcephaly (2). Cernunnos has sequence and structural similarity to XRCC4, to which it binds (1,3,4). In vitro, Cernunnos stimulates ligation of DNA ends by the XRCC4/DNA ligase IV complex (X4L4), particularly noncomplementary ends (5,6). To further define the function of Cernunnos, end joining was examined in Cernunnos-deficient whole-cell extracts, complemented with purified recombinant Cernunnos protein. The results indicate a specific requirement for Cernunnos in gap filling on aligned DSB ends.

METHODS

Cells and extracts

Cernunnos-deficient BuS fibroblasts were isolated from an RS-SCID (severe combined immune deficiency with radiosensitivity) patient, and were immortalized by h-TERT and SV40. The cells were grown in RPMI1640 medium plus 10% fetal bovine serum. These cells harbor a homozygous nonsense mutation at R178 of Cernunnos (2). Monolayers (5000 cm\(^2\) total) were harvested two days after reaching confluence (which appeared to improve the end joining proficiency of extracts), and whole-cell extracts were prepared by Dounce homogenization as described previously (7,8) (see Supplementary Material). Extracts typically contained 10–13 mg/ml protein in 20 mM Tris pH 8, 0.1 M potassium acetate, 1 mM dithiothreitol, 0.5 mM EDTA, 20% glycerol (final dialysis buffer). For some experiments, the extracts were...
immunodepleted of DNA polymerase λ (polλ) and supplemented with recombinant polδ (gift of Drs Kasia Bebenek and Tom Kunkel, NIEHS) or polγ (gift of Luis Blanco, Universidad Autónoma de Madrid) as described previously (9).

Inhibitors

The kinase inhibitors 2-morpholin-4-yl-6-thianthren-1-yl-pyran-4-one (KU-55933) and 2-N-morpholinopheno[8]-dibenzo[2]thienophenyl-chromen-4-one (KU-57788 or NU7441) were obtained from KUDOS and were stored at −20°C in DMSO. KU-55933 inhibits ATM with an IC50 of 13 nM, versus 1.8 μM for DNA-PK (10). KU-57788 inhibits DNA-PK with an IC50 of 14 nM, versus >100 μM for ATM (11). Both are at least 100-fold more potent in inhibiting their target kinase than any of 60 other kinases tested.

Substrates

To generate an internally labeled substrate with partially cohesive 4-base 5’-resected plasmid pRZ56, as described previously (8,12,13). To generate a labeled substrate with cohesive 4-base 5’ overhangs, pUC19 was cut with BsaI, dephosphorylated, and 5’-32P-end-labeled with ATP and T4 polynucleotide kinase. To generate a substrate with 4-base 3’ overhangs, the same labeled DNA was religated and cut with KpnI. In all cases, linear full-length monomers were gel-purified, concentrated by filtration (Amicon Centricon 100) and precipitated. Concentration was determined from A260.

Cernunnos protein and site-directed mutagenesis

For expression in Escherichia coli, the full-length Cernunnos gene was cloned into plasmid pQE80 with an N-terminal 6× His tag. Mutants S245A, S251A and the corresponding double mutant were generated using the QuickChange site-directed mutagenesis kit (Stratagene), and the following primers determined by the QuickChange Primer Design Program (Stratagene). For expression in Escherichia coli, the full-length Cernunnos protein and site-directed mutagenesis were generated with the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA). S9 cells were infected with Cernunnos baculovirus and harvested 60 h after infection. Cells were lysed in 50 mM NaH2PO4, pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and the lysate was sonicated and then centrifuged for 1 h at 150 000g. The supernatant was mixed with Ni-NTA-agarose (Qiagen, Valencia, CA, USA) and incubated for 4 h at 4°C with rotation. The beads were washed with 25 ml of wash buffer (50 mM NaH2PO4, pH 8.0, 0.3 M NaCl, 20 mM imidazole, 1 mM phenylmethyl-sulphonyl fluoride). Bound proteins were then eluted four times with 1 ml of the same buffer containing 0.5 M imidazole. All four fractions were analyzed by SDS-PAGE electrophorosis and peak fractions dialyzed against 20 mM Tris–HCl pH 8.0 overnight. Cernunnos wild-type and mutant proteins were filtered (0.22 μm), loaded on a MonoQ FPLC column (Pharmacia) and eluted with a gradient of 0–0.8 M NaCl in 20 mM Tris–HCl pH 8.0. Fractions were collected and analyzed by SDS–PAGE. Cernunnos-containing fractions eluted at ∼0.75 M NaCl and were stored at −20°C in the elution buffer plus 50% glycerol. Final concentration of the protein was determined using Pierce BCA assay with BSA as standard, and verified by quantification of bands on gels stained with SYPRO orange and scanned with a Typhoon fluorescence imager (GE Healthcare), which agreed within a factor of 1.5×. Stock solutions of Cernunnos were diluted at least 100-fold in end joining reactions.

For baculoviral expression of His-tagged Cernunnos, the cDNA was inserted into pFastBac-HTB vector and recombinant Cernunnos-expressing baculovirus was generated with the Bac-to-Bac baculovirus expression system (Invitrogen, Carlsbad, CA, USA). S9 cells were infected with Cernunnos baculovirus and harvested 60 h after infection. Cells were lysed in 50 mM NaH2PO4, pH 8.0, 0.5 M NaCl, 5 mM β-mercaptoethanol, 10% glycerol, and the lysate was sonicated and then centrifuged for 1 h at 150 000g. The supernatant was mixed with Ni-NTA-agarose (Qiagen, Valencia, CA, USA) and incubated for 4 h at 4°C with rotation. The beads were washed with 25 ml of wash buffer (50 mM NaH2PO4, pH 8.0, 0.3 M NaCl, 20 mM imidazole, 1 mM phenylmethyl-sulphonyl fluoride). Bound proteins were then eluted four times with 1 ml of the same buffer containing 0.5 M imidazole. All four fractions were analyzed by SDS-PAGE electrophorosis and peak fractions dialyzed against 20 mM Tris–HCl pH 8.0 overnight. Cernunnos wild-type and mutant proteins were filtered (0.22 μm), loaded on a MonoQ FPLC column (Pharmacia) and eluted with a gradient of 0–0.8 M NaCl in 20 mM Tris–HCl pH 8.0. Fractions were collected and analyzed by SDS–PAGE. Cernunnos-containing fractions eluted at ∼0.75 M NaCl and were stored at −20°C in the elution buffer plus 50% glycerol. Final concentration of the protein was determined using Pierce BCA assay with BSA as standard, and verified by quantification of bands on gels stained with SYPRO orange and scanned with a Typhoon fluorescence imager (GE Healthcare), which agreed within a factor of 1.5×. Stock solutions of Cernunnos were diluted at least 100-fold in end joining reactions.

End joining reactions

Reactions contained 50 mM triethanolammonium acetate pH 7.5, 1 mM ATP, 1 mM dithiothreitol, 50 μg/ml BSA, 1.3 mM magnesium acetate and dNTPs (or ddNTPs) at 100 μM each. Typically, a 16-μl reaction contained 10 μl of extract, resulting in a final concentration of 8 mg/ml protein, 66 mM potassium acetate and 16% glycerol, and an effective Mg2+ concentration of 1 mM (taking into account ∼0.3 mM EDTA from the extract). Buffer components were first mixed with cell extract at 22°C. Cernunnos was added and the solution mixed by pipeting. In some reactions, KU-57788 in DMSO or DMSO alone (final concentrations of 1 μM and 2%, respectively) was...
added and the sample mixed by pipeting. Finally, substrate (10–100 ng) was added and the reaction again mixed by pipeting, and placed in a 37°C water bath, usually for 6 h. Samples were deproteinized as described (13) and either loaded directly onto 0.8% agarose gels, or cut with BstXI and AvaI and analyzed on 20% polyacrylamide DNA sequencing gels. Storage phosphor screens were exposed to frozen polyacrylamide or dried agarose gels, and images were analyzed with ImageQuant 3.3 software.

RESULTS

Joining of partially complementary DNA ends in cell extracts is completely dependent on Cernunnos

Previous work showed that whole-cell extracts from several cell lines harboring Cernunnos mutations had a severe deficiency in end joining of an EcoRI-cut plasmid, and that the deficiency could be corrected by stable transfection of Cernunnos cDNA (2). We have shown that a variety of human cell extracts can join DNA ends bearing partially complementary –ACG/–ACG 3’ overhangs, and that they do so almost exclusively by a mechanism involving annealing of the terminal self-complementary –CG/–CG, filling of the 1-base gap in each strand, and ligation (8,13). Using an internally labeled plasmid substrate, these end joining events are detected after BstXI/ AvaI cleavage as 43-base (head-to-tail) and 24-base (head-to-head) labeled products on a denaturing gel (Figure 1A).

Within the limits of detection of phosphorimaging, there was no generation of either 43- or 24-base fragments (<0.1%) when the substrate was incubated in extracts of Cernunnos-deficient fibroblasts. This apparent repair deficiency could be complemented by mixing the Cernunnos-deficient extract with a similar extract from DNA-PKcs-deficient M059J cells (Figure 1B), or by addition of purified recombinant Cernunnos (Figure 1C). As with other human cell extracts, only two major repair products, of 43 and 24 bases, were detected, corresponding to accurate annealing, patching and ligation of the -ACG overhangs. Treatment with BsaHI in addition to BstXI and AvaI converted most of the 43-mer and about half of the 24-mer to a 13-mer, confirming the predicted GAC GTC repair joint (Figure 1C). The remaining uncleaved 43- and 24-base products may have been resistant to BsaHI due to incomplete repair of the opposite strand. Cernunnos produced in E. coli was as effective in supporting end joining as that produced in baculovirus-infected insect cells (Figure 1D). A titration with limiting amounts of Cernunnos revealed greater-than-linear concentration dependence between 25 and 100 nM, consistent with multiple molecules of Cernunnos being required, as suggested by structural studies (Figure 1E).

Cernunnos is required for gap filling on aligned DSB ends by polδ or polτ

To directly determine whether Cernunnos is involved in gap filling, end joining reactions were performed in the presence of ddTTP, which supports gap filling by polymerases λ and μ, but blocks the final ligation step. In such reactions, the predicted 16-mer corresponding to the elongated but unligated intermediate was detected only in extracts supplemented with Cernunnos (Figure 2A), implying that gap filling in these extracts is completely dependent on Cernunnos. Some ligation products were still generated in the presence of ddTTP, probably due to residual dTTP in the extracts, which are dialyzed for only 3 hr after ultracentrifugation (7). End joining of the partially complementary substrate in the presence of ddTTP (Figure 2B), as well as gap filling in the presence of ddTTP, was almost completely abolished by immunodepletion with antibodies to polδ, and restored by addition of recombinant polδ, suggesting that polδ is the dominant Cernunnos-dependent gap-filling polymerase (Figure 2C). However, addition of polτ promoted gap filling and end joining to the same extent as polδ (ratio 1.1 ± 0.1, N = 3, for gap filling), but again only in the presence of Cernunnos, indicating that Cernunnos is required for gap filling by either polymerase. Taken together, these results indicate that there was insufficient polτ activity in the extracts to support end joining; however, inasmuch as earlier results suggested that polτ is more susceptible than polδ to nonspecific inactivation during immunodepletion (9), the results do not exclude a significant role for polτ in gap filling in vivo.

Cernunnos is required for efficient joining of cohesive 3’ and 5’ overhangs

Because gap filling is Cernunnos-dependent and must necessarily precede ligation, the above results do not indicate whether the final ligation step also required Cernunnos. To address this question, end joining of substrates bearing fully cohesive ligatable overhangs was examined. End joining of either a KpnI-cut plasmid bearing cohesive 4-base 3’ overhangs (Figure 3A) or a BsaI-cut plasmid bearing cohesive 5’ overhangs (Figure 3B) was almost completely dependent on Cernunnos, with a concentration dependence similar to that for the partially cohesive overhang. However, in contrast to the partially cohesive substrate, a trace of joining of the fully cohesive substrates was detected in the absence of Cernunnos, typically ~0.5% with 20 ng substrate and ~2% with 100 ng. Nevertheless, end joining of the cohesive substrates was stimulated as much as 75-fold by Cernunnos. While the DNA-PK inhibitor KU-55933 blocked Cernunnos-dependent end joining, the residual Cernunnos-independent end joining was unaffected (Figure 3C). As reported previously for DNA-PK-complemented M059J extracts (8), the ATM inhibitor KU-55933 had no detectable effect on end joining at a concentration sufficient to block all ATM-catalyzed phosphorylation (Figure 3C). Overall, the results suggest that the final ligation step of DNA-PK-dependent end joining is also completely dependent on Cernunnos, but that there is a low level of residual end joining that does not require either Cernunnos or DNA-PK.
Phosphorylation at S245 and S251 is not essential for Cernunnos function in end joining in vitro

Recently, the dominant in vivo phosphorylation sites of Cernunnos have been identified as S245, a DNA-PK-dependent site, and S251, an ATM-dependent site (14). To assess the possible functional role of these phosphorylations, mutant Cernunnos proteins harboring S→A mutations at one or both sites were produced in E. coli. All these proteins supported end joining of the substrates bearing either partially cohesive or fully cohesive 3' overhangs (Figure 4). These data imply that neither phosphorylation at S245 nor at S251 is required for either gap filling or ligation of 3' overhangs. The efficiency of end joining with the mutant proteins was comparable to that with wild-type, and there was no change in the fidelity of repair. The S245 and S251 phosphorylation sites also appear to be dispensable for DSB repair in intact cells (14). The finding that phosphorylation of the major sites on Cernunnos is not essential, raises the possibility that Cernunnos might only be required at a later stage in the end joining pathway, after the essential phosphorylations have been completed. However, order-of-addition experiments (Supplementary Figure 1) indicated that in the absence of Cernunnos there was little or no accumulation of products.

Figure 1. Requirement for Cernunnos in joining of partially complementary DNA ends. (A) Internally labeled (*) substrate and head-to-tail repair product. Following alignment, single-base gap filling (bolded T's) and ligation, the repaired top strand yields a labeled 43-base Aval/BstXI fragment. Similar intermolecular joining of two left-hand ends yields a 24-base product. (B) Complementation of end joining deficiency in Cernunnos-deficient BuS cell extracts by mixing with DNA-PK-deficient M059J extracts (1:2, 1:1 or 2:1 ratio) or by addition of Cernunnos protein (100 nM). The substrate (10 ng) was incubated in extracts for 6 hr, deproteinized and cut with BstXI and Aval. (C) Partial cleavage of repair products with BsaHI, to yield a 13-mer. (D) Titration of end joining with various concentrations of Cernunnos, prepared in E. coli or in baculovirus-infected insect cells as indicated. (E) Quantitation of data from (D). (F) Coomassie-stained gels showing purity of His-tagged recombinant Cernunnos proteins (*) (expected Mr = 35 kDa). Cernunnos protein in (B and C) was from E. coli.
Figure 2. Requirement for Cernunnos and polλ or polμ in alignment-based gap filling. (A) The substrate shown in Figure 1A was incubated in BuS cell extracts with or without recombinant Cernunnos for 6 h as in Figure 1, except that ddTTP was added instead of dTTP in some samples as indicated. The 16-mer band represents the gap-filled but unligated intermediate. (B) The same extracts were preincubated with beads coated with antibodies against polλ (+) or preimmune antibodies (−), then incubated with the substrate as in (A), but in the presence of all four dNTPs plus recombinant recombinant polλ (70 ng), polμ (70 ng) and/or Cernunnos as indicated. (C) Same as (B), except that all samples contained ddTTP to trap unligated intermediates. Cernunnos in these experiments was isolated from E. coli.

Figure 3. Cernunnos dependence of joining of cohesive 5′ and 3′ overhangs. (A) A labeled plasmid (20 ng) bearing cohesive 3′ overhangs (–GTAC/–GTAC) was incubated for 6 h in BuS extracts supplemented with the indicated concentrations of Cernunnos. (B) Same as (A) except that the substrate (20 or 100 ng) had cohesive 5′ overhangs (CGGA–TCCG–). (C) The 5′-overhang substrate (100 ng) was incubated for 6 h in extracts containing 0 or 100 nM Cernunnos and either the DNA-PK inhibitor KU-57788 (1 μM) or the ATM inhibitor KU-55933 (1 μM). Reactions without inhibitor contained an equivalent concentration of DMSO. End joining was analyzed by agarose gel electrophoresis.
Figure 4. Effect of S→A mutations in Cernunnos on complementation of end joining. (A) Sequence context of the S245 and S251 phosphorylation sites. (B) The partially complementary substrate shown in Figure 1A was incubated for 6 h in BuS cell extracts supplemented with wild-type or mutant Cernunnos protein from E. coli, as indicated. (C) Quantitation of data from (A) and two replicate experiments. End joining for the mutant proteins was normalized to wild-type. (D) Same as (B), except that the substrate was KpnI-cut pUC19.

of active, appropriately phosphorylated repair complexes that could proceed to gap filling and ligation in the presence of DNA-PK inhibitor when Cernunnos was added. Instead, successful end joining appears to require the simultaneous presence of Cernunnos and catalytically active DNA-PK.

DISCUSSION

Cernunnos was discovered as the gene defective in a subset of SCID patients with accompanying microcephaly (2), and independently as XLF, a binding partner of XRCC4 (1). It has a structure similar to that of XRCC4, with a globular head and long α-helical tail (3,4). Cernunnos binds to Ku as well as to XRCC4, and induces supershifts of both Ku-DNA and XRCC4-DNA complexes in electrophoretic mobility shift assays (3,15,16). The cellular phenotype for Cernunnos deficiency is similar to that for Ku or XRCC4, and includes radiosensitivity and failure to rejoin a substantial fraction of radiation-induced DSBs (2). In reactions with purified proteins, Cernunnos stimulates ligation of DNA ends by X4L4, and confers an ability to join mismatched ends and protruding single strands, albeit at low efficiency (5,6). All these results suggest that Cernunnos is a core factor in the end joining repair complex. The finding that alignment-based gap filling in whole-cell extracts is completely dependent on Cernunnos (Figure 2) suggests that, in addition to stimulating ligase IV, Cernunnos is also important for DNA end alignment.

Previously, we (17) and others (18) have shown that in extracts of CHO (hamster) cells, end joining events requiring gap filling are completely dependent on Ku, while resection-based joining events are not. We also showed directly that X4L4 is required for gap filling, both in hamster whole-cell extracts (19), and in human nuclear extracts (9); furthermore, at least in the hamster extracts, XRCC4 alone could not substitute for the X4L4 complex. Given that these various extract systems show the same specificity in gap filling and the same requirement for either polα or polμ, it appears reasonably certain that they share the same basic gap filling process. Thus, from all these data we infer that gap filling for nonhomologous end joining requires the full complex of Ku, XRCC4, DNA ligase IV and Cernunnos. The requirement for Cernunnos in gap filling is consistent with a recent proposal that end alignment is promoted by assembly of a filament of alternating homodimers of Cernunnos and XRCC4 (3). In principle, the Cernunnos dependence of gap filling could be due to more specific interactions between Cernunnos and polα or polμ, but gel mobility shift experiments indicate that Ku and X4L4 are sufficient to efficiently recruit either polymerase to DNA ends (20–22). Nevertheless, a model in which Cernunnos is required for proper positioning of the polymerase within the repair complex, rather than for end alignment as such, remains a formal possibility.

The polymerase complementation results (Figure 2) are consistent with previous work showing that polα and polμ have distinct but overlapping specificities. Either polymerase can efficiently fill in one-base gaps in aligned DSB ends (22,23), but only polα can fill longer gaps (9) and only polμ can fill gaps with no base pairing, for example, when noncomplementary single-base 3′ overhangs are present on both ends (23). Nevertheless, as shown previously with HeLa nuclear extracts (9), immunodepletion of polα, almost completely eliminates gap filling, suggesting that the level of polμ in the extracts is too low to support robust gap filling. However, we previously found that the activity of polμ (but not polα) was substantially reduced when the recombinant enzyme was mock-depleted from solution with pre-immune rabbit IgG (9), and thus it is possible that polμ in the extract is more susceptible than polα to nonspecific loss or inactivation during the extraction procedure.

Previous studies with purified DNA-PKcs, Ku, Artemis, XRCC4 and ligase IV indicate that all of these proteins except ligase IV are extensively phosphorylated by DNA-PK when they are incubated together in the presence of DNA ends (24). However, only phosphorylation of DNA-PKcs itself has been shown to be important for end joining in cells, and phosphorylation of XRCC4 and Ku is apparently dispensable (24). Our results with mutant Cernunnos proteins not only confirm a previous report that S245A and S251A mutations do not affect repair efficiency (14), but also indicate that they do not affect repair fidelity.

As yet, we have been unable to detect gap filling on aligned DSB ends when the partially complementary (−ACG/−ACG) DSB substrate is incubated with the combination of Ku, DNA-PKcs, X4L4, Cernunnos, and polα, either with or without inositol hexakisphosphate.
(25), even though we have shown that each of these recombinant proteins is able to complement gap filling and end joining of the same substrate in the corresponding repair-deficient extracts (8,9,13,19). Although it has been reported that alignment-based gap filling and ligation can be achieved with only purified recombinant Ku, X4L4 and pol α or pol γ, this reaction requires high concentrations of substrate, as well as polyethylene glycol to promote macromolecular crowding. Moreover, these reactions are inhibited by Cernunnos as well as DNA-PKcs, both of which are required for the same reactions in cell extracts (23,26). Thus, while it is difficult to rigorously eliminate the possibility that there is some artifact associated with combining multiple recombinant proteins, the simplest explanation of the available data is that there are additional components in cell extracts which, even if they are not part of the core end-joining complex, are nevertheless required for it to function properly.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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