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Lysine 271 but not lysine 210 of XRCC4 is required for the nuclear localization of XRCC4 and DNA ligase IV

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ABSTRACT

XRCC4 and DNA Ligase IV (LIG4) cooperate to join two DNA ends at the final step of DNA double-strand break (DSB) repair through non-homologous end-joining (NHEJ). However, it is not fully understood how these proteins are localized to the nucleus. Here we created XRCC4^{K271R} mutant, as Lys271 lies within the putative nuclear localization signal (NLS), and XRCC4^{K210R} mutant, as Lys210 was reported to undergo SUMOylation, implicated in the nuclear localization of XRCC4. Wild-type and mutated XRCC4 with EGFP tag were introduced into HeLa cell, in which endogenous XRCC4 had been knocked down using siRNA directed to 3′-untranslated region, and tested for the nuclear localization function by fluorescence microscopy. XRCC4^{K271R} was defective in the nuclear localization of itself and LIG4, whereas XRCC4^{K210R} was competent for the nuclear localization with LIG4. To examine DSB repair function, wild-type and mutated XRCC4 were introduced into XRCC4-deficient M10. M10-XRCC4^{K271R}, but not M10-XRCC4^{K210R}, showed significantly reduced surviving fraction after 2 Gy γ-ray irradiation as compared to M10-XRCC4^{WT}. The number of γ-H2AX foci remaining 2 h after 2 Gy γ-ray irradiation was significantly greater in M10-XRCC4^{K271R} than in M10-XRCC4^{WT}, whereas it was only marginally increased in M10-XRCC4^{K210R} as compared to M10-XRCC4^{WT}. The present results collectively indicated that Lys271, but not Lys210, of XRCC4 is required for the nuclear localization of XRCC4 and LIG4 and that the nuclear localizing ability is essential for DSB repair function of XRCC4.

1. Introduction

DNA double-strand break (DSB) is considered the most deleterious type of DNA damage, leading to cell death or tumorigenesis unless repaired properly. Eukaryotic cells repair DSB mainly through two pathways, *i.e.*, homologous recombination (HR) and non-homologous end joining (NHEJ) [1]. NHEJ is considered less precise than HR but is available throughout the cell cycle. NHEJ plays an essential role also in V(D)J recombination in vertebrate immune systems. Six molecules are thought to play pivotal roles in NHEJ in vertebrate: Ku70, Ku86 (also known as Ku80), DNA-dependent protein kinase catalytic subunit (DNA-PKcs), XRCC4, DNA ligase IV (LIG4) and XLF (also known as Cernunnos) [1,2]. Ku70 and Ku86 first bind to DSB and then recruit DNA-PKcs. LIG4, in cooperation with XRCC4 and XLF, finally joins two DSB ends [1].

XRCC4 was originally identified as the gene, which can rescue high radiosensitivity and V(D)J recombination defect of Chinese hamster ovary-derived XR-1 cell, which belongs to the complementation group 4 of ionizing radiation-sensitive mutants [2]. It was subsequently found that XRCC4 interacts with and stimulates the activity of LIG4 [3,4]. Human XRCC4 consists of 334 or 336 amino acids (because of the alternative splicing inserting 6 nucleotides in the latter, replacing lysine 298 in the former with asparagine-serine-arginine triplet), assembled into N-terminal globular head domain (amino acids 1–115), middle stalk domain

Abbreviations: DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; D-PBS(−), Ca^{2+}- and Mg^{2+}-free Dulbecco's Phosphate-Buffered Saline; DSB, double-strand break; FBS, fetal bovine serum; NHEJ, non-homologous end-joining; NLS, nuclear localization signal; LIG4, DNA ligase IV; UTR, untranslated region.

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(amino acids 119–203) and C-terminal domain, which is structurally disordered [5–7] (Fig. 1). The stalk domain mediates its homodimerization and interaction with LIG4. The interface of XRCC4 to interact with LIG4 lies between amino acids 173–195. In LIG4, the region linking two BRCT domains, *i.e.*, amino acids 755-782, interacts with XRCC4 [6] and the second BRCT domain is shown to strengthen this interaction [7].

XRCC4 has a putative nuclear localization signal (NLS) sequence, RKRRQR, corresponding to amino acids 270–275 in human XRCC4. In support of the functionality of this putative NLS, Grawunder et al. showed that XRCC4 lacking amino acids 250–300 ($XRCC4^{\Delta 250-300}$) failed to be localized in the nucleus [8]. Girard et al. also showed defective nuclear localization of truncated XRCC4 lacking amino acids 266–334 ($XRCC4^{1-265}$) [9]. There are several lines of evidence that nuclear localization of LIG4 and/or its stability therein depend on the interaction with XRCC4. Bryans et al. showed LIG4 is diminished to an undetectable level in XRCC4-deficient XR-1 cell [10]. Berg et al. showed that XRCC4 is required for efficient import into and stable existence of LIG4 in the nucleus [11]. On the other hand, there are several studies indicating that LIG4 is required for the nuclear localization and the recruitment to DSB of XRCC4. Drouet et al. demonstrated that DNA damage-induced mobilization of XRCC4 as well as LIG4 into the insoluble fraction, containing chromatin and nuclear matrix, was decreased in fibroblasts from a patient of LIG4 syndrome (R278H) [12]. In our recent study, the chromatin binding of XRCC4 was absent in LIG4-disrupted cells derived from human pre-B leukemia cell Nalm-6 [13]. Additionally, Francis et al. very recently demonstrated that XRCC4 existed in the cytoplasm and was absent in the nucleus in LIG4-disrupted Nalm-6 [14]. They also showed that XRCC4 in the nucleus was decreased in fibroblasts from two LIG4 syndrome patients (R278H and R814X, respectively) [14]. Girard et al. identified two tandem NLS, *i.e.*, KKRK and KMKK, which span amino acids 626-629 and 633-636, respectively [9]. LIG4 could be localized to the nucleus in a manner dependent on this NLS, when expressed in XRCC4-deficient XR-1 cell [9]. Co-expression of wild-type XRCC4 with NLS-mutated LIG4 (6 lysine residues changed into threonine) could restore the nuclear localization of LIG4 and co-expression of wild-type LIG4 with $XRCC4^{1-265}$ could restore the nuclear localization of XRCC4. These results collectively indicated that both of LIG4 and XRCC4 have NLS, either one of which might be sufficient for the nuclear localization of the XRCC4-LIG4 complex.

Yurchenko et al. reported that XRCC4 undergoes SUMOylation at Lys210 [15]. $XRCC4^{K210R}$ failed to be localized in the nucleus but conjugating SUMO at C-terminal of $XRCC4^{K210R}$ restored nuclear localization, indicating that SUMOylation at Lys210 is necessary and sufficient for the nuclear localization of XRCC4 [15]. They further showed that $XRCC4^{K210R}$ is devoid of the ability to restore normal radiosensitivity and V(D)J recombination ability to XR-1, whereas SUMO-conjugated $XRCC4^{K210R}$ could restore normal radiosensitivity to XR-1 [15].

Thus, the current knowledge on the mechanisms of nuclear localization of XRCC4 and LIG4 is complicated, necessitating further studies. In this study, we analyzed the roles of Lys271 and Lys210 in the nuclear localization of XRCC4 and LIG4.

2. Materials and methods

2.1. Plasmid construction and mutagenesis

Human XRCC4 cDNA had been obtained by polymerase chain reaction (PCR) from the cDNA pool of human T cell leukemia MOLT-4 and integrated into p3XFLAG-CMV-10 vector (Sigma–Aldrich; St. Louis, MO, USA) [16]. To express XRCC4 as a fusion protein with enhanced green fluorescent protein (EGFP), XRCC4 cDNA was excised from p3XFLAG-CMV-10 vector using restriction enzymes *Eco*RI and *Xho*I and inserted into pEGFP-C1 vector (Clontech; Mountain View, CA, USA) using *Eco*RI and *Sal*I. Point mutations were introduced using PrimeSTAR Mutagenesis Kit (Takara Bio; Otsu, Shiga, Japan). Sequences of PCR primers for mutagenesis are as follows (underlined nucleotides correspond to mutated amino acids: K210R–F, GAC ATC AGG CAA GAA GGG GAA ACT GCA; K210R-R, TTC TTG CCT GAT GTC CTT TTC TCG TTC; K271R–F, AGT AGA AGG AGG AGA CAG CGA ATG CAA; K271R-R, TCT CCT CCT TCT ACT TGG TGC AAT ATC. Correctness of the sequence of entire XRCC4 open reading frame was verified for all the constructs. The vector expressing human LIG4 was constructed as described recently [13].

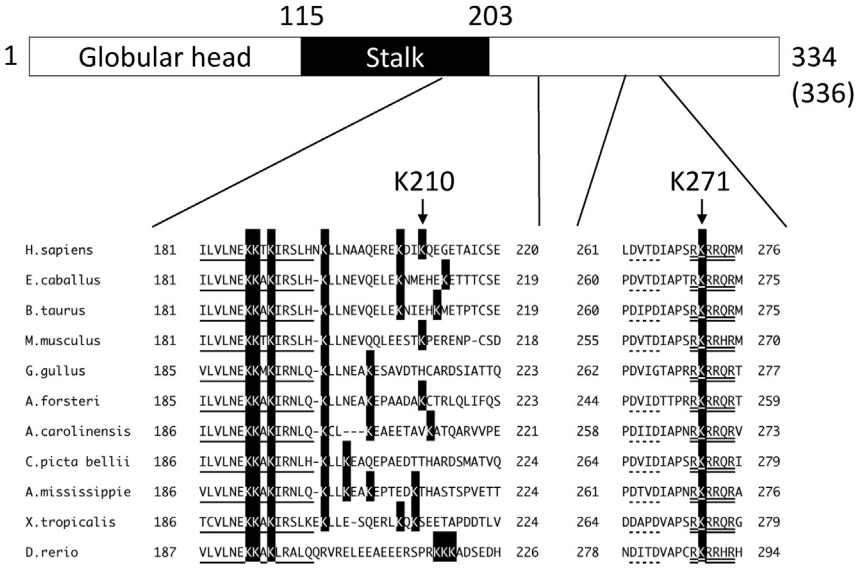

Fig. 1. Schematic presentation of XRCC4 structure and conservation of amino acid sequence surrounding Lys210 and Lys271. Single underlines indicate the LIG4 binding region. Dotted underlines indicate the caspase cleavage motif. Double underlines indicate the putative nuclear localization signal (NLS). Lysines are highlighted by black and white reversal.