PARP and CSB modulate the processing of transcription-mediated DNA strand breaks

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Topoisomerase 1 (Top1)-DNA cleavage complexes induced by camptothecin (CPT) cause DNA strand breaks during DNA replication or transcription. Although the cellular responses to replication-mediated DNA double-strand breaks have been well studied, the responses to transcription-mediated DNA strand breaks have not. Here, we show that poly (ADP-ribose) polymerase (PARP) and cockayne syndrome group B protein (CSB) modulate the CPT-induced formation of discrete p53-binding protein 1 (53BP1) nuclear foci at sites of transcription-mediated DNA strand breaks. Inhibition of PARP activity enhanced the formation of these foci, while knockdown of essential components of the base excision repair (BER) pathway did not. These findings suggest that PARP suppresses transcription-mediated 53BP1 foci formation, but that this does not occur through the BER pathway. In addition, knockdown of CSB, one of the key factors of transcription-coupled repair, slowed the kinetics of 53BP1 foci formation. These data suggest that PARP and CSB modulate the formation of 53BP1 foci during the processing of transcription-mediated DNA strand breaks.

Key words: 53BP1, base excision repair, camptothecin, DNA strand breaks, transcription-coupled repair

INTRODUCTION

Camptothecin (CPT) is a topoisomerase 1 (Top1) inhibitor whose clinically relevant derivatives are used in anticancer chemotherapy (Hsiang et al., 1985; Pommier et al., 2010). Top1 binds covalently to DNA and enzymatically cleaves single-strand DNA to relax supercoiled DNA structure, after which the ends are quickly rejoined and Top1 is released from the DNA. The reactive intermediate of this process is called the “Top1-DNA cleavage complex (Top1-cc)”. CPT prevents the dissociation of Top1-cc from DNA by stabilizing the covalent Top1-DNA interaction (Pommier, 2006).

Top1-cc trapped by CPT is thought to be resolved by base excision repair (BER) via a mechanism similar to that of DNA single-strand break (SSB) repair (Caldecott, 2008). BER is accomplished by the coordinated actions of multiple proteins, including poly (ADP-ribose) polymerase (PARP), x-ray repair cross-complementation group 1 (XRCC1) and tyrosyl-DNA phosphodiesterase 1 (TDP1) (Caldecott, 2003). PARP is known to be a multifunctional protein, although its primary role is in BER. PARP recognizes SSBs and promotes the recruitment of other BER factors, such as XRCC1 (de Murcia and Menissier de Murcia, 1994; Masson et al., 1998; Schreiber et al., 2002), a scaffolding protein that forms a complex with several BER proteins including PARP and TDP1 (Plo et al., 2003). TDP1 then catalyzes the removal of Top1 linked to the 3′ DNA terminus by hydrolyzing the covalent bond between Top1 and DNA (Debethune et al., 2002). It has been shown that XRCC1-deficient cells exhibit increased sensitivity to CPT (Plo et al., 2003). In addition, the cell killing effect of CPT is promoted by combination with inhibition of PARP (Debethune et al., 2002; Patel et al., 2012).

Cells deficient in cockayne syndrome group A (CSA) or
group B (CSB) proteins also show sensitivity to CPT (Squires et al., 1993). Both CSA and CSB are required for transcription-coupled repair (TCR), a subpathway of nucleotide excision repair (NER), in which CSB recognizes stalled RNA polymerase II at the DNA damage site and promotes the recruitment of DNA repair proteins, including CSA complexes and NER factors (Fousteri et al., 2006). The increased sensitivity of CSA- and CSB-deficient cells to CPT suggests that TCR proteins are also involved in the response to DNA damage caused by CPT-triggered Top1-cc.

The major cytotoxic effect of CPT is believed to be due to the secondary DNA damage that is caused by conversion of single-strand breaks into double-strand breaks (DSBs) when the replication fork collides with Top1-cc. DNA replication-mediated DSBs are potentially cytotoxic, and are the primary cause of cell death induced by CPT in mitotic cells (Holm et al., 1989). In addition, transcription-mediated DNA strand breaks induced by CPT are also thought to have significant cytotoxic effects. XRCC1-deficient cells treated with aphidicolin to abrogate DNA replication still exhibited increased sensitivity to CPT compared with XRCC1-complemented cells (Plo et al., 2003). However, the cellular responses to transcription-mediated DNA strand breaks remain to be defined.

We reported previously that CPT induces two types of p53 binding protein 1 (53BP1) nuclear foci in HeLa cells (Sakasai et al., 2010). The first are called “Type I” foci, and form large 53BP1 foci related to transcription. The second type foci, called “Type II”, are diffusely distributed smaller foci that occur in cells in S-phase. Type I foci formation is dependent on ataxia-telangiectasia mutated (ATM) (Sakasai et al., 2010). In addition, ATM-dependent phosphorylation of histone H2AX was observed in response to CPT in post-mitotic cells (Sordet et al., 2009). These results suggest that the ATM-53BP1 pathway is activated by transcription-mediated DNA strand breaks induced by CPT.

In this study, we monitored the formation of Type I 53BP1 foci in an effort to identify factors that influence the response to transcription-mediated DNA strand breaks. Our results revealed that PARP and CSB act as regulatory factors of this response. Inhibition of PARP increased the formation of Type I 53BP1 foci caused by CPT, but the suppression of essential BER factors, such as XRCC1 and TDP1, did not. In addition, we also determined that CSB is involved in the formation of Type I 53BP1 foci. These findings implicate PARP and CSB as modulators of the response to transcription-mediated DNA damage, and provide new insights into the response to DNA strand breaks associated with transcription.

**MATERIALS AND METHODS**

**Cell line and reagents**

HCT116 cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. CPT, 5,6-Dichlorobenzimidazole 1-β-D-ribofuranoside (DRB) and PJ-34 were purchased from Sigma-Aldrich. NU1025 was purchased from Calbiochem.

**Immunofluorescence staining**

Cells grown on 12-mm coverslips in 24-well plates were pre-extracted with 0.1% Triton X-100/PBS for 1 min on ice. The cells were fixed with 4% paraformaldehyde for 15 min following permeabilization with 0.5% Triton X-100/PBS for 5 min. The fixed cells were incubated with primary antibodies specific for 53BP1 (Bethyl Laboratories, A300-272A) and RPA34 (GeneTex, GTX16855), followed by incubation with secondary antibodies conjugated to a fluorescent dye (Alexa 488 or Alexa 594). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI). A Biorevo BZ-9000 fluorescence microscope (Keyence) was used to visualize the samples.

**Analysis of transcription by EU incorporation**

The rate of transcription in the nucleus was analyzed using a Click-iT™ RNA Imaging Kit (Invitrogen). Cells grown on 12-mm coverslips were incubated with 1 mM 5-ethyl-2'-deoxyuridine (5-ethyluridine) (Fousteri et al., 2006) for 30 min. Cells were pre-extracted with 0.1% Triton X-100 for 1 min on ice and then fixed with 4% paraformaldehyde. After permeabilization with 0.5% Triton X-100/PBS for 5 min, incorporated EU was stained according to the manufacturer’s protocol. Nuclei were counterstained with DAPI and cells were observed with a Biorevo BZ-9000 fluorescence microscope. Quantification of EU intensity was performed using Image J (NIH).

**siRNAs and transfection**

The following siRNAs were used to knock down the expression of XRCC1, PARP1 and CSB: XRCC1, 5'-CUCCAGACUCUCUGGCAGAAUU-3' (Huehls et al., 2011); PARP1, 5'-AAGAUAGGCGU- GAAAGCGA-3' (Kameoka et al., 2004); CSB#1, 5'- CCACUGAUACGAGAAUCA-3'; CSB#2, 5'-CACCC- CUCAUCCGAUCUAUC-3'; siRNA control, 5'-UCUUAAUCGCGGAUAAGGC-3'. These siRNAs were purchased from Gene Design, Takara Bio, or Sigma-Aldrich. For TDP1 knockdown, siRNA from siGENOME SMARTpool (Thermo Scientific Dharmacon) was used and siGENOME Non-Targeting siRNA #1 was used as a negative control. HCT116 cells were transfected with 10 pmol siRNA using Lipofectamine RNAiMAX (Invitrogen) following manufacturer’s protocol and incubated for 48 h. For XRCC1 knockdown, cells were transfected with 25 pmol siRNA and incubated for 72 h.

**Western blotting**

Cells were lysed with SDS sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 5% glycerol, 5% 2-mercaptoethanol and bromophenol blue) and separated...
Modulation of transcription-mediated 53BP1 foci by SDS-PAGE. For Western blotting, the following antibodies were used: anti-PARP1 (Kamiya Biomedical Company, MC-710 (C2-10)), anti-TDP1 (Abnova, H00055775-A01), anti-XRCC1 (Sigma, X0629), anti-CSB (Santa Cruz, sc-25370), and β-actin (Sigma, A5316). Chemiluminescent signals were visualized and captured with an ImageQuant™ LAS-4000 (GE Healthcare).

RESULTS AND DISCUSSION

Characterization of CPT-induced 53BP1 foci mediated by transcription 53BP1 is a DNA damage response protein that is rapidly recruited to sites of CPT-induced DNA damage (Rappold et al., 2001). As reported previously (Sakasai et al., 2010), CPT treatment of HCT116 cells induced the formation of Type I 53BP1 foci that did not colocalize with RPA34, the 34-kDa subunit of the heterotrimeric replication protein A (RPA) complex (Fig. 1A). To further characterize the behavior of Type I 53BP1 foci, the kinetics of Type I 53BP1 foci formation were determined following CPT treatment. First, HCT116 cells were treated with different concentrations of CPT for 1 h and the formation of Type I 53BP1 foci was analyzed by immunofluorescence. As shown in Fig. 1B, the numbers of Type I 53BP1 foci increased in a CPT dose-dependent manner. Interestingly, at 1 nM CPT, induced DNA damage (Rappold et al., 2001). As reported previously (Sakasai et al., 2010), CPT treatment of HCT116 cells induced the formation of Type I 53BP1 foci that did not colocalize with RPA34, the 34-kDa subunit of the heterotrimeric replication protein A (RPA) complex (Fig. 1A). To further characterize the behavior of Type I 53BP1 foci, the kinetics of Type I 53BP1 foci formation were determined following CPT treatment. First, HCT116 cells were treated with different concentrations of CPT for 1 h and the formation of Type I 53BP1 foci was analyzed by immunofluorescence. As shown in Fig. 1B, the numbers of Type I 53BP1 foci increased in a CPT dose-dependent manner. Interestingly, at 1 nM CPT,
Type II 53BP1 foci were observed in RPA-positive cells, whereas Type I foci were not. Type I foci were clearly observed in 1000 nM CPT, but not significantly in 10 or 100 nM CPT. This suggests that the concentration of CPT required for the formation of Type I foci is higher than that for Type II foci.

![Fig. 2](image)

Fig. 2. PARP inhibition promotes the CPT-induced formation of Type I 53BP1 foci. (A) Representative images of 53BP1 foci in CPT- and/or PARP inhibitor-treated cells. HCT116 cells were pretreated with a PARP inhibitor (PJ34, 10 μM or NU1025, 10 μM) for 30 min followed by CPT treatment (1 μM, 1 h). After fixation, cells were stained with anti-53BP1 antibody. (B) The percentage of Type I 53BP1 foci-positive cells. HCT116 cells were treated with CPT (1 μM, 1 h) with or without PJ34 pretreatment (10 μM, 30 min). Cells with over four foci were counted as foci-positive cells. Error bars represent S. D. calculated from three independent experiments (*P<0.05). (C) PARP inhibition increased the number of Type I 53BP1 foci per cell. HCT116 cells were treated with CPT (1 μM, 1 h) with or without PJ34 pretreatment (10 μM, 30 min) and stained with anti-53BP1 antibody. The number of Type I 53BP1 foci per cell were counted and classified as 0–3, 4–10 or >10, as indicated. (D) Western blotting for PARP1 in PARP1 knockdown cells. HCT116 cells transfected with control (siCTL) or PARP1 siRNA (siPARP1) were treated with CPT (1 μM, 1 h) and PARP1 protein levels were analyzed by Western blotting. β-actin was used as a loading control. (E) PARP1 knockdown increased the number of Type I 53BP1 foci per cell. HCT116 cells transfected with control or PARP1 siRNA were treated with CPT (1 μM, 1 h) and stained with anti-53BP1 antibody. Classification based on the number of 53BP1 foci was performed as described in (C). The graphs show each percentage among cells with Type I 53BP1 foci. Error bars represent S. D. calculated from three independent experiments (*P<0.05) (C, E). P-values were calculated by two-sided Wilcoxon rank sum test.
Next, the kinetics of Type I 53BP1 foci formation were monitored after the addition and removal of CPT. While the formation of Type I 53BP1 foci was slower than that of Type II 53BP1 foci (Fig. 1C), dissolution of Type I 53BP1 foci was significantly more rapid than that of Type II foci (Fig. 1D). The differences in the kinetics of Type I and Type II foci formation and dissolution may reflect the different processes that are involved in the cellular responses to DNA damage mediated by transcription and replication. Transcription-mediated DNA damage may require an unknown processing step for 53BP1 accumulation and may be more quickly and easily repaired than replication-mediated DNA damage.

**PARP inhibition enhances Type I 53BP1 foci formation induced by CPT** In active transcription, RNF8 and ATM are required for the formation of Type I 53BP1 foci (Sakasai et al., 2010). To further understand the cellular response to transcription-mediated DNA strand breaks, a search was performed to identify other factors that affect CPT-induced Type I 53BP1 foci formation. It is known that inhibition of PARP activity or PARP deficiency enhances sensitivity to CPT cytotoxicity (Bowman et al., 2001; Hochegger et al., 2006). In addition, Top1-cc is resolved by the BER pathway, in which PARP plays a pivotal role (Caldecott, 2008). To investigate the involvement of PARP in CPT-induced DNA strand breaks mediated by transcription, 53BP1 were immunostained in HCT116 cells treated with CPT and/or PARP inhibitors. Cells were then classified by the percentage of cells with Type I 53BP1 foci and the number of foci per cell. Inhibition of PARP activity by the inhibitor PJ34 significantly increased both the number of Type I foci (Fig. 2, A and C) and the percentage of Type I foci-positive cells (Fig. 2B) when treated in combination with CPT treatment. A similar effect was observed in cells treated with the PARP inhibitor NU1025 (Fig. 2A). The increase in Type I 53BP1 foci induced by PARP inhibition was suppressed by pretreatment with DRB, a suppressor of transcription (Supplementary Fig. S1A), confirming that the effect of PARP inhibition was the result of transcription-mediated DNA damage induced by CPT. EU incorporation confirmed that the rate of transcription was not increased by inhibition of PARP, in fact, it was partially decreased (Supplementary Fig. S1B). Similar to the effect of PARP inhibitors, knockdown of PARP1 expression also increased the number of Type I 53BP1 foci per cell (Fig. 2, D and E). These results suggest that transcription-mediated DNA strand breaks induced by CPT are increased by inhibition of PARP. These results are consistent with those of a previous report (Zhang et al., 2011), which showed that inhibition of PARP enhanced CPT-induced γH2AX foci formation. PARP may be involved in the process by which CPT-induced Top1-cc induces DNA strand break during transcription, resulting in the formation of Type I 53BP1 foci.

**CPT-induced 53BP1 foci are not affected by base excision repair** The effects of PARP inhibition raised the possibility that the BER pathway plays an influential role in transcription-mediated DNA strand breaks induced by CPT. To explore the effects of BER factors on Type I 53BP1 foci formation, the expression of XRCC1 and TDP1 was knocked down using siRNA. Unexpectedly, knockdown of XRCC1 or TDP1 did not affect the percentage of cells with Type I 53BP1 foci after CPT treatment (Fig. 3, A and C). Furthermore, there was no difference in the number of Type I 53BP1 foci per cell between TDP1 or XRCC1 knockdown cells and control siRNA-transfected cells (Fig. 3, B and D). In addition to XRCC1 and TDP1, the effect of PNKP, which produces the 3'-hydroxyl DNA terminus after Top1 removal, was examined using the specific inhibitor, A12B4C3 (Freschauf et al., 2010). However, no significant effect on Type I 53BP1 foci formation was observed (data not shown). Based on these findings, it appears that BER is probably not involved in the pathway for the formation of Type I 53BP1 foci, and that PARP might affect formation of DNA strand breaks through transcription in a pathway that is distinct from BER.

PARP is also reported to be involved in the recruitment of nucleosome remodeling and histone deacetylation (NuRD) complexes to sites of DNA damage (Chou et al., 2010). Inhibition of PARP may prevent successful recruitment of the NuRD complex to DNA damage sites, resulting in a failure to repress transcriptional activity and a subsequent increase in transcription-mediated DNA strand breaks. However, no increase in the rate of transcription was observed following the inhibition of PARP activity. Taken together, these data suggest that PARP suppresses transcription-mediated 53BP1 foci formation in a pathway distinct from BER or transcriptional repression.

**CSB is involved in promoting Type I 53BP1 foci formation** In addition to components of the BER pathway, cells deficient in CSA or CSB also show increased sensitivity to CPT treatment (Squires et al., 1993). This suggested that TCR factors contribute to transcription-mediated DNA strand breaks induced by CPT. To test this possibility, CSB expression was knocked down in HCT116 cells and the formation of CPT-induced 53BP1 foci was analyzed. In CSB knockdown cells, the percentage of Type I foci-positive cells was partially decreased (Fig. 4, A and B). The percentage of cells with over 10 Type I 53BP1 foci was significantly decreased by CSB knockdown (Fig. 4C). This effect was not due to a decreased rate of transcription in CSB knockdown cells (Fig. 4D). Moreover, in CSB knockdown cells, the kinet-
ics of Type I 53BP1 foci formation was delayed compared to control siRNA-transfected cells (Fig. 4E). These results suggest that CSB is involved in promoting 53BP1 foci formation during transcription. The comparatively slow response to transcription-mediated DNA strand breaks observed in CSB knockdown cells probably reflects a requirement for the “processing of DNA damage” by CSB in which the collision between Top1-cc and the transcription machinery is converted to DNA damage that activates ATM and subsequently promotes 53BP1 foci formation. However, we observed even levels of ATM autophosphorylation at Ser1981 induced by CPT among control and both CSB knockdown cells (Fig. 4A). In addition, recent report showed no effect of CSB on Top1-cc resolution (Horibata et al., 2011). These results suggest that CSB can be involved in the process of Type I 53BP1 foci formation, which is downstream of ATM activation.

The data presented here suggest that PARP suppresses this DNA damage processing, while CSB is required for it. As reported previously (Squires et al., 1993), transcription is effectively suppressed by CPT treatment (Supplementary Fig. S2). Although the rate of transcription was
almost completely recovered at 1 h after CPT washout, many cells still exhibited Type I 53BP1 foci (Supplementary Fig. S2). This may imply that there is no direct correlation between transcriptional recovery and the dissolution of Type I 53BP1 foci. To clarify the biological significance of transcription-mediated 53BP1 foci, further examination will be necessary. However, the findings presented here strongly support the contribution of PARP and CSB to the processing of DNA damage caused by Top1-cc during transcription. The mechanisms by which these factors are involved in this process remain to be elucidated.

Fig. 4. CSB knockdown decreased the number of 53BP1 foci per cell in response to CPT. (A) HCT116 cells transfected with control or CSB siRNA (siCSB#1 and siCSB#2) were treated with DMSO or CPT (1 μM, 1 h). CSB, ATM, and autophosphorylated ATM at Ser1981 were analyzed by Western blotting. (B, C) CSB knockdown suppressed the formation of Type I 53BP1 foci. Cells prepared as described in (A) were fixed and stained with anti-53BP1 antibody. Type I 53BP1 foci were counted and the percentage of Type I foci-positive cells was calculated (B) and the number of Type I foci per cell was classified and graphed each percentage among cells with Type I 53BP1 foci (*P<0.05) (C). (D) Rates of transcription in CSB knockdown cells. HCT116 cells were transfected with control or CSB siRNA (siCSB#1, siCSB#2) and transcriptional rates were measured based on the amount of EU incorporation. (E) The kinetics of Type I 53BP1 foci formation were delayed by CSB knockdown. HCT116 cells transfected with control or CSB siRNA were treated with 1 μM CPT. At the indicated time points, cells were fixed and stained with anti-53BP1 antibody, and the percentage of cells with Type I 53BP1 foci was calculated (*P<0.05). Error bars show S. D. calculated from three independent experiments. P-values were calculated by two-sided Wilcoxon rank sum test.
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