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Lysines 3241 and 3260 of DNA-PKcs are important for genomic stability and radioresistance

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ABSTRACT

DNA-dependent protein kinase (DNA-PK) is a serine/threonine kinase that plays an essential role in the repair of DNA double-strand breaks (DSBs) in the non-homologous end-joining (NHEJ) pathway. The DNA-PK holoenzyme consists of a catalytic subunit (DNA-PKcs) and DNA-binding subunit (Ku70/80, Ku). Ku is a molecular sensor for double-stranded DNA and once bound to DSB ends it recruits DNA-PKcs to the DSB site. Subsequently, DNA-PKcs is activated and heavily phosphorylated, with these phosphorylations modulating DNA-PKcs. Although phosphorylation of DNA-PKcs is well studied, other post-translational modifications of DNA-PKcs are not. In this study, we aimed to determine if acetylation of DNA-PKcs regulates DNA-PKcs-dependent DSB repair. We report that DNA-PKcs is acetylated *in vivo* and identified two putative acetylation sites, lysine residues 3241 and 3260. Mutating these sites to block potential acetylation results in increased radiosensitive, a slight decrease in DSB repair capacity as assessed by γH2AX resolution, and increased chromosomal aberrations, especially quadriradial chromosomes. Together, our results provide evidence that acetylation potentially regulates DNA-PKcs.

1. Introduction

DNA double-stranded breaks (DSBs) are deleterious DNA lesions that are primarily repaired by two pathways; non-homologous end-joining (NHEJ) and homologous recombination (HR) [1]. NHEJ is the prominent pathway responsible for repairing DSBs in human cells [2]. A central player in NHEJ is the DNA-dependent protein kinase (DNA-PK) (see reviews for more details) [3,4]. DNA-PK consists of a DNA binding subunit (Ku70/80, Ku) and a catalytic subunit (DNA-PKcs). DNA-PKcs is composed of HEAT (Huntington-elongation-A-subunit-TOR) repeats in its N-terminus, which produce a pincer-shaped structure that forms a central channel and a C-terminal region that contains the PI3 kinase domain, which is flanked by the FAT (FRAP, ATM, TRRAP) domain at its N-terminal side and by the FATC domain at its C-terminal side [5,6]. Following DSB induction, the Ku heterodimer quickly binds to the DSB ends and recruits DNA-PKcs to the break site, which is mediated by the N-terminal region of DNA-PKcs [7–9]. Upon interacting with the DSB-Ku complex, DNA-PKcs is activated. It is believed that activation of DNA-PKcs is dependent on a conformational change in both the FAT and FATC domains [4]. Once activated, DNA-PKcs phosphorylates a number of substrates with the best characterized being itself.

Following DSB formation, DNA-PKcs is heavily phosphorylated and these phosphorylations are critical for its role in DSB repair [4,10]. DNA-PKcs autophosphorylates itself and is also phosphorylated by the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3 related (ATR) protein kinases [11,12]. A large number of the phosphorylation sites are clustered in different regions of DNA-PKcs [13–16]. Two prominent clusters phosphorylated in response to DSB induction are the T2609 [13,17] and S2056 [11,18,19] phosphorylation clusters. S2056 is an autophosphorylation site [18], whereas phosphorylation of the T2609 cluster can be mediated by DNA-PKcs, ATM, or ATR. Blocking phosphorylation of DNA-PKcs at the T2609 phosphorylation cluster by mutating the serines/threonines to alanine results in blocking the release of DNA-PKcs from DNA ends, reduced DSB repair capacity, and increased radiosensitivity [17,20,21]. Blocking phosphorylation of the S2056 cluster causes increased radiosensitivity and results in increased DNA end processing, suggesting that it is required for NHEJ [19,22]. Although, regulation of DNA-PKcs through its

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phosphorylation has been extensively investigated, other post-translational modifications of DNA-PKcs are not well studied.

Acetylation of lysine residues is a reversible posttranslational modification, which neutralizes the positive charge of this amino acid and changes the functionality of the protein in diverse ways [23]. There are many individual reports of protein acetylation modulating diverse biological processes, suggesting that lysine acetylation has broad regulatory functions [24]. For example, it plays a key role in the regulation of gene expression through the modification of core histone tails by histone acetyltransferases (HATs) or histone deacetylases (HDACs) [25]. Lysine acetylation has also been implicated to play a role in modulating the activity of DSB repair proteins, including Ku70 [26], ATM [27], and CtIP [28]. A recent proteomic study revealed that there are at least 16 lysine residues acetylated in DNA-PKcs [29], but the biological function of DNA-PKcs acetylation is not characterized. Here, we show that DNA-PKcs is acetylated, and we identified two lysine residues (K3241 and K3260) that are potentially acetylated on DNA-PKcs. Mutating these lysine residues to block acetylation results in increased radiosensitivity and chromosomal aberrations, suggesting that these sites are important for DNA-PKcs-dependent DSB repair. Collectively, the data provide initial evidence that acetylation may modulate DNA-PKcs.

2. Materials and methods

2.1. Cell culture and transfections

Chinese hamster ovary (CHO) DNA-PKcs-deficient (V3) cell line [30] and V3 cells stably expressing YFP-tagged DNA-PKcs were cultured in Hyclone MEM media containing 10% Fetal Bovine Serum and Newborn Calf Serum (1:1 mixture), 100 U/mL penicillin and 100 U/mL streptomycin. The cells were incubated at 37 °C in a humidified incubator with 5% CO_2. For generation of stable cell lines, cells were transfected with the linearized expression plasmid using Lipofectamine® 2000 transfection reagent (Invitrogen) according to the manufacturer's procedures. Stable cell lines expressing YFP-tagged DNA-PKcs were maintained with 500 μg/mL of G418.

2.2. Immunofluorescence, western blotting and antibodies

Immunofluorescence was performed as described previously [31]. Nuclear extraction, immunoprecipitation and western blotting were performed as described previously [22]. Anti-γH2AX (07−164; mouse monoclonal, Millipore), anti-pS2056 (ab124918; rabbit monoclonal, abcam), anti-acetyl-lysine (SA615; rabbit polyclonal, Enzo Life Sciences) are commercially available.

2.3. Cell survival and chromosome analysis

Cell survival curves were obtained by measuring the colony forming abilities of cell populations irradiated with varying doses of irradiation as previously described [32]. Chromosome analysis was performed as described previously [33]. Briefly, exponentially growing cells were irradiated, and cultured in a presence of colcemid (1 μg/mL), starting 30 min post-irradiation for 4 hr. Mitotic cells were harvested by trypsinization and then treated with a hypotonic solution. Cells were fixed in methanol:acetic acid (3:1) and chromosomes were spread by air drying. After the slides were stained with Giemsa, chromosome aberrations were scored.

2.4. Live cell imaging and laser micro-irradiation

Live cell imaging combined with laser micro-irradiation was performed as described previously [20]. Fluorescence signal of YFP-DNA-PKcs was monitored by using an Axiovert 200 M microscope (Carl Zeiss, Inc), with a Plan-Apochromat 63X/NA 1.40 oil immersion objective (Carl Zeiss, Inc). A 365-nm pulsed nitrogen laser (Spectra Physics) was directly coupled to the epifluorescence path of the microscope and used to generate DSBs in a defined area of the nucleus. Analysis of acquired images was done as previously described [34]. Briefly, fluorescence intensity (IN) of each time point was based on pre-laser background intensity using the formula: $IN(t) = Id_t/Ib_t \times Ib_{preIR}$ [Id_t: the difference between the accumulation spot intensity and the undamaged site background intensity of each time point; Ib_t: the background intensity of each time point; Ib_{preIR}: the background intensity before irradiation. Relative fluorescence intensity (RF) was calculated using the formula: $RF(t) = (IN_t\text{-}IN_{preIR})/(IN_{max}\text{-}IN_{preIR})$ [IN_{preIR}: IN of the micro-irradiated area before laser damage; IN_{max}: the maximum IN in the micro-irradiated area of all time points]. Each data point is the average of 10 independent measurements.

2.5. Statistics

Statistical analysis was performed utilizing the student's *t*-test (paired, 1-sided). We refer to statistically significant as $p < 0.05$. Each point represents the mean ± SD of three independent experiments unless otherwise stated.

3. Results

3.1. Mutating 8 potential acetylation sites of DNA-PKcs results in increased radiosensitivity

A proteomic study identified 16 potential lysine residues acetylated in DNA-PKcs; therefore, we postulated that acetylation modulates DNA-PKcs activity [29]. To test this hypothesis, we first examined whether DNA-PKcs is acetylated *in vivo*. Immunoprecipitated DNA-PKcs from HeLa nuclear extract was probed with anti-pan-acetyl-lysine antibodies, and we found that DNA-PKcs is acetylated in normal cycling cells (Fig. 1A). Next, we aimed to identify acetylation sites that may modulate the activity of DNA-PKcs. From the 16 lysine residues identified in the proteomic study [29], we initially focused on eight lysines located around the FAT and kinase domains of DNA-PKcs, as we speculated that acetylation at these sites may affect the activity of DNA-PKcs. Approximate position of each lysine is illustrated in Fig. 1B. To examine if these lysines modulate DNA-PKcs function, we blocked potential acetylation at these sites by replacing the lysines (K) with arginines (R), hereafter termed as 8KR. The 8KR mutant was stably expressed in the DNA-PKcs-deficient Chinese hamster ovary (CHO) cell line, V3, and we assessed if mutating these putative acetylation sites affected the repair of DNA damage by monitoring cell survival to varying doses of IR. As shown in Fig. 1C, DNA-PKcs deficient V3 cells are extremely radiosensitive ($D_{10} = 1.8$ Gy). V3 cells complemented with 8KR are a moderately radiosensitive ($D_{10} = 2.8$ Gy) compared to V3 cells completed with wild-type DNA-PKcs (V3-WT) ($D_{10} = 4.0$ Gy), indicating that possible acetylation at these lysine residues plays a role in DNA-PKcs-dependent repair of DNA damage.

3.2. Mutating K3241 and K3260 of DNA-PKcs results in increased radiosensitivity

To delineate which of the eight lysine residues are required for the repair of IR-induced DNA damage, we created three groups of K-to-R mutants: K2702/2703R, K3241/3260R (termed as 2KR), K3608/3621/3638/3642R. As shown in Fig. 2A, V3 cells complemented

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