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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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, and the second pathway, nonhomologous DNA end-joining (NHEJ), 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 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.

To test the significance of the XRCC4-DNA ligase IV interaction for nonhomologous DNA repair and V(D)J recombination in vitro, 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 the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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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. 1a).

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. 1a). Universal upstream primer UG058 was 5′-GTATGGAGCTCATGGAGAAGATGAGTCAAGACTGATGG-3′ and universal reverse primer UG059 (5′-ACAAGAAAATCTGCTTGAGCAGGGCCTCAGTCATG-3′), binding downstream of the Myc epitope, was subcloned as a BamHI/EcoRI fragment.

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and Δ5, whereas DNA ligase IV activity was present in immunoprecipitations using XRCC4 deletion mutants Δ1, Δ2, Δ6, and Δ7.

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. 3b). This was not due to apparent differences in either DNA ligase IV or XRCC4 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. 4a). Although expression levels of stably expressed XRCC4 mutant proteins were relatively uniform between independent clones of
the same mutant (data not shown), protein expression levels varied significantly between different XRCC4 deletion mutants (Fig. 4a). 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. 4e). 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. 4c). 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. 4c). 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. 4b), 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.

We found that XRCC4 deletion mutants X4Δ6 and Δ7 were able to fully reconstitute the DNA repair deficiency of the XR-1 cells (Fig. 5a), 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. 5b). 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.
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
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results. Controls included untransfected XR-1 cells and a stable transfection of ligase IV is essential for both of these processes and that 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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