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PP2A–B56ε complex is involved in dephosphorylation of γ-H2AX in the repair process of CPT-induced DNA double-strand breaks

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ABSTRACT

Phosphorylation of histone H2AX (γ-H2AX) in response to DNA double-strand breaks (DSBs) should be eliminated from the sites of DNA damage to fulfill the DNA repair and release cells from the growth arrest. Previous study showed that protein phosphatase 2A (PP2A) interact with γ-H2AX that lead to the dephosphorylation of γ-H2AX. Here, we examined the effects of suppression of PP2A regulatory subunits on dephosphorylation of γ-H2AX in human embryonic kidney epithelial cells (HEK) treated by topoisomerase I inhibitor camptothecin (CPT). We found that cells with suppression of B55α or B56ε were more sensitive to DNA damage agents. Suppression of B56ε led to persistence of γ-H2AX, resulting in prolonged DSBs repair and increased chromatin instability measured by comet assay. In addition, the deficiency of B56ε impaired the cell cycle regulation and the DNA repair pathway of homologous recombination (HR). Notably, we detected that PP2A B56ε subunit was involved directly in dephosphorylation of γ-H2AX and translocated from cytoplasm to nucleus upon the treatment of CPT. Our findings demonstrate that PP2A holoenzyme containing B56ε is responsible for the dephosphorylation of γ-H2AX and regulation of DNA repair of DSBs induced by CPT.

1. Introduction

Mammalian cells exposed to DNA damaging agents trigger important defensive pathways by inducing multiple proteins involved in DNA repair, cell cycle checkpoint control, protein translocation and degradation. DNA damage induces structural alterations to DNA, which can be detected by DNA repair mechanisms (Jackson, 2002). Once damage is identified, specific DNA repair molecules are recruited at or near the sites of damage, inducing other molecules to bind and form a complex that enables DNA repair (Rogakou et al., 1999). The failure to repair DNA facilitates genomic instability, which in turn may result in cell death or increase the risk of pathological consequences such as cancer development.

In eukaryotic cells, the induction of DNA double-strand breaks (DSBs) in chromatin promptly initiates the phosphorylation of the histone H2A variant, H2AX, at Serine 139 to generate γ-H2AX (Rogakou et al., 1998). The phosphorylation of H2AX is critical in maintaining the genome stability (Shrivastav et al., 2008). H2AX-deficient mice display higher radio sensitivity, chromosome instability, and enhanced cancer susceptibility. In addition, these mice exhibit repair defects and impaired recruitment of DNA repair proteins including NBS1, 53BP1, and BRCA1 to irradiation-induced foci (Celeste et al., 2003, 2002). These observations establish a key role for γ-H2AX in the activation of the DNA damage response (DDR).

The phosphorylation of H2AX by the members of several kinases is one of the early events in response to DNA DSBs (Fernandez-Capetillo et al., 2004; Rogakou et al., 1998; Tomita, 2010). γ-H2AX serves as a signal to promote the recruitment of DNA repair proteins to the site of DSB, activate cell cycle checkpoints, and prevent unrepaired DNA to pass into daughter cell during replication (Kinner et al., 2008). The repair of DSBs must occur in the context of chromatin (Scott and Pandita, 2006). With the cooperation of distinct histone modifications, γ-H2AX foci anchor additional DDR proteins near DSBs to complete DSBs repair (Fillingham et al., 2006; Huertas, 2010; Xu and Price, 2011). The assembly of proteins at the DSB-flanking chromatin occurs in a highly ordered fashion, which can be achieved by regulation of protein–protein interactions triggered by a variety of posttranslational modifications (PTMs) (Kinner et al., 2008). Accordingly, the disrupted histones must be re-deposited onto DNA to restore the

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chromatin structure following the completion of DNA repair (Xu and Price, 2011). To date, the investigation of the Ser/Thr protein kinases that are responsible for γ-H2AX formation have been intensively studied. However, how γ-H2AX is eliminated in mammalian cells after DSBs rejoining and the functional consequences of persistence of phosphorylated H2AX remains largely unknown. The removal of γ-H2AX from sites of DNA damage is evidently tightly regulated to restore chromatin integrity of mammalian genomes upon completion of DNA repair to block the dissociation of repair proteins and release from cell cycle checkpoint (Keogh et al., 2006). H2AX phosphorylation signal is putatively removed by the joint action of histone exchange and phosphatases (Keogh et al., 2006; Kusch et al., 2004). Although many kinases involved in DSBs are known, the comprehensive mechanism by which phosphatases regulate γ-H2AX remain undefined.

PP2A is a large family of holoenzymes that comprises 1% of total cellular proteins and accounts for the majority of Ser/Thr phosphatase activity in eukaryotic cells (Shi, 2009). PP2A enzymes are ubiquitously expressed proteins that are implicated in diverse cell processes (Janssens and Goris, 2001). Most cellular PP2A exists in two general forms, a heterodimeric complex of scaffolding A subunit (PR65) and a catalytic C subunit (PP2Ac subunit) and a heterotrimeric enzyme that also includes B subunits. The association of the core enzyme with a variety of regulatory B-type subunit generates many distinct holoenzymes. Four distinct B-type protein families, named as B (B55 and PR55), B′ (B56 and PR61), B″ (PR72, PR130, PR59 or PR48) and B‴ (PR93/SG2NA or PR110/Striatin), have been identified. The functional diversity, substrate specificity and subcellular localization of this enzyme are conferred by their distinct B-type regulatory subunit (Xu et al., 2006; Zhao et al., 1997). In addition to binding PR65/A subunit, a small proportion of PP2Ac associates with Tap42/α4, an interaction mutually exclusive with that of PR65/A (Di Como and Arndt, 1996; Prickett and Brautigan, 2004). This interaction leads to inactivation of PP2A enzyme activity and protecting PP2Ac from degradation (Kong et al., 2009). Although it has been found that PP2A directly dephosphorylates γ-H2AX (Chowdhury et al., 2005), it is unknown which specific PP2A regulatory B subunit is involved in this process. It also remains unclear how PP2A is regulated during the DNA DSB repair. Here we show that γ-H2AX is a substrate of B56ε containing holoenzymes. The complexes of PP2A–B56ε holoenzymes mediates the removal of H2AX phosphorylation signal and influences the efficiency of DNA repair. These findings provide a new insight into the role of PP2A in DNA DSB repair.

2. Methods

2.1. Reagents and antibodies

Mitomycin C (MMC) and complete protease inhibitor cocktail tablets were purchased from Roche. Camptothecin (CPT), *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) and cytochalasin B (cyt-B) were obtained from Sigma–Aldrich. The following antibodies were used: mouse anti-γ-H2AX and mouse anti-PP2A Cα (1D6) (upstate Biotechnology), rabbit or mouse anti-Flag tag (Cell Signaling Technology). The B55α and B56ε polyclonal antibody were prepared as previously described (Chen et al., 2005).

2.2. Cell lines and establishment of stable cell lines

The human embryonic kidney cells expressing Simian virus 40 LT antigen (LT) and the telomerase catalytic subunit (*hTERT*) (HEK cells) were established as described previously (Hahn et al., 1999). The pLKO.1-puro vectors containing shRNAs targeting specific PP2A subunits were introduced into HEK cells and selected with puromycin (1 μg/ml). Transient transfection experiments were carried out by transfecting HEK 293FT cells with amino-terminus FLAG tagged (DYKDDDK in amino acids) pBabe-Flag-B55α, pBabe-Flag-B56γ, pBabe-Flag-B56ε and empty vector.

2.3. Cell proliferation assays

To measure cell proliferation, 2×10^4 cells were plated in triplicate and harvested at the indicated time. The number of cells was determined by a Z2 Particle Count and Size Analyzer (Beckman-Coulter, Miami, FL). Cell viability was measured by MTT assay, 8×10^3 cells per well were seeded on a 96-well plate in quadruplicates for 24 h, and treated with various concentrations of DNA damage agents, then cultured for an additional 24 h. WST-1 (water-soluble tetrazolium salt-1) Cell Proliferation kit was used for the assay according to the manufacturer's instructions.

2.4. Isolation of cytosolic and nuclear fractions

Harvested cells were resuspended in a hypotonic buffer (20 mM Tris–HCl (pH 7.2), 15 mM KCl, 2.5 mM $MgCl_2$, 0.05% NP-40 and mixed protease inhibitors) on ice for 10 min, and suctioned with a needle. The homogenate was then centrifuged at $3000 \times g$ for 5 min at 4 °C. The supernatants were collected and the nuclear pellets were resuspended in extraction buffer (50 mM Tris–HCl (pH 7.8), 150 mM KCl, 5 mM $MgCl_2$, 250 mM sucrose and mixed protease inhibitors), followed by a MNase digestion for 15 min at 37 °C. The nuclear extracts were placed on top of a cushion containing 0.88 M sucrose, which was prepared in the same extraction buffer as described above. The mixture was centrifuged at $16{,}000 \times g$ for 15 min and the supernatant was collected as the nuclear fraction.

2.5. Immunoblotting and co-immunoprecipitation

HEK or 293FT cells were treated with CPT or DMSO for 1 h and allowed to recover for indicated times before harvested. For analysis of γ-H2AX levels, cells were lysed directly on the plate using 2× SDS sample buffer (125 mM Tris-base, 138 mM SDS, 10% β-mercaptoethanol, 20% glycerol, bromophenol blue (pH 6.8)). Soluble proteins (30 μg) were subjected to 8–16% gradient acrylamide gel for SDS-PAGE before immunoblotting. For immunoprecipitation analysis, each antibody (PP2A Cα, Flag-tag or γ-H2AX) was incubated with 2–3 mg nuclear fraction overnight at 4 °C. 100 μl of prewashed 1:1 slurry of protein G sepharose was added and incubated for additional 2 h. The protein G beads/protein complexes were washed three times and eluted in 2× SDS loading buffer, followed by SDS-PAGE and immunoblotting.

2.6. Immunofluorescence

HEK cells were grown overnight on coverslips. HEK cells were treated with CPT and fixed with 3.7% paraformaldehyde at indicate times. Cells were incubated with rabbit anti γ-H2AX antibody (1:1000) overnight and goat anti-rabbit IgG (1:1000) for 1 h. After staining with DAPI for 5 min, the nuclei were visualized by fluorescence microcopy (Nikon, ECLIPSE Ti) and digitized. The foci number was qualified by analyzing at least 100 cells for each condition. Cells with five or more discrete bright dots were judged as positive for γ-H2AX foci.

2.7. Neutral comet assay

HEK cells were treated with CPT (2 μM) or DMSO for 1 h and recovered for indicated time points. Cells at a density of 5×10^4 cells/ml were mixed gently with pre-melted low-temperature-