Length-dependent Binding of Human XLF to DNA and Stimulation of XRCC4-DNA Ligase IV Activity

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–9.

An XRCC4-like factor, called XLF or Cernunnos, was recently identified as another important factor in the non-homologous DNA end joining (NHEJ) process. NHEJ is the major pathway for the repair of double-strand DNA breaks. The similarity in the DNA end joining (NHEJ) process. NHEJ is the major pathway for the repair of double-strand DNA breaks (DSBs) and physiological DSBs is repaired by the nonhomologous DNA end joining (NHEJ) pathway. Given that XLF binds to DNA but with unusually long length requirements that are most consistent with a requirement that the C-terminal α helices run parallel to the DNA helix. To better understand the function of XLF, we purified an XLF mutant (R57G), which was identified in patients with NHEJ deficiency and severe combined immunodeficiency. Surprisingly, the mutant protein retained its ability to stimulate XRCC4-DNA ligase IV but failed to translocate to the nucleus, and this appears to be the basis for the NHEJ defect in this patient.

Many pathologic double-strand DNA breaks arise when ionizing radiation passes near DNA or when replication forks encounter a nick. Physiologic double-strand breaks are generated in lymphocytes during V(D)J recombination and class switch recombination (1, 2). Many pathologic double-strand DNA breaks (DSBs) and physiologic DSBs are repaired by the nonhomologous DNA end joining (NHEJ) pathway. Given this, it is not surprising that individuals born with defects in NHEJ are sensitive to ionizing radiation and have severe combined immunodeficiency (SCID) (for review, see Ref. 3). Many factors involved in NHEJ have been identified based on analysis of human and mouse SCID (4–8), and about 15–20% of human SCID is due to NHEJ defects. The proteins involved in NHEJ include Ku70, Ku80, DNA-PKcs, Artemis, XRCC4, DNA ligase IV, and most recently, XLF (or Cernunnos) (for review, see Ref. 1). NHEJ is thought to begin with Ku binding to the two newly created DNA ends at the double-strand break. Ku improves the affinity of a nuclease complex (Artemis–DNA–PKcs), a ligase complex (XRCC4–DNA ligase IV), and POL X polymerases (pol μ and pol λ). XLF (or Cernunnos) is the most recent factor, and it is suspected to be part of the ligase complex (5–7).

Here we have generated purified human XLF and studied its function in a variety of assays. We find that XLF does not form a stable complex with DNA ligase IV in the absence of XRCC4 but does interact with the XRCC4–DNA ligase IV complex. We find that XLF binds to DNA but with unusually long length requirements that are most consistent with a requirement that the C-terminal α helices run parallel to the DNA helix. Finally, XLF stimulates the ligation activity of XRCC4–DNA ligase IV in intramolecular and intermolecular direct ligation assays. Analysis of one point mutant from an XLF mutant SCID patient demonstrates that disruption of nuclear import may be the primary basis of the defect.

EXPERIMENTAL PROCEDURES

Oligonucleotide Substrate—The ligation substrate used was generated by annealing 5′ 32P-labeled oligonucleotide YM-216 (95 nt) with YM-194 (18 nt) and YM-219 (22 nt). This generates a substrate that is single-stranded except for the 18 and 22 nt at the ends of the 95-nt oligonucleotide. The single-strand portion consists entirely of Ts (sequences of oligos were described previously (9)). The 95-nt single-strand DNA probe used in the supplemental material was YM-218, consisting of mostly Ts with occasional Cs (9). Oligos for gel shift assay: HL-63 (83 nt), 5′-TCGACTGC-AGGCCGTTTGGTTCACTTCGTAAGACATTCGCT- GAGTCGACGAGCATGACGGTCTTTGACAGCTAGAAGAGGCAGCTTCGTTACCACTTCG- CACC-3′; HL-110 (83 nt), 5′-TCGACTGCAGGGGTTCGTGGTTCACTTCGTAAGACATTCGCT- GAGTCGACGAGCATGACGGTCTTTGACAGCTAGAAGAGGCAGCTTCGTTACCACTTCG- CACC-3′; HL-111 (90 nt), 5′-GGGTACCTCAAGTAAATGATCAGATACG- GAAACACAGCTGGACAGTGGTACTACACTCCACTCTGTTCGTCACAAAACACCTCAGGAATC- TGCTGTCAG-3′; HL-112 (90 nt), 5′-AGATCCCGAGGGTTTTTGTACAGCCAGACAGTGG-
XLF Stimulation of XRCC4-DNA Ligase IV

AGTACTACACTGTGCCAGCTGTGGTCCGTATCTGACGGTAGCCC-3’.

Construction of GST-XLF and XLF-Myc-Expressing Plasmids—Full-length human XLF cDNA was amplified by recombinant Pfu DNA polymerase (Stratagene, catalog #600154) using a human thymus matchmaker cDNA library (Clontech, catalog #HL4057AH) as the template (primers are available upon request). A GST-XLF-expressing plasmid was constructed by cloning into the pEBG vector after BamHI/NotI digest. An XLF-Myc-His-expressing plasmid was generated by ligating into the pcDNA6/Myc-His vector (Invitrogen, catalog #V221–20) after KpnI/NotI digest. The XLF bound protein G beads were fractionated by centrifugation. Cleared cell lysate was incubated with anti-Myc Ab for XLF and anti-XRCC4 Ab for XRCC4. The amount of bound protein was quantified with Quantity One software.

Protein Expression and Purification—Plasmids expressing ligase IV-Myc and XRCC4-His were expressed in 293T cells by calcium phosphate precipitation. Recombinant plasmids were co-transfected into 293T cells by calcium phosphate precipitation. Cells were lysed, and the cell lysates were incubated with anti-Myc Ab and protein G-Sepharose as described in the purification of bead-bound XLF-Myc-His. The beads were then washed four times with the lysis buffer (salt concentration as indicated under “Results”). Cell lysate and bound and unbound (sup) proteins were fractionated on 8% SDS-PAGE and detected by Western blot with anti-Myc Ab for DNA ligase IV and anti-GST Ab for XLF.

Immunopurification of XRCC4 from XLF-transfected 293T Cells—Wild type or mutant XLF-Myc-His-expressing plasmids were co-transfected into 293T cells by calcium phosphate precipitation. Immunopurifications were carried out as described in the above paragraph. Bound proteins were fractionated on 8% SDS-PAGE and detected by Western blot with anti-Myc Ab for DNA ligase IV and anti-GST Ab for XRCC4.

Immunostaining of XLF-transfected 293T Cells—Wild type or mutant XLF-Myc-His-expressing 293T cells were fixed in 1.5% paraformaldehyde in phosphate-buffered saline (PBS) permeabilized in 0.1% Nonidet P-40 with 0.25% gelatin and 0.01% saponin. Intracellular XLF was revealed by anti-Myc mAb and goat-anti-mouse IgG-fluorescein isothiocyanate conjugate as green. The nucleus DNA was stained with piperidine iodide as red.

Electrophoretic Mobility Shift Assay—The gel shift assay and the 60-bp DNA probe were described previously (9). The 83-bp DNA probe was generated by annealing 5’-32P-labeled oligonucleotide HL-62 and HL-110. The 90-bp DNA probe was annealed from HL-111 and HL-112. The 212-bp DNA probe was a purified PCR fragment from plasmid pEF-BOS.

In Vitro DNA Ligation Assay—The DNA oligonucleotides were gel-purified, and only the long oligonucleotide in each substrate was end-labeled by T4 polynucleotide kinase and [γ-32P]ATP. The labeled long oligonucleotide was annealed to the two specified short oligonucleotides in equimolar ratios. 0.5 pmol of the substrate was incubated with XRCC4-DNA ligase IV (in 25 mM Tris, pH 8.0, 50 mM KCl, 2.5 mM MgCl2, 1 mM DTT, 10% polyethylene glycol (PEG), 0.5 mM ATP, and 0.05 mg/ml bovine serum albumin (BSA)) or T4 DNA ligase (in 66 mM Tris-HCl, pH 7.5, (20 °C), 5 mM MgCl2, 5 mM DTT, 1.5 mM ATP, 10% PEG, and 0.05 mg/ml BSA) with or without XLF for 20 min at 37 °C. Purification by reconstitution with substrate on ice, incubated at room temperature for 15 min, and chilled on ice for 3 min before the substrate was added to initiate the ligation. The ligation prod-
Like XRCC4, XLF can also form homomultimers (5, 7, 13). It remains unclear whether XLF homodimer can bind DNA ligase IV without XRCC4 (5). We co-transfected DNA ligase IV-Myc and GST-XLF into 293T cells. Ligase IV was immunoprecipitated with anti-Myc protein G-Sepharose using 0.3 m NaCl and 0.5 m KCl for incubation and washing, respectively. Associated proteins were detected by Western blotting. Although the beads pulled down most of the ligase IV from the cell lysate, XLF could not be detected in the bound fraction (Fig. 1B). Low salt conditions (0.15 m NaCl) were also tested, but only ligase IV-independent association of GST-XLF to the immunobeads was observed (supplemental Fig. 4A, left panel). As a control, XRCC4-HA and GST-XLF were co-expressed in 293T cells, and XRCC4 was pulled down by anti-HA immunobeads using 0.15 m NaCl (supplemental Fig. 4A, right panel). Co-precipitation of XLF with XRCC4 suggests that the interaction between XLF and ligase IV, if any, is unstable and much weaker than the XRCC4-XLF interaction. Similar results were obtained using cell lysates from insect cells that overexpress DNA ligase IV, XLF, and XRCC4 (supplemental Fig. 4). Given that the interaction between XRCC4 and DNA ligase IV is extremely strong, XLF is probably recruited to the complex by XRCC4.

**XLF Stimulates Ligation of XRCC4-DNA Ligase IV**—Cell lysates from XLF-deficient cell lines have been shown to be unable to carry out NHEJ in vitro (6, 7). Because XLF interacts with XRCC4-DNA ligase IV directly, it is possible that XLF has an important role in the ligation of double-strand DNA breaks by the XRCC4-DNA ligase IV complex. XLF-Myc-His was purified using Ni-NTA followed by Mono Q (supplemental Fig. 2). Using the soluble XLF-Myc-His protein, we tested the effect of XLF in the ligation reactions by XRCC4-DNA ligase IV. The DNA substrate consists of a 95-nt oligonucleotide paired with two short oligonucleotides of 22 and 18 nt positioned near the termini (Fig. 2A). Hence, the substrate consists of a molecule that has two duplex DNA ends tethered by a region of single-stranded DNA. The open double-stranded DNA ends have 4-nt 3' overhangs that are fully compatible. The DNA substrate can self-annex and be ligated into a circular monomer (intramolecular ligation) or multiple substrate molecules can anneal with each other (at their ends) and be ligated as multimers. After ligation at 37 °C, the products were resolved on 5% denaturing PAGE as a direct readout.

Soluble XLF improved intermolecular ligation, indicated by mutimer formation. Using an equimolar amount of DNA ligase IV and DNA, the addition of a 2-fold molar amount of XLF increased the multimer ligation about 4-fold (Fig. 2B), similar to the extent of increase of ligation by XRCC4 observed previously (10). The increase in intermolecular ligation raised the possibility that XLF may bind DNA and facilitate the synapsis between different DNA molecules.

To assess any effect of the affinity tag on the recombinant protein, we purified XLF using the GST tag on the N terminus (supplemental Fig. 3) and tested the GST-XLF protein in the same ligation reactions. Similar to the soluble XLF-Myc-His, GST-XLF increased the dimer ligation 7- and 11-fold at 3- and 10-fold ratios of GST-XLF to XRCC4-DNA ligase IV (Fig. 2D, lanes 1 versus 2 and 3).
Bead-bound XLF-Myc-His was also tested in the ligation. Stimulation was observed at a lower concentration of XRCC4-DNA ligase IV, 8 nM, which is \( \frac{1}{6} \) that of the DNA substrate. Intramolecular ligation was improved 2.3- and 5.2-fold by the addition of 16 and 40 nM XLF, respectively (supplemental Fig. 5A). As a control, anti-Myc protein G-Sepharose was added to the ligation reaction, and this did not alter the ligation activity (supplemental Fig. 5B, lane 1 versus 3), whereas XLF-Myc-His protein G-Sepharose increased the ligation 8-fold (supplemental Fig. 5B, lane 1 versus 2). The level of intermolecular ligation (multimer formation) appeared unaffected by the addition of XLF. Because the XLF protein used here was purified as bead-bound protein, the XLF-XRCC4-DNA ligase IV complex formed is also bound to the beads. This may influence productive interaction of this ligation complex with multiple DNA substrates for intermolecular ligation.

One possible reason for the increase in ligation could simply be that XLF can stabilize DNA end annealing. To test this possibility we replaced XRCC4-DNA ligase IV with the T4 DNA ligase. At the same concentration of XRCC4-DNA ligase IV, T4 DNA ligase showed higher ligation activity (supplemental Fig. 6, lanes 1 and 4). The addition of XLF did not affect ligation activity (supplemental Fig. 6, lanes 2 and 3 versus 4). Even lower concentrations of T4 DNA ligase were used, and XLF still had no effect (data not shown). Therefore, the stimulation of ligation by XLF is specific to the XRCC4-DNA ligase IV complex.

Enhanced Ligation Is Not Due to Stimulated Charging of the DNA Ligase IV—A ligation reaction involves three steps. In the first step the ligase reacts with ATP to form the covalent enzyme-AMP-charged form of the ligase (14). In the second step this charged ligase transfers the AMP to the 5' phosphate group of the DNA 5' end to form a phosphoanhydride. This activates the 5' phosphate of one DNA strand by providing a good potential leaving group (AMP). In the third step the 3' hydroxyl of the other DNA strand attacks the phosphoanhydride to displace the AMP, thereby forming a 3',5'-phosphodiester bond. It is possible that the XLF could affect the recharging of the XRCC4-DNA ligase IV and thereby facilitate turnover of the XRCC4-DNA ligase IV.

To assess this we examined the time course of the ligation reactions. The reactions were assembled on ice, and the substrates were added last. Then the first aliquots (ice) were immediately taken out and mixed with reaction stop dye. The ligation reactions started rapidly even on ice (Fig. 2C, lane 1 and 4). The remainder of the reaction occurred at 37 °C, and aliquots were taken out at the time points indicated. As expected, soluble XLF improved intermolecular ligation (Fig. 2C, lanes 4–6 versus lanes 1–3). The level of ligation reached a plateau at 5 min and remained stable for 1 h (data for 20, 40, and 60 min not shown) despite an ample amount of unligated substrate. The ligase may conduct only one round of ligation. The addition of XLF did not change the time course of the reaction. The apparent reduction in circular monomer formation in the reactions with XLF may be due to depletion of ligase because of multimer ligation.

We also tested XLF for any effect on the charging of XRCC4-DNA ligase IV complex. Purified (precharged) XRCC4-DNA ligase IV was treated with NaPPi (to strip the AMP from the ligase) and then incubated with \( [\alpha^{32}P]ATP \). The addition of XLF did not alter the extent of charging of XRCC4-DNA ligase IV (supplemental Fig. 7) or ligase IV alone (data not shown). Given that XLF did not help recovery of the XRCC4-DNA ligase IV during the reaction, possibly

![Diagram](image.png)
XRCC4-DNA ligase IV cannot recharge after one round of ligation even though it is able to recharge after being stripped with NaPPi. Alternatively, it may have recharged but lost its ligation capability.

**XLF Binding to DNA Is Length-dependent**—The enhancement of ligation raised the possibility that XLF may bind DNA. We tested the affinity of XLF for DNA using electrophoretic mobility shift assay. The first probe that we used was a 60-bp fragment with 2- and 4-nt overhangs at the ends (Fig. 3A). XLF did not show any binding to this DNA. In other studies, 38- and 50-bp fragments were also not bound by XLF (data not shown).

XLF is homologous to XRCC4 (5, 7, 12, 13, 15). XRCC4 is known to have a long alpha-helical C-terminal region (16), and by homology, XLF is presumed to have such an alpha-helical C-terminal region as well. XRCC4 is known to bind 200 bp but not 100 bp of DNA (17). We reasoned that XLF might function similarly, and hence, we tested 83-, 90-, and 212-bp fragments (Fig. 3, B and C). We find that XLF binds to all of these lengths of DNA. To compare equivalent amounts of XLF per unit length of DNA, we also calculate the ratio of XLF to DNA in terms of molecules of XLF/10 bp of DNA. At 0.5 molecules of XLF/10 bp of DNA, XLF binds to all of the longer fragments. However, at this same concentration and even higher concentrations, XLF does not bind to the 60-bp DNA.

Hence, like XRCC4, the binding of XLF is length-dependent. The shorter alpha-helical C-terminal region of XLF (relative to XRCC4) means that the minimum size for binding is smaller (83 bp for XLF versus more than 100 bp for XRCC4) (Fig. 3 and Ref. 17).

**DISCUSSION**

Several aspects of XLF function have been elucidated by these studies. First, XLF improves the intermolecular ligation activity of XRCC4-DNA ligase IV by between 8- and 11-fold in direct assays of ligation. The bead-bound XLF stimulated mutant XLF-Myc was expressed in 293T cells, immunoprecipitated with anti-Myc protein G-Sepharose, and Western-blotted with anti-XRCC4 antibody to detect the endogenous XRCC4. The interaction between mutant XLF (R57G) and XRCC4 was much weaker than the wild type (WT) XLF, although the level of expression was very similar (Fig. 4B). The patient with the R57G mutation in the XLF protein had an NHEJ defect. The discrepancy between the in vivo and in vitro activity of mutant XLF led us to test the ability of XLF to interact with XRCC4-DNA ligase IV in vivo. The patient with the R57G mutation in the XLF protein had an NHEJ defect. The discrepancy between the in vivo and in vitro activity of mutant XLF led us to test the ability of XLF to interact with XRCC4-DNA ligase IV in vivo. The
FIGURE 3. XLF binding to DNA is length-dependent. A, XLF does not bind to DNA of 60 bp in length. The 60-bp DNA probe is illustrated on top of the gel. For all reactions DNA was added to 4 ng. Increasing amounts of XLF were added to lanes 2–4. The molar ratio of XLF to DNA as well as the amount in molecules of XLF per 10 bp DNA were indicated on the top of each lane. B and C, XLF can interact with DNA of 83 and 90 bp in length. An 83-bp DNA probe (B, lanes 2 and 3, and C) and a 90 bp DNA probe (B, lanes 5 and 6) were both shifted by XLF. D, XLF can associate with DNA of 212 bp in length. At the molar ratio of 20 XLF/DNA molecule, most of the DNA was shifted by bound XLF. E, binding curves corresponding to the data in A, C, and D and from replicates of these experiments (to permit determination of the deviations from the mean).
A mutant XLF (R57G) strongly stimulates XRCC4-DNA ligase IV activity in vitro but fails to translocate to the nucleus. A, XLF (R57G) exhibited greater stimulation of DNA ligase IV/XRCC4 when used in equivalent amounts to the wild type XLF (lanes 5 versus 4). The mutant protein XLF (R57G)-Mycl-His was purified the same way as bead-bound wild type XLF, binding of mutant XLF to XRCC4 is much weaker compared with wild type. 293T cells transfected with wild type XLF (WT), mock-treated, or transfected with mutant XLF (R57G) was immunoprecipitated (IP) with anti-Myc Ab and Western-blotted (WB) with anti-XRCC4 Ab to examine the association with the DNA ligase IV/XRCC4 complex in vivo. The amount of XLF protein (wild type or mutant) pulled down by the anti-Myc protein G-Sepharose was detected greater stimulation of DNA ligase IV by XLF under physiological conditions suggests that it exists within human cells predominantly in association with smaller molecular mass proteins and that the total molecular mass of the complex is ~175 kDa in 1 M NaCl (18). This is consistent with XRCC4/DNA ligase IV or XLF-XRCC4/DNA ligase IV configurations. Admittedly, the XLF association with XRCC4-DNA ligase IV may be less stable and less abundant. Hence, XLF may not be present in the predominant species of ligase IV within such analyses. Our findings that XLF association with XRCC4 ligase IV is sensitive to 650 mM KCl also raises this possibility (data not shown).

Our activity stimulation studies are done under physiologic salt conditions. The stimulation of XRCC4-ligase IV by XLF occurs even when XLF is bound to beads. However, this stimulation by immobilized XLF requires a preincubation between the XLF and the XRCC4-ligase IV (the DNA was not present). This suggests a reconfiguration between the immobilized XLF and the XRCC4-ligase IV. The pulldown of XRCC4 and DNA ligase IV by XLF under physiological conditions suggests that both XRCC4 and ligase IV are present in complexes with the XLF (Fig. 1A). Moreover, the XLF is immobilized at its C terminus. Hence, the C terminus mobility cannot be critical for this association with XRCC4-DNA ligase IV and cannot be important for the XLF stimulation of XRCC4-DNA ligase IV activity.

Within a putative complex containing all three proteins, the interactions between XRCC4 and DNA ligase IV and between with endogenous XRCC4 in crude extracts (Fig. 4B), but the purified mutant XLF stimulated XRCC4-ligase IV ligation activity extremely well. This may relate to the efficiency of the mutant XLF to reconfigure appropriately with the XRCC4-ligase IV complexes or with XRCC4 homodimers. Such reconfiguration on DNA ligation substrates (in the activity assays) may differ from reconfiguration without exogenous DNA (in the pulldown assays).

Third, XLF binds to DNA. The small size of XLF (33 kDa) means that 60 bp should be severalfold more than adequate to bind XLF if the binding occurred simply at the globular head domain region (16). The fact that 38, 50, and 60 bp are insufficient but that 83, 90, 212 bp are sufficient for XLF binding to DNA means that there is a minimum length requirement somewhere between 60 and 83 bp. As mentioned earlier, this is reminiscent of the requirement of >100 bp of DNA for the binding of the somewhat larger protein, XRCC4, which has a longer α-helical C-terminal region (17). This suggests that the α-helical C-terminal region of XLF and XRCC4, which both include many basic amino acids, aligns parallel to the DNA helix. Otherwise, the minimum DNA length might be expected to be <15 bp if the globular head domain alone were sufficient for DNA binding. An orientation with the C-terminal helices parallel to the DNA lends itself to many interesting hypotheses regarding the role of XLF and XRCC4. It is known that DNA ligase IV is able to ligate blunt and sticky end configurations without XRCC4 and XLF, but that XRCC4 improves the efficiency of this ligation (10). Perhaps XRCC4 and XLF improve the association of the ligase with DNA ends in vivo but in a less essential way in vitro.

Previous work raises the possibility of a trimeric interaction of XLF, XRCC4, and DNA ligase IV (5, 7). Earlier biochemical work on DNA ligase IV suggests that it exists within human cells predominantly in association with smaller molecular mass proteins and that the total molecular mass of the complex is ~175 kDa in 1 M NaCl (18). This is consistent with XRCC4-DNA ligase IV or XLF-XRCC4-DNA ligase IV configurations. Admittedly, the XLF association with XRCC4-DNA ligase IV may be less stable and less abundant. Hence, XLF may not be present in the predominant species of ligase IV within such analyses. Our findings that XLF association with XRCC4-DNA ligase IV is sensitive to 650 mM KCl also raises this possibility (data not shown).
XLF and XRCC4 appear to be the stronger ones based on the studies here (supplemental Fig. 9). The strength of the XRCC4 interaction with DNA ligase IV may determine the relative ratio of the \((\text{XRCC4})_2\)-DNA ligase IV species to a putative XLF-XRCC4-DNA ligase IV species or a higher order species containing the three proteins. The weak interaction of XLF with DNA ligase IV raises questions of the stability of complexes containing only DNA ligase IV and XLF. In the final stages of preparing this manuscript, another group reported that XLF can stimulate ligation and can bind DNA (13).

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