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C-terminal region of DNA ligase IV drives XRCC4/DNA ligase IV complex to chromatin

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ABSTRACT

DNA ligase IV (LIG4) and XRCC4 form a complex to ligate two DNA ends at the final step of DNA double-strand break (DSB) repair through non-homologous end-joining (NHEJ). It is not fully understood how these proteins are recruited to DSBs. We recently demonstrated radiation-induced chromatin binding of XRCC4 by biochemical fractionation using detergent Nonidet P-40. In the present study, we examined the role of LIG4 in the recruitment of XRCC4/LIG4 complex to chromatin. The chromatin binding of XRCC4 was dependent on the presence of LIG4. The mutations in two BRCT domains (W725R and W893R, respectively) of LIG4 reduced the chromatin binding of LIG4 and XRCC4. The C-terminal fragment of LIG4 (LIG4-CT) without N-terminal catalytic domains could bind to chromatin with XRCC4. LIG4-CT with W725R or W893R mutation could bind to chromatin but could not support the chromatin binding of XRCC4. The ability of C-terminal region of LIG4 to interact with chromatin might provide us with an insight into the mechanisms of DSB repair through NHEJ.

1. Introduction

Ionizing radiation (IR) as well as a subset of chemotherapeutic drugs, which are commonly used for cancer therapy, generate various types of DNA damages, among which DNA double-strand break (DSB) is considered most critical. Eukaryotic cells have evolved two major pathways to repair DSBs: homologous recombination and non-homologous end-joining (NHEJ) [1,2]. In NHEJ, a heterodimer of Ku70 and Ku86 first binds to DSB and in turn recruits DNA-PKcs, which possesses protein kinase activity. When necessary, the DNA end is processed by enzymes, such as Artemis, polynucleotide kinase/phosphatase (PNKP), DNA polymerase λ and μ. Finally, two DNA ends are ligated by DNA Ligase IV (LIG4) in association with XRCC4. XLF (also known as Cernunnos) is thought to stimulate the LIG4 activity toward incompatible or mismatched DNA ends.

XRCC4 was initially found as the human cDNA, which could complement the defective V(D)J recombination and radiosensitivity of XR-1 cells, derived from Chinese hamster ovary cell [3]. Subsequently, XRCC4 was found to be associated with LIG4 [4,5]. XRCC4 stimulates the ligation and adenylation activity of LIG4 [4,6] and is also required for the stabilization of LIG4 [7]. While XRCC4 consists of 336 amino acids, structural studies indicated that N-terminal part, spanning ~200 amino acids, forms globular domain and coiled-coil domain, the latter of which mediates dimerization of XRCC4 and its interaction with LIG4 [6,8,9]. Although the structure of the remaining C-terminal part of XRCC4, spanning ~130 amino acids, has not been determined at high resolution, it is deduced from electron microscopy to form a globular structure at the opposite of N-terminal globular head domain [10]. XRCC4 might also have a scaffold role, as it is shown to interact with other repair enzymes like PNKP [11], aprataxin [12] and APLF (aprataxin- and PNK-like factor, also known as PALF, C2orf13 or XIP1) [13,14]. LIG4 is a 911 amino acid protein and its N-terminal part, spanning ~600 amino acids, contains DNA-binding, adenylation and oligo-binding domains, which are thought important for ligase catalytic function. The remaining C-terminal part contains two BRCT (breast cancer

Abbreviations: IR, ionizing radiation; DSB, double-strand break; NHEJ, non-homologous end-joining; PNKP, polynucleotide kinase/phosphatase; LIG4, DNA ligase IV; APLF, aprataxin- and PNK-like factor; BRCT, breast cancer associated 1 C-terminal; FBS, fetal bovine serum; LIG4-CT, DNA ligase IV C-terminal; XIR, XRCC4-interacting region; LIG4-NT, DNA ligase IV N-terminal.

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associated 1 C-terminal) domains and XRCC4 interacting region (XIR) in between [15]. Recent study showed that XRCC4 and XLF, each as a dimer, interact with their globular head domains to form long, helical filaments, which might bridge or align DNA to facilitate ligation [16].

The mechanisms how these proteins are recruited to DSBs have been explored through various approaches. Nick McElhinny et al. showed Ku-dependent DNA binding of XRCC4/LIG4 by electrophoretic mobility shift assay [17]. Hsu et al. demonstrated binding between Ku and LIG4 and that between XRCC4 and DNA-PKcs by Far-Western analysis [18]. Constantini et al. identified the first BRCT domain of LIG4 as a Ku-binding site [19]. Calsou et al. studied the assembly of proteins on DNA immobilized on paramagnetic beads and showed that Ku and DNA-PKcs were necessary for the recruitment of XRCC4/LIG4 onto DNA [20]. In their later study, they showed DSB-induced insolubilization *in cellulo* of XRCC4/LIG4, which required DNA-PKcs as well as Ku [21]. In these studies, no effects of wortmannin, a potent inhibitor of DNA-PK, were observed, suggesting that the role of DNA-PKcs might be independent of kinase activity [20,21]. We reported that radiation-induced chromatin binding of XRCC4 was not abolished by the treatment with wortmannin or the stable expression of DNA-PKcs siRNA, although some reduction was observed [22].

Another emerging approach is the live cell imaging, tracking the behavior of the fluorescently labeled proteins after laser micro irradiation [2]. Mari et al. demonstrated that the accumulation of XRCC4 in irradiated area was dependent on Ku but not on DNA-PKcs [23]. Yano et al. showed that the accumulation of XRCC4 in irradiated area could be observed, but was significantly reduced, in cells lacking DNA-PKcs, indicating the role of DNA-PKcs in stabilizing XRCC4 on chromatin [24]. Moreover, XRCC4 kinetics in kinase-dead DNA-PKcs-expressing cells were similar to normal DNA-PKcs expressing cells, suggesting that DNA-PKcs might play a scaffolding, rather than a catalytic, role [24]. Recent studies by Rulten et al. and Grundy et al. indicated that APLF is recruited to damage site via interaction with Ku and/or PARP-3 and, in turn, promotes the recruitment or retention of XRCC4 [25,26].

In our recent study, we established a procedure to examine the chromatin binding of XRCC4 using a biochemical fractionation analysis using a detergent Nonidet P-40 [22]. In this study, we investigated the role of LIG4 and its subdomains in the recruitment of XRCC4/LIG4 complex to chromatin.

2. Materials and methods

2.1. Cell culture and irradiation

Human pre-B leukemia cell line Nalm-6 cell and its derivatives were used in this study. *LIG4*$^{-/-}$ derivative of Nalm-6 was described in an earlier publication [27]. Normal LIG4 cDNA or various mutants were introduced into *LIG4*$^{-/-}$ as described below. Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin and 14 μM β-mercaptoethanol at 37 °C in humidified atmosphere containing 5% CO_2. Cell density was maintained between 10^5 and 10^6 cells/ml. FBS was purchased from HyClone and other agents were purchased from Nacalai Tesque.

Cells were irradiated using ^{60}Co γ-ray source [22]. The cellular radiosensitivity was assessed in terms of their colony forming ability in soft agarose. Appropriate number of cells were suspended in 4 ml of RPMI1640 medium supplemented with 15% FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, 14 μM β-mercaptoethanol and 0.2% agarose and plated onto a 60 mm-plastic dish. Two weeks after plating, visible colonies were counted.

2.2. Plasmid construction and transfection

LIG4 cDNA, clone MGC:33819 IMAGE:5259632, was originally obtained from Open Biosystems. Details of the plasmid construction and transfection are described in Supplementary Content. Correctness of the sequence was verified for all the constructs.

2.3. Antibodies and Western blotting

Following commercial antibodies were used: anti-LIG4 rabbit polyclonal antibody from Abcam; anti-XLF rabbit polyclonal antibody from BioVision; anti-FLAG monoclonal antibody M2, conjugated with horseradish peroxidase, from Sigma–Aldrich; anti-rabbit immunoglobulin swine antibody, conjugated with horseradish peroxidase, from DAKO. Anti-XRCC4 rabbit polyclonal antibody was generated in our laboratory as described [22]. Procedures of Western blotting followed our earlier publications with minor modifications [22].

2.4. Chromatin-binding analysis

Chromatin-binding status of XRCC4 and LIG4 proteins was examined by sequential extraction with increasing concentration of Nonidet P-40 to separate chromatin-binding and non-chromatin-binding proteins, as we described earlier [22].

Typically, 10^7 cells were suspended in 150 μl of buffer A (50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1/100 volume each of protease inhibitor cocktail for animal cells (Nacalai Tesque), phosphatase inhibitor cocktail I and II (Sigma–Aldrich)) with 0.2% Nonidet P-40. After standing on ice for 5 min, the suspension was centrifuged at 1000*g* for 5 min and the supernatant was recovered as F-I. The remaining cell pellet, denoted P-I, was resuspended in 150 μl of the same buffer and immediately centrifuged at 1000*g* for 5 min. The supernatant of this step was recovered as F-II. The remaining cell pellet, denoted P-II, was then resuspended in 150 μl of buffer A with 0.5% Nonidet P-40. After standing on ice for 40 min, the suspension was centrifuged at 16,000*g* for 5 min and the supernatant was recovered as F-III. The resultant pellet, denoted P-III, was suspended in equivalent volume of 2 × SDS–PAGE loading buffer and heated in boiling water for 5 min. After centrifugation at 16,000*g* for 5 min, the supernatant was recovered as F-IV. F-IV was regarded chromatin-binding fraction, as we have shown that XRCC4 protein found in P-III was liberated by micrococcal nuclease treatment, indicating that it had been tethered to chromatin DNA.

3. Results

3.1. The chromatin binding of XRCC4 is dependent on the presence of LIG4

The chromatin binding of LIG4 and XRCC4 was examined using human pre-B leukemia cell line Nalm-6 cell and its *LIG4*$^{-/-}$ derivative (Fig. 1A). Chromatin-bound XRCC4, *i.e.*, in F-IV, was seen in wild type Nalm-6 cells but not in *LIG4*$^{-/-}$. The chromatin binding of XRCC4 in *LIG4*$^{-/-}$ was restored in a stable transformant of *LIG4*$^{-/-}$ with human LIG4 cDNA (Fig. 1B). These results indicated that the chromatin binding of XRCC4 is dependent on the presence of LIG4.

Chromatin binding of LIG4 and XRCC4 did not increase appreciably after 20 Gy irradiation (Fig. 1B) and a clear increase was observed only after extremely high dose irradiation, *i.e.*, 100 Gy (Fig. 3A). This is in contrast to our earlier study using murine leukemia cells, where the increase was observed even at 2 Gy [22], but similar to other's results using human cells [21].

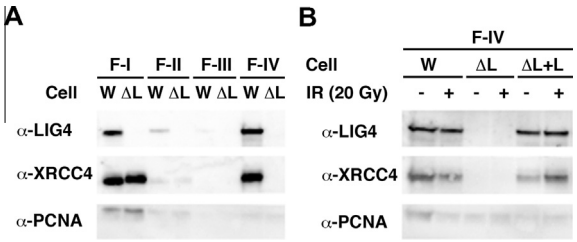

Fig. 1. Chromatin binding of XRCC4 dependent on LIG4. Panels show Western blotting analyses following sequential extraction with increasing concentration of Nonidet P-40 as described in materials and methods. PCNA is shown as a loading control. (A) F-I to F-IV from wild-type (W) and *LIG4*$^{-/-}$ (ΔL) Nalm-6 cells are shown. F-IV represents chromatin binding fraction. Note that F-I, F-II and F-III were diluted 20-, 10- and 2-fold, respectively, by SDS–PAGE sample buffer prior to loading. (B) F-IV, chromatin binding fraction, from wild-type (W), *LIG4*$^{-/-}$ (ΔL) Nalm-6 cells and a stable transformant of *LIG4*$^{-/-}$ with human LIG4 cDNA (ΔL + L). Cells were irradiated with 20 Gy of γ-ray from ^{60}Co source 30 min prior to harvest or left unirradiated, as indicated.

3.2. BRCT domains in LIG4 are essential for the chromatin binding of XRCC4/LIG4 complex

We next sought to determine the subdomain of LIG4 mediating the chromatin binding. We created three point mutants of LIG4 and introduced to *LIG4*$^{-/-}$ (Fig. 2A). N-terminal region contain DNA binding, adenylation and oligo-binding domains, essential for catalytic function. R278H was reported in one of Ligase IV syndrome patients [28] and was shown to decrease the adenylation activity to below 10% [29]. C-terminal region (amino acids 638–911, denoted LIG4-CT hereafter) contains two BRCT domains, BRCT-I and BRCT-II, and XIR (XRCC4-interacting) domain in between (Fig. 2A). To examine possible roles of BRCT-I and BRCT-II domains, we created two point mutants, W725R and W893R. BRCT domain is found in a number of proteins related to DNA repair or DNA damage responses throughout eukaryota and shown to be a binding module for phosphorylated peptide [30,31]. Among BRCT domains of various proteins, amino acid residues corresponding to W725 and W893 are highly conserved. Moreover, changing the corresponding tryptophan residue into arginine in BRCT domains in BARD1 and 53BP1 was shown to compromise the binding to phosphorylated peptide [31]. Therefore, we reasoned that changing W725 and W893 into arginine would compromise the function of BRCT-I and BRCT-II domains, respectively.

In R278H mutant, LIG4 and, possibly, XRCC4 in F-I were somewhat less than those in non-mutated LIG4 (Fig. 2B). This is agreeable with the observation by Riballo et al. that the expression of LIG4 and XRCC4 was reduced in 180BR cell, harboring R278H mutation [28]. However, the chromatin binding of LIG4 and XRCC4 was unaffected in R278H mutant (Fig. 2C). In W725R mutant, the chromatin binding of LIG4 was considerably reduced as compared to non-mutated LIG4 and the chromatin binding of XRCC4 was almost undetectable (Fig. 2C). These results indicated that BRCT-I domain might be important for the chromatin binding of XRCC4/LIG4 complex. In W893R mutant, LIG4 in F-I was diminished (Fig. 2B), suggesting that BRCT-II might be essential for the maintenance of LIG4 stability. The chromatin binding of LIG4 and XRCC4 was at most marginally detectable in W893R mutant (Fig. 2C), but this might be, partially or entirely, due to diminished level of LIG4 itself, which is required for the chromatin binding of XRCC4, as shown above.

3.3. C-terminal fragment of LIG4 is capable of binding to chromatin with XRCC4

We next examined whether LIG4-CT alone, *i.e.*, without N-terminal region (denoted LIG4-NT hereafter), including DNA binding, adenylation and oligo binding domains, can recruit itself and XRCC4 to the chromatin. Expression of LIG4-CT (tagged with triple FLAG epitope) in *LIG4*$^{-/-}$ restored XRCC4 as well as itself in F-IV (Fig. 3A), indicating that C-terminal fragment of LIG4 could drive XRCC4/LIG4 to the chromatin. W725R mutant and albeit to a much lesser extent, W893R mutant could bind to the chromatin (Fig. 3C). However, the chromatin binding of XRCC4 was almost completely abolished in W725R and W893R mutants (Fig. 3C). These results indicated that BRCT-I and BRCT-II domains might be essential for holding XRCC4 on chromatin. It is also noted that W893R in F-I appeared much less than non-mutated LIG4-CT and W725R, as in the case of full-length context (Fig. 3B).

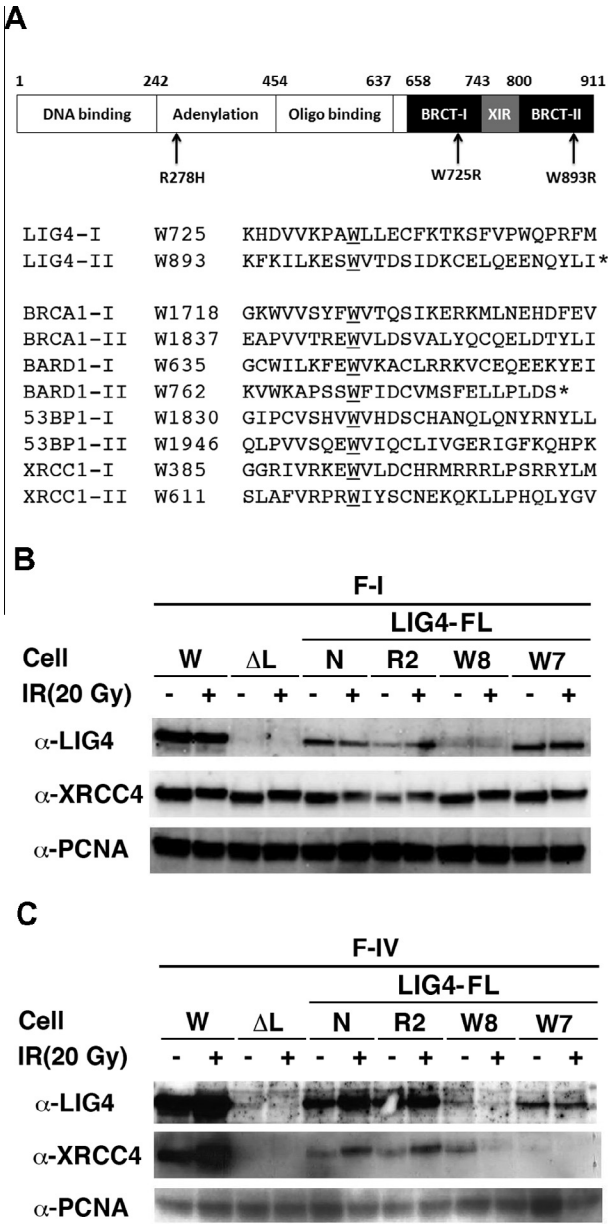

Fig. 2. Chromatin binding of LIG4 mutants with XRCC4. (A) Diagram of LIG4 structure. Below, the sequences of BRCT domains around the conserved tryptophan residues (underlined) of LIG4 and other proteins are aligned. Asterisks indicate the stop codons. (B, C) Western blotting analyses of F-I and F-IV (chromatin-bound fraction). "W" and "ΔL" indicate wild-type and *LIG4*$^{-/-}$ Nalm-6 cells, whereas "N", "R2", "W7" and "W8" indicate stable transformants of *LIG4*$^{-/-}$ with human LIG4 cDNA, R278H, W725R and W893R mutants, respectively. Cells were irradiated with 20 Gy of γ-ray from ^{60}Co source 30 min prior to harvest or left unirradiated, as indicated.

3.4. BRCT mutants are radiosensitive

To examine the importance of BRCT domains in DSB repair, we measured the cellular radiosensitivity by colony formation assay (Fig. 4A). W725R and W893R transformants were more radiosensitive than normal LIG4 transformants. These results indicated that W725R and W893R mutants of LIG4 have reduced DNA repair ability, substantiating the importance of BRCT domains.

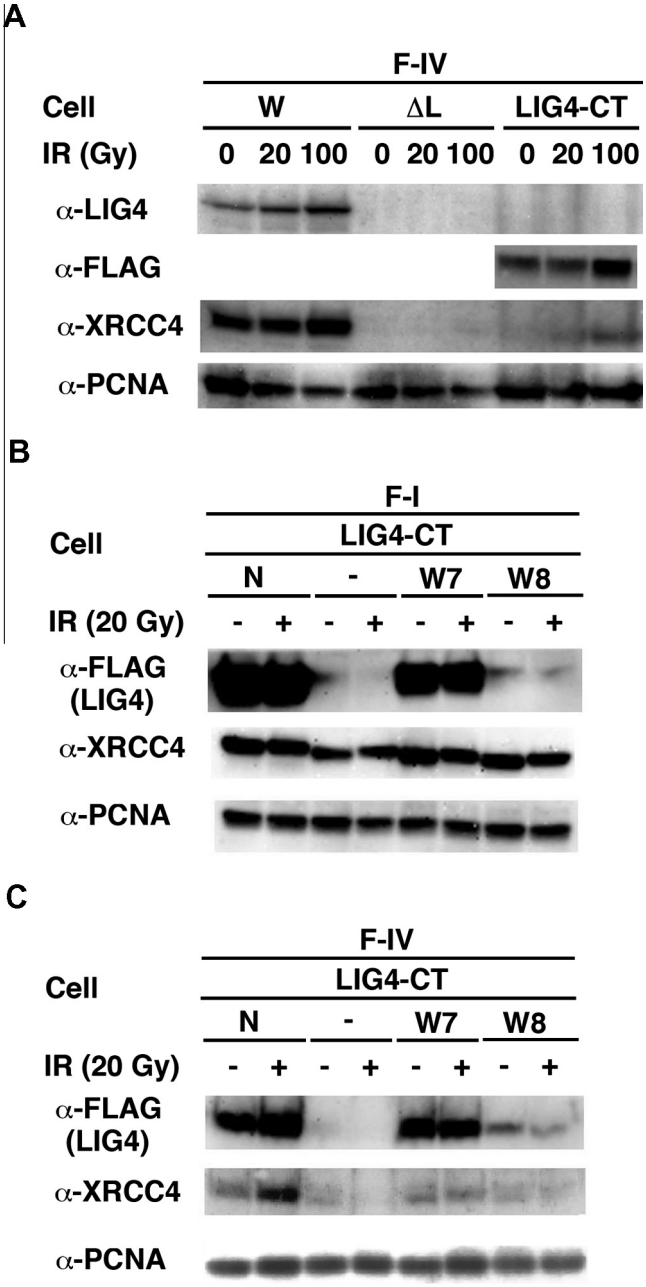

Fig. 3. Chromatin binding of LIG4-CT with XRCC4. (A) Chromatin binding analysis of wild-type (W), *LIG4*$^{-/-}$ (ΔL) and a stable transformant of *LIG4*$^{-/-}$ with LIG4-CT. To detect LIG4-CT, anti-FLAG antibody was used. Cells were irradiated with 20 Gy or 100 Gy of γ-ray from ^{60}Co source 30 min prior to harvest or left unirradiated, as indicated. (B, C) Western blotting analyses of F-I and F-IV (chromatin-bound fraction). "N", "W7" and "W8", respectively, indicate stable transformants of *LIG4*$^{-/-}$ with LIG4-CT, or W725R and W893R mutants, whereas "-" indicate that with the empty vector.

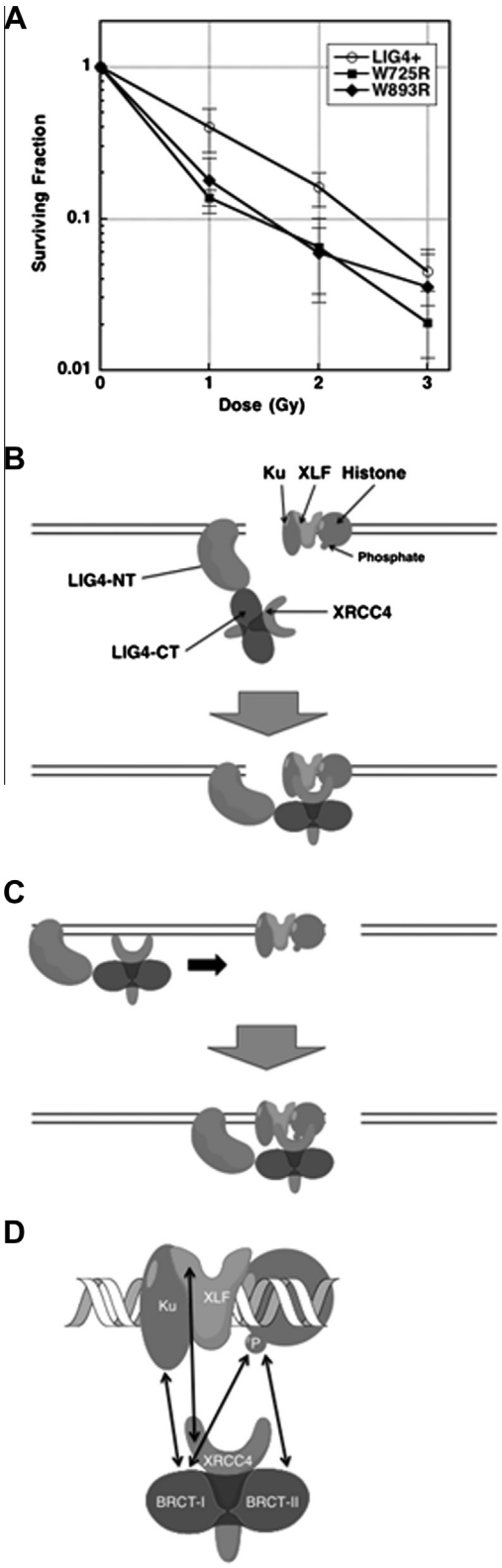

Fig. 4. Role of LIG4 C-terminal region in DSB repair. (A) Radiosensitivity of stable transformants of *LIG4*$^{-/-}$ with full-length, normal LIG4 (○) or mutants W725R (■) and W893R (♦). (B, C) Possible models on the role of LIG4-CT in DSB repair. B: LIG4-CT serves as a "hook" to capture another DSB end. C: LIG4 moves along DNA with LIG4-NT and then anchors at DSB via LIG4-CT. (D) Possible interaction between LIG4-CT/XRCC4 and DSB through other DSB repair molecules. See the text for details.

4. Discussion

In the present study, we first demonstrated that the chromatin binding was diminished in *LIG4*$^{-/-}$ and could be restored by introduction of LIG4 cDNA (Fig. 1). This is in agreement with a study by Drouet et al. showing that the chromatin binding of XRCC4 was reduced in cells from Ligase IV syndrome patient [21]. Their study and the present study indicated that the chromatin binding of XRCC4 is dependent on LIG4. Thus, the mechanisms driving XRCC4/LIG4 complex to the chromatin would lie in LIG4 rather than XRCC4.

LIG4-CT could bind to chromatin and also restore chromatin binding of XRCC4 in *LIG4*$^{-/-}$ (Fig. 3). The ability of C-terminal region, in addition to DNA binding domain in N-terminus, to interact with chromatin might have an implication in the mechanisms of DSB repair through NHEJ. Presence of dual DNA binding domain would enable "hooking" another DNA end (Fig. 4B). It might be noted that a recent electron microscopic study showed the end-bridging by human or yeast XRCC4/LIG4 complex, engaging two Ku-bound DNA ends [32]. Alternatively, LIG4 may move along DNA with DNA-binding domain and then anchor at DSB via C-terminal region (Fig. 4C).

In both of these models, we infer that LIG4-CT might bind selectively or preferentially to the DSB. This is based on the observation that the chromatin binding of LIG4-CT was enhanced by irradiation (Fig. 3A and C) and also on the lines of evidence suggesting the potential of LIG4-CT to interact with proteins, which are assumed to exist at DSB (see the next paragraph and Fig. 4D). However, the chromatin binding of LIG4-CT could be observed even in unirradiated cells. There are two possible explanations for this observation. First, LIG4-CT might bind to spontaneously damaged chromatin. Second, LIG4-CT can bind to and slide along undamaged chromatin but binds more strongly to damaged chromatin. We cannot presently distinguish these possibilities.

LIG4-CT contains two BRCT domains and XIR in between. BRCT domain is shown to be a phosphopeptide binding module [30,31]. ATM and DNA-PKcs are thought to phosphorylate a number of proteins, including histone H2AX, in response to DNA damage, phosphorylated H2AX or other protein might be a bait of BRCT domains in LIG4 (Fig. 4D). If this is the case, it is expected that depletion of ATM and/or DNA-PKcs would reduce chromatin binding of XRCC4/LIG4 complex. However, we and others demonstrated that the chromatin binding of XRCC4 was not affected by wortmannin, a potent inhibitor of both of ATM and DNA-PK [21,22]. As another possibility, it was reported that LIG4 interacts with Ku via BRCT-I [19] (Fig. 4D). In the present study, however, W725R mutation in BRCT-I did not affect the chromatin binding of LIG4-CT but the chromatin binding of XRCC4 was diminished (Fig. 3B). It might be expected that the interaction between LIG4-CT and XRCC4 is weakened by W725R mutation. However, the interaction between XRCC4 with W725R mutant and non-mutated LIG4-CT was similar in co-immunoprecipitation experiments (data not shown). Considering this, BRCT-I might have a role in stabilizing XRCC4 on chromatin. XRCC4 can then interact with XLF (Fig. 4D). Nevertheless, as we introduced mutation in putative phosphopeptide binding pocket, it remains possible that another interface of BRCT-I mediates the interaction between LIG4 and Ku. It may appear contradictory that the chromatin binding of LIG4 was considerably reduced by W725R mutation in the full-length context, but not in the C-terminal context. In this regard, we would note that an electron microscopy study suggested the juxtaposition of LIG4-NT to XRCC4 [10]. Although it is currently unclear whether there is a direct, physical interaction between LIG4-NT and XRCC4, possible tertiary interaction among LIG4-CT, XRCC4 and LIG4-NT, which would be lost in W725R mutant, might firm the complex.

Mutation in BRCT-II domain (W893R) either in the full-length context or the C-terminal context greatly reduced the expression of LIG4 (Fig. 2B, Fig. 3B), suggesting the importance of BRCT-II domain in the maintenance of LIG4. In this regard, it might be noted that LIG4 protein was undetectable in Ligase IV syndrome patients harboring compound heterozygote mutation R580X and R814X [33]. The former mutation would result in the production of LIG4 protein completely lacking both of BRCT-I and BRCT-II domains and the latter mutation would result in the production of LIG4 protein lacking most part of BRCT-II domain. Moreover, it was recently reported that BRCT-II domain, in addition to XIR domain, was required for stable interaction between LIG4 and XRCC4 [34].

Thus, the present study demonstrated that the C-terminal region of DNA ligase IV has an ability to drive XRCC4/LIG4 complex to chromatin. Further study is necessary to clarify the mechanisms how the C-terminal region of DNA ligase IV interacts with the chromatin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.068.

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