The EMBO Journal Vol.18 No.7 pp.2008–2018, 1999

DNA binding of Xrcc4 protein is associated with V(D)J recombination but not with stimulation of DNA ligase IV activity

Mauro Modesti, Joanne E.Hesse and Martin Gellert[1]

Laboratory of Molecular Biology, Building 5 Room 241, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892-0540, USA

[1]Corresponding author
e-mail: gellert@helix.nih.gov

Mammalian cells are protected from the effects of DNA double-strand breaks by end-joining repair. Cells lacking the Xrcc4 protein are hypersensitive to agents that induce DNA double-strand breaks, and are unable to complete V(D)J recombination. The residual repair of broken DNA ends in *XRCC4*-deficient cells requires short sequence homologies, thus possibly implicating Xrcc4 in end alignment. We show that Xrcc4 binds DNA, and prefers DNA with nicks or broken ends. Xrcc4 also binds to DNA ligase IV and enhances its joining activity. This stimulatory effect is shown to occur at the adenylation of the enzyme. DNA binding of Xrcc4 is correlated with its complementation of the V(D)J recombination defects in *XRCC4*-deficient cells, but is not required for stimulation of DNA ligase IV. Thus, the ability of Xrcc4 to bind to DNA suggests functions independent of DNA ligase IV.

Keywords: DNA ligase IV/end joining/ V(D)J recombination/XRCC4

Introduction

Cell viability is critically dependent on repair of DNA double-strand breaks (DSBs), which arise during recombination, or result from errors in replication or damage by external agents such as ionizing radiation (IR). In mammalian cells, DSBs are predominantly repaired by a process of illegitimate recombination called end joining (Critchlow and Jackson, 1998; Tsukamoto and Ikeda, 1998), in which broken ends are aligned and re-ligated without using a template. This alignment can be provided either by base-pairing interactions via micro-homologies of one to several nucleotides, or through protein factors that serve to bring termini into close proximity (Roth and Wilson, 1988; Thode *et al*., 1990).

End joining is also required for repair of site-specific DSBs generated during V(D)J recombination (Taccioli *et al*., 1993). Formation of coding joints (CJs) and signal joints (SJs) is dependent on many of the same end joining factors that are involved in the repair of IR damage (Jeggo, 1998). These factors include the DNA-dependent protein kinase (DNA-PK_{CS}) and its associated DNA-binding co-factor Ku (with 80 and 70 kDa subunits), the *XRCC4* gene product and DNA ligase IV.

The Xrcc4 protein plays a critical role in both end joining and V(D)J recombination (Li *et al*., 1995), and also during neurogenesis (Gao *et al*., 1998). In the XR-1 hamster cell line, which is deficient in *XRCC4*, end joining of linearized plasmids with complementary or mismatched ends is as efficient as in wild-type cells, but re-ligation of these broken ends is markedly more dependent on micro-homologies when mismatched ends are used (Kabotyanski *et al*., 1998). During V(D)J recombination of plasmid substrates in *XRCC4*-deficient cells, formation of CJs and SJs is markedly depressed, and the few junctions that are made also rely heavily on micro-homologies and are often accompanied by large deletions (Taccioli *et al*., 1993; Li *et al*., 1995; Gao *et al*., 1998).

In vivo, Xrcc4 can localize to the nucleus (Critchlow *et al*., 1997; Mizuta *et al*., 1997), associate tightly with DNA ligase IV (Critchlow *et al*., 1997; Grawunder *et al*., 1997), and can be phosphorylated (Critchlow *et al*., 1997; Leber *et al*., 1998). *In vitro*, Xrcc4 can stimulate the activity of DNA ligase IV (Grawunder *et al*., 1997). The yeast homologs of Xrcc4 and DNA ligase IV are required for end joining (Schar *et al*., 1997; Teo and Jackson, 1997; Wilson *et al*., 1997; Herrmann *et al*., 1998; Ramos *et al*., 1998). Taken together, these observations indicate that one role of Xrcc4 in end joining is to stimulate DNA ligase IV activity by physical interaction. However, the *in vitro* stimulatory effect of Xrcc4 on DNA ligase IV activity is modest (~5-fold; Grawunder *et al*., 1997) compared with the severe deficiency in CJ and SJ formation (~100-fold) during V(D)J recombination in *XRCC4*-deficient cells (Taccioli *et al*., 1993; Li *et al*., 1995; Gao *et al*., 1998). Moreover, in yeast cells deficient for the *XRCC4* homolog *LIF1*, end-joining efficiency on linear plasmids with cohesive ends is reduced and repair accuracy is lost (Herrmann *et al*., 1998). In contrast, yeast cells deficient for DNA ligase IV are still capable of end joining with accuracy (Schar *et al*., 1997; Teo and Jackson, 1997; Boulton and Jackson, 1998; Herrmann *et al*., 1998). Thus, Xrcc4 might have additional roles in end joining and V(D)J recombination.

In order to elucidate the role(s) of Xrcc4, we have undertaken a biochemical analysis of recombinant human Xrcc4, which has revealed novel molecular properties of the protein. Stimulation of DNA ligase IV by Xrcc4 is shown to involve the adenylation of the enzyme rather than the joining step itself. Xrcc4 also binds to DNA, and this activity appears to be correlated with its biological function, but is not required for its effect on ligase IV activity.

Results

Recombinant human Xrcc4

To investigate the functions of the human *XRCC4* gene product, an *XRCC4* cDNA was cloned from a human

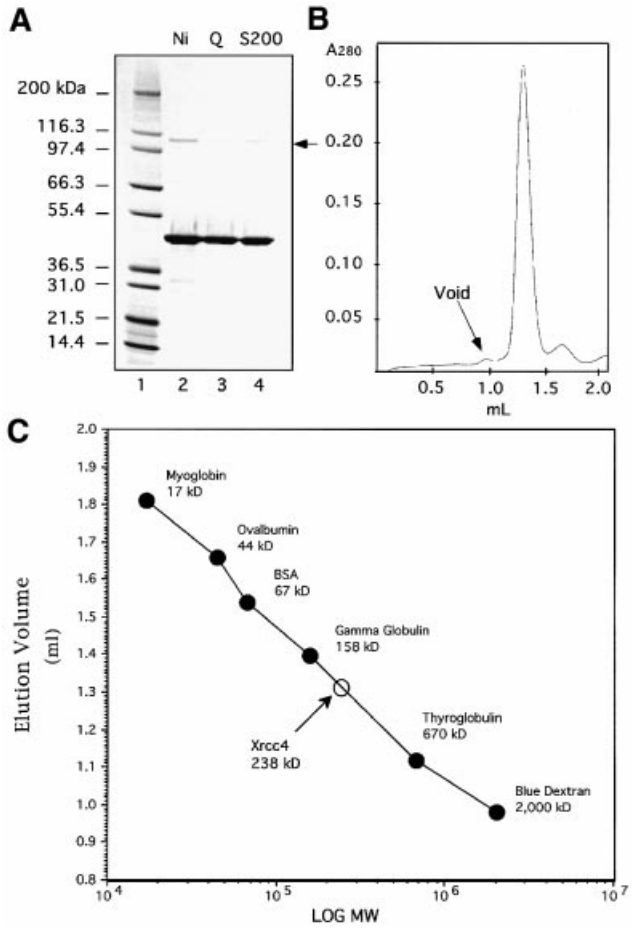

Fig. 1. Purification of recombinant human Xrcc4. (**A**) SDS–PAGE of Xrcc4 in reducing conditions. Xrcc4 protein (2 μg) after nickel affinity (Ni), Mono Q (Q) and gel filtration (S200) chromatography was loaded on the gel in lanes 2, 3 and 4, respectively. The arrow points to a DTT-resistant oligomeric form of Xrcc4. (**B**) Superdex 200 gel filtration of Xrcc4. (**C**) Size elution plot of Xrcc4, with Bio-Rad mol. wt markers.

thymus cDNA library and engineered by PCR for expression in *Escherichia coli*. This C-terminally histidine-tagged protein was purified to near homogeneity by nickel affinity followed by Mono Q chromatography and gel filtration (Figure 1A). When analyzed by SDS–PAGE in the presence of a reducing agent, most of the Xrcc4 protein migrated with an apparent mol. wt of ~50 kDa (the expected mol. wt with tag is 39 kDa). Analytical S-200 gel filtration of the native protein in the presence of 0.5 M KCl, 0.1% Tween 20, 2 mM dithiothreitol (DTT) and 10% glycerol revealed that the majority of the protein elutes at 238 kDa (Figure 1B and C) and thus forms a discrete homogeneous oligomer.

Lipofection of human Xrcc4 protein restores V(D)J recombination in XR-1 cells

V(D)J recombination can be induced to occur in non-lymphoid cell lines by transient expression of the *RAG1* and *RAG2* genes (Oettinger *et al*., 1990).

XR-1, an *XRCC4*-deficient chinese hamster ovary (CHO) cell line (Stamato *et al*., 1983), is severely defective in such assays, and expression of a human *XRCC4* cDNA is sufficient to fully complement the V(D)J defect (Taccioli *et al*., 1993; Li *et al*., 1995). To test the functionality of our Xrcc4 preparations, this V(D)J complementation assay was modified for protein uptake by lipofection. As expected, XR-1 cells were ineffective in formation of V(D)J SJs and CJs when either bovine serum albumin (BSA) or no protein was added (Table I). However, upon addition of recombinant Xrcc4 protein, formation of both SJs and CJs increased at least 100-fold, reaching wild-type recombination frequencies. Among the ampicillin and chloramphenicol-resistant recombinants, restoration of bona fide V(D)J SJ formation was verified by colony-lift and hybridization to an oligonucleotide with the sequence of a perfect SJ. These tests revealed that >99% of SJs tested were perfect. Similarly, restoration of V(D)J CJ formation was verified by screening for hybridization with a battery of six oligonucleotides designed to confirm the junction structure, which proved to be normal in 97% of the junctions tested. No detectable effect of Xrcc4 lipofection was observed in the CHO-K1 wild-type control cell line. Lipofection of Xrcc4 into the xrs-6 and V3 CHO cell lines, defective in the *Ku86* and *DNA-PK$_{CS}$* genes, respectively, did not complement their V(D)J recombination deficiency (data not shown).

Table I. Complementation of plasmid V(D)J recombination by lipofected Xrcc4 protein

Signal joints: substrate pJH200

Cell line	Protein lipofected	No. plasmids screened	Recomb. freq. (%)	Perfect joints (%) (*n*)[a]
XR-1	none	20 524	<0.005	–
	BSA	2550	<0.04	–
	Xrcc4	12 007	4.46	99.6 (255)
CHO-K1	none	10 618	1.34	95.3 (108)
	BSA	700	2.14	100.0 (15)
	Xrcc4	4685	1.45	100.0 (7)

Coding joints: substrate pJH290

Cell line	Protein lipofected	No. plasmids screened	Recomb. freq. (%)	Normal joints (%) (*n*)[a]
XR-1	none	1488	<0.07	–
	BSA	4495	<0.02	–
	Xrcc4	12 982	4.04	96.8 (279)
CHO-K1	none	20 453	0.95	95.8 (144)
	BSA	5765	0.88	100.0 (44)
	Xrcc4	14 980	0.71	99.0 (97)

[a]*n*, number of ampicillin and chloramphenicol resistant recombinants analyzed.

Recombinant human Xrcc4 interacts with DNA

The *XRCC4* gene product is required for completion of V(D)J recombination and resistance to agents that induce DNA DSBs (Stamato *et al*., 1983; Taccioli *et al*., 1993; Li *et al*., 1995). The function of Xrcc4 in the late stages of V(D)J recombination and DSB repair is thought to be due to its physical interaction with DNA ligase IV (Critchlow *et al*., 1997; Grawunder *et al*., 1997). In fact, all of the DNA ligase IV purified from HeLa cell nuclei is tightly associated with Xrcc4 (Robins and Lindahl, 1996). However, the mechanism of action of Xrcc4 in DNA repair is not fully understood. As an initial biochemical characterization, the ability of Xrcc4 to interact with DNA was tested by electrophoretic mobility-shift assay (EMSA).

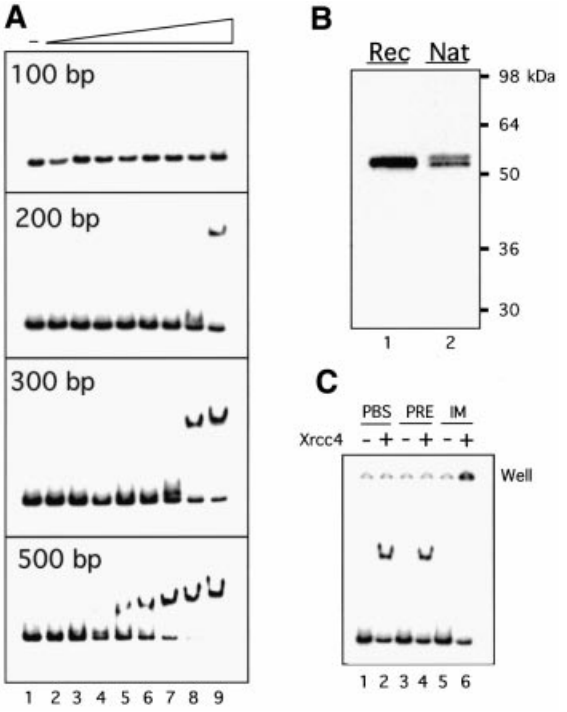

Fig. 2. Xrcc4 interacts with DNA. (**A**) EMSA with ϕX174 double-stranded DNA fragments of increasing size. Xrcc4 at concentrations increasing by 2-fold factors from 3 ng in lane 2 to 400 ng in lane 9 was incubated with 3 fmol of each radiolabeled DNA. (**B**) Immunodetection of native Xrcc4 in 10 μg of HeLa cell soluble protein extract (Nat) compared with 5 ng of recombinant Xrcc4 (Rec), with Xrcc4 affinity purified NIHC2 polyclonal antibody. The doublet detected in the HeLa cell protein extract is probably due to *in vivo* modification of Xrcc4 by phosphorylation. (**C**) Antibody super-shifting of Xrcc4–DNA complexes. A radiolabeled 300 bp ϕX174 double-stranded DNA fragment (3 fmol) was pre-incubated with 200 ng of Xrcc4 (lanes 2, 4 and 6) prior to addition of phosphate-buffered saline (PBS) pH 7.4 (lane 2, PBS), 0.9 μg of pre-immune IgG (lane 4, PRE) or 0.9 μg of immune Xrcc4 affinity-purified NIHC2 IgG (lane 6, IM), and incubated for 30 min at 37°C.

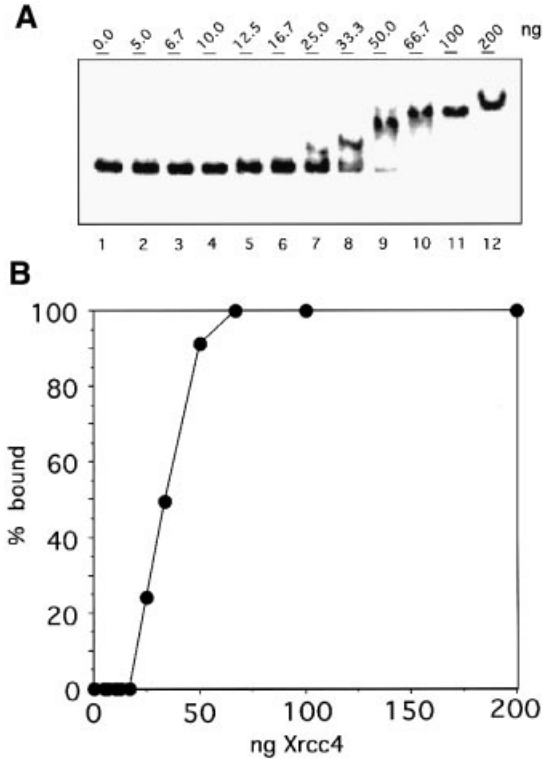

Fig. 3. Co-operative DNA binding of Xrcc4. (**A**) Titration of Xrcc4 with 3 fmol of a radiolabeled 543 bp DNA fragment. (**B**) Quantitation of the DNA-binding reactions shown above.

Linear fragments of ϕX174 double-stranded DNA of various sizes were radiolabeled and incubated with increasing amounts of Xrcc4 protein at a physiological salt concentration. Binding reaction mixtures were fractionated by PAGE. Stable DNA–protein complexes were detected (Figure 2A). For a given protein concentration, the amount of complex formed was a function of the DNA size (Figure 2A, compare lanes 7, 8 and 9), with greater stability on larger DNA molecules. To demonstrate that the complexes are dependent on Xrcc4 and not a fortuitous DNA-binding contaminant in the protein preparations, the complexes were incubated with a highly specific affinity-purified chicken polyclonal antibody raised against the full-length Xrcc4 protein (Figure 2B). Addition of immune antibody caused supershifting or aggregation of complexes in the well, while the same amount of pre-immune antibody had no effect on complex mobility (Figure 2C).

The DNA–Xrcc4 complexes were observed at KCl concentrations between 50 and 300 mM, and were formed independently of the presence of divalent cations (Mg, Mn, Ca, Zn), reducing agent or ATP (data not shown). Similar Xrcc4–DNA interactions were observed with DNA fragments from four different sources with unrelated sequences, indicating that the interaction is not sequence specific (data not shown). No interaction of Xrcc4 with single-stranded DNA was detected (data not shown).

Quantitative titration curves showed a sharp increase in the amount of complex formed over a narrow range of protein concentration and a progressive mobility decrease of the complexes with increasing amount of protein (Figure 3). This behavior is indicative of a co-operative mode of DNA binding.

Preferential binding of Xrcc4 to linear or nicked circular DNA

The affinity of Xrcc4 for various forms of DNA was tested in competition experiments. Protein–DNA complexes were first assembled on a linear 543 bp radiolabeled DNA fragment. After equilibrium was reached, unlabeled DNA was added to the binding reactions and the results are shown in Figure 4. Linear DNA was a more effective competitor than supercoiled or relaxed covalently closed DNA (Figure 4A and C–F), but the end structure of the linear DNA competitor did not affect the interaction (Figure 4D–F). Single-stranded DNA was not an effective competitor when tested similarly (data not shown). Interestingly, nicked circular DNA was the most effective competitor, 2-fold better than linear DNA and at least five times better than supercoiled DNA (Figure 4B).

Phosphorylated Xrcc4 still binds to DNA ligase IV, but not to DNA

Because Xrcc4 is a very effective substrate for DNA-PK phosphorylation (Critchlow *et al.*, 1997; Leber *et al.*, 1998; Figure 5A), we asked whether the DNA binding of Xrcc4 would be affected by DNA-PK phosphorylation *in vitro*. Recombinant Xrcc4 expressed and purified from

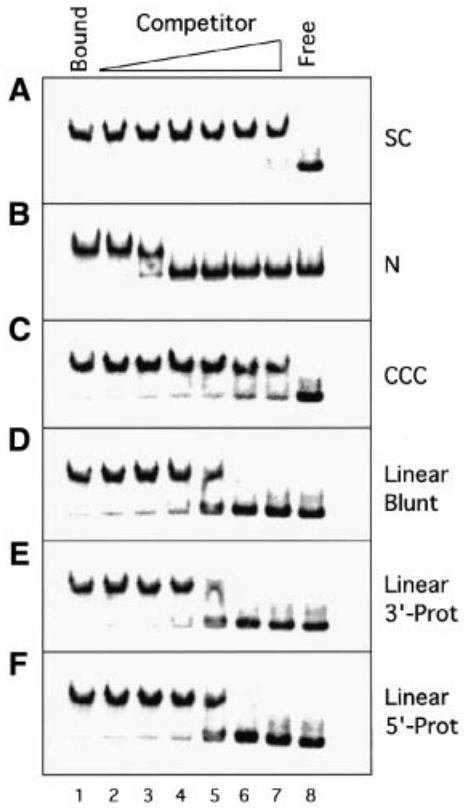

Fig. 4. Affinity of Xrcc4 for linear and nicked circular DNA. Complexes were pre-formed by incubation of 200 ng of Xrcc4 with 3 fmol of a radiolabeled 543 bp blunt end DNA fragment for 20 min at 37°C (lane 1). To compete for binding of Xrcc4 increasing amounts of various unlabeled ϕX174 double-stranded DNA species were added in 2-fold increments from 15.6 ng (lane 2) to 500 ng (lane 7) at 37°C for 30 min. Competitors were: (**A**) 90% negatively supercoiled, 10% nicked circles; (**B**) 100% nicked circles; (**C**) 90% relaxed covalently-closed circles, 10% nicked circles. (D–F) ϕX174 double-stranded DNA digested with: (**D**) *Stu*I, Blunt; (**E**) *Pst*I, 3′-protruding; and (**F**) *Apa*lI, 5′-protruding.

bacteria (Figure 5C, lane 2), was phosphorylated in a DNA-dependent manner by DNA-PK (Figure 5A) and repurified (Figure 5C, lane 3). An aliquot of the phosphorylated Xrcc4 was then treated with lambda protein phosphatase (Figure 5B) and repurified (Figure 5C, lane 4). The three Xrcc4 preparations (Figure 5C) were then tested for DNA binding by EMSA (Figure 5D). No complex formation was detected with phosphorylated Xrcc4, but complex formation was restored after phosphatase treatment, indicating that *in vitro* phosphorylation by DNA-PK abolishes DNA binding by Xrcc4.

The complex of DNA ligase IV with Xrcc4 is known to involve the C-terminal domain of ligase IV (amino acids 624–911; Critchlow *et al*., 1997; Grawunder *et al*., 1998a,b). This binding was unaffected by phosphorylation of Xrcc4 as shown by a simple protein EMSA (Figure 6). Xrcc4 was incubated with or without purified DNA ligase IV C-terminal domain (see Materials and methods), and proteins were resolved by native PAGE and detected by Coomassie Blue staining. Xrcc4 alone ran, as expected, as a single species, while the DNA ligase IV C-terminal domain did not enter the gel (Figure 6A, lanes 1 and 6). In contrast, incubation of Xrcc4 with the DNA ligase IV C-terminal domain yielded complexes of slower mobility than Xrcc4 alone (Figure 6A, lanes 2–5). The presence of both proteins in the complexes was verified by Western blotting (Figure 6B). Phosphorylated Xrcc4, which migrated faster than the non-phosphorylated form under these conditions (Figure 6C, compare lane 1 with 4), was as efficient as the non-phosphorylated form at assembling stable complexes with the DNA ligase IV C-terminal domain (Figure 6C, lanes 3 and 6).

Xrcc4 assists the binding of DNA ligase IV to DNA

Perhaps Xrcc4, which binds both DNA and DNA ligase IV, recruits ligase IV to DNA and thus facilitates access of ligase IV to sites of DNA damage. When tested alone by EMSA, the ligase IV C-terminal domain did not bind to DNA (Figure 7A, lane 4). However, when incubated with Xrcc4, 'ternary' complexes formed having even lower mobility than the binary Xrcc4–DNA complexes (Figure 7A, compare lanes 2 and 3). In a second test, full-length active human DNA ligase IV was expressed and purified from bacteria (see Materials and methods). When incubated alone with DNA, DNA ligase IV did not form stable complexes (Figure 7B, lane 4), while in the same conditions, co-incubation with Xrcc4 induced the formation of stable ternary complexes (Figure 7B, lane 3). The presence of both proteins in the ternary complexes was verified by supershifting the complexes with antibodies specific to Xrcc4 or the DNA ligase IV C-terminal domain (antibodies NIHC2 and TL18, respectively; data not shown). Thus, *in vitro*, Xrcc4 can facilitate the recruitment of DNA ligase IV to DNA.

DNA binding of Xrcc4 is not required for stimulation of DNA ligase IV joining activity

It has been reported that the addition of Xrcc4 to DNA ligase IV stimulates the ligase activity *in vitro* (Grawunder *et al*., 1997). This effect was reproduced here with recombinant human DNA ligase IV and Xrcc4 expressed and purified from bacteria. A 5-fold stimulation of nick repair (Figure 8B) and a 2.5-fold stimulation of cohesive end joining (Figure 8C) by DNA ligase IV was observed upon addition of Xrcc4.

However, we found that this stimulatory effect did not depend on the presence of DNA. First, the adenylation of DNA ligase IV (in the absence of DNA) was stimulated 3-fold during incubation with Xrcc4 (Figure 8A). Kinetic analysis revealed that the initial rate of adenylation increased 3-fold when Xrcc4 was present, and that adenylation was not complete at 30 min (data not shown). Perhaps stimulation of the DNA ligase IV joining activity by Xrcc4 occurred during adenylation, not directly requiring the ternary DNA-binding complex. Secondly, if enhanced DNA binding was a key factor, we might expect that phosphorylated Xrcc4 would not be able to stimulate the joining activity of DNA ligase IV since phosphorylated Xrcc4 does not bind DNA (even when bound to the C-terminal domain of DNA ligase IV; data not shown). In fact, just as with unphosphorylated Xrcc4, both the adenylation (data not shown) and cohesive end joining activity of DNA ligase IV (Figure 8D) were stimulated 3-fold by phosphorylated Xrcc4. Hence, in this system, the stimulation of DNA ligase IV joining activity by Xrcc4 appears to depend on its effect on adenylation rather than on the enhancement of DNA binding.

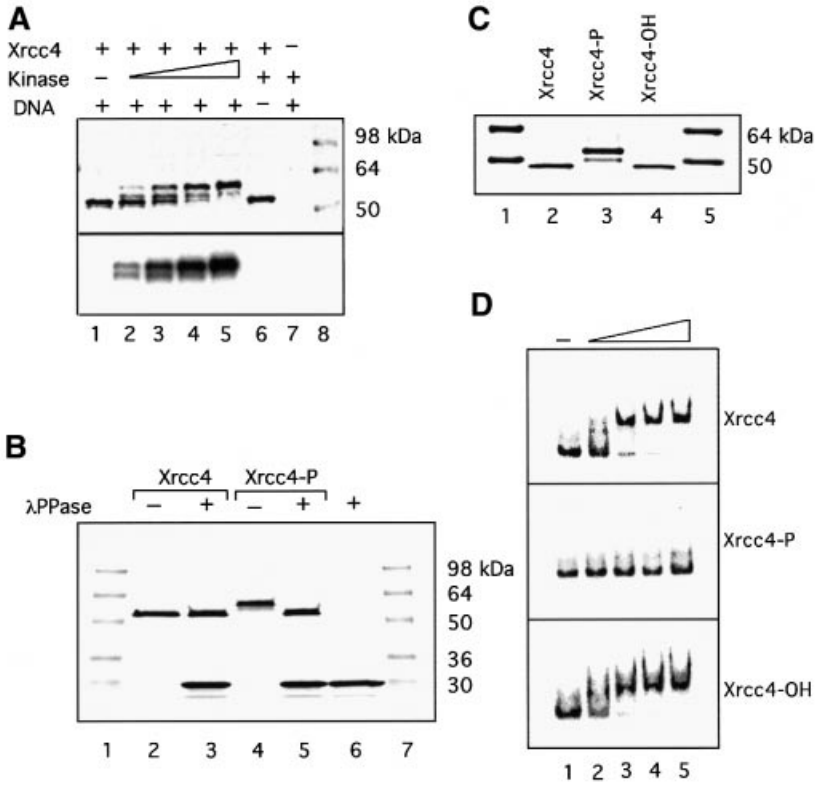

Fig. 5. Xrcc4 DNA binding is inhibited by *in vitro* DNA-PK phosphorylation. (**A**) Phosphorylation of Xrcc4 by DNA-PK *in vitro*. Xrcc4 (1 μg) was treated with 0, 50, 100, 200 or 400 U of DNA-PK in the presence of linear DNA (lanes 1–5), or without DNA (lane 6, 400 U of DNA-PK). Top panel, Coomassie-stained SDS–PAGE gel. Bottom panel, autoradiogram. (**B**) Phosphatase treatment of Xrcc4. One microgram of Xrcc4 (non-phosphorylated) or Xrcc4-P (phosphorylated) were mock-treated (lanes 2 and 4) or treated with 200 U of lambda protein phosphatase (lanes 3 and 5), resolved by SDS–PAGE and detected by Coomassie staining. (**C**) SDS–PAGE and Coomassie staining analysis of 200 ng of non-phosphorylated (lane 2, Xrcc4), phosphorylated (lane 3, Xrcc4-P) and dephosphorylated (lane 4, Xrcc4-OH) purified Xrcc4. (**D**) Gel mobility shift assay with a 543 bp radiolabeled DNA fragment. Three femtomoles of DNA were incubated with 0, 25, 50, 100 or 200 ng (lanes 1–5) of each of the Xrcc4 preparations shown in (C).

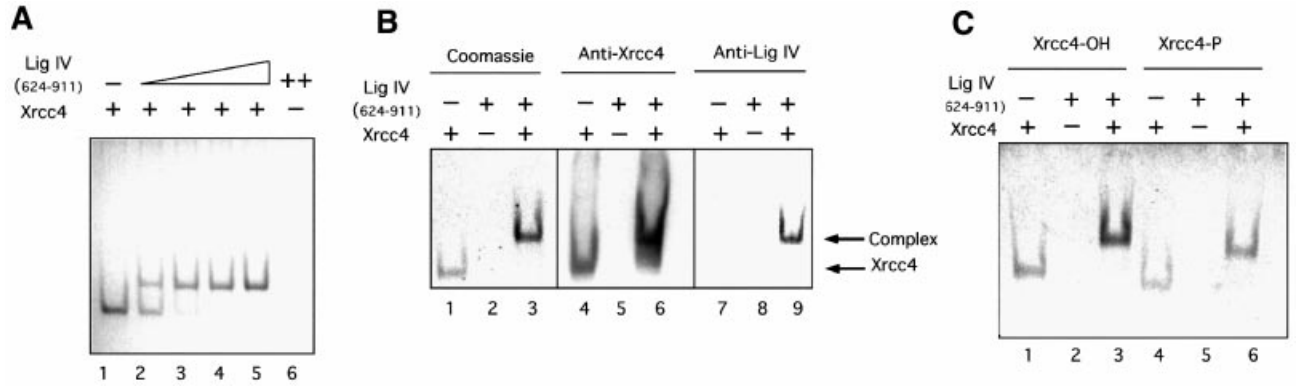

Fig. 6. *In vitro*-phosphorylated Xrcc4 interacts with the DNA ligase IV C-terminal domain. (**A**) Interaction of Xrcc4 with the DNA ligase IV C-terminal domain. Xrcc4 (1 μg) was incubated with increasing amounts of the DNA ligase IV C-terminal domain (0, 200, 400, 600 or 800 ng, lanes 1–5). Under the same conditions, 800 ng of DNA ligase IV C-terminal fragment did not enter the gel (lane 6). (**B**) Immunoblotting analysis demonstrating the presence of both proteins in the complexes. Binding reactions were performed using Xrcc4 (1 μg), either alone (lanes 1, 4 and 7) or in the presence of 1 μg of DNA ligase IV C-terminal domain (lanes 3, 6 and 9), or using 1 μg of DNA ligase IV C-terminal fragment alone (lanes 2, 5 and 8). After fractionation by native PAGE, proteins were detected by Coomassie staining (left panel), immunoblotting with anti-Xrcc4 polyclonal antibody NIH13 (middle panel) or with anti-DNA ligase IV polyclonal antibody TL18 (right panel). (**C**) *In vitro*-phosphorylated Xrcc4 still binds to the C-terminal fragment of DNA ligase IV. Non-phosphorylated Xrcc4 (1 μg, Xrcc4-OH) or phosphorylated Xrcc4 (1 μg, Xrcc4-P) was incubated either alone (lanes 1 and 4, respectively) or in the presence of 1 μg of DNA ligase IV C-terminal fragment (lanes 3 and 6, respectively).

Different portions of Xrcc4 support different activities

We wished to determine which parts of Xrcc4 are necessary to support its observed activities, including DNA binding, interaction with and stimulation of DNA ligase IV, and support of V(D)J recombination. Truncated polypeptides missing portions of either the N- or C-terminus of Xrcc4 were purified and tested (Figure 9A). Complementation of the V(D)J defect in XR-1 cells by the truncated Xrcc4 polypeptides was assessed by protein lipofection in the plasmid V(D)J assay described earlier. We found that the first 200 residues of Xrcc4 were sufficient to restore

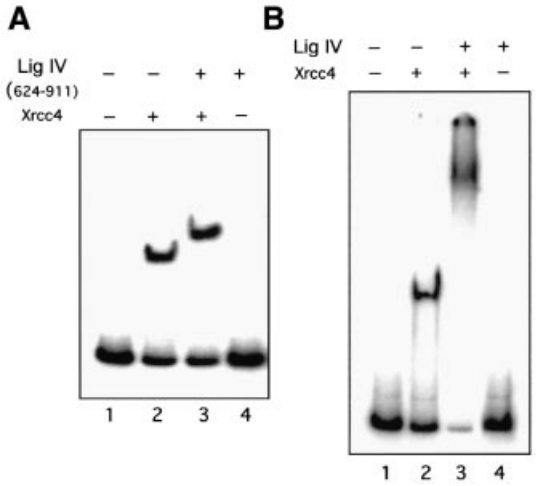

Fig. 7. Xrcc4 stimulates the DNA interaction of DNA ligase IV. (**A**) Gel mobility supershift of Xrcc4–DNA complex by the C-terminal domain of DNA ligase IV. Xrcc4 (200 ng, lanes 2 and 3) was incubated with 3 fmol of a 300 bp radiolabeled ϕX174 double-stranded DNA fragment prior to addition of 100 ng of the C-terminal domain of DNA ligase IV (lanes 3 and 4). (**B**) Gel mobility supershift of Xrcc4–DNA complex by full-length DNA ligase IV. Conditions are as in (A) except that 100 ng of recombinant DNA ligase IV was added (lanes 3 and 4).

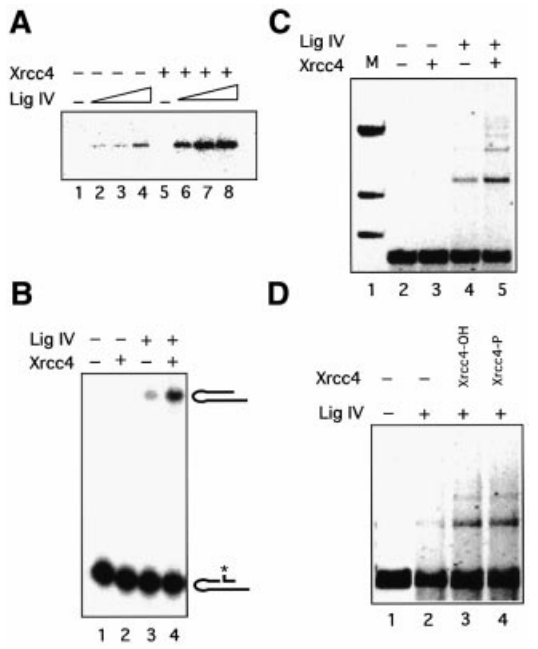

Fig. 8. Xrcc4 stimulates the adenylation and joining activity of DNA ligase IV. (**A**) Stimulation of the adenylation of recombinant DNA ligase IV by Xrcc4. Recombinant DNA ligase IV (20, 50 and 100 ng) was incubated with radiolabeled ATP in the absence of Xrcc4 (lanes 2, 3 and 4) or in the presence of 200 ng of Xrcc4 (lanes 6, 7 and 8). (**B**) Stimulation of nick-closing activity of DNA ligase IV by Xrcc4. Nicked substrate (0.02 pmol) was incubated with 10 ng of recombinant DNA ligase IV without (lane 3) or with (lane 4) addition of 20 ng of Xrcc4. (**C**) Stimulation of cohesive end ligation of DNA ligase IV by Xrcc4. *Apa*LI digested double-stranded ϕX174 DNA (100 ng) was incubated with DNA ligase IV (100 ng) without (lane 4) or with (lane 5) addition of 200 ng of Xrcc4. M, DNA size markers: 23.1, 9.4 and 6.5 kb. (**D**) Stimulation of cohesive end ligation of DNA ligase IV by phosphorylated Xrcc4. Reactions were performed as in (C), without addition of Xrcc4 (lane 2), or in the presence of 200 ng of unphosphorylated Xrcc4 (lane 3) or 200 ng of phosphorylated Xrcc4 (lane 4).

normal efficiency and accuracy of SJ and CJ formation in XR-1 cells (Table II, mutant 1–200), similar to a previously defined core consisting of the first 204 residues (Mizuta *et al.*, 1997; Leber *et al.*, 1998). However, N-terminal deletion of only the first 28 residues resulted in 70-fold decreased efficiency of SJ formation, and at least 80-fold decreased efficiency of CJ formation (Table II, mutant 29–336), similar to a mutant lacking the first 32 residues (Mizuta *et al.*, 1997) and to a mutant lacking the first 50 residues (Grawunder *et al.*, 1998a). No dominant-negative effects were detected in the V(D)J assay when these truncated proteins were tested in wild-type CHO-K1 cells (data not shown).

Is interaction of Xrcc4 with DNA ligase IV correlated with complementation of a V(D)J defect? We found that residues 168–200 of Xrcc4 were critical for binding of any of the truncated polypeptides to the C-terminal domain of DNA ligase IV (data summarized in Figure 9A). Surprisingly, the 29–336 protein, while unable to complement the V(D)J defects in XR-1 cells, was not only still competent to bind to the C-terminal domain of DNA ligase IV, but also to stimulate both the adenylation and joining activity (Figure 9A). Thus, binding of Xrcc4 to DNA ligase IV and stimulation of its activity is not sufficient for complementation of plasmid V(D)J recombination in XR-1 cells. This is consistent with an earlier study (Grawunder *et al.*, 1998a).

On the other hand, DNA binding of Xrcc4 did correlate with complementation of the V(D)J defect. When the proteins were tested for DNA binding, stable and discrete protein–DNA complexes were detected with full-length Xrcc4 as well as with the 1–200, 1–232 and 1–265 truncations, all of which can complement the plasmid V(D)J recombination defects in XR-1 cells (Figure 9A and B; not all data shown). The remaining mutants, none of which rescues V(D)J recombination in XR-1 cells, formed either no protein–DNA complexes (1–133 and 1–167) or only low levels of unstable complexes (29–336, 201–336, 234–336 and 267–336). These results suggest that polypeptides must include residues 1–28 as well as residues 168–200 to form stable protein–DNA complexes (Figure 9B, compare lanes 3 and 6), and that stable DNA binding of Xrcc4 is required for complementation of plasmid V(D)J recombination in XR-1 cells.

A titration of the 1–200 protein showed that its DNA binding still exhibits co-operativity, although to a lesser extent than the full-length protein (compare Figure 9C with 3A). The unstable binding of the 201–336, 234–336 and 267–336 truncations (as shown in Figure 9B, lane 4 for mutant 201–336) may suggest that residues 201–336 can enhance the co-operative DNA binding of Xrcc4.

Finally, the truncations were tested for *in vitro* phosphorylation by DNA-PK, and the results defined two phosphorylation regions; one between residues 233 and 265 and one between residues 267 and 336 (data summarized in Figure 9A). However, restoration of V(D)J recombination appeared to be independent of Xrcc4 phosphorylation; the C-terminal third of Xrcc4, including the two phosphorylation regions, is entirely dispensable for complementation of the plasmid V(D)J recombination defects in XR-1 cells. In addition, full-length Xrcc4 protein that had been phosphorylated by DNA-PK *in vitro*, restored plasmid V(D)J recombination with the same efficiency

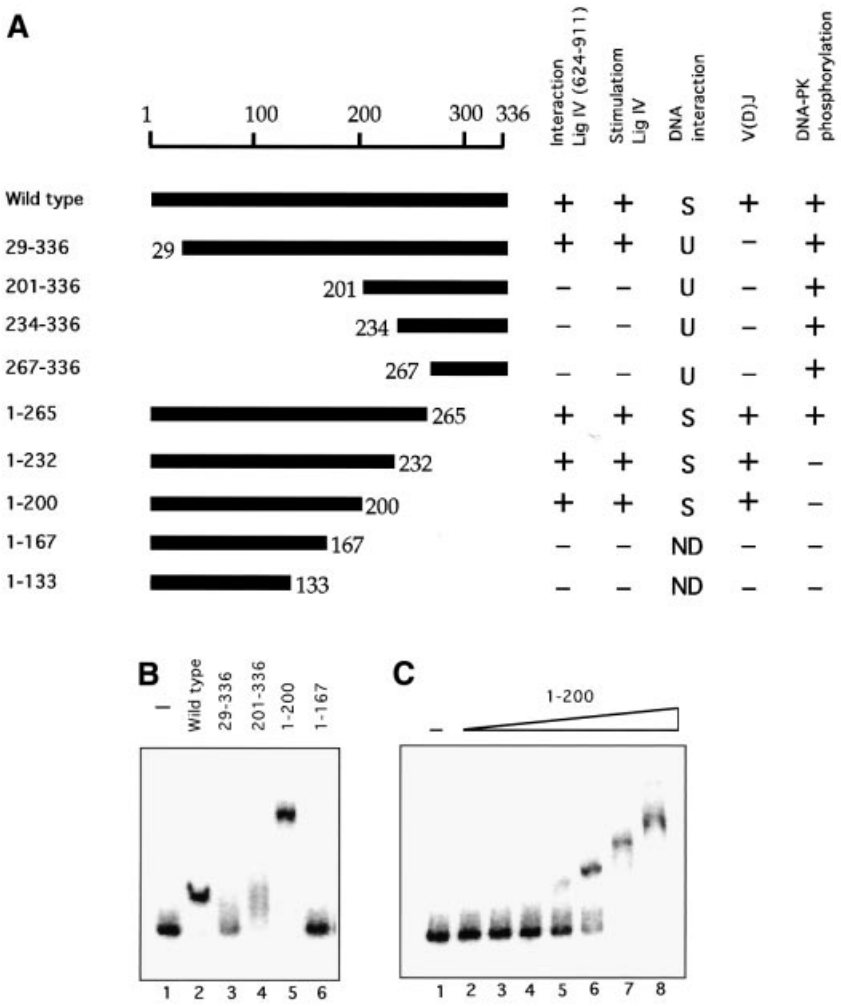

Fig. 9. Analysis of truncated forms of Xrcc4. (**A**) Summary of the properties of truncated forms of Xrcc4. The different truncated polypeptides are indicated as solid bars representing the residues preserved in the constructions. Binding to the DNA ligase IV C-terminal domain was tested by co-immunoprecipitation (see Materials and methods). Stimulation of DNA ligase IV was tested by adenylation and end joining assays as in Figure 8A and C. Binding to DNA was analyzed by EMSA using a 300 bp radiolabeled DNA fragment as shown in (B) (S, stable DNA binding; U, weak unstable DNA binding; ND, not detected). Complementation of the V(D)J recombination defects by lipofection in XRCC4-deficient cells is summarized from the results shown in Table II. DNA-PK phosphorylation was performed as indicated in Figure 5A. (**B**) A radiolabeled 300 bp DNA fragment (3 fmol) was incubated in the absence of Xrcc4 or in the presence of 100 ng full-length Xrcc4, or 200 ng of the 29–336, 201–336, 1–200 or 1–167 fragment (lanes 1–6, respectively), and analyzed by EMSA as in Figure 2. (**C**) Titration of the 1–200 Xrcc4 polypeptide incubated with DNA as in (B) and analyzed by EMSA. The protein was diluted in 2-fold increments starting with 200 ng in lane 8 to 3 ng in lane 2.

Table II. Complementation of V(D)J recombination by truncated Xrcc4 proteins

	Protein	Signal joints pJH200			Coding joints pJH290		
		No. plasmids screened	Rec. freq. (%)	Fraction perfect joints	No. plasmids screened	Rec. freq. (%)	Fraction normal joints
1	1–336 (WT)	2855	7.7	69/69	750	2.8	18/18
2	29–336	18 410	0.1	0/6	2960	<0.03	NA
3	201–336	35 030	0.03	0/12	6050	0.03	2/2
4	234–336	22 940	0.01	1/3	14 180	0.03	2/5
5	267–336	2420	<0.04	NA	2646	<0.04	NA
6	1–265	8340	7.1	51/51	8,700	3.9	31/32
7	1–232	14 910	7.2	41/44	11 590	3.7	30/30
8	1–200	11 900	7.2	36/38	6490	2.1	74/74
9	1–167	17 050	0.04	0/7	22 050	0.01	0/1
10	1–133	20830	0.01	0/3	27 150	0.01	0/3
11	Xrcc4-P	654	5.8	36/36	864	2.0	16/17

NA, not applicable.

and accuracy as the non-phosphorylated form when lipofected into XR-1 cells (Table II, Xrcc4-P). However, it is not certain that the phosphorylation was retained inside the cells.

Discussion

The *XRCC4* gene product is required for DSB repair by end joining and for SJ and CJ formation during V(D)J recombination (Li *et al*., 1995). The discovery that Xrcc4 can associate with and stimulate the *in vitro* joining activity of DNA ligase IV was an important advance in the understanding of its function (Critchlow *et al*., 1997; Grawunder *et al*., 1997). However, several observations indicated that Xrcc4 might have additional roles that are independent of its interaction with DNA ligase IV (see Introduction).

In this study, we found that Xrcc4 has a DNA-binding activity and stimulates the adenylation of DNA ligase IV.

Xrcc4 binds DNA

When we tested truncated Xrcc4 proteins by lipofection for their effect on plasmid V(D)J recombination in *XRCC4*-deficient cells (Figure 9A), we found that a polypeptide still capable of binding to ligase IV and stimulating its end-joining activity is not sufficient for complementation of the V(D)J recombination defects in these cells (Figure 9A, mutant 29–336). Another study has obtained a similar result with a different *XRCC4* mutation (Grawunder *et al*., 1998a). Hence, Xrcc4 has additional roles *in vivo* [at least for plasmid V(D)J recombination] that may not be associated with the function of DNA ligase IV.

In this biochemical analysis, we found that Xrcc4 binds co-operatively and in a sequence-independent manner to DNA (Figures 2 and 3) without any requirement for divalent cations or ATP. The formation of oligomeric protein complexes in solution (Figure 1B), the higher affinity for large DNA molecules (Figure 2A) and the co-operative DNA binding (Figure 3) indicate that the Xrcc4 DNA-binding mode is rather elaborate, involving multiple Xrcc4 molecules per DNA molecule. At the primary sequence level, we found by truncation mutant analysis that DNA binding involves several regions of the Xrcc4 polypeptide. The N-terminal region (1–28) and a central portion (168–200) are both required for stable DNA binding (Figure 9B). Interestingly, the 168–200 region has been shown to be necessary for self-interaction of Xrcc4 (Mizuta *et al*., 1997), suggesting that Xrcc4 protein–protein interactions are required for DNA binding. Furthermore, the C-terminal region (201–336), which is dispensable for complementation of the plasmid V(D)J recombination defects in *XRCC4*-deficient cells, does not form stable complexes with DNA, but when present appears to favor the co-operative DNA binding of Xrcc4 (compare Figure 9C with 3).

The role of DNA binding by Xrcc4 in DSB repair remains to be elucidated at the molecular level. However, Xrcc4 has been shown to stimulate DNA binding of the Ku protein, which is known to bind DNA ends (Leber *et al*., 1998). Interestingly, Ku stimulates the joining of blunt-ended DNA (but not cohesive ends) by mammalian DNA ligases (Ramsden and Gellert, 1998). Perhaps Xrcc4 promotes an end-joining pathway that is independent of micro-homologies, either directly via its own DNA binding and/or indirectly via stimulation of Ku DNA binding. In addition, by competition experiments (Figure 4), we have found that Xrcc4 has higher affinity for linear and nicked circular DNA than for closed-circular DNA, indicating that Xrcc4 may bind preferentially to sites of DNA damage. Further analysis of the Xrcc4–DNA interaction in the context of chromatin will be required to understand its function.

In order to test the biological relevance of the Xrcc4 DNA binding, we have performed a complementation analysis in *XRCC4*-deficient cells by mutant protein lipofection. The ability of Xrcc4 to bind DNA did correlate with its ability to complement the V(D)J defects in *XRCC4*-deficient cells (Figure 9A), suggesting that this property is required *in vivo*. However, further mutational studies will be required to establish this point definitively.

Xrcc4 stimulates adenylation of DNA ligase IV

As reported previously (Grawunder *et al*., 1997) and confirmed in this study (Figure 8B and C), Xrcc4 stimulates DNA joining by DNA ligase IV. In addition, Xrcc4 enhances DNA binding of DNA ligase IV (Figure 7). Thus, it was reasonable to postulate that the improved DNA binding explains the stimulation of DNA joining. Unexpectedly, we found that Xrcc4 stimulates the adenylation of DNA ligase IV in the absence of DNA. The initial rate of adenylation of DNA ligase IV is increased 3-fold when Xrcc4 is present, while we measured a 2.5- and 5-fold stimulation of cohesive end joining and nick closing, respectively. This raised the possibility that enhancement of ligation by Xrcc4 is due to its effect on the adenylation step and not related to the improved DNA binding of ligase IV. This explanation is made more plausible by the fact that ligation is still stimulated to the same extent by the phosphorylated form of Xrcc4, which no longer binds to DNA. Furthermore, the 29–336 protein, which is unable to complement the plasmid V(D)J recombination defects in *XRCC4*-deficient cells, stimulates both the adenylation and end-joining activity of DNA ligase IV in spite of the fact that it cannot bind DNA in a stable manner. It remains possible, of course, that the effect of Xrcc4 on DNA binding of ligase IV will prove to be more significant intracellularly.

In contrast, a previous study (Grawunder *et al*., 1997) concluded that Xrcc4 has no effect on adenylation of DNA ligase IV. However, in that report, adenylation of recombinant DNA ligase IV overexpressed and purified from *XRCC4*-deficient cells (and therefore devoid of Xrcc4) was compared with adenylation of endogenous DNA ligase IV that was already complexed with Xrcc4. This was a more oblique approach than the one we have used, where the effect of Xrcc4 is tested directly by addition of Xrcc4 to the ligase preparation.

The Xrcc4 protein binds to DNA ligase IV via a domain in the C-terminal region of the ligase that does not contain the active site for joining (Critchlow *et al*., 1997; Grawunder *et al*., 1998b). Consequently, the stimulatory effect of Xrcc4 on DNA ligase IV adenylation might suggest a conformational change induced at the active site upon binding of Xrcc4 to the DNA ligase IV C-terminal domain. In the cell, one role of Xrcc4 might be to ensure

that the pool of DNA ligase IV remains in a more fully adenylated state.

In light of the observation that heterochromatization factors are required for end joining in yeast (Tsukamoto *et al*., 1997; Boulton and Jackson, 1998), the full extent of the role of the Xrcc4 DNA-binding activity in end joining and V(D)J recombination will probably only be revealed in the context of chromatin or higher order chromosomal structures at DSB sites.

Potential roles of Xrcc4 phosphorylation

Xrcc4 is a nuclear phosphoprotein and is an effective *in vitro* substrate for the serine/threonine-specific DNA-PK (Critchlow *et al*., 1997; Leber *et al*., 1998; Figure 5A). It is interesting to note that the best substrates for *in vitro* phosphorylation by DNA-PK are DNA-binding proteins (Anderson and Carter, 1996). Consistent with a previous report (Leber *et al*., 1998), we show in this study that the C-terminal region of Xrcc4 (amino acids 233–336), dispensable for complementation of the V(D)J defects in *XRCC4*-deficient cells, is the target for DNA-PK phosphorylation. In addition, we found that within this C-terminal region, there are multiple phosphorylation sites, including at least one residue in the 233–265 interval and at least one residue in the 267–336 interval (Figures 2B, 5A and 9A).

To investigate the role of phosphorylation, we looked for interactions between the *in vitro* phosphorylated form of Xrcc4 and DNA ligase IV. We found that phosphorylated Xrcc4 binds to DNA ligase IV (Figure 6) and stimulates its adenylation and cohesive end joining activity to the same extent as non-phosphorylated Xrcc4 (Figure 8D). However, phosphorylated Xrcc4 was not able to bind DNA, either alone or when associated to the C-terminal domain of DNA ligase IV (Figure 5D; data not shown). This analysis suggests that DNA-PK can regulate the DNA–protein interaction of Xrcc4 or the Xrcc4–DNA ligase IV complex. In contrast, neither the protein–protein interactions nor the *in vitro* joining activity of the Xrcc4–DNA ligase IV complex appear to be affected by phosphorylation of Xrcc4 via DNA-PK.

Nevertheless, in the more complex cellular environment, the inhibitory effect of phosphorylation on the DNA binding of Xrcc4 could regulate the accessibility of Xrcc4 or the Xrcc4–DNA ligase IV complex to the DNA substrate, controlled by interplay between protein phosphatases and kinases. Alternatively, serine phosphorylation of Xrcc4 might provide a means for protein–protein interaction (Stone *et al*., 1994; Muslin *et al*., 1996) allowing association with other DNA repair factors such as the *MRE11–RAD50–NBS1* complex (Carney *et al*., 1998; Varon *et al*., 1998). These possibilities remain to be tested.

Materials and methods

Plasmid constructions

The human *XRCC4* open reading frame (ORF) was cloned and polyhistidine tagged at the 3′ end by *Pfu* PCR from a human thymus cDNA pool (Clontech), and subcloned into pET28a (Novagen) to generate pBMM42. The constructs allowing the expression of the truncated Xrcc4 polypeptides (Figure 9A, p29–336, p201–336, p234–336, p267–336, p1–265, p1–232, p1–200, p1–167 and p1–133) were generated from pBMM42 by *Pfu* PCR using 5′-end phosphorylated primers oriented in an outward direction relative to the *XRCC4* ORF in order to create in-frame deletion of *XRCC4* sequences. The DNA ligase IV C-terminal domain construct, pBMM126, includes amino acids Q624 to I911 (Grawunder *et al*., 1997). The partial ORF was cloned by *Pfu* PCR from a human DNA ligase IV cDNA (Wei *et al*., 1995), tagged with a hemagglutinin (HA) epitope (Wilson *et al*., 1984) and six histidine triplets, and subcloned into pET-17b (Novagen). The full-length DNA ligase IV expression construct, pDR119 (911 amino acids; Grawunder *et al.*, 1997) was generated by fusing a polyhistidine tag at the C-terminus and subcloning into pET28a.

All constructions were verified by DNA sequencing. Our *XRCC4* ORF has two additional triplets when compared with the previously published sequence. The lysine triplet 298 (Li *et al*., 1995) is replaced by the sequence AATTCTAGG, thus predicting a 336 residue polypeptide instead of 334.

Protein purification

All recombinant proteins in this work were produced in *E.coli* BL21(DE3)/pLysS cells (Novagen). For Xrcc4 purification, expression was induced by addition of IPTG to a final concentration of 1 mM and incubation for 2 h at 37°C. Proteins were extracted in 20 mM Tris–HCl pH 8.0, 0.5 M KCl, 20 mM imidazole pH 7.0, 20 mM β-mercaptoethanol, 10% glycerol, 0.2% Tween 20, 1 mM PMSF and 0.1 mg/ml lysozyme. After clarification by centrifugation at 30 000 *g* for 1 h, Xrcc4 was purified by nickel affinity chromatography (Qiagen). Proteins were eluted with a 40 ml linear gradient from 60 to 500 mM imidazole (elution at ~200 mM imidazole). Peak fractions were pooled and dialyzed against buffer A (20 mM Tris–HCl pH 8.0, 150 mM KCl, 2 mM DTT and 10% glycerol). The preparation was loaded on a Mono Q HR 5/5 column (Pharmacia) in buffer A. Proteins were eluted with a 40 ml linear gradient from 150 to 500 mM KCl. The Xrcc4 peak eluted at ~350 mM KCl. Peak fractions were pooled and dialyzed against buffer A (yield 10–12 mg/6 l of induced culture). Analytical gel filtration was performed on a Superdex 200 column in 0.5 M KCl, 20 mM Tris–HCl pH 8.0, 2 mM DTT, 0.1% Tween 20, 10% glycerol and 2 mM DTT. Fractions were analyzed by SDS–PAGE and Coomassie staining. For the DNA ligase IV C-terminal domain purification, the induction was performed overnight at 15°C. Purification by nickel affinity (elution at ~150 mM imidazole) and Mono Q chromatography (elution at ~250 mM KCl) was as described above (yield 20 mg/6 l induced culture). For the full-length DNA ligase IV purification, induction was as described for the C-terminal domain. The lysis buffer was modified by addition of 2% Triton X-100 and 0.5 M NaCl instead of KCl. The detergent was kept present during adsorption to the nickel affinity resin. However, during FPLC nickel affinity chromatography, the detergent was omitted in the buffers. The full-length DNA ligase IV eluted as a broad peak from 80 to 250 mM imidazole. Peak fractions were pooled and adsorbed on Blue Sepharose 6 Fast Flow resin (Pharmacia). Proteins were eluted by increasing the NaCl concentration in 0.25 M increments. Most DNA ligase IV eluted at 1 M NaCl and was dialyzed against buffer A. The preparation was further purified by Mono Q chromatography as described above (elution at ~350 mM KCl). Peak fractions were pooled and dialyzed against buffer A (yields were 0.1 mg/l of induced culture).

Transient V(D)J recombination assay

The hamster cell lines CHO-K1, Xrs6, V3 and XR-1 (Jeggo and Kemp, 1983; Stamato *et al*., 1983; Whitmore *et al*., 1989) were maintained in a humidifier chamber at 37°C with 5% CO_2 in F12 medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 20 mM HEPES pH 7.5, 200 μg/ml streptomycin and 200 U/ml penicillin (all reagents were from Gibco-BRL).

For transient V(D)J recombination assay by lipofection, DNA (0.5 μg of each plasmid: pJH548, *RAG*-1, pJH549, *RAG*-2, and either pJH200 for SJ assays or pJH290 for CJ assays) and Xrcc4 protein (2 μg) were incubated with 10 μl of liposome suspension (LipofectAMINE, Gibco-BRL) and processed according to the Bethesda Research Laboratory instructions for lipofection. Plasmids were extracted at 48 h post-transfection and analyzed for recombination. Signal and coding joints were qualitatively analyzed by colony lift hybridization as previously described (Oettinger *et al*., 1990; Sadofsky *et al*., 1994).

Gel mobility shift assays

The DNA substrates were radiolabelled by PCR and gel purified. RFI, RFII and single-stranded ϕX174 DNA were obtained from New England Biolabs. Standard DNA-binding reactions were performed in 150 mM KCl, 20 mM Tris–HCl pH 7.5, 2 mM DTT, 0.1% Triton X-100, 100 μg/ml acetylated BSA and 5% glycerol in 10 μl for 30 min at 37°C. DNA-binding reactions were performed in the linear range (trace amount of

DNA) by verifying that the percentage protein bound did not change after further dilution of the DNA substrate. The binding reactions were directly loaded on 6% acrylamide gels (Novex), and separated by electrophoresis at 120 V in 45 mM Tris–borate pH 8.3 and 1 mM EDTA for 80 min. Data were analyzed using a PhosphorImaging system (Molecular Dynamics). Protein mobility shifts were performed as described above except that no BSA was added in the binding reaction buffer. The gels were stained with Coomassie Blue, and the data analyzed by video imaging.

DNA ligase assays

Adenylation reactions were performed in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 5 mM DTT, 50 μg/ml BSA and addition of 5 μl of [α-^{32}P]ATP (800 Ci/mmol, 10 mCi/ml) in a final volume of 20 μl for 15 min at 37°C. Reactions were stopped by addition of 20 μl of protein loading buffer (20% glycerol, 4% SDS, 100 mM Tris–HCl pH 7.0, 100 mM DTT, 0.02% Bromophenol Blue). The samples were boiled and separated by SDS–PAGE. The gels were stained with Coomassie Blue to verify equal loading, dried and exposed. The nick-closing assay substrate was prepared by 5′-end labeling of 5′-AAATGTAAAACG-ACGGCCAGTG and annealing it to 5′-GCTTGGCACTGGCCGT-CGTTTTACATTTGAGACGTCCCAGCTTTTTTGCTGGGACGTCTC. Reactions were performed with 0.02 pmol of substrate in 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 μg/ml BSA in 10 μl for 10 min at 37°C. Reactions were stopped by addition of one volume of 95% formamide, 20 mM EDTA and separated by urea–PAGE. The cohesive end ligation assay substrate was prepared by digestion of double-stranded ϕX174 DNA with *Apa*LI. The resulting linear product was purified by phenol extraction and ethanol precipitation. Assays were performed with 100 ng of substrate in 50 mM Tris–HCl pH 7.5, 5 mM DTT, 0.1% Triton X-100, 120 mM potassium glutamate, 100 μg/ml BSA, 0.5 mM ATP, 5 mM $MgCl_2$ and 10% PEG-8K in 10 μl final volume for 2 h at 37°C. Reactions were stopped by addition of 2 μl of 0.1% SDS, 10 mM EDTA, 5% glycerol, 0.01% Bromophenol Blue and treated with proteinase K for 10 min at 37°C. The samples were separated by agarose gel electrophoresis in Tris–acetate buffer and stained with ethidium bromide.

Protein kinase and phosphatase assays

Standard phosphorylation assays were performed with 1 μg of Xrcc4 in 10 mM Tris–HCl pH 7.5, 1 mM DTT, 50 mM KCl, 10 mM $MgCl_2$, 5 mM ATP, 10 ng/μl DNA (BRL 100 bp ladder) and addition of 0.5 μl of [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/ml) and 200 U of DNA-PK (Promega) in 20 μl at 30°C for 20 min. Reactions were stopped by addition of 1 vol. of protein loading buffer and separated by SDS–PAGE. For purification of the phosphorylated form of Xrcc4, reactions were scaled up (50-fold) and stopped by addition of Wortmannin (Sigma) to a final concentration of 10 μM. The samples were diluted 10-fold prior to purification by nickel affinity chromatography using spin Ni-NTA columns (Qiagen). Phosphatase treatments were performed using 50 μg/ml of phosphorylated Xrcc4 and 200 U of lambda protein phosphatase as recommended (New England Biolabs). The sample was diluted 20-fold and purified by nickel affinity chromatography as described above.

Antibodies and immunodetection

Affinity-purified antibody TL18, raised against a C-terminal domain of human DNA ligase IV, was a gift from T.Lindahl (Wei *et al*., 1995). The polyclonal antibodies NIHC2 (chicken), NIH13 and NIH14 (rabbit) were raised against the full-length recombinant Xrcc4 using their standard protocol (Cocalico Biologicals, PA). Xrcc4 affinity purification was performed using Affigel 15 resin as recommended (Bio-Rad). Immunoblots were performed by transfer of SDS–PAGE gels on PVDF membranes in 25% methanol in 1× Tris–glycine buffer. After blocking overnight at 4°C in PBS pH 7.4, 0.2% Tween 20 and 3% non-fat dry milk, the blots were incubated for 2 h at room temperature with a 1/1000 dilution (0.9 μg/ml) of the NIHC2 Xrcc4 affinity purified primary antibody in blocking buffer. The blots were washed three times for 10 min with blocking buffer and incubated with an anti-chicken IgG secondary antibody coupled to horseradish peroxidase (Pierce), diluted 1/5000 in blocking buffer for 1 h at room temperature. The blots were washed three times with PBS pH 7.4, 0.2% Tween 20 and revealed using SuperSignal chemiluminescent substrates (Pierce). The interaction between the Xrcc4 truncations and the HA epitope-tagged C-terminal domain of DNA ligase IV was assayed by co-immunoprecipitation. Binding reactions were performed by incubation of 1 μg of the DNA ligase IV C-terminal fragment with 1 μg of full-length Xrcc4 or each of the truncated Xrcc4 polypeptides in 1 ml of PBS pH 7.4, 0.2% Tween 20 for 1 h at room temperature in a rotary shaker. Then, 4 μg of anti HA epitope mouse monoclonal antibody (Boehringer Mannheim) was added to each reaction and incubated for 1 h as above. Immune complexes were incubated with protein G–agarose for 1 h and collected by low speed centrifugation. After six washes with 1 ml of PBS pH 7.4/0.2% Tween 20, the immune complexes were resuspended in 100 μl of protein loading buffer, boiled and fractionated by SDS–PAGE. Xrcc4 polypeptides were detected by immunoblotting using rabbit polyclonal NIH13, which was previously tested for detection of all the Xrcc4 truncated polypeptides.

Acknowledgements

We thank Tomas Lindahl for the gift of antibody TL18 and a DNA ligase IV cDNA clone, and Dale Ramsden for the construction of plasmid pDR119. We are grateful to our colleagues in the Laboratory of Molecular Biology for valuable discussions.

References

Anderson,C.W. and Carter,T.H. (1996) The DNA-activated protein kinase: DNA-PK. *Curr. Top. Microbiol. Immunol.*, **217**, 91–111.

Boulton,S.J. and Jackson,S.P. (1998) Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing. *EMBO J.*, **17**, 1819–1828.

Carney,J.P., Maser,R.S., Olivares,H., Davis,E.M., Le Beau,M., Yates,J.R.,III, Hays,L., Morgan,W.F. and Petrini,J.H. (1998) The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair to the cellular DNA damage response. *Cell*, **93**, 477–486.

Critchlow,S.E., Bowater,R.P. and Jackson,S.P. (1997) Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV. *Curr. Biol.*, **7**, 588–598.

Critchlow,S.E. and Jackson,S.P. (1998) DNA end-joining: from yeast to man. *Trends Biochem. Sci.*, **23**, 394–398.

Gao,Y. *et al*. (1998) A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. *Cell*, **95**, 891–902.

Grawunder,U., Wilm,M., Wu,X., Kulesza,P., Wilson,T.E., Mann,M. and Lieber,M.R. (1997) Activity of DNA ligase IV stimulated by complex formation with XRCC4 protein in mammalian cells. *Nature*, **388**, 492–495.

Grawunder,U., Zimmer,D., Kulesza,P. and Lieber,M.R. (1998a) Requirement for an interaction of XRCC4 with DNA ligase IV for wild-type V(D)J recombination and DNA double-strand break repair *in vivo*. *J. Biol. Chem.*, **273**, 24708–24714.

Grawunder,U., Zimmer,D. and Lieber,M.R. (1998b) DNA ligase IV binds to XRCC4 via a motif located between rather than within its BRCT domains. *Curr. Biol.*, **8**, 873–876.

Herrmann,G., Lindahl,T. and Schar,P. (1998) *Saccharomyces cerevisiae* LIF1: a function involved in DNA double-strand break repair related to mammalian XRCC4. *EMBO J.*, **17**, 4188–4198.

Jeggo,P.A. (1998) Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. *Radiat. Res.*, **150**, S80–S91.

Jeggo,P.A. and Kemp,L.M. (1983) X-ray-sensitive mutants of Chinese hamster ovary cell line. Isolation and cross-sensitivity to other DNA-damaging agents. *Mutat. Res.*, **112**, 313–327.

Kabotyanski,E.B., Gomelsky,L., Han,J.O., Stamato,T.D. and Roth,D.B. (1998) Double-strand break repair in Ku86- and XRCC4-deficient cells. *Nucleic Acids Res.*, **26**, 5333–5342.

Leber,R., Wise,T.W., Mizuta,R. and Meek,K. (1998) The *XRCC4* gene product is a target for and interacts with the DNA-dependent protein kinase. *J. Biol. Chem.*, **273**, 1794–1801.

Li,Z., Otevrel,T., Gao,Y., Cheng,H.L., Seed,B., Stamato,T.D., Taccioli,G.E. and Alt,F.W. (1995) The *XRCC4* gene encodes a novel protein involved in DNA double-strand break repair and V(D)J recombination. *Cell*, **83**, 1079–1089.

Mizuta,R., Cheng,H.L., Gao,Y. and Alt,F.W. (1997) Molecular genetic characterization of XRCC4 function. *Int. Immunol.*, **9**, 1607–1613.

Muslin,A.J., Tanner,J.W., Allen,P.M. and Shaw,A.S. (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell*, **84**, 889–897.

Oettinger,M.A., Schatz,D.G., Gorka,C. and Baltimore,D. (1990) *RAG-1* and *RAG-2*, adjacent genes that synergistically activate V(D)J recombination. *Science*, **248**, 1517–1523.

Ramos,W., Liu,G., Giroux,C.N. and Tomkinson,A.E. (1998) Biochemical and genetic characterization of the DNA ligase encoded by *Saccharomyces cerevisiae* open reading frame YOR005c, a homolog of mammalian DNA ligase IV. *Nucleic Acids Res.*, **26**, 5676–5683.

Ramsden,D.A. and Gellert,M. (1998) Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks. *EMBO J.*, **17**, 609–614.

Robins,P. and Lindahl,T. (1996) DNA ligase IV from HeLa cell nuclei. *J. Biol. Chem.*, **271**, 24257–24261.

Roth,D.B. and Wilson,J.H. (1988) Illegitimate recombination in mammalian cells. In Kucherlapati,R. and Smith,G.R. (eds), *Genetic Recombination*. American Society for Microbiology, Washington, DC, pp. 621–654.

Sadofsky,M.J., Hesse,J.E. and Gellert,M. (1994) Definition of a core region of RAG-2 that is functional in V(D)J recombination. *Nucleic Acids Res.*, **22**, 1805–1809.

Schar,P., Herrmann,G., Daly,G. and Lindahl,T. (1997) A newly identified DNA ligase of *Saccharomyces cerevisiae* involved in RAD52-independent repair of DNA double-strand breaks. *Genes Dev.*, **11**, 1912–1924.

Stamato,T.D., Weinstein,R., Giaccia,A. and Mackenzie,L. (1983) Isolation of cell cycle-dependent γ ray-sensitive Chinese hamster ovary cell. *Somatic Cell Genet.*, **9**, 165–173.

Stone,J.M., Collinge,M.A., Smith,R.D., Horn,M.A. and Walker,J.C. (1994) Interaction of a protein phosphatase with an *Arabidopsis* serine-threonine receptor kinase. *Science*, **266**, 793–795.

Taccioli,G.E., Rathbun,G., Oltz,E., Stamato,T.D., Jeggo,P.A. and Alt,F.W. (1993) Impairment of V(D)J recombination in double-strand break repair mutants. *Science*, **260**, 207–210.

Teo,S.H. and Jackson,S.P. (1997) Identification of *Saccharomyces cerevisiae* DNA ligase IV: involvement in DNA double-strand break repair. *EMBO J.*, **16**, 4788–4795.

Thode,S., Schafer,A., Pfeiffer,P. and Vielmetter,W. (1990) A novel pathway of DNA end-to-end joining. *Cell*, **60**, 921–928.

Tsukamoto,Y. and Ikeda,H. (1998) Double-strand break repair mediated by DNA end-joining. *Genes Cells*, **3**, 135–144.

Tsukamoto,Y., Kato,J. and Ikeda,H. (1997) Silencing factors participate in DNA repair and recombination in *Saccharomyces cerevisiae*. *Nature*, **388**, 900–903.

Varon *et al*. (1998) Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell*, **93**, 467–476.

Wei *et al*. (1995) Molecular cloning and expression of human cDNAs encoding a novel DNA ligase IV and DNA ligase III, an enzyme active in DNA repair and recombination. *Mol. Cell. Biol.*, **15**, 3206–3216.

Whitmore,G.F., Varghese,A.J. and Gulyas,S. (1989) Cell cycle responses of two X-ray sensitive mutants defective in DNA repair. *Int. J. Radiat. Biol.*, **56**, 657–665.

Wilson,I.A., Niman,H.L., Houghten,R.A., Cherenson,A.R., Connolly, M.L. and Lerner,R.A. (1984) The structure of an antigenic determinant in a protein. *Cell*, **37**, 767–778.

Wilson,T.E., Grawunder,U. and Lieber,M.R. (1997) Yeast DNA ligase IV mediates non-homologous DNA end joining. *Nature*, **388**, 495–498.

Received January 22, 1999; revised and accepted February 12, 1999