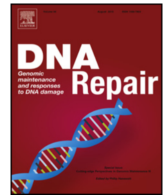
DNA Repair 48 (2016) 8–16

Contents lists available at ScienceDirect

DNA Repair

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



DNA maintenance following bleomycin-induced strand breaks does not require poly(ADP-ribosyl)ation activation in *Drosophila* S2 cells

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ARTICLE INFO

Article history:
Received 15 December 2015
Received in revised form 5 September 2016
Accepted 9 October 2016
Available online 21 October 2016

Keywords:
DNA repair
DNA breaks
PARP
Poly(ADP-ribosyl)ation
PARG

ABSTRACT

Background: Poly-ADP ribosylation (PARylation) is a post translational modification, catalyzed by Poly(ADP-ribose)polymerase (PARP) family. In *Drosophila*, PARP-I (human PARP-1 ortholog) is considered to be the only enzymatically active isoform. PARylation is involved in various cellular processes such as DNA repair in case of base excision and strand-breaks.
Observations: Strand-breaks (SSB and DSB) are detrimental to cell viability and, in Drosophila, that has a unique PARP family organization, little is known on PARP involvement in the control of strand-breaks repair process. In our study, strands-breaks (SSB and DSB) are chemically induced in S2 Drosophila cells using bleomycin. These breaks are efficiently repaired in S2 cells. During the bleomycin treatment, changes in PARylation levels are only detectable in a few cells, and an increase in PARP-I and PARP-II mRNAs is only observed during the recovery period. These results differ strongly from those obtained with Human cells, where PARylation is strongly activating when DNA breaks are generated. Finally, in PARP knock-down cells, DNA stability is altered but no change in strand-breaks repair can be observed.
Conclusions: PARP responses in DNA strands-breaks context are functional in *Drosophila* model as demonstrated by PARP-I and PARP-II mRNA increases. However, no modification of the global PARylation profile is observed during strand-breaks generation, only changes at cellular levels are detectable. Taking together, these results demonstrate that PARylation process in *Drosophila* is tightly regulated in the context of strands-breaks repair and that PARP is essential during the maintenance of DNA integrity but dispensable in the DNA repair process.

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1. Introduction

DNA strand breaks, and especially double-strand breaks (DSBs), are highly detrimental to genome stability and by extension to cell life. These genomic alterations have various causes, ranging from intrinsic ones such as reactive oxygen species or defective replication machinery to extrinsic ones like chemotherapeutic agents or ionizing radiations (for review see [1]). Prokaryotic and eukaryotic cells have developed a large range of mechanisms to counteract this biological issue and to lead up to a complex DNA damage response (DDR). This fully-integrated response includes sensing, signalling and promotion of DNA repair in the cell cycle context in order to coordinate an efficient DNA repair [2].

Among all the biological responses leading to DNA repair, post-translational modifications (PTM) constitute a tight regulator for activation or inactivation of DNA damage signalling and repair mechanisms [3]. The most characterized PTM in the context of DSBs repair are phosphorylations of several molecular actors including the well-known ATM signalling molecules (i.e. H2AX, 53BP1, Rad17, DNA-PKcs, Rad52 etc.). Beside phosphorylations, Poly (ADP-ribosyl)ation (PAR) plays also a unique role in DNA breaks repair. It consists of polymers of ADP-ribose moieties linked by 2′,1′′-O-glycosidic ribose-ribose bonds or even branched structures through 2′′,1′′′-O-glycosidic ribose-ribose bonds. Beside this extended modification, a single ADP moiety can be added to the protein resulting

Abbreviations: PARP, poly(ADP-ribose)polymerase; PARG, poly(ADP-ribose)glycohydrolase; PAR, poly(ADP-ribosyl)ation; SSB, single-strand break; DSB, double-strands break.

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http://dx.doi.org/10.1016/j.dnarep.2016.10.002
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into mono ADP ribosylation. ADP-ribose (either as a monomer or a polymer) is covalently bound to specific target proteins [4]. The extent of this modification on acceptor proteins, reaches few hundreds ADP-ribose moieties (for review, see [5]). This PTM is the result of nicotinamide adenine dinucleotide (NAD^+) hydrolysis by specific enzymes such as the Poly ADP-ribose polymerase (PARP) family. This large family (17 members in Human) is involved in various cellular processes such as gene transcription, chromatin remodelling or cell death pathways. Poly (ADP-ribose) polymerase-1 (PARP-1), a major member of the PARP family, is the main isoform involved in the promotion of DNA repair. In response to genotoxic agents, PARP-1, mainly located in nucleus, is activated through recognition of damaged DNA. As a result, PARP auto-modification and ADP-ribose polymers incorporation to a number of nuclear proteins is stimulated. In response to low levels of DNA lesions, PARP-1 activity favours repair and survival. Knock-out of PARP-1 in mice and fibroblasts leads to genomic instability after gamma irradiation and alkylating agent treatment [6,7]. Several studies suggest a central role of PARP-1 in replication fork restart by homologous recombination. PARP-1, as a sensor of disrupted replication forks, promotes the recruitment of Mre11 and/or RAD51 for end processing that is required for subsequent recombination repair and restart of replication forks [8]. However, PARP-1 inactivation in embryonic stem cells or embryonic fibroblasts results in hypersensivity to the replication inhibitor hydroxyurea (HU) but does not impair normal recombination repair of genomic DSBs [9] suggesting the fact that PARP could be dispensable for homologous recombination but remains essential for DNA repair. Various studies support a role for PARP-1 in Non Homologous End Joining (NHEJ) process via its interaction with DNA-PKcs or its partner ku70/ku80 [10]. However, PARP-1 does not appear to constitute a core factor in the C-NHEJ pathway [11]. On the other hand, when C-NHEJ is impaired, cells shift to alternative end-joining pathway (A-NHEJ), a PARP-dependant pathway. PARP-1 acts at the initiation step by binding to DNA ends and by ADP-ribosylating itself and other proteins [12]. This repair mode has also been shown to operate in some human cancers and during later stages of embryonic development.

In contrast, PARP-1 is well characterized in the resolution of strand breaks as an intermediate part of the BER/SSBR pathway. It has affinity to single strand that stimulate its catalytic activity. Also, PARP-1 plays a main role in BER actors recruitments as polymerase β [13] and APE1[14]. In *Drosophila*, PARP superfamily is limited to only one unique *parp* gene [15], encoding three different isoforms [16,17]. PARP enzymatically active isoform in fruit fly is encoded by PARP-I transcript.

The main objectives of this study were to determine the link between PARylation and DNA damages during DNA breaks production and recovery in *Drosophila* model. We show that, in contrast to what is observed in human cells, PARylation levels are slightly affected after DNA breaks induction with bleomycin. Moreover, PARP-I, II and PARG mRNA levels remain unchanged upon DNA breaks. However, PARP-I and PARP-II mRNA accumulations are dramatically increased during the recovery period while the PARylation levels remain stable, suggesting a tight control of this PTM during breaks repair in *Drosophila*. This unique behavior was only observed with DNA breaks and was different during oxidative stress induction.

2. Materials and methods

2.1. S2 cell culture

Drosophila melanogaster S2 cells were grown in Schneider medium (Biological industries) supplemented with 10% fetal calf serum and 1% penicillin/streptomycin solution (PAA) at 24 °C under normal atmosphere.

2.2. Bleomycin treatment

S2 cells were grown for 48 h in 75 cm^2 culture flasks. After two washes with PBS, the medium was discarded and replaced by fresh medium containing 75 μg/mL bleomycin (Bellon) at 24 °C for 5, 15 and 30 min. After three washes with PBS to eliminate residual bleomycin, cells were incubated in fresh medium between 30 min and 1 h, at 24 °C. For all samples and kinetics experiment, DNA, RNA and proteins were extracted immediately.

2.3. Western blotting

Cells were lysed either by adding immediately to cell pellet Laemmli solution buffer containing 5% (v/v) β-mercaptoethanol and heated at 95 °C for 5 mins or by resuspending cells pellet in lysis buffer (50 mM Tris HCl pH7.8, 150 mM NaCl, 1% Nonidet P-40 with protease inhibitors (Roche). Proteins concentrations were determined by Bradford assay (BioRad).

Proteins were separated on 8% (w/v) SDS-PAGE and transferred to nitrocellulose membrane (GeHealthcare) as previously described [18].

Membranes were blocked in TBST (50 mM Tris pH 7.4, 20 mM NaCl, 0.1% Tween 20) with 10% nonfat milk for 2 h or overnight. The membranes were incubated with the following antibodies: mouse anti-phosphorylated H2Av (1:100, UNC993-5.2.1, DSHB), rabbit-anti-PAR polyclonal (1:2000, TREVIGEN), mouse-anti-tubulin (1:20,000, Sigma). UNC93-5.2.1 was deposited to the DSHB by Hawley, R.S. (DSHB Hybridoma Product UNC93-5.2.1). The presence of different proteins was detected using LuminataTM forte western HRP Substrate (Millipore)

2.4. Southern blot

DNA extraction was carried out according to Genomic DNA purification from tissue protocol (MACHEREY-NAGEL). For each DNA extraction, 2×10^7 cells were used. Nucleic acid concentration and purity were spectrophotometrically measured (Nanodrop ND-1000, Thermo scientific).

For Southern blot, 2 μg DNA digested by EcoRV were loaded onto 0.8% agarose gel. DNA fragments were transferred onto nylon membrane. The membrane were hybridized with radiolabelled nuclear probe (18SrDNA, [19]). After autoradiography, the hybridization signals were analysed by phosphoimager (Biorad).

2.5. RT-qPCR analysis

For each RNA extraction, 2×10^6 cells were used. RNA extraction was carried out according to the manufacturer instructions using TRI REAGENT (Molecular Research Center, Inc.). RNA concentration and purity were measured by Nanodrop 1000 (Thermo Scientific). RNA (2 μg) from each sample was used to synthesize cDNA using M-MLV transcriptase Kit (Promega). After cDNA synthesis, samples were incubated with RNAse A (Sigma Aldrich) for 20 min at 37 °C.

Quantitative PCR analysis was performed using Mastercycler PCR apparatus (Eppendorf) with MESA GREEN qPCR Mastermix Plus for SYBR Assay (Eurogentec) according to the manufacturer's protocol. The relative abundance of mRNAs was calculated by the comparative cycle of threshold (Ct) method with Rp49 mRNA as the invariant control.

The primers used are as follows. ***Rp49***: 5′-GCTAAGCTGTCGCACAAATG-3′, 5′-TCTGCATGAGCAGGACCT-3′; ***parg***: 5′-ACATCAACTTTAACAGGCTTTA-3′, 5′-GCACTTTGGCTCCAGTCG-3′; ***parp-II***:

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