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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**

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The *XRCC4* gene is required for the repair of DNA double-strand breaks in mammalian cells. Without XRCC4, cells are hypersensitive to ionizing radiation and deficient for V(D)J recombination. It has been demonstrated that XRCC4 binds and stimulates DNA ligase IV, which has led to the hypothesis that DNA ligase IV is essential for both of these processes. In this study deletion mutants of XRCC4 were tested for their ability to associate with DNA ligase IV *in vitro* and for their ability to reconstitute XRCC4-deficient cells *in vivo*. We find that a central region of XRCC4 from amino acids 100–250 is necessary for DNA ligase IV binding and that deletions within this region functionally inactivates XRCC4. Deletions within the C-terminal 84 amino acids neither affect DNA ligase IV binding nor the *in vivo* function of XRCC4. The correlation between the ability or inability of XRCC4 to bind DNA ligase IV and its ability or failure to reconstitute wild-type DNA repair *in vivo*, respectively, demonstrates for the first time that the physical interaction with DNA ligase IV is crucial for the *in vivo* function of XRCC4. Deletions within the N-terminal 100 amino acids inactivate XRCC4 *in vivo* but leave DNA ligase IV binding unaffected. This indicates further DNA ligase IV-independent functions of XRCC4.

Cells have developed mechanisms for the repair of chromosomal DNA breaks that can be generated either randomly (*e.g.* by ionizing radiation) or site-specifically during V(D)J recombination. Two distinct DNA double-strand break repair pathways are operative in eukaryotic cells: homologous recombination, which employs stretches of homologous DNA to replace a region containing a DNA double-strand break. The second pathway, nonhomologous DNA end-joining (NHEJ),[1] allows cells to directly religate a broken chromosome.

The latter mechanism is an integral part of the DNA end-joining phase in V(D)J recombination, the mechanism that assembles coding regions for the variable domains of immunoglobulin and T cell receptors in developing lymphocytes (1). The DNA double-strand breaks in this process are generated by two lymphoid-specific proteins, RAG-1 and RAG-2 (2, 3), that cleave DNA at conserved recombination signal sequences flanking all V (variable), D (diversity), and J (joining) gene segments (4). Cleavage of DNA by RAG proteins is followed by a DNA end-joining phase involving many activities that are also essential for general NHEJ. This is evidenced by a variety of mutations resulting in increased x-ray sensitivity as well as a defect in V(D)J recombination.

One example is the *scid* mutation in mice that results in hypersensitivity to ionizing radiation as well as a severe combined immunodeficiency (5). *Scid* cells carry a mutation in DNA-dependent protein kinase affecting its kinase activity (6–9). Deficiencies in DNA double-strand break repair and V(D)J recombination are also caused by mutations in the genes encoding the Ku70 and Ku86 proteins (10, 11). The Ku70·86 complex has DNA end binding activity and is able to stimulate DNA-dependent protein kinase *in vitro*. Mutations in either of the Ku subunits affect both signal and coding joint formation in V(D)J recombination (12–15).

The cDNA for another factor, XRCC4, with a dual role in DNA double-strand break repair and V(D)J recombination has recently been cloned (16). This cDNA is able to complement the DNA repair defect in the Chinese hamster ovary cell line XR-1 carrying a deletion of the *XRCC4* gene. The biochemical function of the putative XRCC4 protein remained initially unknown, since it did not display significant homology to any other known protein.

It has recently been demonstrated that the XRCC4 protein associates with DNA ligase IV in mammalian cells (17, 18), and that complex formation stimulates DNA ligase IV activity *in vitro* (17). Together with the finding that the yeast homologue of DNA ligase IV is essential for nonhomologous DNA end-joining (19–21), this has led to the hypothesis that DNA ligase IV is also essential for DNA repair and V(D)J recombination in mammalian cells (22). Despite these findings, it has been suggested that DNA ligase I and not IV is involved in V(D)J recombination, as only DNA ligase I has been found to stimulate signal and coding joint formation in an *in vitro* assay (23).

To test the significance of the XRCC4-DNA ligase IV interaction for nonhomologous DNA repair and V(D)J recombination *in vivo*, we have generated a series of XRCC4 deletion mutants. The XRCC4 mutants were assayed for their ability to associate with DNA ligase IV *in vitro*, and stable transfectants of these mutants were analyzed for their V(D)J recombination and DNA repair phenotypes.

MATERIALS AND METHODS

Generation of XRCC4 Deletion Mutants—XRCC4 deletion mutants were generated from XRCC4 expression vector pUG14 (17), which contained the human XRCC4 cDNA cloned into pCDNA3 (Invitrogen) with

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[1] The abbreviations used are: NHEJ, nonhomologous DNA end-joining; IP, immunoprecipitation; RAG, recombination activating gene; *scid*, severe combined immunodeficiency; XRCC, x-ray cross complementing; PCR, polymerase chain reaction; HA, hemagglutinin.

a C-terminal nine-His tag and three Myc epitopes. For the N-terminal truncation (XRCC4Δ1), a PCR product primed by an internal primer (5′-GCTCGGATCCATGGAGATTTCCCAAGAAGCTGATGACATGG-3′) and universal reverse primer UG059 (5′-ACAAGAATTCTCTA-GAGTCGCGGCCGCTTATAAC-3′), binding downstream of the Myc epitope, was subcloned as a *Bam*HI/*Eco*RI fragment.

Internal XRCC4 deletion mutants were generated by fusing an N-terminal piece of XRCC4 to a C-terminal piece by a two-step PCR approach (Fig. 1*a*). Universal upstream primer UG058 was 5′-GTATG-GATCCATGGAGAGAAAAATAAGCAGAATCC-3′. PCR products were cloned as *Bam*HI/*Eco*RI-digested PCR fragments into pCDNA3. All PCRs were carried out with KlenTaqLA enzyme (24), which has a 10-fold reduced error rate ($\sim 10^{-5}$ mutations/base pair) compared with *Taq* polymerase (24). For a 1-kilobase pair template, the probability for a point mutation in every PCR cycle with KlenTaqLA is therefore $p = 0.01$. The probability of a point mutation in a single PCR clone after i PCR cycles is given by Equation 1.

$$p = 0.01 \sum_{n=1}^{i} \frac{1}{2^{n-1}} \qquad \text{(Eq. 1)}$$

Hence, with $i = 40$, the probability is approximately 0.02. Internal primers for the various deletion mutants were as follows. XRCC4Δ2, 5′-GGGACAGTTTCTGAATCACTGAAAGATGTCTCATTCAGACTTG-G-3′, 5′-GAATGAGACATCTTTCAGTGATTCAGAAACTGTCCCAGTC-CATG-3′; XRCC4Δ3, 5′-TTCTTCTTTGAGAAAAACCTTCTGAGAGAT-TGGAATGATGTTCAAGG-3′, 5′-ATTCCAATCTCTCAGAAGGTTTTT-CTCAAAGAAGAAATAACAAGACTC-3′; XRCC4Δ4: 5′-CAGAAAGAA-AATGAAAGGGCAGCTCAAGAACGAGAAAAGGACATCAAAC-3′, 5′-TTCTCGTTCTTGAGCTGCCCTTTCATTTTCTTTCTGCAGGTGCTC-ATTTTTGG-3′; XRCC4Δ5: 5′-CTGCAGAAAGAAAATGAAAGGGTAA-GTAAAGATGATTCCATTATTTCAAGTC-3′, 5′-GGAATCATCTTTAC-TTACCCTTTCATTTTCTTTCTGCAGGTGCTCATTTTTGG-3′; XRCC-4Δ6: 5′-GGGTTGGCTTCAGCTGCTTCTTCACTACCTGAGACGTCGA-AAAAGGAG-3′, 5′-CGTCTCAGGTAGTGAAGAAGCAGCTGAAGCCA-ACCCAGAGAGATCAG-3′; XRCC4Δ7: 5′-CAAGAAAAGGAAAAGCCT-GATCACCATCACCATCACCATCACCATCACGCGTCCGAG-3′, 5′-AT-GGTGATGGTGATGGTGATCAGGCTTTTCCTTTTCTTGAAGCTGAT-TCTC-3′.

Transient Expression of Proteins, Immunopurifications, Western Blotting—Proteins were transiently expressed in XR-1 Chinese hamster ovary or HeLa cells using the vaccinia virus overexpression system (25). Cells were harvested 18–24 h post-transfection/infection.

Western blot analyses were performed as described (26). Western blots were probed with either purified anti-HA monoclonal antibodies 12CA5 or anti-Myc monoclonal 1–9E10.2 as a primary reagent and developed with a horseradish peroxidase-labeled polyclonal goat anti-mouse IgG antibody by ECL.

Immunopurifications of HA- or Myc-tagged proteins were done as described (27) with 50 μg of monoclonal anti-HA or anti-Myc antibodies and 50 μl of protein G-Sepharose beads/1–5 × 10^7 cells. Immunoprecipitations were washed 5 times with ice-cold 25 mM HEPES/KOH, 0.1% Nonidet P-40, 10 mM $MgCl_2$, 650 mM KCl, pH 7.9, and eventually twice in ligation buffer (see below).

In Vitro Ligation Assays—Nick ligations were performed in 60 mM Tris/Cl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 5 μg/ml acetylated bovine serum albumin, pH 7.9. Ligations were carried out with 0.1 pmol of nick ligation substrate (17) for 30 min at 37 °C. Ligated products and unligated substrates were separated by denaturing polyacrylamide gel electrophoresis and detected by autoradiography.

Immunocytochemistry—Intracellular staining of stable transfectants for the Myc-tagged XRCC4 deletion mutants was carried out as described (28). Purified, monoclonal anti-Myc antibody 1–9E10.2 was used as a primary reagent and was developed with a fluorescein isothiocyanate-labeled polyclonal goat anti-mouse IgG antibody.

X-ray and V(D)J Recombination Assays—Cells grown to confluency were γ-irradiated and plated into 96-well plates under limiting dilution conditions. The percent survival was calculated by dividing the number of colonies at a given dilution of irradiated cells by the number of colonies at the same dilution of nonirradiated cells ×100. Transient cellular V(D)J recombination assays were performed as described (29). Recombination substrates retaining either coding or signal joints upon V(D)J recombination were pGG121 and pGG124, respectively (30).

RESULTS

Generation and Expression of XRCC4 Deletion Mutants—XRCC4 deletion mutants were generated from an XRCC4 expression construct encoding human XRCC4 fused to a nine-His-tag and three Myc epitopes at the C terminus (17). One N-terminal deletion mutant (X4Δ1) was generated by a PCR using an internal upstream primer and a universal downstream primer amplifying a 50-amino acid truncated mutant of XRCC4. Internal deletion mutants were generated using specific internal primers and a two-step PCR approach (see Fig. 1*a*). The location of the deletions for, altogether, seven XRCC4 mutants are depicted in Fig. 1*b*. XRCC4 deletion mutant Δ5 contained a 100-amino acid deletion instead of the regular 50- or 34-amino acid deletions, because primers that would be required to generate a 50-amino acid XRCC4 deletion mutant spanning the gap between deletion mutants Δ4 and Δ6 repeatedly did not result in detectable PCR products (probably the result long A/T-rich stretches in the primer binding sites). Expression contructs encoding the various XRCC4 deletion mutants were tested by 3–4 diagnostic restriction enzyme digests. All PCRs were performed with high fidelity KlenTaq polymerase (24). The probability of introducing point mutations by PCR on a 1-kilobase pair template (the length of the XRCC4 cDNA) with this enzyme is $p = 0.02$ (see "Materials and Methods"), which we consider low enough to assume that PCR errors are insignificant for our deletional mutagenesis.

The expression of XRCC4 mutants from these constructs was first tested using the transient vaccinia virus overexpression system, and protein expression was detected by Western blotting with against the C-terminal Myc-epitope tag (Fig. 2). Immunoreactive proteins were detectable for all XRCC4 deletion mutants with apparent molecular weights below that of the wild-type full-length XRCC4 control. As expected, the construct for the 100-amino acid deletion resulted in the smallest detectable protein product. The fact that the Myc-epitope tags are located at the C termini of all deletion mutants demonstrated that neither frameshifts nor stop-codons were introduced in any of the mutants. In stable transfectants, full-length XRCC4 is detected as a double band migrating at 60 and 65 kDa, which represents a significantly larger molecular weight as predicted from its cDNA sequence (17). Likewise, XRCC4 deletion mutants expressed transiently were found to migrate at larger molecular weights (Fig. 2). Additional immunoreactive proteins migrating below the major protein bands in some of the samples are most likely the result of proteolytic degradation that is characteristic for proteins transiently expressed in the vaccinia virus system (25).

Association of XRCC4 Deletion Mutants with DNA Ligase IV—We next assayed the various XRCC4 deletion mutants for their ability to interact with DNA ligase IV. This was performed by transient co-expression of the Myc-tagged XRCC4 deletion mutants with full-length, HA-tagged DNA ligase IV followed by anti-Myc immunoprecipitation. Direct anti-HA and anti-Myc Western blotting was performed to control for comparable expression levels of all epitope-tagged proteins (data not shown). Association of DNA ligase IV with the XRCC4 mutants was then analyzed by anti-HA Western blotting with all of the anti-Myc immunoprecipitates (Fig. 3*a*). These experiments show that interaction of DNA ligase IV with XRCC4 is not affected by deletions within either the first 100 N-terminal or the last 84 C-terminal amino acids. However, deletions within the central region of XRCC4 from amino acid positions 100–250 disrupted the interaction of DNA ligase IV with XRCC4 (see XRCC4 deletion mutants Δ3, 4, and 5). Anti-HA and anti-Myc immunoprecipitates of the same samples were further assayed for DNA ligase activity using nick-ligation substrates (Fig. 3*b*). In agreement with the Western blotting data, no significant activity was detectable in anti-Myc co-immunoprecipitates using XRCC4 deletion mutants Δ3, Δ4,

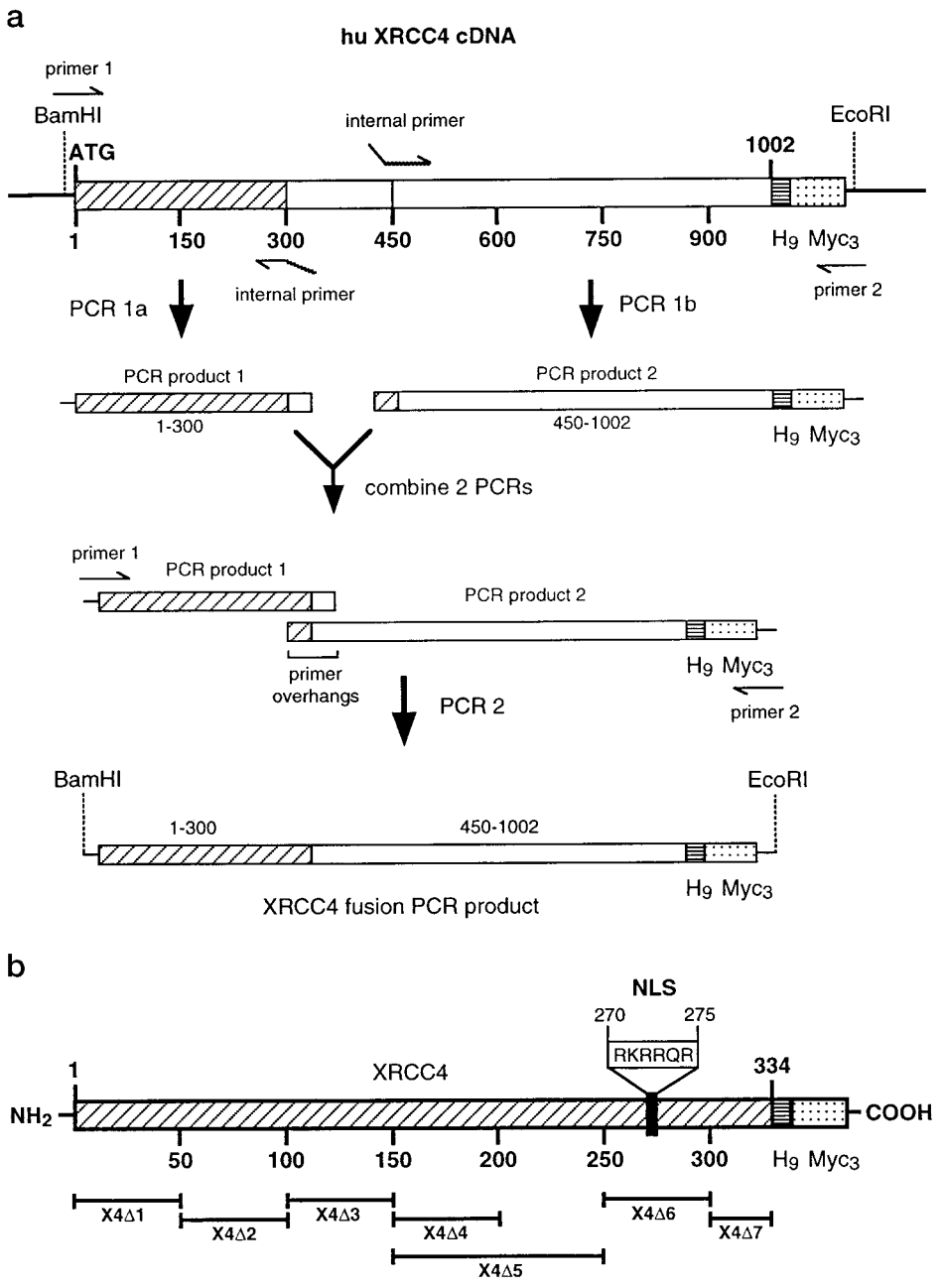

FIG. 1. *a*, two-step PCR approach to generate internal deletion mutants. In a first step, fragments of the XRCC4 cDNA encoding an N-terminal and a C-terminal piece of the protein are PCR-amplified. The internal primers are designed such that they contain complementary 5′ overhangs (18 to 21 nucleotides). The resulting PCR products therefore contain 36–42 base pairs of identical sequence that can anneal in a secondary PCR step and result in a fusion product upon PCR amplification with universal forward and reverse cloning primers. *b*, schematic representation of the N-terminal, C-terminal, and internal XRCC4 deletion mutants that were functionally analyzed in this study. The position of a putative nuclear localization signal (*NLS*) as published (16) is indicated. Also indicated are the location of the C-terminal 9 × histidine tag (*H9*) followed by three Myc-epitopes (*Myc3*).

and Δ5, whereas DNA ligase IV activity was present in immunoprecipitations using XRCC4 deletion mutants Δ1, 2, 6, and 7.

Anti-HA IPs (*i.e.* IPs against DNA ligase IV) demonstrated that DNA ligase IV activity was present in all samples; however, at very low levels in the control sample lacking XRCC4 protein and in samples containing XRCC4 deletion mutants that failed to associate with DNA ligase IV. This confirms previous data demonstrating a requirement of XRCC4 for optimal *in vitro* ligation activity of DNA ligase IV (17).

Interestingly, we reproducibly observed a roughly 2-fold lower level of DNA ligase IV stimulation with XRCC4 deletion mutant Δ1 as *e.g.* compared with XRCC4 deletion mutants Δ2, Δ6, Δ7, or the full-length wild-type control (Fig. 3*b*). This was not due to apparent differences in either DNA ligase IV or X4Δ1 expression levels in this sample as judged by Western blotting (data not shown).

Generation of Cells Stably Expressing XRCC4 Deletion Mutants—The expression constructs for the various XRCC4 deletion mutants were then stably transfected into the XRCC4-deficient Chinese hamster ovary cell line XR-1 to assay the mutants for reconstitution of the DNA repair defect in XR-1 cells. Several clones were identified for each mutant expressing Myc immunoreactive proteins of approximately the same sizes as found in transient expression experiments (Fig. 4*a*). Although expression levels of stably expressed XRCC4 mutant proteins were relatively uniform between independent clones of

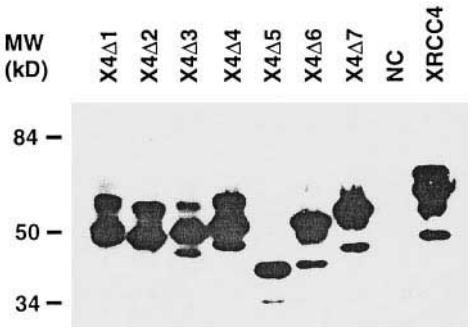

FIG. 2. **Western blot analysis of transiently expressed XRCC4 deletion mutants in total cell lysates.** Proteins were expressed in HeLa cells using the vaccinia virus overexpression system and detected with the anti-Myc monoclonal antibody. Extracts from 5×10^4 cells were loaded per *lane*. *NC* designates a negative control where XR-1 cells were transfected with an empty expression vector. The XRCC4 *lane* contains lysates from cells transfected with a full-length human XRCC4 cDNA expression vector. *MW*, molecular mass.

the same mutant (data not shown), protein expression levels varied significantly between different XRCC4 deletion mutants (Fig. 4*a*). XRCC4 mutants carrying deletions within the first 150 amino acids (X4Δ1, Δ2, and Δ3) were expressed at approximately 10-fold lower levels compared with deletion mutants X4Δ4–Δ7 or to wild-type XRCC4 (Fig. 4*a*). The finding that the expression levels of the XRCC4 mutants were found to be identical by transient vaccinia virus overexpression (Fig. 2) indicates that mutations within the N-terminal 150 amino acids decrease the half-life of these XRCC4 mutants. As expected, stably transfected mutant XRCC4 proteins displayed less proteolytic degradation as those transiently expressed in the vaccinia virus system.

To analyze the subcellular distribution of the XRCC4 deletion mutants and to ascertain that all cells of a given clone would expressed the XRCC4 mutant protein, the stable transfectants were analyzed by immunocytochemistry (Fig. 4*c*). In agreement with the Western blotting data, it was found that the expression levels of XRCC4 deletion mutants X4Δ1, Δ2, and Δ3 was significantly lower as compared with all other mutants and the wild-type controls. However, in each of the selected clones, all cells were found to express the transfected XRCC4 mutants, demonstrating that the differences in protein expression levels were not the result of expression in only a fraction of the cells.

Immunocytochemistry further demonstrated that three of the deletion mutants (X4Δ2, 3, and 6) had lost their capacity to properly localize to the nucleus, resulting in a diffuse staining pattern with proteins being equally distributed between cytoplasm and nucleus (Fig. 4*c*). Although this could be expected for deletion mutant X4Δ6 carrying a deletion of the putative nuclear localization signal, it is unclear why mutants X4Δ2 and Δ3 do not properly localize to the nucleus.

Anti-Myc immunoprecipitations from the stable transfectants were assayed for nick ligation activity. In agreement with results obtained with transient coexpression of the XRCC4 deletion mutants with recombinant DNA ligase IV, we only found ligase activity in immunoprecipitates with XRCC4 deletion mutants X4Δ1, Δ2, Δ6, and Δ7 (Fig. 4*b*), confirming that deletions within the central region of XRCC4 (amino acid positions 100–250) disrupted association with the endogenously expressed DNA ligase IV.

Analysis of X-ray Sensitivity and V(D)J Recombination in Stable Transfectants of XRCC4 Mutants—Stable transfectants expressing the XRCC4 deletion mutants were assayed for their DNA repair phenotype and for their ability to confer V(D)J recombination on extrachromosomal recombination substrates.

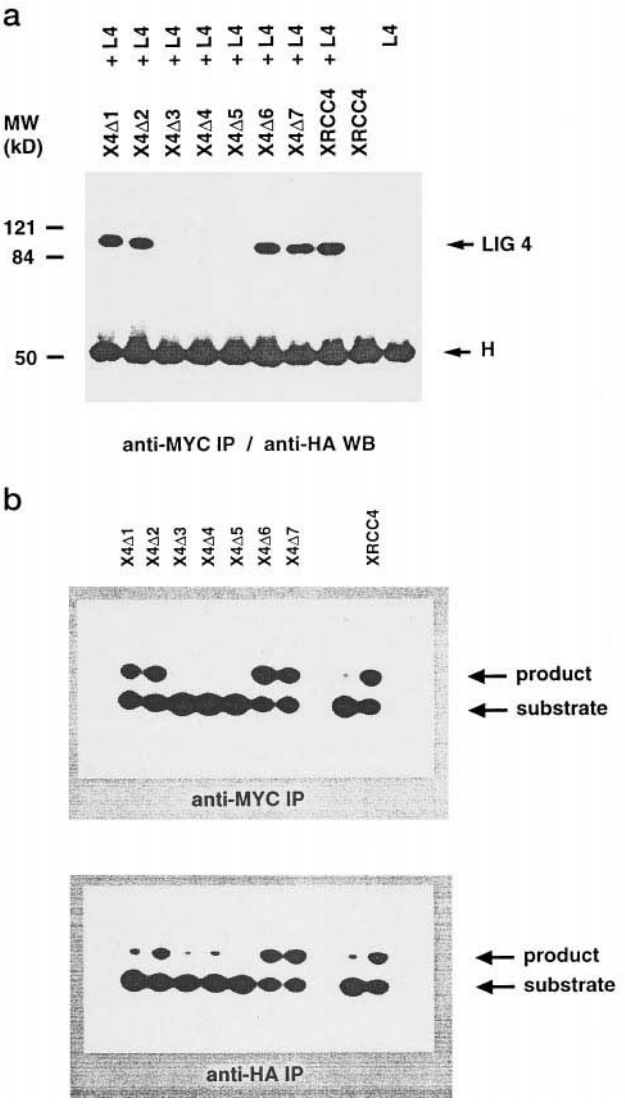

FIG. 3. *a*, co-immunoprecipitation of HA-tagged DNA ligase IV with XRCC4 deletion mutants. Myc-tagged XRCC4 deletion mutants (as indicated) were transiently coexpressed with HA-tagged DNA ligase IV and were anti-Myc-immunoprecipitated. The IPs were fractionated by SDS-PAGE, Western blotted, and detected with the anti-HA antibody. The position of the co-immunoprecipitated DNA ligase IV (LIG 4) is indicated. The cross-reactive IgH chain of the anti-Myc antibody is also indicated. Proteins were expressed in the XRCC4-deficient cell line XR-1. *b*, ligase activity associated with XRCC4 deletion mutants. Nick ligase activity in anti-Myc IPs (*top panel*) and anti-HA IPs (*lower panel*) from transfections in which HA-tagged DNA ligase IV (present in all samples) was co-expressed with various Myc-tagged XRCC4 deletion mutants. Negative and positive controls (*two rightmost lanes*) include samples containing no XRCC4 and full-length XRCC4, respectively. The *top panel* displays DNA ligase IV activity associating with XRCC4 deletion mutants, whereas the *lower panel* displays the DNA ligase IV activity in the presence of the various XRCC4 deletion mutants.

We found that XRCC4 deletion mutants X4Δ6 and Δ7 were able to fully reconstitute the DNA repair deficiency of the XR-1 cells (Fig. 5*a*), whereas expression of all other XRCC4 deletion mutants did not reconstitute wild-type DNA repair.

V(D)J recombination was analyzed for both signal and coding joint formation using extrachromosomal recombination substrates. In agreement with data from the x-ray sensitivity assay, only deletion mutants X4Δ6 and Δ7 reconstituted wild-type V(D)J recombination, whereas all other deletion mutants were ineffective (Fig. 5*b*). We infer from this that the C-terminal 84 amino acids of XRCC4 are dispensible for both nonhomologous DNA end-joining as well as V(D)J recombination *in vivo*.

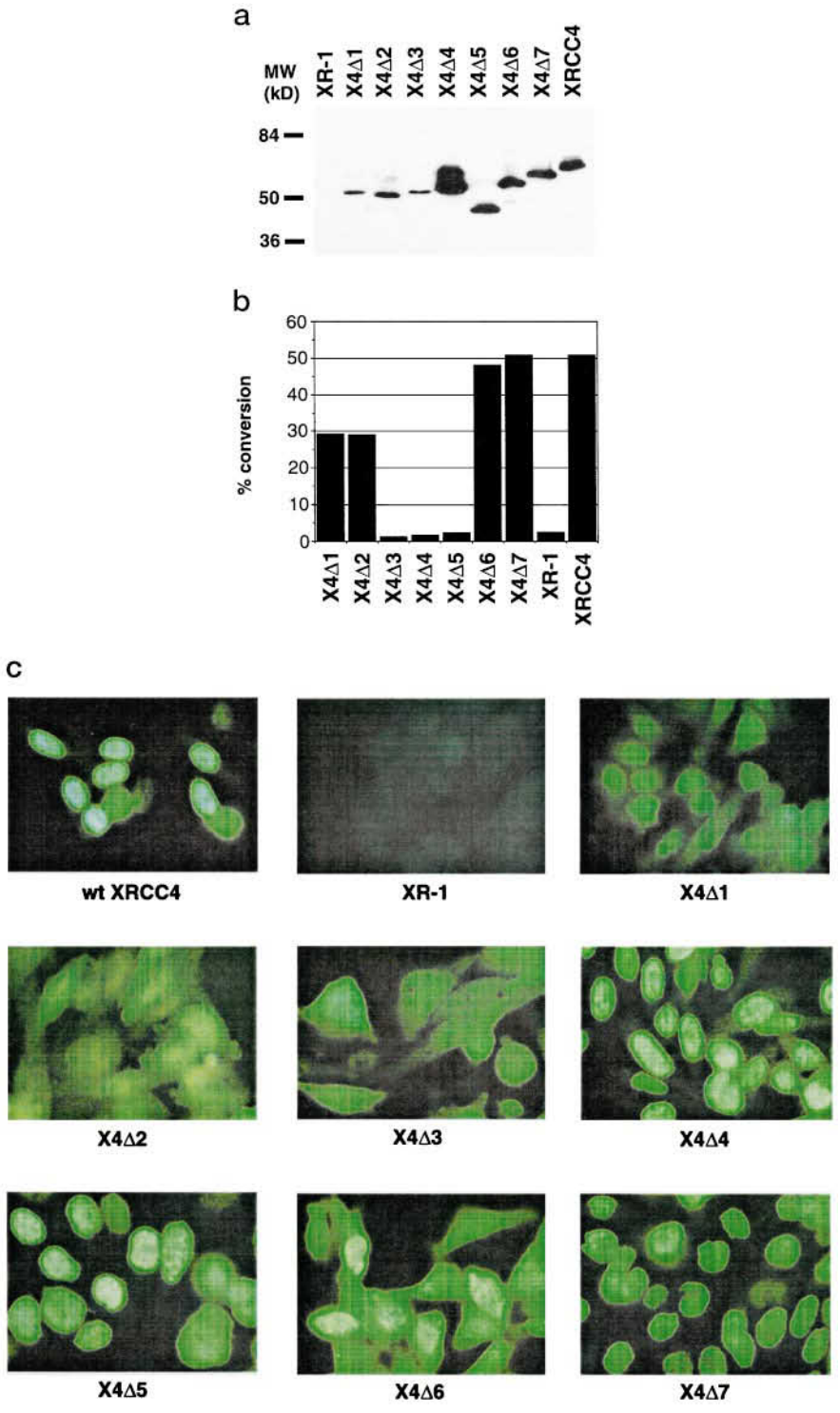

FIG. 4. *a*, anti-Myc Western blot analysis of XRCC4 deletion mutants stably transfected into XR-1 cells including untransfected cells (left) as a negative control and a stable transfectant of full-length XRCC4 (right) as a positive control. Lysates from equal numbers of cells (2×10^5/*lane*) were analyzed. *b*, Anti-Myc co-immunoprecipitation of endogenous DNA ligase activity from stable transfectants with XRCC4 mutants. IPs were performed from identical number of cells, and immunoprecipitates were assayed for nick-ligation activity. The percent conversion of substrate to product was determined by PhosphorImager analysis. *c*, immunocytochemical detection of stably transfected XRCC4 deletion mutants in XR-1 cells. Exposure times for the negative XR-1 control and XRCC4 deletion mutants X4Δ1, Δ2, and Δ3 were three times longer than for all other cell lines. Distinct nuclear staining is characterized by a round-shaped staining pattern with the stained areas separated from each other (by the surrounding cytoplasm of the cells), whereas loss of nuclear localization leads to uniform staining of the spindle-shaped cells with no separation between the stained areas.

DISCUSSION

We and others have recently demonstrated that the DNA repair protein XRCC4 forms a complex with DNA ligase IV (17, 18) and that complex formation stimulates DNA ligase IV activity *in vitro* (17). This has led to the hypothesis that DNA ligase IV is involved in nonhomologous DNA end-joining in mammalian cells. This notion is supported by the finding that the *Saccharomyces cerevisiae* DNA ligase IV homologue is an essential component for NHEJ in yeast cells (19–21). However, using a cell-free *in vitro* V(D)J recombination assay, it has been demonstrated that DNA ligase I and not IV was able to stimulate signal and coding joint formation, leaving it controversial which of the known mammalian DNA ligases might be essential for this process (23).

To test the physiologic significance of the XRCC4·DNA ligase IV complex for NHEJ and V(D)J recombination, we have created a set of XRCC4 deletion mutants that were analyzed for their ability to interact with DNA ligase IV *in vitro* as well as for their capacity to reconstitute wild-type DNA repair and V(D)J recombination upon stable expression *in vivo*. We find a correlation between the inability of XRCC4 deletion mutants to associate with DNA ligase IV and a failure of these mutants to reconstitute wild-type DNA repair or V(D)J recombination in cells. In addition, two of the four XRCC4 deletion mutants that were able to associate with DNA ligase IV reconstituted the DNA repair and V(D)J recombination defect of XR-1 cells back

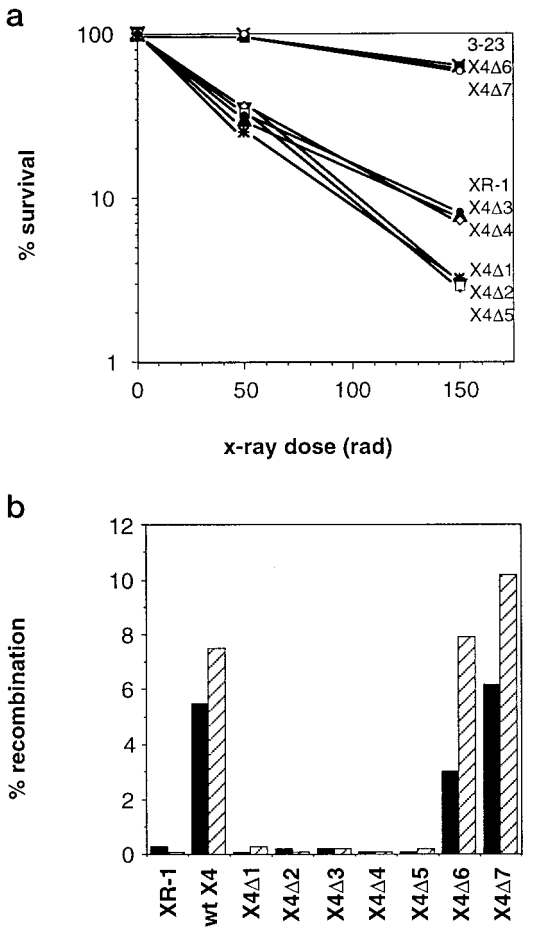

FIG. 5. *a*, x-ray sensitivity assay of stable transfectants expressing XRCC4 deletion mutants. ●, XR-1; ○, 3-23; □, X4Δ1; *, X4Δ2; ◇, X4Δ3; ▲, X4Δ4; ▼, X4Δ5; ×, X4Δ6; ■, X4Δ7. *b*, frequency of signal (*striped bars*) and coding joint (*black bars*) formation with V(D)J recombination substrates transfected into cells stably expressing XRCC4 deletion mutants X4Δ1 to Δ7 (as indicated). The *figure* displays one data set for the x-ray sensitivity and V(D)J recombination assays in which all mutants were analyzed in the same experiment. Individual or subsets of stable transfectants were analyzed at least once more for both x-ray sensitivity and V(D)J recombination in separate experiments with identical results. Controls included untransfected XR-1 cells and a stable transfectant (3–23) expressing full-length XRCC4 (as indicated).

to wild-type levels. It cannot be excluded that deletions, which disrupt binding to DNA ligase IV, might also affect some unidentified DNA repair functions of XRCC4. However, we consider the correlation between the ability or inability to interact with DNA ligase IV and the ability or failure to reconstitute wild-type DNA repair, respectively, compelling and suggest that a physical interaction of XRCC4 with DNA ligase IV is required for both NHEJ and V(D)J recombination in mammalian cells. As an extension from this we conclude that DNA ligase IV is essential for both of these processes and that it cannot function without its co-factor XRCC4.

At this point, we do not have an explanation why XRCC4 deletion mutants X4Δ1 and X4Δ2 failed to reconstitute wild-type DNA repair upon stable transfection in XR-1 cells despite their ability to associate with DNA ligase IV. For one, it is possible that the approximately 10-fold lower XRCC4 expression levels in stable transfections of these mutants were not sufficient to reconstitute wild-type DNA repair function. Furthermore, mutant X4Δ2 does not properly localize to the nucleus, which might affect function of this XRCC4 deletion mutant. However, loss of nuclear localization at high expression levels, as seen for mutant X4Δ6, did not interfere with the reconstitution of a wild-type phenotype, arguing that improper nuclear localization can potentially be compensated by high expression levels.

The fact that XRCC4 deletion mutant X4Δ1 localizes to the nucleus is able to bind DNA ligase IV but fails to confer a wild-type DNA repair phenotype suggests that XRCC4 exhibits functions beyond binding to DNA ligase IV (17, 31). One of the possibilities is a failure to properly stimulate DNA ligase IV activity. However, our data indicate that XRCC4 deletion mutant X4Δ1 can stimulate DNA ligase IV, although with a slightly decreased efficiency compared with that of all other mutants that bind DNA ligase IV.

Our study is in good agreement with recently published data showing that a core region of XRCC4 from amino acid position 18 to 204 is required for its *in vivo* function (31, 32). It is interesting to note that XRCC4 forms homodimers (31, 32) and that the region required for homodimerization overlaps with the region that is essential for DNA ligase IV binding (32). It will be interesting to determine whether homodimerization of XRCC4 is a prerequisite for the association with DNA ligase IV.

We and others have demonstrated that the *S. cerevisiae* homologue of DNA ligase IV is essential for NHEJ in yeast, and it was hypothesized that mammalian DNA ligase IV is therefore also involved in NHEJ and V(D)J recombination. This notion is in apparent contrast to a study demonstrating a role of DNA ligase I and not IV for signal and coding joint formation using a cell-free *in vitro* V(D)J recombination system (23). The latter finding is also in conflict with data demonstrating that V(D)J recombination has been found to occur normally in DNA ligase I-deficient cell lines (33, 34).

Further studies are needed to define the precise physiologic role of the XRCC4·DNA ligase IV complex for DNA repair and V(D)J recombination. However, the correlation between the ability or inability of XRCC4 mutants to bind DNA ligase IV with the respective ability or failure to reconstitute DNA repair and V(D)J recombination provides the strongest *in vivo* data, to date, that the physical association of XRCC4 with DNA ligase IV is essential for both processes.

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