DNA Repair 21 (2014) 131–139

Contents lists available at ScienceDirect

DNA Repair

journal homepage: www.elsevier.com/locate/dnarepair

DNA-PK phosphorylation of RPA32 Ser4/Ser8 regulates replication stress checkpoint activation, fork restart, homologous recombination and mitotic catastrophe

Amanda K. Ashley[a,1], Meena Shrivastav[b], Jingyi Nie[a], Courtney Amerin[a], Kyle Troksa[a], Jason G. Glanzer[c], Shengqin Liu[c], Stephen O. Opiyo[c], Diana D. Dimitrova[c], Phuong Le[a], Brock Sishc[a], Susan M. Bailey[a], Greg G. Oakley[c,d], Jac A. Nickoloff[a,b,*]

[a] Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO, United States
[b] Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM, United States
[c] Department of Oral Biology, University of Nebraska Medical Center, Omaha, NE, United States
[d] Eppley Cancer Center, University of Nebraska Medical Center, Omaha, NE, United States

ARTICLE INFO

Article history:
Received 3 November 2013
Received in revised form 17 March 2014
Accepted 14 April 2014
Available online 10 May 2014

Keywords:
DNA repair
Replication stress
Checkpoint regulation
Homologous recombination

ABSTRACT

Genotoxins and other factors cause replication stress that activate the DNA damage response (DDR), comprising checkpoint and repair systems. The DDR suppresses cancer by promoting genome stability, and it regulates tumor resistance to chemo- and radiotherapy. Three members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, ATM, ATR, and DNA-PK, are important DDR proteins. A key PIKK target is replication protein A (RPA), which binds single-stranded DNA and functions in DNA replication, DNA repair, and checkpoint signaling. An early response to replication stress is ATR activation, which occurs when RPA accumulates on ssDNA. Activated ATR phosphorylates many targets, including the RPA32 subunit of RPA, leading to Chk1 activation and replication arrest. DNA-PK also phosphorylates RPA32 in response to replication stress, and we demonstrate that cells with DNA-PK defects, or lacking RPA32 Ser4/Ser8 targeted by DNA-PK, confer similar phenotypes, including defective replication checkpoint arrest, hyper-recombination, premature replication fork restart, failure to block late origin firing, and increased mitotic catastrophe. We present evidence that hyper-recombination in these mutants is ATM-dependent, but the other defects are ATM-independent. These results indicate that DNA-PK and ATR signaling through RPA32 plays a critical role in promoting genome stability and cell survival in response to replication stress.

1. Introduction

Cells respond to genotoxic stress by activating the DNA damage response (DDR), a network of damage sensor, signal transducer, and effector proteins that arrest the cell cycle and stimulate DNA repair. During S phase, replication forks stall at fragile sites, telomeres, DNA lesions, and when the replication machinery is disrupted by topoisomerase inhibitors or nucleotide pool depletion by hydroxyurea (HU) [1–5]. Prolonged fork stalling can result in fork collapse to one-ended double-strand breaks (DSBs) that promote genome instability and cancer. Collectively these events are termed "replication stress" and cells respond to replication stress by activating checkpoint and repair processes.

Replication checkpoints arrest the cell cycle, promote fork stabilization and repair, and prevent further encounters of replication forks with damage, thereby promoting cell survival and genome stability [6–8]. Key upstream checkpoint factors are replication protein A (RPA), a heterotrimeric single-stranded DNA (ssDNA) binding complex with critical roles in replication and DNA repair, and members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, ATR, ATM, and DNA-PK. Although early studies indicated that ATR and ATM respond to replication stress and replication-independent DSBs, respectively [9,10], and DNA-PK functions in DSB repair by nonhomologous end-joining (NHEJ) [11], it is now

* Corresponding author at: Department of Environmental and Radiological Health Sciences, Colorado State University, Fort Collins, CO 80523, United States. Tel.: +1 970 491 5222; fax: +1 970 491 0623.
E-mail address: J.Nickoloff@colostate.edu (J.A. Nickoloff).
[1] Present address: Cell & Molecular Biology Laboratory, Center for Animal Health, Food Safety, & Bio-Security, New Mexico State University, Las Cruces, NM, United States.

http://dx.doi.org/10.1016/j.dnarep.2014.04.008
1568-7864/

clear that PIKKs have overlapping roles and display crosstalk in various DNA damage response pathways [12–23].

DSBs are also repaired by homologous recombination (HR), and HR proteins also play key roles in replication fork stabilization and restart [7,8]. HR can result in accurate repair, but sometimes it leads to genome rearrangements including deletions, amplifications, and translocations through crossovers and strand-transfer reactions between non-allelic homologous sequences [24,25]. Genome stability is maintained, in part, by crossover suppression [26–28]. Sister chromatid exchange (SCE) is mediated by HR and can be detected by cytogenetic methods [29,30]. Most SCEs have no genetic consequence because sister chromatids typically have identical sequences. However, mammalian genomes comprise ~50% repeated sequences (e.g., Alu elements), and strand exchange can occur between linked repeats in equal or unequal fashion, with the latter resulting in genome rearrangement. While cytogenetic approaches cannot distinguish these outcomes, direct repeat HR substrates allow detection of unequal exchange events that produce a functional selectable marker, including gene conversion and repeat deletions, whereas equal exchange events are not detected (Fig. S1). Thus, all SCE events are detected cytogenetically, but HR substrates reveal additional information about HR accuracy.

RPA bound to ssDNA at stalled forks recruits and activates ATR through a Rad17-RFC, 9-1-1, MRN, and TopBP1-dependent pathway. ATR phosphorylates/activates Chk1 which signals downstream factors that stabilize and repair forks, arrest active forks, and stimulate dormant origin firing to complete replication adjacent to stalled forks [8,31]. The RPA32 subunit of RPA is phosphorylated on Ser23 and Ser29 by CDK cyclically during the cell cycle, and in response to replication stress on Ser33 by ATR, and Ser4/Ser8, Ser12, and Thr21 by one or more PIKKs depending on the replication stress agent [13,20,32–36]. Certain replication stress-induced phosphorylation events in RPA32 are subject to "priming" by phosphorylation of other residues [13,33]. DNA-PK phosphorylates RPA32 Ser4/Ser8, and defects in DNA-PK or RPA32 Ser4/Ser8 residues suppress replication stress-induced Chk1 activation, checkpoint arrest, and fork repair (revealed as persistent γ-H2AX foci) [33,37]. Liaw et al. [37] showed that blocking RPA32 Ser4/Ser8 phosphorylation increases SCEs; as noted above, SCE analysis cannot distinguish accurate vs inaccurate HR. We previously showed that DNA-PK suppresses replication-associated (spontaneous) direct repeat HR (inaccurate HR) [38], suggesting that DNA-PK suppresses inaccurate HR, perhaps by modulating the interaction between p53 and RPA [39]. ATM/ATR-mediated phosphorylation of p53, and DNA-PKcs-mediated phosphorylation of RPA32, dissociates the p53-RPA complex which promote HR and cell cycle arrest [40].

In this study we define novel PIKK and RPA32 Ser4/Ser8 roles in replication stress responses. We demonstrate that DNA-PKcs phosphorylation of RPA32 Ser4/Ser8 regulates replication fork restart, new origin firing, HR, mitotic catastrophe, and cell survival in response to replication stress. These results indicate that in addition to its direct role in NHEJ, DNA-PKcs (along with ATM and ATR) helps maintain genome stability after replication stress by regulating checkpoint activation and suppressing inaccurate HR through RPA32 phosphorylation.

2. Materials and methods

2.1. Cell lines

Human UM-SCC-38 oral squamous carcinoma cells expressing HA-tagged wild-type or Ser4 → Ala/Ser8 → Ala (S4A/S8A) mutant RPA32 were described previously [33,41]. WT and S4A/S8A RPA32 are expressed at similar levels in the UM-SCC-38 derivatives (Fig. S2A). CHO V3 derivatives lacking DNA-PKcs (DNA-PKcs null) or complemented with wild-type (WT) or kinase-dead (KD; Lys3792 → Arg) and carrying neo direct repeat HR substrates were described previously [38,42]. Human and CHO cells were cultured in DMEM or α-MEM, respectively, supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. ATR was downregulated in UM-SCC-38 cells as described [33].

2.2. Induction of replication stress, PIKK inhibition, and Western immunoblotting

Replication stress was induced by treating cells with etoposide, camptothecin (CPT), HU, or cis-platin at indicated concentrations/times, then growth medium was replaced and cells were harvested at various times after release from stress to analyze checkpoint arrest, RPA phosphorylation, DNA replication, cell survival, and homologous recombination as described below. In the case of etoposide, cells were treated for 2 h, and untreated cells are designated "-2 h." To inhibit specific PIKKs, cells were pretreated with ATM inhibitors (ATMi) KU55933 (20 μM) or KU60019 (3 μM), DNA-PKi NU7026 (40 μM) or NU7441 (10 μM) (EMD Biosciences), or ATRi VE-821 (10 μM) (MedChem Express), before treatment with replication stress agents for specified periods, released from stress, and harvested for specific endpoints noted above. ATR was also inhibited by siRNA knockdown. Where tested, similar results were obtained with the two ATMi; the two DNA-PKi; and with siRNA knockdown of ATR and ATRi VE-821 (data not shown). UM-SCC-38 cell lysates were separated by SDS-PAGE, transferred to PVDF membranes, immunoblotted using RPA32 and β-actin primary antibodies (ThermoFisher Scientific) and horseradish peroxidase-conjugated secondary antibodies (GE Healthcare). RPA32 and β-actin signals were detected with a Typhoon 9410 (Molecular Dynamics) or Odyssey Infrared scanner (LI-COR), respectively.

2.3. Flow cytometry and immunofluorescence microscopy analysis

Cell cycle profiles were determined by flow cytometry after release from replication stress for indicated times and fixation in 70% ethanol overnight. Cells were incubated in 50 μg/ml propidium iodide (PI) and 100 μg/ml RNase A for 30 min and 10,000 cells per sample were analyzed on a BD FACSarray (BD Biosciences); data were analyzed using WinList™ (Verity Software House). Abnormal mitoses (i.e., anaphase bridges) were scored by immunofluorescence microscopy in CHO cells grown on 4-well chamber slides overnight and fixed in 100% ice-cold methanol for 10 min at −20 °C. Cells were briefly permeabilized in 0.1% Triton X-100, washed and blocked in 1% milk in PBS for 30 min at room temperature. An anti-α tubulin antibody (Santa Cruz Biotechnology) was applied in blocking solution for 1 h at room temperature, then an appropriate Alexa Fluor-488 conjugated secondary antibody was incubated in blocking solution for 1 h at room temperature (Invitrogen). Cells were mounted in PermaFluor (Fisher) supplemented with 0.5 μg/ml DAPI (Roche). Images were acquired with a Zeiss Axiovert 200 M microscope, randomized, and scored blindly. Human cells were fixed, and nuclei stained with DAPI, and imaged as described [33].

2.4. BrdU incorporation and DNA fiber analysis

BrdU incorporation was analyzed following cell growth in six-well culture dishes and treatment with 5 mM HU or PBS control in growth medium for 1 h 37 °C. After 3 PBS washes, 100 μM BrdU was added in fresh growth medium. Samples were harvested at various times following HU release, fixed and processed with the FITC BrdU Flow Kit (BD Biosciences) according to manufacturer's directions. To correlate BrdU incorporation with cell cycle phase,