DNA ligase IV (Lig4), x-ray cross-complementation group 4 (XRCC4), and DNA-dependent protein kinase (DNA-PK) are essential mammalian nonhomologous end joining proteins used for V(D)J recombination and DNA repair. Previously a Lig4 peptide was reported to be an in vitro substrate for DNA-PK, but the phosphorylation state of Lig4 protein in vivo is not known. In this study, we report that a full-length Lig4 construct was expressed as a phosphoprotein in the cell. Also the full-length Lig4 protein, in complex with XRCC4, was an in vitro substrate for DNA-PK. Using tandem mass spectrometry, we identified a DNA-PK phosphorylation site at Thr-650 in human Lig4 and a potential second phosphorylation site at Ser-668 or Ser-672. Phosphorylation of Lig4 per se was not required for Lig4 DNA end joining activity. Substitution of these amino acids with alanine, individually or in combination, led to changes in Lig4 protein stability of mouse Lig4. The phosphomimetic mutation S650D returned Lig4 stability to that of the wild-type protein. Furthermore DNA-PK was found to negatively regulate Lig4 protein stability. Our results suggest that Lig4 stability is regulated by multiple factors, including interaction with XRCC4, phosphorylation status, and possibly Lig4 conformation.

Nonhomologous end joining (NHEJ) is an efficient mechanism used by mammalian cells to repair DNA double strand breaks (1, 2) and is also required for the process of V(D)J recombination, the rearrangement of immunoglobulin and T-cell receptor genes essential for the generation of a diverse immune response (3). XRCC4 and DNA ligase IV (Lig4), which form a ligation complex in the cell (4, 5), are two critical proteins involved in these two processes (6–8).

The five most well studied proteins that are required for both NHEJ and V(D)J recombination in mammalian cells are Ku70, Ku80, DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, and Lig4. The heterodimeric Ku protein, comprising subunits Ku70 and Ku80, binds strongly to double-stranded DNA ends (9). When bound to DNA, Ku recruits DNA-PKcs and activates its kinase activity (10, 11). The XRCC4-Lig4 protein complex is recruited to the DNA ends to complete ligation (4, 5, 12). An additional protein, Artemis, which has nuclease activity and can open DNA hairpin intermediates with DNA-PKcs during V(D)J recombination (13), has also been shown to be involved in both NHEJ and V(D)J recombination (14, 15).

Mice lacking NHEJ components have abnormal lymphocyte development due to defective V(D)J recombination (16). Mice deficient in Ku70, Ku80, XRCC4, or Lig4 also exhibit growth defects, premature senescence, and hypersensitivity to ionizing irradiation. Deficiency of XRCC4 or Lig4 is most severe, resulting in significant neuronal apoptosis and embryonic lethality (7, 8, 17). Embryonic fibroblast cells that are deficient for either DNA-PKcs or Artemis also exhibit cell type-specific ionizing irradiation hypersensitivity (16). Defects in the NHEJ factors have also been implicated in the generation of chromosome instability and cancer development. Deficiency of XRCC4 or Lig4 in mice has been reported to be associated with genomic instability (18, 19), pro-B-cell lymphoma formation (20, 21), and medulloblastoma formation (22). Hypomorphic mutations in Lig4 were identified in some human patients displaying immunodeficiency and developmental delay (23). Polymorphisms in human XRCC4 and Lig4 have been associated with variations in the risk of cancer development (24–27). Lig4 contains one ATP-dependent DNA ligase domain at the N-terminal region (28) and two BRCA1 C terminus (BRCT) domains at the C-terminal region (29). The BRCT domain is often implicated in protein-protein interactions and is found predominantly in proteins involved in cell cycle checkpoint functions responsive to DNA damage. The BRCT domains of some proteins have been identified as phosphopeptide binding motifs (30–32). Lig4 binds XRCC4 via a region between the two BRCT domains (4, 33, 34). XRCC4 functions in multiple ways to potentiate DNA double strand break repair by Lig4, including the enhancement of Lig4 intracellular stability (35–37) and the stimulation of the enzymatic activity of Lig4 by potentiating the formation of the activated enzyme-adenylate complex (38). XRCC4 also has intrinsic DNA binding activity and may target Lig4 specifically to sites of double strand breaks in a manner that depends on functional DNA-PK (38–40). It is still unclear how XRCC4 enhances Lig4 stability and enzyme activity and how the XRCC4-Lig4 DNA-PK complex is regulated before and after DNA double strand break repair.
DNA-PKcs is a serine/threonine kinase in the phosphoinositide 3-kinase family and is believed to be a critical signaling molecule in response to cellular stress. While there are multiple in vitro targets of DNA-PK, very few of these have been proven to be phosphorylated in vivo. The most clearly established in vivo target is DNA-PKcs itself (41–43). Artemis is a strong candidate for DNA-PK phosphorylation (13). XRCC4 has been shown to be an effective substrate for DNA-PK, and there is a DNA-PK-dependent phosphorylation of XRC4C after DNA damage (4, 38, 44, 45). However, the function of DNA-PK-dependent phosphorylation of XRC4C is not clear. Furthermore, little is known about Lig4 protein modification and regulation. There is one report indicating that a Lig4 fragment is an in vitro substrate for DNA-PK (46). Here we report that DNA-PK can phosphorylate Lig4 in vitro when present as a complex with XRC4C. The in vitro sites were identified, and a series of mutations of the phosphorylation sites were generated. Our results suggest that DNA-PK may influence Lig4 stability through phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The plasmid pCMV-Tag-5+-mXRC4C is a C-terminal Flag-tagged mouse XRC4 construct that was derived from an EcoRI-BglII XRC4C fragment from plasmid pcDNA3-mXRC4C (5). Oligonucleotides synthesized to generate the XRC4C 3’ end of the open reading frame without stop codon (GATCCTTCTCTGC ACTGAGTCGAACAACTCAGC) and 3’-TdT (TATACGACTCATTAGGGCGC) and the following specific set of primers: S650A-N, d(GTTTACGTTAGCAAGGTTGGGTGCTTTTAAG); S650D-N, d(GTTTACGTTAGCAAGGTTGGGTGCTTTTAAG); S650A-C, d(GTTTACGTTAGCAAGGTTGGGTGCTTTTAAG); S650D-C, d(GTTTACGTTAGCAAGGTTGGGTGCTTTTAAG). All single mutants were generated by performing two-step PCR. Multiple mutants of mLig4 were generated using the plasmid DNA of the single mutants as the template.

**Antibodies**—The following antibodies were used: anti-FLAG M2 monoclonal antibody (Sigma), rabbit anti-XRC4C (AHP387, Serotec, Raleigh, NC), and anti-DNA-PKcs (clone 1-82, Kamiya Biomedical Company, Seattle, WA).

**Cell Lines**—The human cell lines CHO-K1, V3, and XR-1 (48–50) were maintained in a humidified chamber at 37 °C with 5% CO2 in F-12K medium supplemented with 10% fetal bovine serum, 4 mM t-glutamine, 20 mM HEPES, pH 7.5, 20 μg/ml streptomycin, and 200 units/ml penicillin (all reagents were from Invitrogen).

**Metabolic Labeling**—Cells were transfected with Superfect according to the manufacturer’s conditions (Qiagen, Valencia, CA). After 48–72 h, cells were washed in essential Eagle’s medium supplemented with 10% dialyzed calf serum and then labeled with 0.3 mCi/ml [3H]orthophosphate (Amersham Biosciences) for 3 h at 37 °C. The cells were lysed in Tris lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A). The lysates were cleared by centrifugation, and the supernatant was fractionated on 10 μl of anti-FLAG M2-agarose (Sigma) for 2 h at 4 °C with rotation. The beads were washed six times with Tris lysis buffer, resuspended in Laemmli buffer, and boiled for 5 min. The proteins were separated by 7% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Billerica, MA), and visualized by autoradiography and then Western blot.

**In Vitro Kinase Assay**—Typically 0.5 μg of purified human Histagged Lig4/untagged XRCC4 proteins were incubated with 50 units of DNA-PK (Promega, Madison, WI) with or without 0.1 μg of linearized pBluescript plasmid DNA (Stratagene) in a total volume of 50 μl of DNA-PK reaction buffer (50 mM HEPES, pH 7.4, 100 mM KCl, 10 mM MgCl2, 1 mM dithiothreitol) with or without 10 μM MnCl2. The proteins were expressed in *Escherichia coli* and purified as described previously (51). The mixtures were incubated with 10 μg of γ32P-ATP for 1 h at 37 °C and then purified with Ni-NTA-agarose (Qiagen). Samples were loaded onto SDS-polyacrylamide gels. Following electrophoresis, gels were stained with Coomassie Blue R-250, dried, and then exposed to Kodak X-ray film. Alternatively, cells transfected with FLAG-M2-agarose and XRCC4 in 10-cm dishes were lysed in immunoprecipitation buffer (25 mM HEPES, 100 mM MgCl2, 300 mM KCl, 1% Triton X-100 supplemented with protease inhibitors) and immunoprecipitated with anti-FLAG M2-agarose. The immunoprecipitates were incubated with DNA-PK. In some cases, wortmannin was included at a final concentration of 10 μM.

**Identification of Phosphorylation Sites**—In vitro phosphorylated Lig4 and XRC4 bands were excised, subjected to trypsin digestion, and sequenced by microcapillary reverse-phase high pressure liquid chromatography nanoelectrospray tandem mass spectrometry on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer. Tandem mass spectra were acquired with a relative collision energy of 30% and isolation width of 2.5 Da. Interpreting and assigning tandem mass spectra of the peptides was facilitated with the algorithm SEQUEST and programs developed in the Harvard Microchemistry and Proteomics Facility and then confirmed by manual inspection.

**DNA Ligase Assay**—The assay was performed as described in Ref. 38 with minor modifications. The cohesin沉迷 ligation assay substrate was prepared by digestion of plasmid pCMV-Tag2A (Stratagene) with EcoRI. The resulting linear product was purified by phenol extraction and ethanol precipitation. Assays were performed with 100 ng of substrate in 50 mM HEPES, pH 7.4, 5 mM dithiothreitol, 300 mM KCl, 100 μg/ml bovine serum albumin, 0.5 mM ATP, 10 mM MgCl2, 10 mM MnCl2, 0.5 μg of purified Histagged Lig4/untagged XRC4C, with or without 50 units of DNA-PK, for 20 h at 30 °C in the presence of 10% polyethylene glycol 8000 in a 20-μl final volume. Reactions were incubated at 37 °C for 2 h, stopped by addition of 2 μl of 0.1% SDS, 10 mM EDTA, 5 μl of 5% glycerol, 0.1% bromophenol blue, and then treated with proteinase K for 10 min at 37 °C. The samples were separated by agarose gel electrophoresis in Tris borate/EDTA buffer and stained with ethidium bromide.

**Lig4 Adenylation Assay**—XR-1 cells were transfected with FLAG-tagged Lig4 and FLAG-tagged XRC4C, and cell lysates were immunoprecipitated with anti-FLAG M2 antibody. Adenylation assays were performed as described in Ref. 5.

**VDJ Recombination Assay**—Transient VDJ recombination assays were performed as described in Ref. 6 with minor modifications. In brief, 1 μg of recombination substrate plasmid pPG51 (52) and 1 μg of XRC4C and Lig4 expression plasmids were transfected into 5 × 106 Nalm6 or N114 cells with a BTX electroporator (Harvard Apparatus, Inc., Holliston, MA). Electroporation procedures involved electroporating cells in 0.4-cm cuvettes at 315 V with 10-ms pulse length at room temperature. After transfection and a 48-h incubation, plasmids were recovered by alkaline lysis, extracted with phenol/chloroform, and digested with DpnI. After a second extraction with phenol/chloroform, the DNA was then transferred into E. coli, and the resulting samples were assayed in parallel on ampicillin/chloramphenicol and ampicillin plates.

**Immunoprecipitation**—For Fig. 2A, XR-1 cells in a 10-cm2 dish were harvested in Tris lysis buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml pepstatin A). The lysates were cleared by centrifugation, and the supernatants were precipitated with mouse IgG for 1 h. Next 30 μl of protein A-agarose were added and the minimal incubation was performed for 1 h. The samples were centrifuged, and 30 μl of anti-FLAG M2-agarose were added to the supernatant followed by incubation for 2 h at 4 °C with rotation. The beads were washed six times with Tris lysis buffer, one time with phosphate-buffered saline, resuspended in Laemmli buffer, and boiled for 5 min. The proteins were then separated by 7% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore), and visualized by Western blot.

For Fig. 7, cell extraction and immunoprecipitation were performed as described above except that no ethidium bromide was added in the lysis buffer. To detect XRC4C without the interference of Ig heavy chain.
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RESULTS

Lig4 Is a Phosphoprotein and Potential in Vivo Target of DNA-PK—To determine whether Lig4 is a phosphoprotein, we co-transfected Lig4 and XRCC4 into the XRCC4-deficient XR-1 cell line and then labeled cells with $^{32}$P orthophosphate. As can be seen in Fig. 1A, both Lig4 and XRCC4 were phosphorylated. The XRCC4 phosphorylation signal was much stronger than the Lig4 signal.

A Lig4 fragment had been reported to be an in vitro substrate for DNA-PK (46), so we next asked whether DNA-PK is the kinase responsible for Lig4 phosphorylation. We examined Lig4 phosphorylation in wild-type CHO-K1 cells and DNA-PK-deficient V3 cells. FLAG-tagged mouse Lig4 was transiently transfected along with FLAG-tagged mouse XRCC4. The cells were metabolically labeled with $^{32}$P orthophosphate. Next the proteins were immunoprecipitated with anti-FLAG antibody followed by autoradiography and then Western blot. As shown in Fig. 1B, XRCC4 phosphorylation was detected in both types of cells. However, Lig4 phosphorylation was only detected in the DNA-PK wild-type CHO-K1 cells, not in the DNA-PK-deficient V3 cells. These data suggest that DNA-PK is required for Lig4 phosphorylation.

We next determined whether the endogenous DNA-PK formed a complex with the XRCC4-Lig4 proteins expressed from the constructs that were used in the transfection, which is the prerequisite for DNA-PK phosphorylation. After transfection of mouse FLAG-tagged Lig4 and XRCC4 constructs into the XRCC4-deficient XR-1 cells, cell lysates were immunoprecipitated with anti-FLAG antibody, and Western blots were performed to examine for the presence of DNA-PKcs. As shown in Fig. 2, when either XRCC4 or Lig4 was transfected alone, no visible DNA-PKcs was pulled down. However, when XRCC4 and Lig4 were transfected together, Lig4 expression increased significantly, and DNA-PKcs was detected. This result indicates that the presence of Lig4 could facilitate DNA-PKcs contact with the Lig4/XRCC4 complex. The DNA-PKcs antibody can react with intact DNA-PKcs as well as its degradation products (13), explaining the multiple bands seen in our DNA-PK blots.

To further examine DNA-PK phosphorylation of the full-length Lig4 protein, we performed an in vitro kinase assay. Human Lig4 and XRCC4 were expressed in E. coli and purified as described previously (51). The purified human Lig4/XRCC4 complex was incubated with purified DNA-PK, with Mg$^{2+}$, in the presence or absence of DNA, and with or without Mn$^{2+}$. As shown in Fig. 3A, Lig4 was most obviously phosphorylated in the presence of Mn$^{2+}$ and DNA. In the absence of DNA, the Lig4 phosphorylation signal was very faint and almost indiscernible from background. As an additional analysis, the mouse FLAG-tagged XRCC4-Lig4 complex was immunoprecipitated from V3 cells after transient transfection and then phosphorylated in vitro by adding DNA-PK. As shown in Fig. 3B, the FLAG-tagged mouse Lig4 could still be phosphorylated by DNA-PK, and this phosphorylation was inhibited by wortman-
Ser-672 is only found in human (Fig. 4A). This indicated that Lig4 phosphorylation per se did not significantly influence the ligase activity of Lig4. This result also suggests that DNA-PK stimulation of Lig4 activity is not dependent on its kinase activity (Fig. 5A, compare lanes 3 and 5). The mechanism of stimulation could function by facilitating Lig4 recruitment to ends or by DNA-PK-supported end synthesis.

We next mutated the Ser-650 and Ser-668 sites in mouseFLAG-tagged Lig4 to alanine individually or in combination. Mutants were tested for adenylation activity and V(D)J recombination activity. Lig4 is an ATP-dependent ligase that reacts with ATP to form a covalent bond with AMP. Addition of \([\alpha-^{32}P]ATP\) to the Lig4 immunoprecipitates allows for the formation and detection of ligase-AMP intermediates by autoradiography. XRCC4-deficient XR-1 cells were transfected with FLAG-tagged Lig4 and XRCC4, and cell lysates were immuno-precipitated with anti-FLAG antibody, incubated with \([\alpha-^{32}P]ATP\), and then resolved by SDS-PAGE. As can be seen in Fig. 5B, both S668A and S650A/S668A mutants were adenylationed as efficiently as the wild-type Lig4. The adenylation of the S650A mutant was not detected (data not shown); however, this mutant also had lower expression than the other constructs (Fig. 6B). We then investigated whether the mutations would affect V(D)J recombination. V(D)J recombination assays were performed by transfecting a coding joint substrate pGG51, which replicates in human cells, and a hXRCC4 construct, with or without the Lig4 constructs, into the Lig4-deficient human pre-B-lymphocyte cell line N114. As shown in Table I, the recombination efficiency measured by this assay was not influenced significantly by mutation of the phosphorylation site. Furthermore, we also mutated Ser-650 to aspartic acid, a phosphomimetic mutation. Similar to the alanine substitution, this aspartic acid did not significantly influence the V(D)J recombination efficiency (data not shown). These results further support that Lig4 phosphorylation is not required for its ligase activity.

**Phosphorylation Site Mutation Influences Lig4 Stability**—When comparing the expression of Lig4 mutant constructs with or without mouse XRCC4, an interesting phenomenon was found. In the absence of XRCC4 (Fig. 6A), the expression level was almost the same among all the constructs. In the presence of XRCC4, the S650A mutant expression level was lower than the others (Fig. 6B and C). Wild-type Lig4 and Lig4 mutants were detected in several heterogeneous forms: form A (\(\sim 105\) kDa), form B (\(< 90\) kDa), and form C (\(< 75\) kDa). Form A is the major form presented in wild-type Lig4, the S668A mutant, and the S650A/S668A mutant. Form B is the major form seen in the S650A mutant. In the absence of XRCC4, the S650A mutant form A band was not detected (Fig. 6C).

XRCC4 is known to be able to stabilize Lig4 at the protein level, not at the transcription level (35–37). Because the S650A mutant was expressed at the same level as the other constructs in the absence of XRCC4, the transcription and translation of the S650A-containing construct appears to have no significant difference from the wild-type construct. We speculated that the lower level of the S650A mutant expression in the presence of XRCC4 was due to a stability change in the S650A mutant. Two possibilities were considered. One possibility is that the S650A mutation might influence its interaction with XRCC4, which would make the XRCC4 protection less efficient. The other possibility is that the S650A mutation might directly influence the processing of Lig4 by an unknown degradation pathway.

The interaction between the mutants and XRCC4 was analyzed by transfection of the FLAG-mouse Lig4 and the human XRCC4-his construct into XR-1 cells followed by immunoprecipitation with an anti-FLAG antibody and immunoblotting.
with an anti-XRCC4 antibody. As a control, the total amount of XRCC4-his protein was monitored by immunoprecipitation with Ni-NTA-agarose, and the expression level of XRCC4-his was constant regardless of which FLAG-Lig4 mutant was transfected (Fig. 7, bottom panel). XRCC4-his co-immunoprecipitated with each of the FLAG-Lig4 mutants. The affinity between the S650A mutant and XRCC4 appeared to have no significant change when compared with wild-type Lig4 (Fig. 7A, middle panel). However, the S668A mutant was expressed at a lower level than other constructs (Fig. 7A, top panel). In contrast, the mutant S650A/S668A appeared to have lower affinity for XRCC4, but the level of expression was the same as that of the wild-type Lig4. These results suggest that the cause of the decreased S650A expression is not a change in the affinity for XRCC4.

Another possibility for the lower level of S650A mutant expression in the presence of XRCC4 is that the mutation may facilitate Lig4 processing by its degradation pathway. In that case, a shorter half-life would be expected, so we characterized the half-life of the mutants. As shown in Fig. 8, A–C, the S668A mutation alone did not affect half-life of Lig4 in the presence or absence of XRCC4. Two effects were observed using the protein containing the S650A mutation. The half-life of the Lig4 form A was shortened, and the half-life of the Lig4 form B was increased. The S650A/S668A mutation had a complex influence on the half-life of Lig4. In the absence of XRCC4, the S650A/
tated with anti-FLAG M2-agarose (with XRCC4-his plasmid for 72 h. Cell extracts were immunoprecipi-
tated with XR-1 cells were transfected with FLAG-Lig4 mutant constructs and
antibody was used to detect wild-type Lig4 and Lig4 mutants (panel
and middle panels). Samples were run in
the same gel and analyzed together. For clarity, irrelevant samples
were removed during image processing.

**TABLE I**

| Lig4 construct | RS<sup>a</sup> | Cell type | Exp. | DAC<sup>b</sup> | DA<sup>b</sup> | %R<sup>c</sup> | Relative level<sup>f</sup> |
|----------------|-------------|-----------|------|--------------|-------------|----------|-----------------|
| No Lig4        | pGG51       | Nalm6     | 1    | 174,500      | 412,000     | 42       |                 |
|                |             |           | 2    | 650          | 3,600       | 18       |                 |
|                |             |           | 3    | 177,000      | 1,236,500   | 14       |                 |
| No Lig4        | No RS       | Nalm6     | 1    | 0            | 300         | 0.0      |                 |
|                |             |           | 2    | 0            | 3,400       | 0.0      |                 |
|                |             |           | 3    | 0            | 3,400       | 0.0      |                 |
| Wild type      | pGG51       | N114      | 1    | 20,533       | 79,550      | 26       | 1.0             |
|                |             |           | 2    | 3,285        | 21,250      | 15       | 1.0 (1.0)       |
|                |             |           | 3    | 21,600       | 702,500     | 3.1      | 1.0             |
| S650A          | pGG51       | N114      | 1    | 21,500       | 172,000     | 12       | 0.46            |
|                |             |           | 2    | 1,237        | 20,267      | 6.1      | 0.41 (0.48)     |
|                |             |           | 3    | 12,533       | 680,500     | 1.8      | 0.58            |
| S668A          | pGG51       | N114      | 1    | N/A          | N/A         | N/A      |                 |
|                |             |           | 2    | 2,505        | 19,067      | 13       | 0.87 (1.3)      |
|                |             |           | 3    | 25,967       | 500,500     | 5.2      | 1.7             |
| S650A/S668A    | pGG51       | N114      | 1    | 21,200       | 152,500     | 14       | 0.54            |
|                |             |           | 2    | 2,477        | 30,100      | 8.2      | 0.55 (0.80)     |
|                |             |           | 3    | 35,050       | 847,000     | 4.1      | 1.3             |

<sup>a</sup> Coding joint recombination substrate.
<sup>b</sup> Numbers of ampicillin-chloramphenicol-resistant *E. coli* transformants after DpnI treatment.
<sup>c</sup> Numbers of ampicillin-resistant transformants after DpnI treatment.
<sup>d</sup> Recombination.
<sup>e</sup> Average relative level in parentheses.
<sup>f</sup> N/A, not applicable.

S668A mutation increased the half-life of form A and form B. However, in the presence of XRCC4, this mutation did not affect Lig4 half-life. These results suggest that the phosphorylation of these residues may be important for Lig4 stability.

The effects of the S650A mutation that we observed could be caused by either a loss of the serine hydroxyl or a failure of phosphorylation at this residue. To distinguish between these possibilities, we constructed a phosphomimetic mutation, S650A. This mutation reversed the S650A mutation phenotype (Fig. 9). In the presence or absence of XRCC4, the major form detected in S650D was form A, as seen for the wild-type protein, instead of form B in S650A (Fig. 9A). The half-life of the form A in S650D was extended to more than 24 h (Fig. 9B) similar to the wild-type Lig4 half-life (Fig. 8). In contrast, the form A half-life in S650A was only about 8–12 h in the presence of XRCC4. These results strongly suggest that the phosphorylation status may be the main reason for the change in Lig4 stability.

**DISCUSSION**

In this report, we have shown that Lig4 is a phosphoprotein when transiently expressed in cell lines, and we suggest that the endogenous protein is likely also phosphorylated. Moreover Lig4 in complex with XRCC4 can be phosphorylated by DNA-PK in *vitro*. Our data suggests that the phosphorylation of Lig4 may be less complex than for its partner XRCC4, which is possibly phosphorylated by multiple kinases (44). In DNA-PK-deficient cells, the phosphorylation of Lig4 was not detected, indicating that DNA-PK is the main kinase required for Lig4 phosphorylation.

Our analysis of Lig4 associations within the NHEJ complex demonstrated that Lig4 can facilitate DNA-PKcs binding to the Lig4/XRCC4 complex (Fig. 2). In the absence of Lig4, DNA-PKcs and XRCC4 do not bind efficiently. DNA-PKcs has been reported to enhance Lig4/XRCC4 interactions with Ku (55). These data suggest that the components of the NHEJ complex may have a positive feedback regulation for their assembly. We used ethidium bromide in our assay, which should disrupt DNA-protein interactions (56). This indicated that DNA might not be required for the complex formation as was reported previously (55). Further studies are needed to fully understand the NHEJ complex formation before and after DNA repair.
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the nuclear matrix and a change in protein-protein interactions following a signal of DNA damage (57). We speculate that phosphorylation modification of Lig4 and XRCC4, as well as of the other NHEJ proteins, may affect the protein-protein interactions that are made in response to DNA damage.

Our in vitro kinase assay included Mn²⁺/H11001 (Fig. 3), which is not usually used in the DNA-PK kinase assay, but ataxia telangiectasia mutated and ATR (ataxia telangiectasia and rad3-related kinase), which are in the same kinase family as DNA-PK, require Mn²⁺/H11001 absolutely (46). Lig4 was reported to require Mn²⁺/H11001 for DNA duplex ligation (5). However, how our in vitro system mimics the in vivo condition needs to be further assessed. DNA-PK itself is an interesting kinase. Although initially DNA-PK was depicted as a DNA-dependent kinase that required Ku70/80 for its holoenzyme activity (58), recent data has indicated that DNA-PK may have kinase activity without DNA (59, 60) and also that DNA-PK kinase does not absolutely require Ku for its function in some instances (13, 61). There is still much more to learn about DNA-PK.

Overexpression of Lig4 revealed several different forms of the protein. The expression level of form A, with a molecular mass of about 105 kDa, increased more than 30-fold in the presence of XRCC4. It was also the only band detected when labeled with [α-³²P]ATP in the adenylation assay, so this form should be the major native Lig4 seen in cells. Form B, with a molecular mass of 95 kDa, could be derived from form A by dephosphorylation, or it may be a Lig4 degradation product.
Treatment with λ protein phosphatase did not change form A band into form B band (data not shown), so we speculate that form B is more likely to be a Lig4 degradation product. Differences in other post-translational modifications could also potentially account for the differences between forms A and B. Form C, with a molecular mass of about 75 kDa, is most likely to be a Lig4 degradation product. The levels of both forms B and C increased only about 2–4 times in the presence of XRCC4.

Using mass spectrometry, we identified a clear DNA-PK phosphorylation site at residue Thr-650 in human Lig4 and a potential second phosphorylation site at Ser-668 or Ser-672. However, these sites may still influence the Lig4 stability. Because XRCC4 interacted with a Lig4 mutant in /H9261 treatment with 2 M NaCl or 7 M urea (4, 34). XRCC4 has been shown to stabilize the Lig4 protein (35). The affinity of XRCC4 for Lig4 has been reported to be very strong. The two proteins co-purify over a range of chromatographic techniques, and their association withstands buffer conditions that include 2 M NaCl or 7 M urea (4, 34). XRCC4 has been shown to stabilize the Lig4 protein (35–37). More recent data examining the complex during the cell cycle suggests that Lig4 and XRCC4 interactions and localization may change (46). The identification of phosphorylation sites using a full-length protein is more likely to reflect the in vivo phosphorylation pattern. The mutation of mouse Ser-650, homologous to the human Thr-650, resulted in two effects: a decreased form A stability and an increased form B stability. Although the Ser-668 residue itself had no influence on Lig4 stability when mutated to alanine, Lig4 containing mutation of both Ser-650 and Ser-668 had less affinity with XRCC4 and a longer half-life in the absence of XRCC4. Due to these two effects, the S650A/S668A mutation manifests almost the same half-life as wild-type Lig4 when XRCC4 is present. Residue Ser-668 is within the first BRCT domain of Lig4, while Ser-650 is next to the BRCT domain. Recent reports have shown that the tandem BRCT domains can act as phosphopeptide binding motifs (30–32). We speculate that the tandem BRCT domains of Lig4 might bind the phosphorylated Ser-650 residue so that when Ser-650 is mutated to alanine, the structure of Lig4 might change accordingly. The Ser-668 residue is within the BRCT domain, and when Ser-668 is mutated alone, it has no effect because it is buried in the tandem BRCT pocket. However, when Ser-650 is also mutated, the BRCT domain might be exposed and allow the S668A mutation to show an effect. This model provides a hypothesis about Lig4 stability to be tested in the future. The effect of Lig4 phosphorylation in an animal has yet to be characterized. However, because the phosphomimetic mutation S650D can reverse the effect of the S650A mutation on the stability and the prevalence of the A form of Lig4 (Fig. 9), these results strongly suggest that this site might also be phosphorylated in vivo. It will be very interesting to examine this potential in vivo phosphorylation site by using a phosphospecific antibody to study its possible effect on DNA repair.

The affinity of XRCC4 for Lig4 has been reported to be very strong. The two proteins co-purify over a range of chromatographic techniques, and their association withstands buffer conditions that include 2 M NaCl or 7 M urea (4, 34). XRCC4 has been shown to stabilize the Lig4 protein (35–37). More recent data examining the complex during the cell cycle suggests that Lig4 and XRCC4 interactions and localization may change during the cell cycle (62). In that case, Lig4 stability would not be solely dependent on an interaction with XRCC4. Our data indicated that in the presence of XRCC4, Lig4 half-life could be extended from less than 3 to 30–48 h in XR-1 cells (compare Fig. 8, A and B). XRCC4 plays an important role in Lig4 stability. Because XRCC4 interacted with a Lig4 mutant in which both potential phosphorylation sites were mutated, our results indicated that these sites are not absolutely required for XRCC4 interaction. However, these sites may still influence the Lig4-XRCC4 interaction as S650A/S668A mutants showed a lower affinity for XRCC4.

Our data indicate that phosphorylation is not required for Lig4 ligase activity either in the DNA ligation and adenylation assays or in the transient V(D)J recombination assay. However, this phosphorylation might still play an important role in the DNA repair process. One possibility is that the phosphorylation might play a role in protein-protein interactions. For example, it might influence the interaction with DNA polymerase α (63). Another possibility is that the phosphorylation might influence the DNA repair complex disassembly, after Lig4 has completed the ligation of the broken DNA ends, through the regulation of DNA-PKcs disassembly. In effect, DNA-PKcs autophosphorylation has been reported to facilitate DNA-PKcs disassembly from DNA (64). DNA-PKcs phosphorylation of XRCC4 can cause XRCC4 to dissociate from DNA (38). It is likely that phosphorylation is important to regulate the NHEJ complex on several levels.

Mutation of Ser-650 to alanine shortened half-life of Lig4 form A and increased the half-life of form B, suggesting that Lig4 degradation may be a multistep process. Regulation of Lig4 degradation may contribute to the regulation of DNA repair activity within the cells. The presence of too much or too little repair activity may cause problems (65, 66). This is suggested by the examination of a tumor-prone mouse strain (ink4a/arf−/−) in which haploinsufficiency of Lig4 promoted the development of soft-tissue sarcomas that possess chromosomal aberrations including clonal amplifications, deletions, and translocations (67).

In summary, this study revealed that DNA-PK is an in vitro kinase for the Lig4 protein and that DNA-PK can negatively influence Lig4 stability. The data presented here also suggest that Lig4 stability is finely tuned by multiple factors, including interaction with XRCC4, phosphorylation status, and possibly Lig4 conformation. Further study of the regulation of Lig4 will help to elucidate the molecular mechanism of the DNA-PK-dependent NHEJ pathway.

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