Mammalian DNA double-strand break repair protein XRCC4 interacts with DNA ligase IV

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Background: Mammalian cells deficient in the XRCC4 DNA repair protein are impaired in DNA double-strand break repair and are consequently hypersensitive to ionising radiation. These cells are also defective in site-specific V(D)J recombination, a process that generates the diversity of antigen receptor genes in the developing immune system. These features are shared by cells lacking components of the DNA-dependent protein kinase (DNA-PK). Although the *XRCC4* gene has been cloned, the function(s) of XRCC4 in DNA end-joining has remained elusive.

Results: We found that XRCC4 is a nuclear phosphoprotein and was an effective substrate *in vitro* for DNA-PK. Human XRCC4 associated extremely tightly with another protein(s) even in the presence of 1 M NaCl. Co-immunoprecipitation and adenylylation assays demonstrated that this associated factor was the recently identified human DNA ligase IV. Consistent with this, XRCC4 and DNA ligase IV copurified exclusively and virtually quantitatively over a variety of chromatographic steps. Protein mapping studies revealed that XRCC4 interacted with ligase IV via the unique carboxy-terminal ligase IV extension that comprises two tandem BRCT (BRCA1 carboxyl terminus) homology motifs, which are also found in other DNA repair-associated factors and in the breast cancer susceptibility protein BRCA1.

Conclusions: Our findings provide a function for the carboxy-terminal region of ligase IV and suggest that BRCT domains of other proteins may mediate contacts between DNA repair components. In addition, our data implicate mammalian ligase IV in V(D)J recombination and the repair of radiation-induced DNA damage, and provide a model for the potentiation of these processes by XRCC4.

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Background

One of the most dangerous forms of damage that can befall a cell is the DNA double-strand break, which is the principal lethal lesion induced by ionising radiation and radiomimetic agents. Consequently, cells have evolved highly effective systems for recognising this type of DNA damage and ensuring that this damage is repaired efficiently. Two major pathways have evolved to repair DNA double-strand breaks in eukaryotes — homologous recombination and DNA non-homologous end-joining — and the available evidence suggests that both pathways are highly conserved from yeast to man. Much of what is currently known about DNA non-homologous end-joining in mammalian systems has been obtained from studies of mutant rodent cell lines that are hypersensitive to ionising radiation and display defects in DNA double-strand break repair (reviewed in [1–3]). Characterisation of these cell lines has revealed that they fall into three complementation groups, termed IR4, IR5 and IR7. The hamster cell line XR-1 defines IR4, IR5 comprises several independently isolated hamster cell mutants, and IR7 includes the hamster cell line V3 and cells derived from the severe combined immune-deficient (*scid*) mouse. The *scid* immune defect arises through a deficiency in the DNA non-homologous end-joining step of site-specific V(D)J recombination — a genomic rearrangement process that generates the vast range of antigen-binding sites of antibody and T-cell receptor proteins in developing lymphoid cells [4,5]. Transient transfection studies have shown that cells of the IR4 and IR5 groups are also defective in V(D)J recombination. Together, these results reveal that the genes defined by the IR4, IR5, and IR7 groups play crucial roles in the repair of ionising radiation-induced DNA damage and in the generation of antigen-binding diversity in the vertebrate immune system.

In light of the above findings, considerable effort has been directed towards establishing the nature of the gene products that are defective in cells of the IR4, IR5 and IR7 complementation groups, and determining how they function in DNA non-homologous end-joining. Cells of the IR5 and IR7 groups have been found to be deficient in components of the DNA-dependent protein kinase (DNA-PK). DNA-PK is a nuclear protein serine/threonine

kinase that is activated upon binding to DNA double-strand breaks or other perturbations of the DNA double-helix [6]. DNA-PK is a multiprotein complex that can be fractionated into two components: one of these components confers a DNA end-binding activity and corresponds to the heterodimeric Ku protein, comprising subunits of approximately 70 kDa and 80 kDa (Ku70 and Ku80, respectively); the other DNA-PK component, the catalytic subunit (DNA-PK_{cs}), is a polypeptide of ~465 kDa that, in association with DNA-bound Ku, mediates DNA-PK kinase catalytic function [7]. Notably, cells of the IR5 group lack DNA-PK activity because of a specific defect in Ku80 [8–11]. Conversely, cells of the IR7 group are deficient in DNA-PK activity because of the absence of functional DNA-PK_{cs} [12–14]. In contrast, XR-1 cells of the IR4 group are not deficient in DNA-PK activity [8,13,15,16]. Instead, DNA from human chromosome region 5q13–14 complements the deficiency in XR-1 cells, and the complementing gene has been termed *XRCC4* [17]. Moreover, Li *et al.* [18] have identified the *XRCC4* gene by its ability to confer normal V(D)J recombination activity and partially restore the double-strand break repair defect in XR-1 cells, and have demonstrated that the *XRCC4* locus is deleted in XR-1 cells. *XRCC4* encodes a small protein of 334 amino acid residues with a calculated molecular weight of 38 kDa, and the human and mouse XRCC4 homologues are approximately 75% identical [18]. Sequence analyses have revealed that XRCC4 is not significantly related to any previously characterised proteins. Therefore, although XRCC4 plays a crucial role in DNA double-strand break repair and V(D)J recombination, the cloning and sequencing of the cDNA for this factor have so far provided little information regarding its mechanism of action.

It appears highly unlikely that XRCC4, DNA-PK_{cs} and Ku80 are the only components of the mammalian DNA double-strand break repair apparatus. Given that Ku80 is invariably found in association with Ku70 and appears to require Ku70 for DNA binding, it is predicted that Ku70 will also function in DNA double-strand break repair. In addition, it is clear that a DNA ligase must act in DNA non-homologous end-joining, yet sequence analyses suggest that such a function is not mediated by DNA-PK_{cs}, Ku70, Ku80, or XRCC4. Mammalian cells contain at least four biochemically distinct DNA ligases, termed ligase I, II, III and IV and, although these enzymes have similar catalytic domains, they have unique carboxy-terminal and/or amino-terminal extensions and different functional specificities *in vitro*. Evidence suggests that the ligases perform specialised functions within the cell. For example, whereas DNA ligase I plays an essential role in joining Okazaki fragments during lagging-strand DNA replication [19–22], DNA ligase III interacts with the repair protein XRCC1 and is thought to function in DNA base excision repair [23–26]. Furthermore, a testis-specific splice variant of DNA ligase III is thought to play a role in meiotic recombination [27,28]. At present, it is unknown whether DNA ligase II arises from a separate gene or by alternative splicing of the ligase III transcript, and the physiological role for ligase II remains unclear [29–31]. Recent work has identified an additional mammalian DNA ligase, termed ligase IV, that has distinct biochemical properties and substrate specificities from the other ligases [23,32]; however, the biological function of DNA ligase IV remains unknown. In this present study, we show by a combination of immunological and biochemical approaches that ligase IV associates tightly and specifically with XRCC4. We discuss the implications of this finding in terms of XRCC4 and ligase IV function and in relation to the mechanism of DNA non-homologous end-joining.

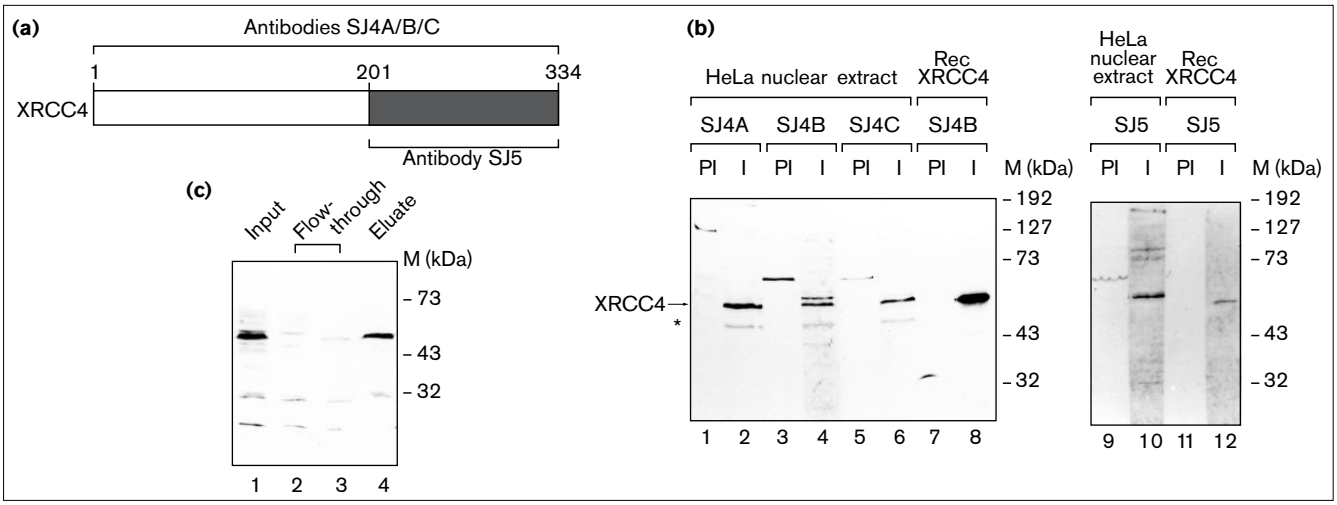
Results

Generation of antisera that recognise XRCC4

To investigate the mechanism of XRCC4 action, we decided to characterise the human protein biochemically. Full-length human XRCC4 and the carboxy-terminal region of XRCC4 comprising residues 201–344 were expressed in *Escherichia coli* as hexa-histidine-tagged proteins (Figure 1a). After purification to homogeneity, each protein was used as an immunogen to raise polyclonal antisera in rabbits. During these studies, we observed that recombinant full-length XRCC4 runs anomolously upon SDS–PAGE with an apparent molecular mass of ~55 kDa, which is considerably larger than the predicted molecular weight of 38 kDa (for example, Figure 1b, lane 8 and Figure 2b). Untagged and histidine-tagged versions of XRCC4 behave similarly (data not shown). Although the reason for this electrophoretic behaviour is not fully clear, it may reflect the fact that XRCC4 contains a high proportion of acidic residues, which may reduce the amount of SDS bound to the protein and decrease its mobility upon SDS–PAGE [33].

Western blot analyses revealed that each of the anti-XRCC4 antisera raised could recognise less than 1 ng of recombinant XRCC4 (data not shown and Figure 1b). To establish whether these antisera could detect endogenous XRCC4 in mammalian cell lysates, crude nuclear extracts from HeLa cells were subjected to SDS–PAGE followed by western immunoblot analysis. Each antiserum but none of the pre-immune sera recognised a HeLa cell protein of 55–60 kDa, in agreement with the size of recombinant XRCC4 (Figure 1b; the band marked with an asterisk probably represents an amino-terminal XRCC4 proteolytic product because it is recognised by all sera raised against full-length XRCC4 but not by serum SJ5 which was raised against the XRCC4 carboxy-terminal region). Interestingly, we have been unable to detect XRCC4 in extracts of mouse or hamster cells by direct western blotting using these antibodies (data not shown). One possibility is that this reflects low immunological

Figure 1



Generation and purification of anti-XRCC4 antisera. **(a)** Generation of antisera recognising full-length XRCC4 (residues 1–344) and the XRCC4 carboxy-terminal domain (residues 201–344) to produce SJ4 (rabbits SJ4A/B/C) and SJ5, respectively. A representation of the regions of XRCC4 to which antisera were raised is shown. **(b)** Detection of XRCC4 in HeLa cell nuclear extracts. HeLa cell nuclear extract (50 μg per lane; lanes 1–6,9,10) was subjected to western immunoblot analysis using pre-immune (PI) or immune (I) sera derived from rabbits SJ4A, SJ4B, SJ4C, or SJ5, as indicated. Each immune serum recognises endogenous HeLa XRCC4 as a ~55 kDa protein. Recombinant XRCC4 (5 ng) was also run alongside (lanes 7,8,11,12). The position of XRCC4 is indicated by an arrow, and an asterisk marks the position of a possible amino-terminal XRCC4 proteolytic product. **(c)** Affinity purification of antiserum SJ4B. Crude SJ4B serum (input) was passed over the XRCC4 affinity column. The depleted serum that flowed through was collected (flow-through) and the column was then washed extensively. Finally, bound antibody was eluted at low pH (eluate). Samples of input, flow-through and eluate were tested for their ability to detect XRCC4 in 50 μg of whole cell HeLa protein extract by immunoblotting.

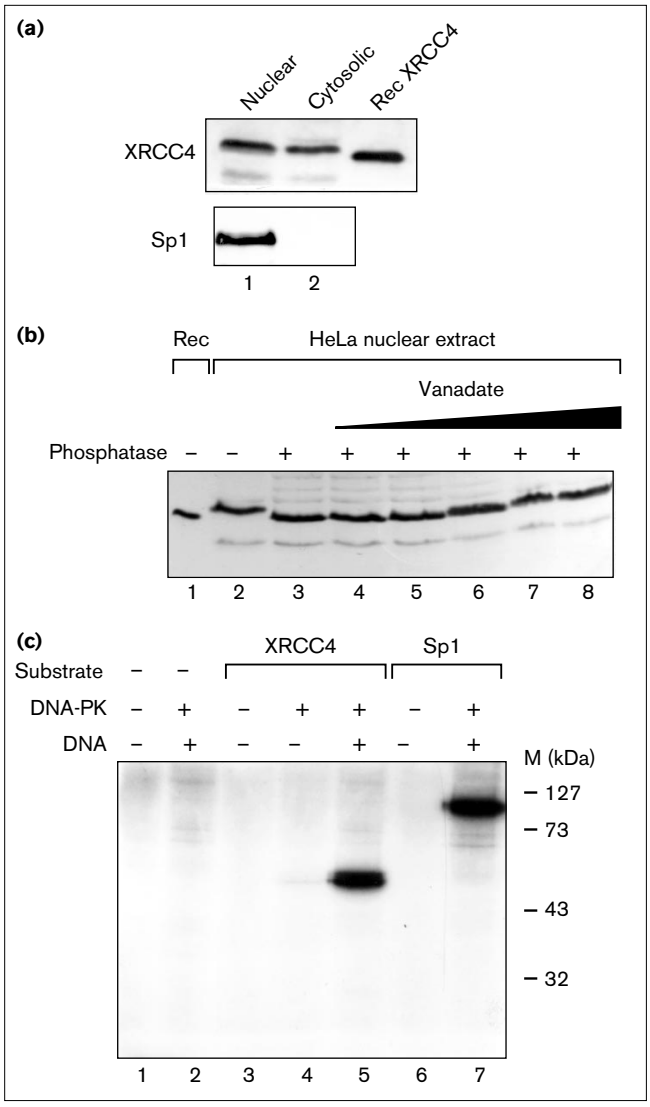
cross-reactivity between the human and rodent proteins. Given the high degree of sequence conservation between rodent and human XRCC4 [18], however, the model that we favour is that XRCC4 is expressed at lower levels in rodent cells than in human cells, as is the case for other DNA double-strand break repair factors, such as Ku and DNA-PK_{cs} [13,34]. The specificity of anti-XRCC4 antiserum SJ4B was further enhanced by subjecting it to immunoaffinity chromatography using XRCC4 covalently attached to Sepharose beads. Significantly, whereas the crude serum recognises a number of polypeptides in HeLa whole cell extracts in addition to full-length XRCC4 (Figure 1c, input), much of the reactivity towards the other proteins is recovered only in the flow-through fractions, resulting in the affinity-purified material (eluate) having improved specificity and selectivity for full-length XRCC4 compared with the unfractionated serum (Figure 1c).

XRCC4 is a nuclear phosphoprotein and serves as an effective substrate for DNA-PK *in vitro*

As a first step towards establishing the biochemical function of XRCC4, we determined its subcellular localisation. Nuclear and cytosolic fractions were prepared from HeLa cells and were subjected to western blot analysis using the affinity-purified XRCC4 antibody SJ4B (Figure 2a). The integrity of the fractions was established by additionally probing with antiserum against Sp1, which is located predominantly in the nucleus. These studies revealed that XRCC4 is present in the nuclear extract, with some protein also being detectable in the cytosolic fraction. The presence of XRCC4 in the nucleus is consistent with models in which this factor serves as part of a DNA double-strand break repair apparatus.

During the course of the above studies, we observed that HeLa XRCC4 reproducibly migrated more slowly than recombinant XRCC4 upon SDS–PAGE (for example, Figures 1b and 2a), suggesting that human XRCC4 is modified post-translationally. To determine whether this is due to phosphorylation of XRCC4, HeLa nuclear extract was either mock-treated, treated with λ protein phosphatase, or treated with λ phosphatase in the presence of the phosphatase inhibitor sodium orthovanadate (Figure 2b). Western analysis of these samples revealed that treatment with λ phosphatase increased the electrophoretic mobility of HeLa XRCC4 to that of the recombinant protein, and this effect was abrogated by treatment with the phosphatase inhibitor. These data therefore reveal that XRCC4 is phosphorylated to a high level in HeLa cell extracts and suggest that this modification might modulate XRCC4 activity *in vivo*. In the light of this phosphorylation and the similarity between XRCC4-deficient cells and those defective in components

Figure 2



HeLa XRCC4 is a phosphoprotein and is phosphorylated efficiently by DNA-PK *in vitro*. **(a)** XRCC4 is present in nuclear and cytoplasmic extracts. Equivalent amounts (10 μg) of nuclear and cytosolic extracts were resolved by SDS–PAGE, and XRCC4 was detected by western blotting with affinity-purified SJ4B antiserum. To determine the purity of the extracts, blots were reprobed with an antibody against the transcription factor Sp1, which is predominantly nuclear. **(b)** XRCC4 is phosphorylated to high stoichiometry in HeLa cell nuclear extracts. Untreated HeLa nuclear extract (50 μg; lane 2), HeLa nuclear extract that had been treated with λ protein phosphatase (lane 3), HeLa nuclear extract that had been treated with λ protein phosphatase in the presence of 0.1 μM, 1 μM, 10 μM, 0.1 mM and 1 mM of the phosphatase inhibitor sodium orthovanadate (lanes 4–8), or recombinant XRCC4 as a size control (lane 1) were analysed by immunoblotting using anti-XRCC4 antiserum (SJ4B), as indicated. **(c)** XRCC4 is phosphorylated by DNA-PK *in vitro* in a DNA-dependent manner. DNA-PK was used in kinase reactions containing either 50 ng of Sp1 or XRCC4 in the absence or presence of linear DNA as indicated.

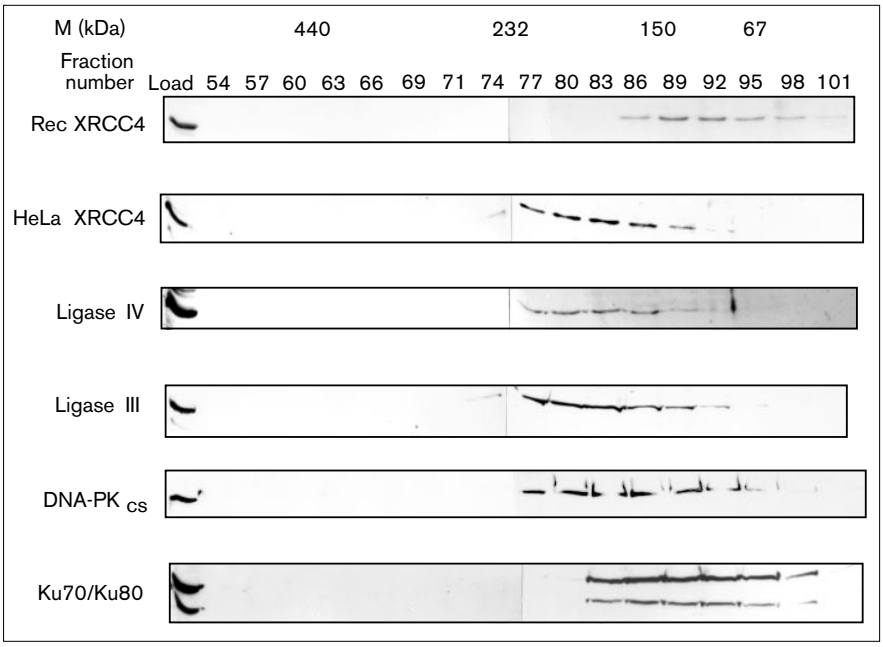
of DNA-PK, we tested whether DNA-PK could phosphorylate XRCC4 *in vitro*. As shown in Figure 2c, XRCC4 is as an effective substrate for DNA-dependent phosphorylation by DNA-PK, suggesting that DNA-PK might control XRCC4 activity.

Endogenous XRCC4 is complexed with another protein(s)

There are various ways in which XRCC4 might function in DNA double-strand break repair and V(D)J recombination. One possibility is that XRCC4 might interact directly with DNA; however, we have been unable to detect binding of recombinant XRCC4 to various DNA species in electrophoretic mobility shift assays (data not shown). Furthermore, when HeLa nuclear extracts are passed through DNA–agarose columns under salt concentrations that retain many DNA-binding proteins, the majority of endogenous XRCC4 protein flows through the column (data not shown). These data therefore argue that XRCC4 does not bind avidly to DNA. Another possible role for XRCC4 is to interact with another component of the DNA double-strand break repair apparatus. To address this possibility, we investigated the biochemical fractionation of XRCC4 and other known and potential DNA double-strand break repair factors upon gel-filtration chromatography using a Superose-6 column. These experiments were performed under stringent conditions of 1 M NaCl to disrupt possible non-specific protein–protein associations. The elution profile from gel-filtration analysis of pure recombinant untagged XRCC4 was consistent with a mass of around 100 kDa (Figure 3), which is larger than the predicted molecular weight of the XRCC4 monomer and its apparent mass determined by SDS–PAGE (Figure 1b). These data suggest that XRCC4 is either a monomeric protein that behaves anomolously upon gel-filtration analysis, or exists as a multimer.

Gel-filtration analysis of HeLa nuclear extract in the presence of 1 M NaCl revealed that endogenous XRCC4 fractionated in a manner consistent with a molecular mass of around 200 kDa, which is markedly higher than that of recombinant XRCC4 (Figure 3). HeLa XRCC4 might therefore be associated with another protein(s), possibly a component of the double-strand break repair apparatus. To evaluate this proposal, the same set of HeLa extract gel-filtration fractions were examined for the presence of Ku, DNA-PK_{cs}, and DNA ligases I, III and IV. Although some overlap was evident in each case, the XRCC4 elution profile did not parallel the profiles exhibited for DNA ligase I or Ku (Figure 3 and data not shown): DNA ligase I peaked at ~150 kDa, which is slightly larger than the predicted monomer molecular weight of 102 kDa (data not shown), and Ku elution peaked at ~150 kDa, consistent with the predicted size of a Ku70/Ku80 heterodimer. These data therefore argue against a stable interaction between XRCC4 and DNA ligase I or Ku. XRCC4 had a similar elution profile to DNA-PK_{cs} (465 kDa), which eluted at a lower molecular weight than expected (~200 kDa). Furthermore, the elution profile of XRCC4

Figure 3



Gel-filtration analysis of recombinant and HeLa XRCC4. Recombinant untagged (Rec) XRCC4 was loaded onto a Superose-6 gel filtration column in the presence of 1 M NaCl. Fractions were analysed by SDS–PAGE and Coomassie-blue staining. An identical gel-filtration run was performed with HeLa nuclear extract and fractions were analysed by immunoblotting with antibodies specific for XRCC4, DNA ligase III, DNA ligase IV, Ku70, Ku80 or DNA-PK_{cs}. The elution profiles of the molecular weight standards (Pharmacia) ferritin (460 kDa), catalase (232 kDa), aldolase (150 kDa) and albumin (67 kDa) are indicated.

was found to be virtually identical to those of DNA ligases III and IV. These data therefore raise the possibility that XRCC4 interacts stably with DNA-PK_{cs}, DNA ligase III or DNA ligase IV.

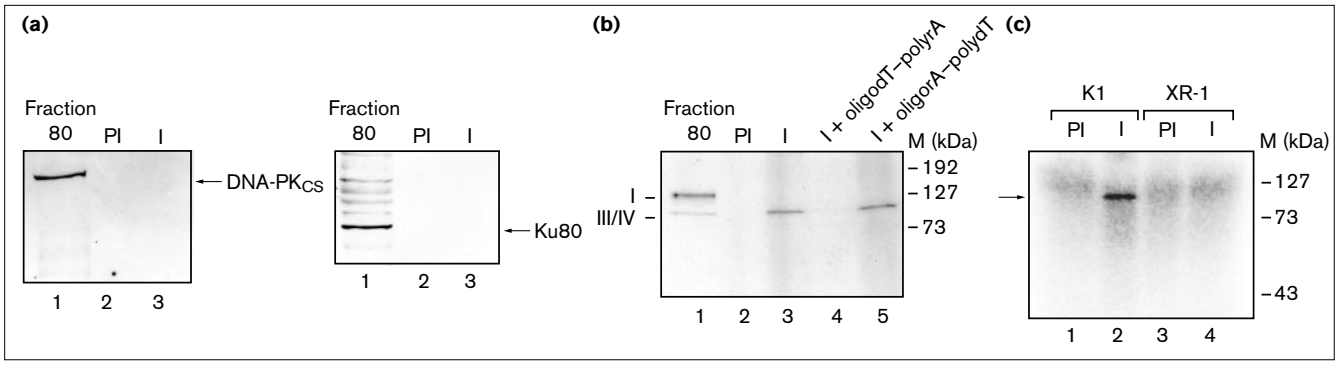
HeLa cell XRCC4 co-immunoprecipitates with DNA ligase IV

To test for possible interactions between XRCC4 and the factors described above, we immunoprecipitated XRCC4 from its peak gel-filtration fractions in the presence of 1 M NaCl and 50 μg/ml ethidium bromide (to abolish non-specific interactions mediated via DNA), and examined whether Ku, DNA-PK_{cs}, and DNA ligases I, III, and IV were present in the resulting precipitated material. Western immunoblot analyses revealed that DNA-PK_{cs} and Ku did not co-immunoprecipitate with XRCC4 (Figure 4a).

To assay for possible interactions between XRCC4 and a DNA ligase, we utilised the fact that mammalian DNA ligases form covalently-linked adenylate complexes [23,32,35]. As shown in Figure 4b, incubation of the XRCC4-containing gel-filtration fraction with α-[^{32}P]ATP led to the formation of adenylylated proteins of approximately 120 kDa and 100 kDa, which correspond to DNA ligase I and a combination of DNA ligases III and IV, respectively. To investigate the association between these ligases and XRCC4, unlabelled extract was incubated with pre-immune or anti-XRCC4 antisera in the presence of 1 M NaCl then, after stringent washing, the immunoprecipitated material was incubated with α-[^{32}P]ATP and tested for radioactively-labelled proteins. An adenylylated protein species of ~100 kDa, corresponding to DNA ligase III and/or IV was immunoprecipitated efficiently by the affinity-purified XRCC4 antiserum but not by pre-immune sera (Figure 4b); by contrast, the adenylylated species corresponding to DNA ligase I was not recovered. Consistent with the fact that the adenylate moiety of adenylylated DNA ligase complexes is discharged in the presence of ligatable polynucleotide substrates, the radiolabel associated with the XRCC4-precipitated material was lost upon incubation with DNA that had been nicked by DNase I treatment (data not shown). To exclude the possibility that the immunoprecipitated ligase was recognised directly by the anti-XRCC4 antiserum, we performed parallel immunoprecipitation reactions on extracts derived from the hamster cell lines K1 and XR-1, which express XRCC4 and lack XRCC4, respectively. As shown in Figure 4c, the ~100 kDa adenylylated ligase species was recovered from K1 extracts but not from XR-1 extracts, revealing that the DNA ligase was not recognised by the antiserum but instead was immunoprecipitated via its association with XRCC4.

The above results show that XRCC4 forms a tight salt-stable interaction with DNA ligase III and/or DNA ligase IV. We were able to establish which of these two enzymes was associated with XRCC4 because DNA ligases III and IV have different abilities to join single-strand breaks in polynucleotide substrates: whereas DNA ligase III can catalyse joining in both oligo(rA)–poly(dT) and oligo(dT)–poly(rA) substrates, DNA ligase IV is only able to mediate joining of the latter [32]. We therefore

Figure 4



XRCC4 co-immunoprecipitates with DNA ligase IV. **(a)** XRCC4 does not co-immunoprecipitate DNA-PK_{cs} or Ku80. Gel-filtration fraction 80 (lane 1) was subjected to immunoprecipitation using either pre-immune serum (PI, lane 2), or affinity-purified XRCC4 antiserum (I, lane 3). Precipitated proteins were resolved by SDS–PAGE and, after transfer to nitrocellulose, filters were probed with antibodies raised against DNA-PK_{cs} or Ku80. **(b)** XRCC4 co-immunoprecipitates with a DNA ligase. After immunoprecipitation with XRCC4 antiserum as in (a), samples were incubated with α-[^{32}P]ATP and, where indicated, then further incubated with unlabelled polynucleotide substrate. Gel filtration fraction 80 (lane 1), material precipitated with pre-immune serum (PI, lane 2) or the material immunoprecipitated with anti-XRCC4 antiserum (I, lanes 3–5) were incubated with α-[^{32}P]ATP, then analysed by SDS–PAGE and autoradiography. Adenylylated proteins were incubated with oligo(dT)–poly(rA) (lane 4) or oligo(rA)–poly(dT) (lane 5) before electrophoresis. **(c)** Anti-XRCC4 antiserum immunoprecipitates a protein that forms an adenylylated complex from K1 but not XR-1 cells. K1 (lanes 1,2) or XR-1 (lanes 3,4) whole cell extract was subjected to immunoprecipitation using either pre-immune serum (PI, lanes 1,3) or affinity-purified anti-XRCC4 antiserum (I, lanes 2,4) prior to incubation with α-[^{32}P]ATP.

performed adenylylation assays on material immunoprecipitated with anti-XRCC4 antiserum and incubated the labelled immunoprecipitates with either oligo(dT)–poly(rA) or oligo(rA)–poly(dT). Only oligo(dT)–poly(rA) could dissociate the adenylate group from the ligase that was immunoprecipitated with XRCC4 (Figure 4b, compare lanes 4 and 5). These results therefore suggest that XRCC4 interacts tightly and specifically with DNA ligase IV but not with DNA ligase III.

XRCC4 and DNA ligase IV copurify extensively

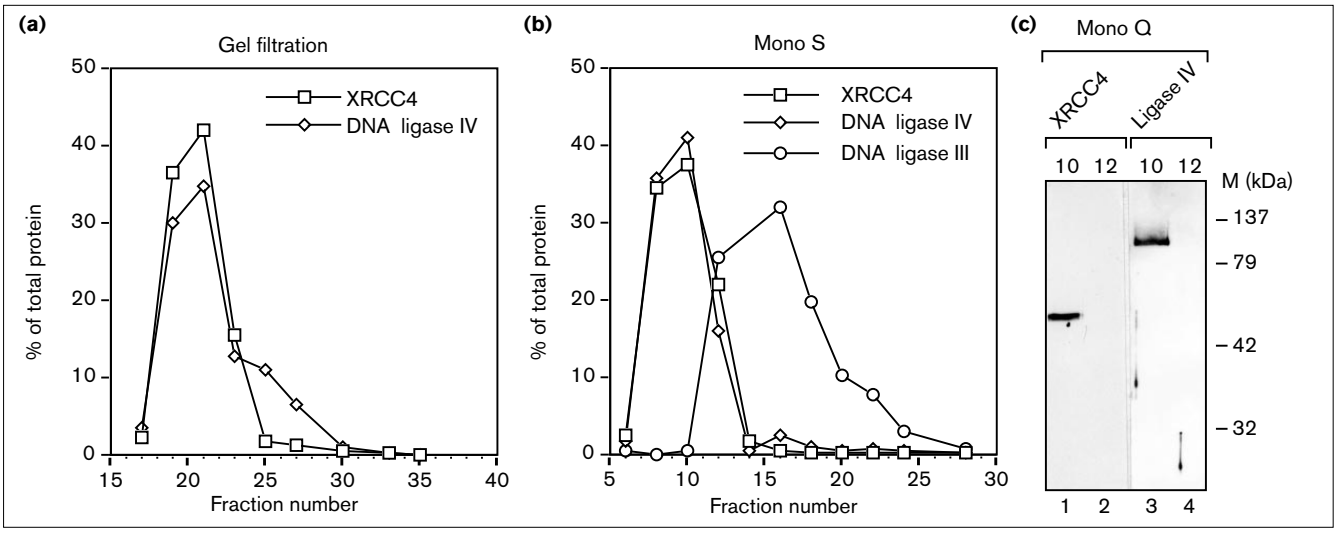
To confirm the interaction between XRCC4 and DNA ligase IV, and to determine the proportion of the two proteins in the complex, we purified DNA ligase IV using established protocols [32] and tested for the presence of DNA ligase IV and XRCC4 at each chromatographic stage by quantitative western immunoblot analyses. As demonstrated previously, DNA ligases III and IV co-elute during gel-filtration chromatography (Figures 3 and 5a) but can be resolved by chromatography on Mono S (Figure 5b). XRCC4 tracked with DNA ligase IV throughout these purification procedures but was separated from DNA ligase III at the Mono S chromatography step (Figure 5a,b). XRCC4 was also present in more highly purified samples of DNA ligase IV generated via subsequent chromatography on Mono Q (Figure 5c). In contrast, DNA ligase III was not present in the Mono Q-purified samples of XRCC4 and DNA ligase IV (data not shown). In additional studies, we observed that XRCC4 and DNA ligase IV copurify on phenyl Sepharose (data not shown). In fact, we have only been able to separate these two proteins by incubation with harsh ionic detergents. Interestingly, the XRCC4 protein that copurified with DNA ligase IV corresponded to the phosphorylated form as shown by its SDS–PAGE mobility and by the fact that this mobility was increased by phosphatase treatment (data not shown). XRCC4 and DNA ligase IV copurified almost quantitatively, and no free pools of either factor were evident (Figure 5a,b).

XRCC4 interacts with the carboxy-terminal region of DNA ligase IV

To investigate the basis for the binding of DNA ligase IV to XRCC4, we determined which region(s) of ligase IV were involved in this interaction. As depicted in Figure 6a, DNA ligase I, III and IV display high levels of sequence similarity within the core ligase catalytic domain [23]. In addition, each DNA ligase possesses discrete amino-terminal and/or carboxy-terminal extensions that have been proposed to confer unique properties on the three enzymes. Significantly, although the carboxy-terminal extensions of DNA ligases III and IV show very little homology with one another at the primary sequence level, they possess one and two copies, respectively, of the BRCA1 carboxyl terminus (BRCT) homology domain that was recently identified in the BRCA1 breast cancer susceptibility protein and a series of other proteins ([36,37]; see Discussion).

To test which region(s) of DNA ligase IV interacts with XRCC4, we divided the ligase IV protein into three parts:

Figure 5



Copurification of HeLa cell DNA ligase IV and XRCC4. Using the protocol described previously [32], DNA ligase IV was purified from HeLa cells. Fractions collected were analysed on SDS–polyacrylamide gels and subjected to western blot analysis using anti-DNA ligase III, anti-DNA ligase IV or anti-XRCC4 antisera. The amount of a specified protein in each fraction was quantitated from densitometric scans of the autoradiographs and the percentage of protein in each analysed fraction (as a proportion of its total amount) was plotted. **(a)** XRCC4 and DNA ligase IV co-elute during gel-filtration chromatography. Fraction 21 was loaded on to the Mono S column (see below). **(b)** XRCC4 copurifies with DNA ligase IV but not DNA ligase III upon Mono S chromatography. **(c)** XRCC4 is present in essentially pure DNA ligase IV fractions. Samples 10 and 12, which had been further purified by Mono Q chromatography [32], were analysed by immunoblotting to test for the presence of XRCC4 (lanes 1,2) or DNA ligase IV (lanes 3,4).

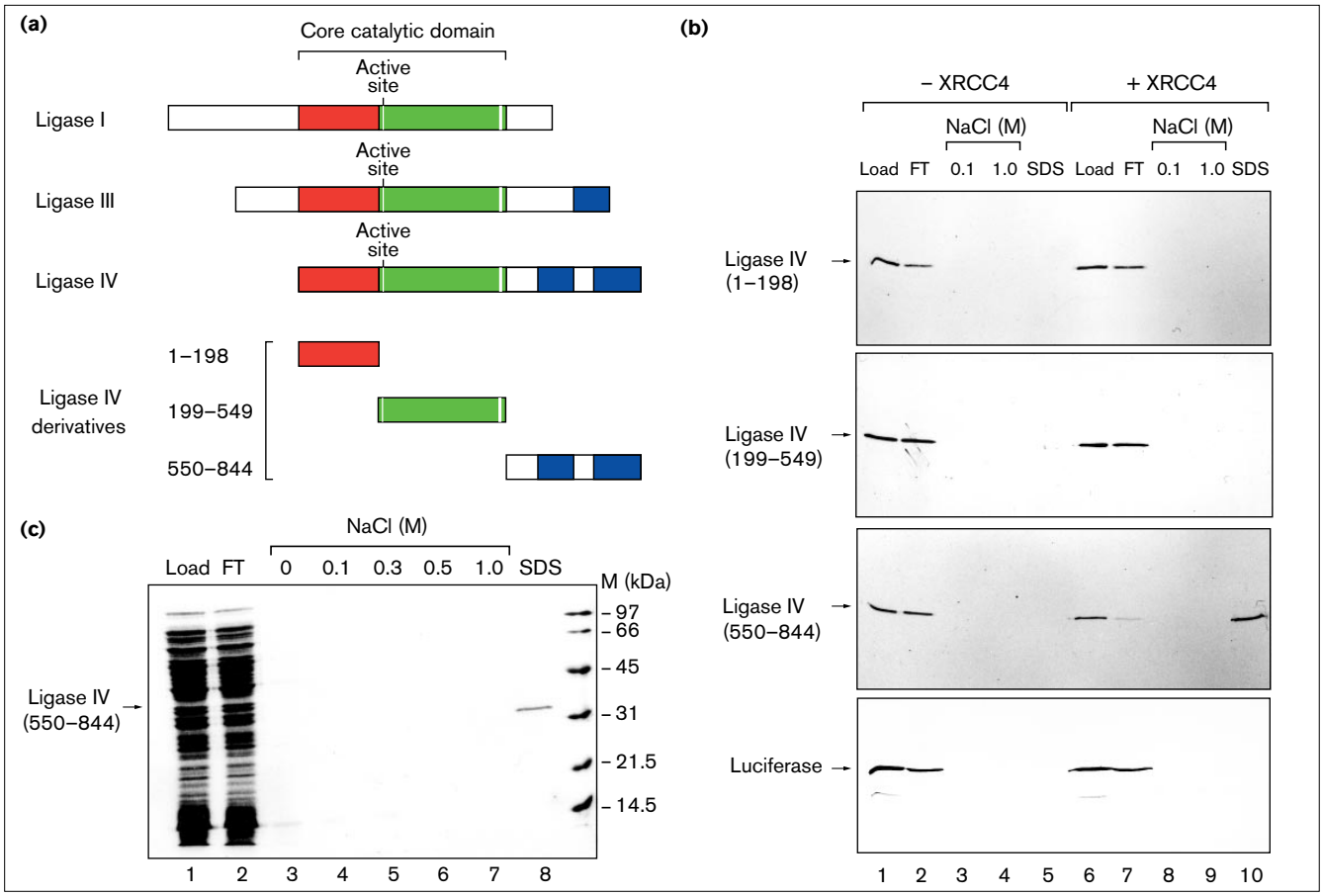
an amino-terminal region (amino acid residues 1–198) which exhibits homology with DNA ligase I and III; a central region (amino acid residues 199–549) which shows highest levels of homology with DNA ligase I and III and contains the ligase catalytic site; and a carboxy-terminal region (amino acid residues 550–844) which contains the two BRCT homology domains (Figure 6a). The three regions were transcribed and translated separately *in vitro*, and tested for their ability to bind to Sepharose beads or to Sepharose beads containing covalently attached XRCC4. As shown in Figure 6b, the amino-terminal and central fragments of DNA ligase IV failed to bind detectably to the XRCC4–Sepharose beads, as was the case for the luciferase protein used as a control. In marked contrast, the ligase IV carboxy-terminal fragment was retained almost quantitatively on the XRCC4–Sepharose beads but not on control beads lacking XRCC4 (Figure 6b). Moreover, the binding of the carboxy-terminal portion of DNA ligase IV to XRCC4 was very strong, as shown by the fact that the ligase IV carboxy-terminal region was not eluted by washing at 1 M NaCl (Figure 6b, lane 9) and was only recovered following addition of the ionic detergent SDS (Figure 6b, lane 10).

To further address the specificity of the above interaction, we assessed whether XRCC4–Sepharose beads could be used to purify the carboxy-terminal region of DNA ligase IV from crude bacterial lysates. An unfractionated extract from *E. coli* cells expressing fairly low levels of this region of ligase IV was incubated with XRCC4–Sepharose beads and the bound material was eluted with increasing salt concentrations, followed by a final elution in the presence of SDS. As shown by total Coomassie-blue staining of an SDS–polyacrylamide gel containing these fractions, this method resulted in the purification of the carboxy-terminal region of DNA ligase IV to virtual homogeneity in a single step (Figure 6c; the identity of this polypeptide as the ligase IV carboxyl terminus was confirmed by western blotting, and this protein was not retained by Sepharose beads alone). Taken together, these results demonstrate the extreme strength and specificity of the interaction between the ligase IV carboxy-terminal region and XRCC4.

Discussion

Previous work has established that cells deficient in XRCC4 are hypersensitive to ionising radiation, defective in DNA double-strand break rejoining and unable to perform site-specific V(D)J recombination. Although these findings reveal that XRCC4 plays a crucial role in DNA double-strand break repair, its mechanism of action has remained obscure. Another previously unresolved question regarding DNA non-homologous end-joining is which DNA ligase(s) is (are) involved. Here, we have expressed XRCC4 in bacteria, raised antisera against this factor and used these antisera to characterise endogenous mammalian cell XRCC4. Consistent with XRCC4 serving

Figure 6

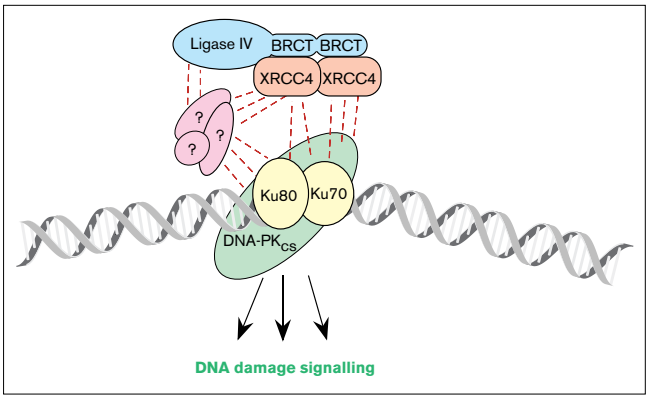


(a) Schematic representation of the various eukaryotic DNA ligases. The core ligase catalytic domain is divided into two regions: the red box indicates homology between DNA ligase I, III and IV and the green box represents the highest levels of homology between the mammalian DNA ligases and contains the catalytic site (active site) and conserved peptide (indicated by solid white bars). The putative BRCT domains [36,37] of DNA ligase III and IV are represented by blue boxes. **(b)** Interaction between the carboxy-terminal portion of DNA ligase IV comprising the BRCT domains and XRCC4. The fragments ligase IV (1–198), ligase IV (199–549), ligase IV (550–844) and a luciferase control were translated in the presence of [^{35}S]methionine, then were applied to XRCC4–Sepharose beads or negative control beads (load, lanes 1,6) and unbound proteins were collected (FT, lanes 2,7). After sequential washes with 0.1 M NaCl (lanes 3,8) and 1.0 M NaCl (lanes 4,9), bound proteins were eluted with SDS–gel loading buffer (SDS, lanes 5,10). After SDS–PAGE, the [^{35}S]methionine-labelled proteins were detected by autoradiography. **(c)** Recombinant DNA ligase IV (550–844) binds tightly and selectively to XRCC4. Bacterial extract containing ligase IV (550–844) was applied to XRCC4–Sepharose beads (load, lane 1) and unbound proteins collected (FT, lane 2). After washes with stepwise increases of NaCl from 0–1.0 M (lanes 3–7), proteins were eluted with SDS–gel loading buffer (SDS, lane 8).

as a component of the DNA double-strand break repair apparatus, we found that it was present in nuclear extracts. Interestingly, however, we also detected a substantial proportion of the XRCC4 protein in cytosolic fractions. Moreover, through a variety of approaches, we have demonstrated that XRCC4 mediates extremely tight and specific interactions with DNA ligase IV; for example, these two components co-immunoprecipitated highly specifically from HeLa cell extracts, even in the presence of 1 M NaCl. These interactions were not abrogated by ethidium bromide, suggesting that the interaction between XRCC4 and DNA ligase IV is not mediated by a DNA intermediate. Indeed, we have shown that bacterially expressed XRCC4 and ligase IV bind to one another tightly, revealing that their interaction is direct. In addition, XRCC4 and ligase IV copurified over every chromatographic fractionation procedure we employed, and we have only been able to resolve these two proteins by the addition of harsh ionic detergents.

The interaction of XRCC4 with DNA ligase IV but not with other DNA ligases led us to investigate the basis for this binding specificity. Although all characterised mammalian DNA ligases contain a common highly related core catalytic region, each has unique amino-terminal and/or carboxy-terminal extensions. Previous studies of DNA

Figure 7



Model in which XRCC4 serves as a molecular bridge to target DNA ligase IV to a DNA double-strand break. In this model, Ku binds to the free DNA ends and recruits DNA-PK_{cs}, activating the kinase catalytic function of the latter in the process. A DNA ligase IV–XRCC4 complex is then recruited to the DNA double-strand break, either by direct interactions with DNA-PK_{cs} and/or Ku, or indirectly via other as yet uncharacterised components of the double-strand break repair machinery. Active DNA-PK may also trigger DNA-damage signalling events or may phosphorylate other DNA double-strand break repair components, such as XRCC4, thus regulating their activities. The stoichiometry of the XRCC4–DNA ligase IV complex is currently unknown but we have chosen to depict it as 2:1 because DNA ligase IV has two BRCT homology domains.

ligases I and III have revealed that such regions are involved in interactions with other factors. The amino-terminal portion of DNA ligase I, for example, is important for enzyme function *in vivo* and targets DNA ligase I to specific nuclear structures termed replication factories [38]. In addition, the carboxy-terminal extension of the predominant form of mammalian DNA ligase III binds to the DNA base excision repair factor XRCC1 [27,39]. In line with these data, we have found that the unique carboxy-terminal domain of DNA ligase IV interacts with XRCC4. This region contains two tandem copies of the BRCT homology domain [36,37], leading to the speculation that either one or both of these domains mediates the interaction with XRCC4. BRCT domains also exist in a variety of other factors, including XRCC1, DNA ligase III, and a group of yeast DNA repair and DNA-damage checkpoint proteins [36,37]. It is noteworthy that the breast cancer susceptibility protein BRCA1 contains a BRCT domain and has been implicated recently in DNA repair processes [40].

Given that XRCC4 functions in DNA non-homologous end-joining, our data suggest that DNA ligase IV also plays a crucial role in this process. Indeed, it is tempting to speculate that XRCC4 serves as a molecular bridge to link DNA ligase IV with DNA double-strand breaks, perhaps through additional interactions between XRCC4 and other components of the DNA non-homologous end-joining apparatus (Figure 7). XRCC4 could therefore function analogously to XRCC1 which, in addition to interacting with DNA ligase III, has been shown recently to bind to the other DNA repair-associated factors DNA polymerase β and poly-ADP-ribose polymerase [26,41]. In regard to a putative bridging function for XRCC4, immunoprecipitation studies suggest that XRCC4 can interact with Ku and/or DNA-PK_{cs}, although these interactions only occur at low salt concentrations and hence are weak compared to those between XRCC4 and DNA ligase IV (S.E.C. and S.P.J., unpublished data). A possible physical linkage between XRCC4 and DNA-PK is attractive in light of the fact that HeLa XRCC4 is a phosphoprotein and is an effective substrate for DNA-PK *in vitro*. Consistent with the proposal that ligase IV plays an important role in DNA double-strand break repair, a *Saccharomyces cerevisiae* homologue of DNA ligase IV has been identified recently [42,43] and inactivation of this factor debilitates DNA non-homologous end-joining in a manner that is epistatic with mutations in the yeast homologues of Ku70 and Ku80 [43–46]. By contrast, yeast ligase IV does not play essential roles in other DNA repair pathways that have been analysed [42,43]. Taken together, the data therefore suggest that ligase IV is dedicated to DNA non-homologous end-joining and that this function is conserved throughout the eukaryotic kingdom. It therefore seems likely that, as is the case for cells bearing mutations in XRCC4 or DNA-PK components, disruption of the DNA ligase IV gene in mammalian systems would result in deficient V(D)J recombination and an inability to repair ionising radiation-induced DNA damage.

Conclusions

The XRCC4 protein is required for DNA non-homologous end-joining and V(D)J recombination in mammalian cells. We have demonstrated that XRCC4 interacts tightly and specifically with HeLa DNA ligase IV. The *S. cerevisiae* homologue of DNA ligase IV has recently been identified and found to be necessary for DNA non-homologous end-joining in yeast [42,43]. Taken together, these findings strongly implicate DNA ligase IV in the joining of double-strand breaks via the non-homologous end-joining pathway and in the ligation steps of V(D)J recombination. Furthermore, these data suggest that XRCC4 acts as a molecular bridge to target DNA ligase IV to other components of the DNA non-homologous end-joining apparatus. Protein mapping studies revealed that XRCC4 interacts with DNA ligase IV via its unique carboxy-terminal domain. This domain contains two of the recently identified BRCT homology motifs found in other DNA repair-associated factors and BRCA1, raising the intriguing possibility that other BRCT domains may mediate contacts between DNA repair components.

Identification of components of the mammalian non-homologous end-joining apparatus has been facilitated by

studies in cell lines that are defective in double-strand break repair. Determination of the defects in these cell lines has demonstrated that DNA-PK_{cs}, Ku80 and XRCC4 are crucial for non-homologous end-joining. The strong interaction between XRCC4 and DNA ligase IV represents an advance in the identification of as yet unknown components of the double-strand break repair apparatus and provides further insights into the mechanisms of detection and removal of this dangerous form of DNA damage.

Materials and methods

Enzymes, antibodies and DNA

The pET-30b and pQE-30 vectors were obtained from Novagen and Qiagen, respectively. All plasmid constructs were verified by automated DNA sequencing (J. Lester; Biochemistry Department, University of Cambridge). HeLa nuclear and cytoplasmic extracts were obtained from Computer Cell Culture Centre, Mons, Belgium.

Cell culture and preparation of whole cell extracts

The Chinese hamster ovary cell lines K1 (parental) and XR-1 were maintained as monolayers at 37°C in a 5% CO_2 atmosphere in minimal essential medium supplemented with nonessential amino acids, penicillin (100 U/ml), streptomycin (0.1 mg/ml), and 10% foetal calf serum. Whole cell extracts were prepared as described [34].

Expression and purification of XRCC4 derivatives

To generate recombinant untagged XRCC4, the full-length *XRCC4* coding region was amplified from pBlueScript containing the human *XRCC4* gene by PCR and inserted into pET-30a (Novagen) digested with *Nde*I/*Sal*I thereby removing the amino-terminal His/S tags; the *XRCC4* stop codon also prevents the addition of a carboxy-terminal His-tag. BL21(DE3) cells were used to express the *XRCC4* from the plasmid (pET30XRCC4). After lysing by sonication, 30.2 g of ammonium sulphate was added per 100 ml of supernatant and incubated with stirring at 4°C for 30 min. After centrifugation, the pellet was resuspended in TED (50 mM Tris-HCl pH 7.5, 2 mM DTT and 1 mM EDTA) and dialysed against TED. The protein was then loaded onto a heparin Sepharose column and protein was eluted with a 0–0.6 M NaCl linear gradient. Fractions containing XRCC4 were pooled and dialysed against TED containing 1.0 M ammonium sulphate and were then loaded onto a phenyl Sepharose column. Proteins were eluted with a 100 ml linear gradient of 1.0–0 M $(NH_4)_2SO_4$. Fractions containing XRCC4 (typically 95% pure), eluting at ~0.2 M $(NH_4)_2SO_4$, were pooled and dialysed against 50 mM Tris-HCl pH 7.5, 2 mM DTT, 1 mM EDTA and 10% (w/v) glycerol, and stored at −80°C.

Anti-XRCC4 antibody production and purification

Regions of the *XRCC4* gene were amplified by PCR and then inserted in-frame downstream of the hexa-histidine (His) tag of pQE-30 (Qiagen) and were expressed and purified according to the manufacturer's instructions from the soluble fraction of bacterial lysates. Antibodies were raised in rabbits using standard procedures [47] and are available commercially from Serotec, UK. Recombinant histidine-tagged full-length XRCC4 was attached to Sulfolink Coupling Gel (Pierce) and was used to carry out immunoaffinity purification of anti-XRCC4 antibodies from crude SJ4 serum as described previously [48].

Phosphorylation assays

Phosphorylation assays were performed essentially as described in [7]. Assays contained 50 ng of Sp1 or recombinant untagged XRCC4, 100 ng of purified DNA-PK and 200 ng of linearized pBluescript as indicated.

Co-immunoprecipitations and ligase adenylylation assays

HeLa nuclear extract was dialysed into buffer D* (20 mM HEPES-KOH, 20% (w/v) glycerol, 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM sodium metabisulphite and 0.1% NP-40) and then incubated with either pre-immune or anti-XRCC4 serum for 1 h at 4°C in the presence of 50 μg/ml ethidium bromide to disrupt protein–DNA interactions [49]. Immune complexes were bound to protein A Sepharose beads (Pharmacia), followed by extensive washing with buffer D* containing 0.15–1 M NaCl. Protein A Sepharose beads were finally washed in buffer D* containing 0.15 M NaCl prior to analysis. Samples were then tested for the ability to form DNA ligase-adenylylated complexes as described previously [32]. Polynucleotide substrates oligo(dT)–poly(rA) and oligo(rA)–poly(dT) were prepared as described [35]. The reactivity of the enzyme–adenylate intermediates formed was examined by adding 0.8 μg of unlabelled oligo(dT)–poly(rA) or oligo(rA)–poly(dT) for 1 h at 30°C.

Gel-filtration chromatography

Total HeLa nuclear extract (6 mg protein) was dialysed extensively against buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 mM DTT, 10% (w/v) glycerol) containing 1 M NaCl. Material was then loaded onto a Superose 6 (Pharmacia) column (60 × 1.5 cm), pre-equilibrated with buffer A containing 1 M NaCl. On an identical gel-filtration run, 0.2 mg of pure untagged recombinant XRCC4 was analysed in buffer A containing 1 M NaCl.

Purification of DNA ligase IV from HeLa cells

DNA ligase IV was purified from HeLa cells as described previously [32]. Fractions collected from each column were analysed by immunoblots with antibodies specific for XRCC4 and DNA ligases III and IV.

Expression of recombinant ligase IV derivatives

Fragments of the human ligase IV gene coding region were amplified by PCR from reverse-transcribed HeLa RNA. Each PCR product included a *Bam*HI site at the 5′ end and a stop codon followed by a *Sal*I site at the 3′ end. After digestion, the PCR products were ligated into pET30b digested with *Bam*HI/*Sal*I. The 550–844 fragment of ligase IV was also cloned into pQE-30 and the resulting clone was expressed in *E. coli* M15(Rep4). For *in vitro* transcription and translation of ligase IV fragments, 1 μg of pET30LigIV(1–198), pET30LigIV(199–549), pET30LigIV(550–844) or a luciferase control (Promega) were transcribed *in vitro* and translated using the TnT rabbit reticulocyte lysate kit (Promega) according to the manufacturer's instructions. The amino-terminally histidine-tagged ligase IV products were then purified by Ni^{2+}–NTA agarose chromatography.

Interaction assays between recombinant XRCC4 and ligase IV derivatives

Full-length XRCC4 was immobilised on Sepharose-4B gel beads (Pharmacia) using the cyanogen bromide method according to the manufacturer's instructions. As a negative control, coupling was performed without XRCC4. A 30 μl bed volume of beads (with and without XRCC4) was pre-equilibrated with binding buffer (50 mM Tris-HCl pH 7.5, 2 mM DTT, 1 mM EDTA, 10% (w/v) glycerol, 0.1% NP-40 and 0.36 mg/ml BSA) before addition of the purified *in vitro* translated ligase IV products or luciferase. Unbound material was collected after centrifugation and beads were washed with binding buffer containing 0.1 M NaCl and 1.0 M NaCl. Proteins bound to the beads were then analysed by SDS–PAGE and autoradiography. The ability of the recombinant carboxy-terminal fragment of ligase IV (residues 550–844) to bind XRCC4–Sepharose was tested as above, except that analysis was by Coomassie-blue staining and immunoblotting with the anti-ligase IV antibody.

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