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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].