Cloning, localization and focus formation at DNA damage sites of canine Ku70

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ABSTRACT. Understanding the molecular mechanisms of DNA double-strand break (DSB) repair machinery, specifically non-homologous DNA-end joining (NHEJ), is crucial for developing next-generation radiotherapies and common chemotherapeutics for human and animal cancers. The localization, protein-protein interactions and post-translational modifications of core NHEJ factors, might play vital roles for regulation of NHEJ activity. The human Ku heterodimer (Ku70/Ku80) is a core NHEJ factor in the NHEJ pathway and is involved in sensing of DSBs. Companion animals, such as canines, have been proposed to be an excellent model for cancer research, including development of chemotherapeutics. However, the post-translational modifications, localization and complex formation of canine Ku70 have not been clarified. Here, we show that canine Ku70 localizes in the nuclei of interphase cells and that it is recruited quickly at laser-microirradiated DSB sites. Structurally, two DNA-PK phosphorylation sites (S6 and S51), an ubiquitination site (K114), two canonical sumoylation consensus motifs, a CDK phosphorylation motif, and a nuclear localization signal (NLS) in the human Ku70 are evolutionarily conserved in canine and mouse species, while the acetylation sites in human Ku70 are partially conserved. Intriguingly, the primary candidate nucleophile (K31) required for 5'dRP/AP lyase activity of human and mouse Ku70 is not conserved in canines, suggesting that canine Ku does not possess this activity. Our findings provide insights into the molecular mechanisms of Ku-dependent NHEJ in a canine model and form a platform for the development of next-generation common chemotherapeutics for human and animal cancers.

KEY WORDS: canine, companion animal, DNA double-strand break, Ku80, nonhomologous DNA-end joining
and next-generation radiotherapies [10, 25, 30]. Previous studies in human and rodent cells have shown that the localization and function of Ku70 at DSB sites might play a crucial role in modulating the NHEJ repair pathway [3, 14, 20, 26, 28]. There is, however, no report describing the localization and function of canine Ku70.

In this study, we have cloned Ku70 cDNA from a beagle dog testis library and conducted comparative analysis to understand the mechanisms underlying Ku70 regulation and function. Additionally, we examined its expression and localization. We also asked whether canine Ku70 accumulates at DSB sites after laser-microirradiation.

**MATERIALS AND METHODS**

**Cloning of canine Ku70**

Oligonucleotide primers used to amplify canine Ku70 cDNA from a male beagle dog cDNA library (Biochain, Newark, CA, U.S.A.) were designed based on the predicted Ku70 genomic sequence of a female boxer dog, Canis lupus familiaris (XM_531714.3). PCR amplification with sense (F: 5′-CTCAAGCTTGAATTCTGCTAGGGAAGTCTCTTGTGAAG-3′) and antisense (R: 5′-TAGATCCGGTGGATCCTCAGTTCTTCTGGGAAG-3′) primers was performed in a Thermal Cycler using Premix LA Taq, Version 2.0 (Takara Bio Inc., Otsu, Japan). Pre-denaturation was carried out for 1 min at 94°C. This was followed by 40 cycles of PCR amplification. Each cycle consisted of a denaturation step at 94°C for 0.5 min, annealing at 60°C for 0.5 min and extension at 72°C for 2 min. PCR products were subcloned into the EcoRI and ApaI sites of pEYFP-C1 (pEYFP-canine Ku70) using the In-Fusion HD cloning kit (Takara Bio Inc.), and the inserts were validated by sequencing. Other PCR primers used in this study were as follows: dX6 Nseq F1: 5′-GCACAAGGGTGAGCAATAAACAATATG-3′, dX6 Nseq R1: 5′-TGGATACACTGGATGCTCATGTCAAAAG-3′, dX6 Cseq F2: 5′-GGTGGGTTGAAGAAGCAGGAG-3′ and dX6 Cseq R2: 5′-AAAGACACAGCAAAGTGGGAAG-3′.

**Cell lines, cultures and transfections**

A Madin-Darby canine kidney cell line (MDCK) (HSRRB, Osaka, Japan), a canine lung adenocarcinoma cell line (CLAC) (HSRRB), a human cervical carcinoma cell line (HeLa) (Riken Cell Bank, Tsukuba, Japan) and a human colon cancer cell line (HCT116) (Riken Cell Bank) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). pEYFP-canine Ku70 or pEYFP-C1 was transiently transfected into cells using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, U.S.A.). Post-transfection, cells were cultured for 2 days and then monitored under an FV300 confocal laser-scanning microscope (CLSM) (Olympus, Tokyo, Japan) as previously described [16, 17, 22].

**Immunocytochemistry**

Immunocytochemistry was performed using a mouse anti-γH2AX monoclonal antibody (JBW301) (Upstate Biotechnology Inc., Charlottesville, VA, U.S.A.) and an Alexa Fluor 568-conjugated secondary antibody (Molecular Probes, Eugene, OR, U.S.A.) as previously described [20, 21].

**Immunoblotting**

The extraction of total cell lysates and Western blot analysis were performed as described previously [20, 21] with the following modifications. The molecular weight marker was used 3-Color prestained XL-ladder (APRO science, Tokushima, Japan). The membranes were blocked in Blocking One (Nacalai Tesque, Kyoto, Japan) or ECL Prime Blocking reagent (GE Healthcare Bio-Sci. Corp., Piscataway, NJ, U.S.A.) for 30 min at room temperature. The following antibodies were used: rabbit anti-Ku70 polyclonal antibody (347V), rabbit anti-Ku70 polyclonal antibody (AHP316) (Serotec, Oxford, UK), rabbit anti-GFP polyclonal antibody (FL) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) and anti-β-actin monoclonal antibody (Sigma, St. Louis, MO, U.S.A.). The anti-Ku70 antibody (347V) was raised for this study against corresponding to the N-terminal amino acids: 175–188 of canine Ku70, characterized and affinity purified. The amino acids sequence is perfectly conserved between canine and human. The anti-GFP antibody cross-reacts with GFP mutants, such as EYFP [20, 21]. The anti-Ku70 and anti-GFP antibodies were diluted in Signal Enhancer HIKARI (Nacalai Tesque). In accordance with the manufacturer’s instructions, the binding to each protein was detected using a Select Western blotting detection system (GE Healthcare Bio-Sci. Corp.) and visualized using the ChemiDoc XRS system (Bio-Rad, Hercules, CA, U.S.A.).

**Local DNA damage induction using laser and cell imaging**

Local DNA damage induction using laser and subsequent cell imaging was performed as described previously [15, 17]. Briefly, local DSBs were induced using a 3% power scan (for 1 sec) from a 405-nm laser. Images of living or fixed cells expressing EYFP-canine Ku70 or EYFP alone were obtained using an FV300 CLSM system (Olympus).

**RESULTS**

**Sequence analysis of canine Ku70**

Canine Ku70 cDNA was cloned from a beagle dog testis library and sequenced. We isolated an 1,827-nucleotide open reading frame encoding a protein of 608 amino acids (Fig. 1). The cDNA sequence of Ku70 obtained from the beagle dog was identical to its predicted sequence from a female boxer dog genomic sequence (XM_531714.3). The obtained canine sequence has been
Fig. 1. Nucleotide and deduced amino acid sequences of Ku70 cDNA from canine (*Canis lupus familiaris*, GenBank accession number: LC195221) species. The CDS of canine Ku70 is composed of 1,827 bp encoding 608 amino acids residues. Numbers on the right refer to nucleotides (top) and amino acids (bottom). The asterisk after the amino acid sequence shows the position of the termination codon.
motif ([S/T]Px[K/R]: 401TPRR404) [9, 23, 29]. We found that the sequences of these three motifs are conserved in canine and mouse conserved in canine, although it is conserved in mouse species (Fig. 2).

Interestingly, the primary candidate nucleophile (K31 in the Ku70) required for 5′dRP/AP lyase activity [31, 32]. On the other hand, the DNA damage-induced phosphorylation sites (S27, S33 and S155), the putative cyclin B1/CDK1 phosphorylation sites (T401 and T428) and the putative cyclin E1/CDK2 phosphorylation site (T58) in human Ku70 are similarly conserved in canine and mouse species. In addition, seven (K317, K331, K338, K539, K542, K553 and K556) of the eight acetylation sites in humans are perfectly conserved in canine and mouse species. In addition, seven (K317, K331, K338, K539, K542, K553 and K556) of the eight acetylation sites in humans are perfectly conserved in canine and mouse, whereas the acetylation site K54 is not conserved in canine species. Recently, it has been reported that human Ku has a 5′dRP/AP lyase activity [31, 32]. Interestingly, the primary candidate nucleophile (K31 in the Ku70) required for 5′dRP/AP lyase activity is not conserved in canine, although it is conserved in mouse species (Fig. 2).

Expression and subcellular localization of canine Ku70

We wanted to examine the expression and subcellular localization of Ku70 in canine cells. First, we evaluated at the expression of Ku70 in the canine cell lines, MDCK and CLAC, and the human cell lines, HeLa and HCT116. Signals of canine Ku70 were detected in both MDCK and CLAC cells, although Ku70 exhibited lower expression levels in the two canine cell lines compared to in the human cell lines (Fig. 3A). Next, to examine subcellular localization of Ku70 in live canine cells, we generated MDCK cells transiently expressing EYFP-canine Ku70. To this end, the expression vector pEYFP-C1 containing canine Ku70 (pEYFP-canine Ku70) was transfected into MDCK cells (Fig. 3B). Western blotting using anti-Ku70 and anti-GFP antibodies showed that EYFP-canine Ku70 was expressed in the transfected cells (Fig. 3C). Confocal laser microscopy showed that EYFP-canine Ku70 was localized in the nuclei of the EYFP-canine Ku70 transfected cells during interphase (Fig. 3D). EYFP, used as a control, was distributed throughout the cell excluding the nucleolus in EYFP transfectant cells (Fig. 3D), consistent with our previously reports [20, 21]. These data indicate that the canine Ku70 is expressed and localized in the nuclei of canine cells.

EYFP-canine Ku70 accumulates instantaneously at laser-microirradiation-induced DSBs

We next examined whether EYFP-canine Ku70 accumulates immediately at 405 nm laser-induced DSB sites in canine cells (Fig. 4A). Microirradiation led to the accumulation of EYFP-canine Ku70 in live MDCK cells (Fig. 4B). To examine if EYFP-canine Ku70 accumulated at 405 nm laser-induced DSB sites, we immunostained cells with an antibody that detects γH2AX, a gold-standard marker of DSBs. EYFP-canine Ku70 was found to accumulate and colocalize with γH2AX at microirradiated sites in MDCK cells (Fig. 4C). To investigate the temporal dynamics of Ku70 localization, we performed time-lapse imaging of EYFP-canine Ku70 transfected MDCK cells. We observed EYFP-canine Ku70 accumulation at the microirradiated sites 5 sec after irradiation (Fig. 4D). These results reveal that after irradiation, EYFP-canine Ku70 accumulates immediately and forms foci at laser-induced DSBs in live canine cells.

DISCUSSION

It is important to elucidate the underlying molecular mechanisms of DNA repair processes, such as NHEJ, for the development of new chemoradiotherapies and targeted drugs for cancer. Human Ku is the most important DSB sensor protein, and NHEJ is considered to initiate with the binding of Ku to the ends of the DSBs [14, 28]. Hence, Ku70 and Ku80 might be potential target molecules for the development of next-generation radiosensitizers to not only human cancers, but also canine cancers. In this study, we demonstrate that EYFP-canine Ku70 localizes in the nuclei and is recruited to micro-laser induced DSB sites soon after irradiation. Furthermore, sequence alignment revealed that the PTM sites and the protein-protein interaction motifs of Ku70 are not completely conserved in human and canine species. This study, amongst others, contributes to the understanding of the Ku-dependent NHEJ pathway and the development of a combination of radiotherapy and targeted therapy with DNA repair inhibitors.

It is well known that molecular mechanisms underlying protein-protein interactions and PTMs of DNA repair proteins play critical roles in the regulation of various DNA repair pathways [5, 28]. In this study, we found that almost all of these target-motifs and sites in human Ku70 are conserved in canine Ku70. For example, sequence alignment revealed a CDK phosphorylation motif ([S/T]Px[K/R]: 401TPRR404), the cyclin B1/CDK1 phosphorylation sites (T401 and T428) and the putative cyclin E1/CDK2 phosphorylation site (T58) in human Ku70 are conserved in canine and mouse Ku70. In addition, we found that two of the three cyclin A2/CDK2 phosphorylation sites (T401 and T428) in human Ku70 are conserved in canine and mouse species. There are several reports describing cell cycle-dependent Ku70-localization and -function in human and rodent cells [7, 14, 24, 29]. Altogether, we speculate that Ku70-localization and -function in canine cells might be regulated by cell cycle dependent-phosphorylation, which will be validated in the future. Previously, our findings using immunocytochemistry and GFP-technology revealed that Ku70 mainly localizes in the nuclei of various human and murine adhesion cells during interphase [16–19]. In our present study, the data showed that canine Ku70 mainly localizes in the nuclei of canine adhesion cells during...
Fig. 2. Amino acid sequences of Ku70 from canine (Canis lupus familiaris, GenBank accession number: LC195221), human (Homo sapiens, GenBank accession number: NP_001460.1) and mouse (Mus musculus, GenBank accession number: NP_034377.2) species. The location of the nuclear localization signal (NLS) sequence (NLS: 539-556), the two putative canonical sumoylation consensus motifs (ψ-K-X-E: 509PKVE512 and 555PKVE558) and the CDK phosphorylation motif ([S/T]Px[K/R]: 401TPRR404) in human Ku70 are shown [9, 18, 29]. The location of the primary candidate nucleophile required for 5′dRP/AP lyase activity (K31), the DNA-PK phosphorylation sites (S6 and S51), the DNA damage inducible phosphorylation sites (S27, S33 and S155), the putative phosphorylation sites required for Ku’s dissociation from DSB (T305, S306, T307, S314 and T316), the cyclin B1/CDK1 phosphorylation sites (T401 and T428), the cyclin A2/CDK2 phosphorylation sites (T401, T428 and T455), the putative cyclin E1/CDK2 phosphorylation site (T58), the ubiquitination site (K114), the acetylation sites (K317, K331, K338, K539, K542, K544, K553 and K556) and the two putative sumoylation sites (K510 and K556) in human Ku70 [1, 2, 4, 7, 9, 12, 26, 28, 29] are marked with asterisks.
interphase. Previously, we identified the NLS motif in human Ku70 and showed that its structure is conserved among various species, including eight rodents, a chicken and a frog [14, 18, 19]. In this study, we found that this NLS motif is conserved in canine Ku70. On the other hand, accumulating evidence suggests the possibility that human Ku70 is a multifunctional protein possessing functions in the cell membrane and cytoplasm as well as the nucleus [4, 14]. Subcellular localization of human Ku70 dynamically changes during the cell cycle [14, 23, 24]. The nuclear localization of human Ku70 starts during the late telophase / early G1 phase after the nuclear envelope is formed [14, 23]. Previously, we clarified that human Ku70 translocates to the nucleus via interaction between its own NLS and classical NLS receptors [23]. Our sequence alignment showed that the two putative sumoylation consensus motifs in human Ku70 are conserved in canine and mouse Ku70, and one target site lies within the NLS motif. Moreover, we found that seven of the eight acetylation sites in humans are perfectly conserved in canine and mouse species, and four target sites lie within the NLS of three species. These findings suggest that the sumoylation and/or acetylation of lysine residues within the NLS control the nuclear localization of Ku70. Altogether, we speculate that the NLS is critical for the function and regulation of Ku70 not only in humans, but also in other species including canines, although further studies are needed to validate this.

In this study, we demonstrate that canine Ku70 is recruited to micro-laser induced DSB sites soon after irradiation. Binding to the DSB ends of Ku is essential for Ku-dependent NHEJ activity; Ku plays a key role in the recognition and protection of DSB ends and is important for the recruitment of other core NHEJ factors, including DNA-PKcs, XRCC4 and XLF, and other factors that process the ends [6, 8, 14, 17, 28]. In the present study, we found that the DNA-PK phosphorylation sites of Ku70 are conserved in human, mouse and canine species. Moreover, the DNA damage inducible phosphorylation sites and the putative phosphorylation sites required for Ku’s dissociation from DSBs in human Ku70 are partially conserved in canine and mouse species. These findings suggest that canine Ku70 might, at least in part, be regulated by some kinases at DSB sites.

We are interested in understanding whether these PTMs affect the functions of canine Ku70 at DSB sites. Interestingly, it has been reported that human Ku has a 5’dRP/AP lyase activity [31]. This activity is important for excising abasic sites from DSB ends, suggesting that Ku has a novel and direct role in end-processing. Strande et al. identified K31 in Ku70 as the primary candidate...
nucleophile required for catalysis [32]. In addition, they showed that the lysine is not conserved in Xenopus laevis Ku70 and that Ku70 in this species has negligible activity. Moreover, when 5′dRP/AP lyase activity in human Ku70 was inactivated, the inclusion of DNA-PKcs provided some compensating activity. In the present study, our data revealed that the lysine (K31) is not conserved in canine Ku70, suggesting that canine Ku70 does not possess any 5′dRP/AP lyase activity. Further studies may shed light on the functional differences between human and canine Ku70 at DSB sites.

In this study, we cloned and characterized canine Ku70. Sequence alignment provided valuable information for clarifying the regulation mechanism of Ku70 function in canine cells. Moreover, some of the motifs and PTM target sites, at least in part, might play a key role in the regulation of Ku70 localization and its functions in canine cells. Most recently, we cloned two other core NHEJ factors, i.e., canine XLF (GenBank accession number: LC176889) and canine XRCC4 (GenBank accession number: LC168634), and characterized them [20, 21]. Taken together, these basic findings describing the core NHEJ factors contribute to the understanding of molecular mechanisms of underlying NHEJ and provide valuable information for the development of potential targets for next-generation chemotherapeutics in the treatment of human and canine cancers.

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Fig. 4. EYFP-canine Ku70 accumulated immediately at DSBs induced by laser microirradiation. (A) The localization and accumulation of EYFP-canine Ku70 at DSBs induced by 405-nm laser irradiation were examined in MDCK cells. (B) Imaging of EYFP-canine Ku70-transfected live MDCK cells before (left panel) and after (right panel) microirradiation. Arrowheads show the microirradiated sites. (C) Immunostaining of microirradiated EYFP-canine Ku70-transfected cells with anti-γH2AX antibody. Cells were fixed and stained with anti-γH2AX antibody 5 min postirradiation. EYFP-canine Ku70 (left); γH2AX (center panel); merged image (right panel). (D) Time-dependent EYFP-canine Ku70 accumulation in live cells 5–120 sec after irradiation.


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