NEOPLASIA
www.neoplasia.com

Volume 11 Number 10 October 2009 pp. 1012–1021 1012

A Nonhomologous End-joining Pathway Is Required for Protein Phosphatase 2A Promotion of DNA Double-Strand Break Repair[1,2]

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Abstract

Protein phosphatase 2A (PP2A) functions as a potent tumor suppressor, but its mechanism(s) remains enigmatic. Specific disruption of PP2A by either expression of SV40 small tumor antigen or depletion of endogenous PP2A/C by RNA interference inhibits Ku DNA binding and DNA-PK activities, which results in suppression of DNA double-strand break (DSB) repair and DNA end-joining in association with increased genetic instability (i.e., chromosomal and chromatid breaks). Overexpression of the PP2A catalytic subunit (PP2A/C) enhances Ku and DNA-PK activities with accelerated DSB repair. Camptothecin-induced DSBs promote PP2A to associate with Ku 70 and Ku 86. PP2A directly dephosphorylates Ku as well as the DNA-PK catalytic subunit (DNA-PKcs) *in vitro* and *in vivo*, which enhances the formation of a functional Ku/DNA-PKcs complex. Intriguingly, PP2A promotes DSB repair in wild type mouse embryonic fibroblast (MEF) cells but has no such effect in Ku-deficient MEF cells, suggesting that the Ku 70/86 heterodimer is required for PP2A promotion of DSB repair. Thus, PP2A promotion of DSB repair may occur in a novel mechanism by activating the nonhomologous end-joining pathway through direct dephosphorylation of Ku and DNA-PKcs, which may contribute to maintenance of genetic stability.

Neoplasia (2009) 11, 1012–1021

Introduction

Environmental factors and genotoxic products of endogenous metabolic processes pose a constant threat to genome integrity by generating hundreds of thousands of DNA modifications in each cell everyday [1]. Among these modifications, DNA double-strand breaks (DSBs) are particularly detrimental because both strands are damaged [2]. Inability to repair DSBs can lead to the accumulation of genomic rearrangements that potentially promote tumorigenesis [3,4]. DSBs are repaired by homologous recombination or by nonhomologous end-joining (NHEJ). Homologous recombination is the predominant DSB repair mechanism in prokaryotes, and in eukaryotes, it plays a principal role during the S and G_2 phases of the cell cycle. In contrast, NHEJ, which simply pieces together the broken DNA ends, can function in all phases of the cell cycle [2,5]. In higher eukaryotes, the predominant recourse is the NHEJ DSB repair pathway. NHEJ is a versatile mechanism using the Ku heterodimer, DNA-PK catalytic subunit (DNA-PKcs), ligase IV/XRCC4, and a host of other proteins that juxtapose two free DNA ends for ligation [1].

The reversible phosphorylation of proteins, catalyzed by protein kinases and protein phosphatases, is a major mechanism for regulation of many eukaryotic cellular processes including DSB repair [6]. DNA-PK undergoes phosphorylation of all three of its protein components *in vitro*, and phosphorylation of the DNA-PK complex correlates with loss of protein kinase activity and disruption of DNA-PKcs from the Ku-DNA complex [7]. DNA-PKcs can be phosphorylated at multiple sites, and phosphorylation of DNA-PKcs at Ser 2056 inhibits

Abbreviations: NHEJ, nonhomologous end-joining; DSB, DNA double-strand break; PP2A/C, protein phosphatase 2A catalytic subunit; small T, the SV40 small tumor antigen; PFGE, pulsed-field gel electrophoresis; HA, hemagglutinin; siRNA, small interfering RNA; RNAi, RNA interference

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[1]This work was supported by National Cancer Institute, National Institutes of Health grant R01 CA112183, by an Award from Stop Children Cancer, Inc., and by a Flight Attendant Medical Research Institute Clinical Innovator Award (to X.D.).

[2]This article refers to supplementary materials, which are designated by Figures W1 to W7 and are available online at www.neoplasia.com.

Received 6 May 2009; Revised 25 June 2009; Accepted 25 June 2009

DOI 10.1593/neo.09720

DNA end processing [8]. Ku 70 can be phosphorylated by DNA-PK at Ser 6 and Ser 51 [9,10]. Ku 86 is phosphorylated at Ser 577, Ser 580, and Thr 715 [9]. However, the functional role of Ku 70/86 phosphorylation is not fully understood.

Protein phosphatase 2A (i.e., PP2A) is one of the major Ser/Thr phosphatases implicated in the regulation of many cellular processes including cell cycle progression, apoptotic cell death, DNA replication, gene transcription, and DSB repair [11–13]. The AC catalytic complex alone has phosphatase activity, whereas the distinct B subunits can recruit PP2A/C to distinct subcellular locations and then define a specific substrate target [14–16]. The A and C subunits are evolutionary conserved and ubiquitously expressed [17]. These two subunits form a catalytic complex (PP2A/AC) that interacts with at least three families of regulatory subunits (B, B′, and B″) and tumor antigens (i.e., SV40 small tumor antigen [18]). The B subunits determine the substrate specificity of PP2A [14].

PP2A has recently been reported to dephosphorylate γ-H2AX leading to facilitation of DSB repair [11]. A previous report indicates that PP2A can directly dephosphorylate Ku and DNA-PKcs *in vitro*, which leads to increased DNA-PK protein kinase activity *in vitro* [6]. However, it is not clear whether the effect of PP2A on DSB repair results from a direct activation of the NHEJ factors (i.e., Ku and DNA-PKcs) *in vivo*. Here, we provide strong evidence that PP2A facilitates DNA end-joining and DSB repair in a novel mechanism by activating Ku and DNA-PKcs, which contributes to the suppression of genetic instability.

Materials and Methods

Materials

PP2A/C, Ku 70, Ku 86, DNA-PKcs, and tubulin antibodies as well as PP2A/C small interfering RNA (siRNA) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Small T antibody was purchased from Research Diagnostics, Inc (Concord, MA). Anti–γ-H2AX antibody was purchased from Upstate Biotech (Charlottesville, VA). Ku 70/Ku 86 DNA Repair Kit was obtained from Active Motif (Carlsbad, CA). The SignaTECT DNA-PK Assay Kit was purchased from Promega (Madison, WI). Alexa Fluor 594–conjugated goat antimouse immunoglobulin G antibody and Alexa Fluor 488–conjugated goat antirabbit immunoglobulin G antibody were obtained from Invitrogen (Carlsbad, CA). Telomere PNA FISH Kit/Cy3 was obtained from DakoCytomation (DK-2600; Glostrup, Denmark). Purified PP2A was obtained from Calbiochem (San Diego, CA). Purified Ku 70 and Ku 86 proteins were purchased from GenWay Biotech, Inc (San Diego, CA). Camptothecin (CPT) was obtained from Sigma-Aldrich (St Louis, MO). The hemagglutinin (HA)-tagged PP2A/C/pcDNA3 construct was generously provided by Dr. Brain Law (University of Florida). Wild type small T antigen complementary DNA (cDNA) in pCMV5 was kindly provided by Dr. Marc Mumby (University of Texas Southwestern Medical Center, Dallas, TX). Wild type and Ku $86^{-/-}$ mouse embryonic fibroblast (MEF) cells were kindly provided by Dr. David J. Chen (University of Texas Southwestern Medical Center, Dallas, TX). All of the reagents used were obtained from commercial sources unless otherwise stated.

Cell Lines, Plasmids, and Transfections

H69, H82, H157, H1299, and H460 cells were maintained in RPMI 1640 with 10% fetal bovine serum (FBS). A549 cells were maintained in F-12K medium with 10% FBS. Wild type and Ku $86^{-/-}$ MEF cells were maintained in Dulbecco's modified Eagle medium with 10% FBS and 4 mM L-glutamine. The HA-tagged PP2A/C/pcDNA3 or small T/pCMV5 were transfected into H1299 cells using LipofectAMINE 2000 (Invitrogen Life Technology, Carlsbad, CA) according to the manufacturer's instructions.

Phosphorylation/Dephosphorylation Assay

Cells were cultured with 0.5% FBS overnight. The cells were washed with phosphate-free RPMI medium and metabolically labeled with [^{32}P]orthophosphoric acid for 60 minutes. After agonist or inhibitor addition, the cells were washed with ice-cold phosphate-buffered saline and lysed in detergent buffer. Ku 70, Ku 86, or DNA-PKcs was then immunoprecipitated. Phosphorylation of Ku 70, Ku 86 or DNA-PKcs was determined by autoradiography. For dephosphorylation, ^{32}P-labeled Ku 70, Ku 86, or DNA-PKcs was immunoprecipitated as described. The beads were washed and resuspended in 60 μl of phosphatase assay buffer containing 50 mM Tris-HCl, pH 7.0, 20 mM β-mercaptoethanol, 2 mM $MnCl_2$, and 0.1% BSA. Purified PP2A was added, and the samples were incubated at 30°C for 10 minutes as described [19]. The samples were boiled for 5 minutes before loading onto SDS-PAGE. Phosphorylation of Ku 70, Ku 86, or DNA-PKcs was determined by autoradiography.

Preparation of Cell Lysates

Cells were washed with 1× PBS and resuspended in ice-cold EBC buffer (0.5% NP-40, 50 mM Tris, pH 7.6, 120 mM NaCl, 1 mM EDTA, and 1 mM β-mercaptoethanol) with a cocktail of protease inhibitors (EMD Biosciences, Gibbstown, NJ). Cells were lysed by sonication and centrifuged at 14,000*g* for 10 minutes at 4°C. The resulting supernatant was collected as the total cell lysate.

Pulsed-Field Gel Electrophoresis

Pulsed-field gel electrophoresis (PFGE) was performed as described [20,21]. Briefly, cells were harvested and resuspended in ice-cold buffer L (0.1 M Na_2-EDTA, 0.01 M Tris, 0.02 M NaCl, pH 8.0) at a concentration of 5×10^6 cells per milliliter and mixed with an equal volume of 1% low–melting point agarose (Beckman, Fullerton, CA) at 42°C. The mixture was pipetted into a small length of Tygon tubing, clamped tight at both ends, and chilled to 0°C. The solidified agarose "snake" was extruded from the tubing, added to 10× volume of buffer L containing 1 mg/ml proteinase K and 1% sarkosyl, and incubated for 16 hours at 50°C. After lysis, the agarose snake was washed four times with Tris/EDTA buffer and then cut into 0.5-cm plugs. The plugs were inserted into the wells of a precooled 1% low–melting point agarose gel (4°C). PFGE (200-second pulse time, 150 V, 15 hours at 14°C) was performed using the clamped, homogenous electric fields Mapper (Bio-Rad, Hercules, CA). After electrophoresis, the gel was stained with ethidium bromide for photography.

Immunofluorescence

The cells were washed with 1× PBS, fixed with methanol and acetone (1:1) for 5 minutes, and then blocked with 10% normal mouse or rabbit serum for 20 minutes at room temperature. Cells were incubated with a mouse or rabbit primary antibody for 90 minutes. After washing, samples were incubated with Alexa Fluor 488 (green)–conjugated antimouse or Alexa Fluor 594 (red)–conjugated antirabbit