Cellular and Biochemical Impact of a Mutation in DNA Ligase IV Conferring Clinical Radiosensitivity*

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DNA ligase IV functions in DNA non-homologous end-joining, in V(D)J recombination, and during brain development. We previously reported a homozygous mutation (R278H) in DNA ligase IV in a developmentally normal leukemia patient who overresponded to radiotherapy. The impact of this hypomorphic mutation has been evaluated using cellular, biochemical, and structural approaches. Structural modeling using T7 DNA ligase predicts that the activity and conformational stability of the protein is likely to be impaired. We show that wild type DNA ligase IV-Xrc4 is an efficient double-stranded ligase with distinct optimal requirements for adenylate complex formation versus rejoining. The mutation impairs the formation of an adenylate complex as well as reducing the rejoining activity. Additionally, it imparts temperature-sensitive activity to the protein consistent with the predictions of the structural modeling. At the cellular level, the mutation confers a unique V(D)J recombination phenotype affecting the fidelity of signal joint formation with little effect on the frequency of the reaction. These findings suggest that hypomorphic mutations in ligase IV may allow normal development but confer marked radiosensitivity.

Defects in DNA repair mechanisms underlie several hereditary disorders associated with cancer susceptibility including hereditary non-polyposis colon carcinoma, familial breast cancer, ataxia-telangiectasia, and xeroderma pigmentosum. Many of these repair processes or the proteins involved in them have additional functions such as the role of excision repair proteins in transcription and the role of double strand break repair proteins in V(D)J recombination (1). Inactivating mutations in many DNA repair proteins are associated with developmental abnormalities and confer characteristic clinical features such as those found in ataxia-telangiectasia and xeroderma pigmentosum. There is mounting speculation and some evidence that subtle defects or polymorphisms in proteins involved in damage response mechanisms might not be associated with overt clinical features characteristic of a defined syndrome but nonetheless be responsible for some, and possibly the majority, of sporadic cancer incidence. The identification of mutations/polymorphisms in these genes and understanding their impact on cancer predisposition and sensitivity to chemotherapy and radiotherapy is an important challenge ahead. Now that the phenotype conferred by null mutations in many of the DNA repair genes is known a next step is to understand the impact of hypomorphic mutations, and subsequently we may be able to evaluate the significance of more common polymorphic changes.

Previously, we reported a mutation in DNA ligase IV in a 12-year-old leukemia patient who was developmentally normal until the onset of leukemia but dramatically overresponded to radiotherapy (2). This represents a rare case in which the genetic defect in a developmentally normal, radiosensitive individual has been identified and marks DNA ligase IV as a protein contributing to clinical radiosensitivity. DNA ligase IV is one of five identified components that function in DNA non-homologous end-joining (NHEJ), the major mechanism for DNA double strand break (DSB) repair in mammalian cells (3–5). The other components include Xrc4, a protein that interacts strongly with DNA ligase IV, and the three subunits of DNA-dependent protein kinase (DNA-PK), Ku70, Ku80, and the large catalytic subunit termed DNA-PKcs (DNA-PK catalytic subunit) (for reviews, see Refs. 6 and 7). NHEJ also functions to effect rearrangements at site-specific DSBs introduced during V(D)J recombination (8). Cell lines defective in NHEJ proteins display radiosensitivity, a reduced capacity to repair DSBs, and impaired V(D)J recombination. Mice defective in the different NHEJ proteins show somewhat distinct phenotypes suggesting either that the proteins have additional functions or that the loss of the different components differentially impacts upon NHEJ. DNA ligase IV and Xrc4 knock-out mice are embryonic lethal, and mice null for Ku70 or Ku80 are severely growth impaired, whereas DNA-PKcs-null mice grow and develop normally. DNA-PK-defective mice, however, show severe combined immunodeficiency. A cell line (180BR) derived from the radiosensitive patient defective in DNA ligase IV showed marked cellular radiosensitivity and defective DSB repair but surprisingly did not appear to be defective in the ability to carry out V(D)J recombination (9, 10). The patient showed no overt immunodeficiency or developmental abnor-

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1 The abbreviations used are: NHEJ, non-homologous end-joining; DSB, double strand break; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, DNA-PK catalytic subunit; ds, double-stranded; FAR, fraction of activity released.
mality other than the onset of leukemia (11). The homozygous mutation in DNA ligase IV in 180BR cells is the substitution of a histidine for an arginine (R278H) within a motif close to the active site lysine that is highly conserved between AP end-dependent DNA ligases and GTP-dependent mRNA capping enzymes (2). Initial studies of the impact of this mutational change on DNA ligase IV function suggested that it did not confer a null phenotype and that the mutant protein retains low residual adenylation activity (2).

In this study, we have examined further the impact of this mutational change using a combination of cellular and biochemical analysis. We have used the known crystal structure of T7 DNA ligase to model the impact of the R278H mutation (12, 13). By complementation analysis, we confirm that a mutation in DNA ligase IV is responsible for the radiosensitivity of 180BR cells and show that the cells do, in contrast to previous findings, have a subtle V(D)J recombination-defective phenotype that impacts most noticeably on signal joint formation. We have carried out a biochemical analysis of wild type and mutant DNA ligase IV-Xrc4 complexes that provides insight into the activity of the wild type complex and the impact of the R278H mutation. Our findings suggest that a reduced level of DNA ligase IV activity is sufficient to carry out V(D)J recombination efficiently although with impaired fidelity. We suggest, however, that the ligase IV activity in this patient was insufficient to repair efficiently the length of breaks induced by ionizing radiation thus conferring marked radiosensitivity.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture Conditions**—180BRneo is an SV40-transformed but not immortalized cell line derived from primary 180BR cells. Transformed 180BRneo rather than primary 180BR cells were used in all experiments. 1BRneo is an SV40-transformed and immortalized cell line derived from a normal human fibroblast cell line. Cells were cultured in minimum essential medium supplemented with 15% fetal calf serum, penicillin, and streptomycin as described previously (14). Survival following exposure to γ-rays was carried out using a cobalt-60 source (1 gray/min) as described previously (14).

**Retroviral Infection—Histidine-tagged DNA ligase IV cDNA and β-galactosidase cDNA were each cloned into the retroviral vector LZRSpBMM. To create amphotrophic retroviruses, the packaging cell line PT67 (CLONTECH Laboratories, Inc.) was transfected with 2 μg of LZRSpBMM-hisligIV or control LZRSpBMM-Z using the Fugene transfection procedure (Roche Molecular Biochemicals). The cells were incubated at 37 °C for 24 h and then transfected to 30 °C. 48 h after transfection, the amphotropic retrovirus was added to the culture. At 3 days after transfection, virus-containing medium was collected, filtered through 0.45-μm filters, and used to infect 30% confluent 1BRneo or 180BRneo cells in the presence of Polybrene (10 μg/ml).

The transduction efficiency was monitored by staining the cells infected with LZRSpBMM-Z virus supernatant for β-galactosidase. Between 60 and 80% of the cells stained positively for β-galactosidase.

**Double Strand Break Rejoining—** 2–3 × 10⁶ cells were labeled with 0.02 μCi/ml [³²P]thymidine (50–60 μCi/mm, Amersham Pharmacia Biotech) for 48–50 h followed by incubation for 2 h in unlabeled medium. Agarose plugs were prepared from the cells (now in stationary phase) irradiated with 40-gray γ-rays and incubated for repair as described previously (15). The cells were lysed in the plugs, and DNA fragments were separated by pulse field gel electrophoresis using a Bio-Rad CHEF DR3II with forward and backward pulse times of 30 min and a total electrophoresis time of 48 h at 1.5 V/cm. After electrophoresis, the gel was placed onto DE18 paper (Whatman), dried for 3 h at 50 °C, and analyzed on a STORM PhosphorImager using ImageQuant software (Molecular Dynamics).

**V(D)J Recombination Assays—** Assays to examine the frequency of V(D)J recombination were performed essentially as described previously using the recombinase substrates pG4G9 and pG551 (16–18). To assess the fidelity of signal joint formation, colonies bearing rearranged pG4G9 plasmids (i.e. ampicillin/chloramphenicol-resistant bacteria) were subjected to colony-filter hybridization and challenged with a probe (Sj2) (19) specific for a precise signal joint, and the percentage of hybridizing clones was determined. The plasmids from non-hybridizing clones were then chosen for sequence analysis. To sample the coding junctions, rearranged pG551 plasmid DNA was recovered from ampicillin/chloramphenicol-resistant colonies, and the junctional sequence was determined. Three independent transfections were carried out for each experiment, and the mean results are presented.

**Expression and Purification of Ligase IV Wild Type and Mutant Proteins in the Complex with Xrc4d Protein—** Both protein complexes were expressed in the Bac-To-Bac™ Baculovirus expression system (Life Technologies, Inc.) and purified to near homogeneity on metal chelate TALON affinity resin. For the biochemical studies both complexes were further purified and concentrated using MonoQ FC 1.3/5 ion-exchange resin (Amersham Pharmacia Biotech). Aliquots of the protein complexes were stored in −80 °C in the presence of 10% glycerol.

**Adenylase Assay—** Protein complexes were pretreated with 5 μl disodium PPi for 15 min at room temperature in adenylation buffer (60 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 5 mM dithiothreitol). A Vivascipin 500 column (Sartorius) was used to remove PPi, and then [γ-³²P]ATP (0.5 μCi), ICN Pharmaceuticals, Inc.) was added to each reaction and incubated for 10 min at the indicated temperatures. Reactions were stopped by the addition of 1 volume of 2 × standard SDS-polyacrylamide gel electrophoresis buffer. The samples were boiled for 5 min and run on a 9% SDS-polyacrylamide gel. Fixed and dried gels were analyzed and quantified using a STORM PhosphorImager (Molecular Dynamics).

**Nick Ligation Assay—** The nick ligation assay was performed essentially as described previously (2). Briefly, the activity of protein complexes were incubated for 1 h at different temperatures in 20 μl of reaction mixture (50 mM triethanolamine, pH 7.5, 2 mM Mg(OAc)₂, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 12% polyethylene glycol, and the indicated amounts of ATP) with 10 ng of the nicked DNA substrate prepared by the annealing of three complementary synthetic oligonucleotides (53-, 5′-, and 3′-)mers. The 17-mer was labeled at the 5′-end prior to annealing using T4 PNK (Roche Molecular Biochemicals) and [γ-³²P]ATP. The reactions were stopped by the addition of 1 volume of standard formamide sequencing buffer. Samples were boiled for 5 min, and aliquots were loaded onto a 15% denaturing polyacrylamide gel. Fixed and dried gels were analyzed and quantified using a STORM PhosphorImager (Molecular Dynamics).

**ds Ligation Assay—** The indicated amounts of protein complexes were incubated for 2 h in 30 μl of reaction mixture (50 mM triethanolamine, pH 7.5, 2 mM Mg(OAc)₂, 2 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 12% polyethylene glycol, and the indicated amounts of ATP) with 20 ng of the AflIII-PstI fragment of BLUESCRIPT plasmid as a substrate ([γ-³²P]ATP labeled on the 5′-end). After incubation, the reactions were deproteinized, phenol/chloroform-extracted, and precipitated with Pellet-Paint coprecipitant (Novagen). Aliquots of the reactions were run on 0.8% agarose gels. Dried gels were analyzed and quantified using a STORM PhosphorImager (Molecular Dynamics).

**Molecular Modeling of the R278H Mutation—** Molecular modeling studies used the 2.6-Å resolution crystal structure of T7 DNA ligase (12) (Protein Data Bank code 1A0). The structure of the ligase also contains co-ordinates for bound ATP. The arginine 37 of T7 ligase was replaced by a histidine, and its position was initially optimized manually to satisfy both steric and electrostatic constraints. Final optimization of the structures were achieved using energy minimization, and molecular dynamics protocols were carried out using the program Modeller (27) to relieve areas of steric strain.

**RESULTS**

**DNA Ligase IV cDNA Complements the Radiosensitivity and Repair Defect of 180BRneo Cells—** To verify that the R278H mutation is responsible for the repair defects in 180BR cells, we examined the ability of DNA ligase IV (LIGIV) cDNA to correct the chloramphenicol-resistant defect in 180BR cells. Transformed but not immortalized 180BR cells are available (designated 180BRneo). The 180BRneo transformed cells undergo 20–25 divisions before ceasing growth; this is insufficient to select transfectants expressing LIGIV and then carry out survival or repair experiments. We therefore used a retroviral infection procedure that gives a high transduction efficiency avoiding clonal expansion. In control experiments, the transduction frequency based on the number of cells expressing β-galactosidase was estimated to be around 60%. 180BRneo cells transduced with...
The radioactivity that enters the gel divided by the total activity (well gel electrophoresis. The fraction of activity released (FAR) transduced with virus particles expressing cells. Survival was also examined in 1BR3neo and 180BRneo cells survival analysis. 1BR3neo and 180BRneo were non-infected control transduction, cells were exposed to ionizing radiation and plated for ation. At times varying between 1 and 4 weeks following retroviral experiments carried out on three separate transduced populations. 180BRneo transduced with ligase IV, they represent the mean of five survival experiments for 1BR3neo and 180BRneo, respectively (data not shown). The results shown are the mean of six and four similar to that shown for uninfected 1BRneo and 180BRneo, respectively (data not shown). We do not attach any significance to the apparent greater correction of the DSB rejoining defect than of radiosensitivity, which may be the result of the lower sensitivity of the DSB repair procedure. A further feature of 180BRneo cells is an inability to form DNA ligase IV-adenylate complexes. Recovery of adenylation activity was also seen in the transduced cells (Fig. 1C). The recovery of this activity is marked, although it remains decreased relative to control cells, consistent with the 60% transduction frequency and with the intermediate survival response. Taken together these results verify that the R278H mutation in LIGIV is responsible for the radiosensitivity and DSB repair defect of 180BRneo cells. 180BRneo Cells Have Larger Deletions at V(D)J Signal Junctions—During V(D)J recombination, site-specific DSBs are introduced at the border between two recombination signal sequences and their adjacent antigen receptor coding gene segments (for reviews, see Refs. 21 and 22). The resulting DSBs have two distinct structures, blunt signal and hairpin coding ends, that are subsequently rejoined by the NHEJ machinery to form two new junctions, signal and coding joints. In contrast to the marked radiosensitivity and impaired DNA DSB rejoining of 180BR cells, the cells have been reported to show a normal level of coding joint formation (9, 10). Although surpris-ing considering the role of NHEJ in V(D)J recombination, this result was consistent with the fact that the patient displayed no obvious immunodeficiency (11). The rejoining of signal and coding joints differs in details: DNA-PKcs is largely dispensable for signal joint formation but required for the formation of coding joints. Signal joints are normally precise, whereas coding joints frequently involve deletions and insertions of nucle-otides. The previous report on V(D)J recombination in 180BR cells examined only the frequency of coding joint formation (10). Here we examined the frequency and fidelity of both signal and coding joint formation using 180BRneo cells. The frequency of both signal and coding joint formation was slightly (2–3-fold) decreased in 180BRneo relative to control cells (Fig. 2). A more marked phenotype was a decrease in the fidelity of signal joint formation; although the signal junctions from 1BR3neo cells were rejoined accurately (98%), deletions ranging from 2 to 40 nucleotides were observed in around 42% of the 180BRneo signal junctions (Fig. 2). Only 2 of the 20 junctions analyzed showed any evidence of microhomology at the junctions (Fig. 2; junctions 5 and 11). A similar analysis of the fidelity of coding junctions failed to show any marked differ-ence between 1BRneo and 180BRneo cells (data not shown). We plus lane). The results presented are the ratio of the FAR remaining at the specified time compared with the FAR at time 0. The FAR for unirradiated cells is subtracted prior to these estimations. Transduced cells were examined at longer times post-infection (1–4 weeks) com-pared with the survival analysis to allow time to mass sufficient cell numbers. Cells transduced with virus expressing β-galactosidase showed FAR values similar to the respective control cells (data not shown for clarity). The results represent the mean of three experiments. C, formation of ligase IV-adenylate complexes in complemented 180BRneo cells. Whole-cell extracts (1.5 mg of protein) of control or transduced cells were immunoprecipitated using anti-Xrcc4 antibodies. The immunoprecipitated material was treated with PPi, to disrupt preformed DNA ligase IV-adenylate complexes and used in an assay for adenylation activity.
that the active site lysine is positioned adjacent to the ATP
the ATP-dependent bacteriophage T7 DNA ligase has revealed
AMP in the ligase-adenylate complex. The crystal structure of
signal and coding joints was also assessed in the three experiments.

**A**

| Cell          | Frequency | Fidelity | Frequency |
|---------------|-----------|----------|-----------|
| BRneo         | 3.5 +/- 0.4 | 98 +/- 0 | 3 +/- 0.4 |
| 180BRneo      | 1.3 +/- 0.3 | 58 +/- 4 | 1.4 +/- 0.4 |
| 180BRneo + 2.7 (1,833,5) | 71 (69,73) |

**B**

Precise signal joint:

[Formula]

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fig. 2. analysis of v(D)j recombination in 180BR neo. A, v(D)j recombination frequency and fidelity. The frequency and fidelity of signal and coding joint formation was assessed in 1BRneo and 180BR-neo cells. The frequency shown represents the mean and standard deviation of three independent experiments. The level of fidelity of signal and coding joints was also assessed in the three experiments. B, signal junctions formed in 180BR cells. The lower panel shows the sequence analysis of 20 randomly imprecisely rejoined signal junctions from 180BRneo cells.

next examined whether LIGIV cDNA could complement the defect in signal junction fidelity by co-transfecting LIGIV cDNA with substrates and recombination activating genes. Significant correction was observed in both frequency and fidelity of rejoining (Fig. 2). The R278H mutation, therefore, does impact upon V(D)J recombination resulting in imprecise rejoining of signal ends.

**Structural Modeling of the R278H Mutational Change**—The R278H mutational change lies within a motif KDGDXR (designated motif I) that includes the active site lysine, which is highly conserved between ATP-dependent DNA ligases and GTP-dependent mRNA capping enzymes (12, 23–26). The lysine ε-amino group in this motif forms a covalent bond with AMP in the ligase-adenylate complex. The crystal structure of the ATP-dependent bacteriophage T7 DNA ligase has revealed that the active site lysine is positioned adjacent to the ATP γ-phosphate, and the KDGDXR motif forms a loop structure close to the ATP with the arginine residue forming a hydrogen bond with the 3’-OH of the ATP ribose sugar (12, 26). To assess the significance of the R278H mutational change, we used the T7 DNA ligase structure for modeling studies. Fig. 3A shows hydrogen bond formation (hatched line) between Arg-39 (red), which is the equivalent of the Arg-278 in DNA ligase IV, and the 3’-OH group of the ribose of ATP (blue). Although the histidine residue has a very different side chain compared with arginine, it does have the potential to function directly in enzymatic reaction chemistry. Energy minimization studies and molecular dynamics calculations were carried out to assess the effect of the mutation on the structure (27). Fig. 3B shows three possible configurations of the imidazole side chain of which position 3 is the most energetically favorable. In this conformation the histidine is flipped back into a large cavity below the active site loop preventing any clashes with surrounding residues. All three conformations are likely to severely impair but not necessarily abolish hydrogen bond formation with the ribose.

This structural analysis also suggested that the arginine residue in the active site may form a hydrogen bond network with other neighboring sites. Arg-39 of T7 ligase is held in place by a hydrogen bond to the main carbonyl of Phe-9 and a salt bridge to the Asp-91 (Fig. 3C). Asp-91 is in turn hydrogen-bonded with Tyr-228 (Fig. 3C) (12). The PBCV-1 ligase has virtually the same network of protein interactions between the corresponding motif I Arg-32, motif III Asp-65, and the Tyr-172 side chains (Fig. 3D) except that it hydrogen bonds to the main carbonyl of Leu-7 (13). All these interacting residues, with the exception of Phe-9 of T7 ligase, are conserved in all ATP-dependent DNA ligases including human DNA ligase IV. It is likely that this network stabilizes the conformation of the active site loop. These interactions would be lost if the arginine were replaced by a histidine, raising the additional possibility that the active site may have decreased conformational stability.

As a first step, we compared the global conformational stability of recombinant wild type or mutated DNA ligase IV-Xrcc4 complexes using far-UV circular dichroism. Both complexes showed remarkable stability up to high temperatures (95 °C), and no differences could be detected between their spectra (data not shown). These results show that the mutation does not confer a global change in protein stability, but they do not eliminate the possibility of a local conformational change involving the active site.

**Adenylate Complex Formation Is Impaired but Not Abolished in DNA Ligase IV Expressing the R278H Mutation**—The first step of the ligation reaction is the formation of a ligase-adenylate complex, most likely involving hydrogen bond formation with Arg 278 (12). Previous studies have shown that the majority of endogenous DNA ligase IV exists as a preadenylated complex (28). In the presence of DNA ends, AMP is transferred to the 5’-phosphate group at the break site to form a DNA-adenylate complex (step 2), and finally the DNA is ligated with the release of AMP and non-adenylated DNA ligase IV (step 3).

Using baculovirus-expressed wild type DNA ligase IV-Xrcc4 complexes no adenylate complex formation was observed without prior treatment with disodium PPi, indicating that the majority of baculovirus-expressed DNA ligase IV is also preadenylated (Fig. 4A, lane 1, −PPi). Following PPi treatment, DNA ligase IV efficiently formed adenylate complexes (Fig. 4A). We next examined the effect of temperature on this reaction and observed optimal activity at 16 °C that decreased markedly at 37 °C (Fig. 4B). Previously we examined DNA ligase IV adenylation activity in 180BRneo and wild type cell extracts following immunoprecipitation of the complex with Xrcc4 antibodies and were unable to detect any residual activity using 180BRneo cell extracts (2). However, using bacterially expressed R278H mutant protein, low residual adenylation activity (0.5% wild type) was detected providing evidence that the mutant protein is impaired but not abolished in its ability to form a DNA ligase IV-adenylate complex (2). Here we used the baculovirus-expressed complexes for further, more quantitative analysis. As with the wild type complexes, no adenylate complex formation was observed without prior treatment with disodium PPi, indicating that the majority of baculovirus-expressed DNA ligase IV is also preadenylated (Fig. 4A, lane 1, −PPi). Following PPi treatment, DNA ligase IV efficiently formed adenylate complexes (Fig. 4A). We next examined the effect of temperature on this reaction and observed optimal activity at 16 °C that decreased markedly at 37 °C (Fig. 4B). Previously we examined DNA ligase IV adenylation activity in 180BRneo and wild type cell extracts following immunoprecipitation of the complex with Xrcc4 antibodies and were unable to detect any residual activity using 180BRneo cell extracts (2). However, using bacterially expressed R278H mutant protein, low residual adenylation activity (0.5% wild type) was detected providing evidence that the mutant protein is impaired but not abolished in its ability to form a DNA ligase IV-adenylate complex (2). Here we used the baculovirus-expressed complexes for further, more quantitative analysis. As with the wild type complexes, no adenylate complex formation was observed without prior treatment with disodium PPi, indicating that the majority of baculovirus-expressed DNA ligase IV is also preadenylated (Fig. 4A, lane 1, −PPi). Following PPi treatment, DNA ligase IV efficiently formed adenylate complexes (Fig. 4A). We next examined the effect of temperature on this reaction and observed optimal activity at 16 °C that decreased markedly at 37 °C (Fig. 4B). Previously we examined DNA ligase IV adenylation activity in 180BRneo and wild type cell extracts following immunoprecipitation of the complex with Xrcc4 antibodies and were unable to detect any residual activity using 180BRneo cell extracts (2). However, using bacterially expressed R278H mutant protein, low residual adenylation activity (0.5% wild type) was detected providing evidence that the mutant protein is impaired but not abolished in its ability to form a DNA ligase IV-adenylate complex (2).
following PPi treatment. Second, they confirm that the mutant protein is impaired but not ablated in its ability to form an adenylate complex.

Wild Type DNA Ligase IV-Xrcc4 Has Distinct Temperature Optima for Adenylation versus Rejoining: R278H Mutant Protein Is Impaired in Nick Ligation—We next exploited the fact that the majority of recombinant protein is endogenously preadenylated to monitor the rejoining steps (steps 2 and 3) of the ligation reaction separately from the adenylation step (step 1) by examining ligation of a nicked DNA substrate in the absence of exogenous ATP. As anticipated, significant rejoining activity was observed (Fig. 5A). This activity was optimal at 37 °C decreasing ~2-fold at 16 °C in contrast to the temperature optimum required for the adenylation reaction (compare Fig. 5B with Fig. 4B). The difference in temperature optima for these two steps of the ligation reaction suggested that coordinated adenylation/ligation might not be efficient under these in vitro conditions. To test this, we examined the ability of the complex to rejoin a nicked DNA substrate in the presence of ATP to allow readenylation. Titration experiments indicated that 0.2 mM ATP was optimal (data not shown), but only a small stimulation (0.3-fold) was observed when the reaction

FIG. 3. A, ATP binding in the active site of T7 ligase. The conserved residues involved in ATP binding are shown. Lys-34, which forms a covalent phosphoramidite with the nucleotide, is positioned near the α-phosphate of the ATP molecule (blue). Arg-39 (red), the equivalent of Arg-278 in DNA ligase IV, forms a hydrogen bond (hatched line) with the 3′-OH of the ribose moiety. B, structural modeling of the R278H mutation in T7 DNA ligase. Arg-39 has been replaced by three different conformations of histidine (shown in red). Histidine, like arginine, is a basic amino acid thus maintaining the positive charge. The imidazole side chain of histidine is relatively short compared with arginine and can adopt a maximum of six positions (rotamers) in the structure. Some of these are sterically equivalent but have inverted imidazole groups. The three most likely conformations (1–3) are shown in red. The modeling program Modeller was used to determine the most energetically favorable (27). The most favored conformation is position 3, and two different rotamers are possible. C and D, local contacts between the conserved motif I arginine and other residues in the T7 and PBCV-1 ligase structures, respectively. The KXDGXK conserved residues (motif I) form part of the active site loop of all ATP-dependent DNA ligases and are involved in ATP binding. Arg-39 of T7 and Arg-32 of PBCV-1 ligases (red) are the equivalent of Arg-278 in DNA ligase IV and hydrogen bond (hatched lines) with the bound nucleoside moiety. Additional hydrogen bonds and salt bridges are also formed with neighboring conserved residues. These interactions stabilize the conformation of the respective DNA ligase active site loops.
was carried out at 37 °C (Fig. 5C, column II). We next carried out the reaction incorporating a temperature shift. The reactions were first incubated for 1 h at 37 °C to allow a cycle of ligation, cooled to 16 °C, and then incubated for 10 min after the addition of 0.2 mM ATP to allow readenylation. Finally the temperature was increased to 37 °C to allow another cycle of rejoining (for details, see the legend for Fig. 5). Under these conditions, a 2.8-fold increase in ligation was observed (Fig. 5C, column III), verifying that the co-ordinated reaction is ATP-dependent and limited by the differing temperature optima of the two steps.

Because both mutant protein and wild type protein are expressed predominantly as an adenylated complex, we were able to examine steps 2 and 3 of the reaction independently of step 1. Reduced but detectable nick ligation activity was observed in the single turnover reaction (Fig. 5B). To quantify the magnitude of the decrease, we examined wild type and mutant activity under differing protein concentrations at 37 °C. Approximately 30-fold higher levels of mutant protein were required to achieve a level of nick ligation similar to that seen with the wild type protein (under conditions in which the reaction was linearly dependent upon protein concentration). Most significantly, however, the mutant protein had a reverse temperature dependence compared with that found for the wild type protein with 2-fold lower activity being observed at 37 °C compared with 16 °C (Fig. 5, A and B). This temperature sensitivity for ligation suggested that the mutant protein has decreased conformational stability.

We also examined the ability of the mutant protein to carry out the cycling adenylation/ligation reaction. The addition of
Aflc closely resembles its IV-Xrcc4 complex to rejoin dsDNA ends because this more
Next we examined the ability of the DNA ligase Ligation stranded Ligase: R278H Mutant Protein Is Impaired in ds
C rated consistent with a dual defect in adenylation and ligation
activity was observed when a temperature shift was incorpo-
mutant protein under these conditions, and no increase in
ATP alone did not significantly stimulate the reaction with the
mutant protein under these conditions, and no increase in
activity was observed when a temperature shift was incorpo-
mental conditions are as described for the nick ligation reaction. LX, DNA ligase IV-Xrcc4; mut, mutant; wt, wild type.

FIG. 6. Double-stranded ligation using recombinant wild type and mutant DNA ligase IV-Xrcc4 complexes. A shows the ds ligation reaction at increasing protein concentrations (left panel), wild type DNA ligase IV-Xrcc4; right panel, mutant DNA ligase IV-Xrcc4). B shows the effect of temperature on ds ligation using wild type (filled columns) and mutant complexes (crosshatched columns). The experimental conditions are as described for the nick ligation reaction. LX, DNA ligase IV-Xrcc4; mut, mutant; wt, wild type.

ATP alone did not significantly stimulate the reaction with the mutant protein under these conditions, and no increase in activity was observed when a temperature shift was incorporated consistent with a dual defect in adenylation and ligation activity (Fig. 5C).

Wild Type DNA Ligase IV-Xrcc4 Is an Efficient Double-stranded Ligase: R278H Mutant Protein Is Impaired in ds Ligation—Next we examined the ability of the DNA ligase IV-Xrcc4 complex to rejoin dsDNA ends because this more closely resembles its in vivo role in NHEJ. The substrate used was a AflI-PstI Bluescript plasmid fragment. Conditions to optimize the reaction were determined, and in contrast to previous reports, we observed highly efficient ligation into dimers and higher order concatamers (Fig. 6, A and B) (28–30). Under optimal conditions, greater than 90% of the double-stranded substrate was converted into higher order concatamers. Only 2–3-fold higher levels of protein were required to see similar levels of ds ligation compared with nick ligation (Figs. 5A and 6A). The ds rejoining reaction was also temperature-dependent with a profile closely resembling the nick ligation reaction. At 37 °C the reaction was about 2-fold more efficient than at 16 °C with intermediate values for room temperature (Fig. 6B).

For the mutant protein, a 20–25-fold higher protein concentration was required to achieve a similar level of ds ligation compared with the wild type protein. The reaction was signif-

FIG. 7. Ligation reaction using a preadenylated nick DNA substrate. A shows control reactions with bacteriophage T4 ligase on the preadenylated AMP-nick substrate. B shows ligation of AMP-nick substrate with wild type DNA ligase IV-Xrcc4 with and without treatment with PPi, and C shows ligation of the same substrate with mutant DNA ligase IV-Xrcc4 complexes following PPi treatment. LX, DNA ligase IV-Xrcc4; mut, mutant; wt, wild type.

The R278H Mutant Protein Is Defective in Rejoining a Preadenylated DNA Substrate—Taken together these results suggest that the mutant protein is impaired both in its ability to form an adenylylation complex as well as in its ability to rejoin DNA. To verify that the R278H mutation impacts upon the ability of the protein to rejoin DNA and not simply on the formation of an adenylylation complex, we dissected the process further to monitor solely step 3 of the ligation reaction by measuring the conversion of a nicked DNA-adenylate complex to a ligated DNA molecule. A nicked DNA-AMP substrate was prepared, and its conversion to a rejoined linear molecule was verified using T4 ligase (Fig. 7A). Using the wild type DNA ligase IV, significant rejoining was observed following but not without PPi treatment (Fig. 7B). This indicates that for the rejoining step 3 the enzyme needs to be in its unadenylated form. As predicted, this interaction is impaired for the mutant protein because no residual ligation activity could be detected on the AMP-DNA substrate either with or without PPi treatment (Fig. 7C). (Note that this assay is less sensitive than the previous nick ligation assay so that 10% residual activity would not be detected.)

DISCUSSION

Many genes involved in DNA damage response mechanisms are essential, and inactivating mutations in both alleles are frequently incompatible with life. In contrast, mutations conferring residual but impaired function in such genes may contribute significantly to sporadic cancer incidence. We have previously identified a hypomorphic mutation in DNA ligase IV in a developmentally normal leukemia patient who was clinically radiosensitive (2). This represents a rare case in which the defect underlying clinical radiosensitivity in a normal patient has been identified and raises the possibility that hypomorphic mutations in DNA ligase IV could contribute to human radiosensitivity and to sporadic cancer incidence. In this study, we have analyzed the impact of this hypomorphic mutation at the cellular, structural, and biochemical level, and we relate the findings to the clinical features of the patient who was homozygous for this mutation. First, by complementation analysis we have confirmed that a defect in DNA ligase IV is responsible for the radiosensitivity and DSB rejoining defect of a cell line,
180BR, derived from the patient consolidating our previous conclusion that this underlies the clinical radiosensitivity of the patient. Surprisingly, neither 180BR cells nor the patient appeared to display any overt defect in V(D)J recombination, a phenotype anticipated from the role of DNA ligase IV in NHEJ (10, 11, 20). Here we show that 180BR cells display a novel and subtle V(D)J recombination phenotype involving an impairment in the fidelity of signal joint formation with only a small change in the frequency of rejoining. Variability between normal cells in this assay makes the small difference in frequency questionable. However, the decrease in the precision of signal joint formation is marked and reproducible. Because the coding sequences constitute the antigen receptor loci, the results of this plasmid assay are consistent with the development of a normal immune response in the patient. However, an effect on the fidelity rather than the frequency of V(D)J recombination could impact upon the onset of leukemia by enhancing, for example, the possibility of a translocation event.

Our analysis of recombinant wild type DNA ligase IV-Xrcc4 complexes has identified several novel features of the ligation reaction. Our results demonstrate that baculovirus-expressed complexes, like those present in human cells, are endogenously preadenylated (28). The adenylation reaction occurs in vitro more efficiently at a non-physiological temperature of 16 °C than at 37 °C, whereas the rejoining step occurs more efficiently at 37 °C. This suggests that the in vitro reaction may not accurately monitor the process in vivo. Significantly, the majority of the R278H mutant protein is also preadenylated both in human and insect cells, although in vitro the adenylation activity appears to be markedly impaired (2) (data shown here). It is possible that preadenylated mutant protein accumulates with time in vitro or that non-preadenylated protein is unstable. An alternative possibility is that in vivo an additional factor facilitates the formation of a preadenylated DNA ligase IV complex. Our results also provide an explanation for the finding of Lee et al. (29) that in vitro DNA ligase IV activity is limited to a single turnover event because readenylation is inefficient at 37 °C. Recent evidence on the crystal structure of adenylated or non-adenylated ligase molecules (12, 13, 24, 26) has shown that the adenylation and rejoining reactions are not closely coupled and that the enzymes undergo distinct conformational changes following adenylation/deadenylation (26). Our results suggest that hydrogen bonding contacts that stabilize ATP binding in the active site may be favored at 16 °C, whereas interactions required for ligation may be optimal at 37 °C. Finally, in marked contrast to previous reports, our results also show that DNA ligase IV is a highly efficient double-stranded ligase (29, 30). The basis underlying this difference in efficiency is currently under investigation.

To assess the impact of the R278H mutation, we first used T7 ligase for structural modeling. This analysis suggested that there might be two effects of the mutation. First, it was predicted that the adenylation activity would be impaired because of the likelihood of the histidine side chain inhibiting the formation of an adenylate complex. Second, it was suggested that the normal hydrogen bond and salt bridge interactions of the arginine side chain with adjacent residues at the active site might be impaired, thus potentially conferring decreased local conformational stability. Although we could not detect any change in the global structural stability of the mutant DNA ligase IV-Xrcc4 complex, ligation activity of the mutant protein was temperature-sensitive compared with the wild type protein consistent with a local destabilization of the active site loop. By dissecting the steps of the ligation reaction, we have shown that the mutant protein is defective in adenylate complex formation as well as in rejoining a preadenylated DNA substrate. Thus, the R278H mutational change has three distinct impacts on DNA ligase IV function that are consistent with and extend other structure/function analyses of DNA ligases. First, the crystal structures of the T7 and PBCV-1 ligases as well as the PBCV-1 capping enzyme have confirmed the significance of the active site loop (motif I) and the function of the arginine residue for adenylate complex formation by hydrogen bonding to the ribose of the covalently bound nucleotide (12, 13, 24, 26). Replacement of the active site arginine residue by alanine in both PBCV-1 ligase and human ligase I abolished formation of the ligase-adenylate intermediate (step 1) (31, 32). Second, Srisakanda et al. (31) also showed that this mutation prevented ligation at preadenylated nicks (step 3). The crystal structures of T7 and PBCV-1 ligases represent two snapshots along the ligation pathway. In the T7 ligase structure, the enzyme has bound ATP but has not yet undergone the adenylation reaction (step 1). The adenosine nucleoside is in a syn conformation, and the Arg is contacting the ribose 3’-O (Fig. 3C). In the PBCV-1 ligase structure, which represents the next step in the pathway, post-adenylation of the enzyme, the nucleoside has shifted from a syn to an anti conformation and a remodeling of the contacts with the Arg-32 now hydrogen bonding to the 2’-O of the ligase-AMP (Fig. 3D). These structures suggest a role for the conserved arginine, not only in step 1, but also in later steps including step 3 as observed here. Finally, the importance of additional local interactions with Arg-278 is evident from the recent studies on PBCV-1 ligase (13). Asp-65 of PBCV ligase, which makes the bidentate salt bridge to the essential Arg-32 (equivalent to Arg-278 in ligase IV; Fig. 3D), is critical for catalysis (13), and a D63A mutation has no strand-joining activity despite being distinct from the active site. These findings highlight the importance of correctly positioning this conserved arginine for interaction with the nucleoside at the active site.

Taken together these findings confirm that the DNA ligase IV activity is impaired but not ablated in 180BRneo. Such partial activity appears to have a greater impact on γ-ray sensitivity than on V(D)J recombination. This is consistent with results of site-directed mutagenesis of Ku80 in which we have detected significant radiosensitivity in lines expressing hypomorphic mutations in Ku80 that confer only modestly impaired V(D)J recombination activity (data not shown). In vivo T or B lymphocytes undergoing V(D)J recombination may encounter only a few breaks. Similarly in the extrachromosomal V(D)J assay each transfected cell may contain only a few broken plasmid molecules. This contrasts with the larger number of breaks per cell introduced in survival or DSB repair analysis. We therefore suggest that the residual preadenylated activity present in 180BR cells may be sufficient to handle efficiently a few but not excessive DSBs. These findings importantly raise the possibility that hypomorphic mutations in DNA ligase IV may not impair normal development but impair significant radiosensitivity. Our findings also show that another phenotype of partial NHEJ activity is an alteration in the fidelity rather than the frequency of V(D)J recombination.

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