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Poly(ADP-ribose)-binding Promotes Exo1 Damage Recruitment and Suppresses Its Nuclease Activities

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Abstract

Exonuclease 1 (Exo1) has important roles in DNA metabolic transactions that are essential for genome maintenance, telomere regulation and cancer suppression. However, the mechanisms for regulating Exo1 activity in these processes remain incompletely understood. Here we report that Exo1 activity is regulated by a direct interaction with poly(ADP-ribose) (PAR), a prominent posttranslational modification at the sites of DNA damage. This PAR-binding activity promotes the early recruitment of Exo1 to sites of DNA damage, where it is retained through an interaction with PCNA, which interacts with the C-terminus of Exo1. The effects of both PAR and PCNA on Exo1 damage association are antagonized by the 14-3-3 adaptor proteins, which interact with the central domain of Exo1. Although PAR binding inhibits both the exonuclease activity and the 5' flap endonuclease activity of purified Exo1, the pharmacological blockade of PAR synthesis does not overtly affect DNA double-strand break end resection in a cell free *Xenopus* egg extract. Thus, the counteracting effects of PAR on Exo1 recruitment and enzymatic activity may enable appropriate resection of DNA ends while preventing unscheduled or improper processing of DNA breaks in cells.

Keywords

Exo1; poly(ADP-ribosyl)ation; 14-3-3; PCNA; DNA end resection; DNA replication

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1. Introduction

First identified in *Schizosaccharomyces pombe*, Exo1 is an evolutionarily conserved nuclease that participates in DNA replication and repair pathways, including DNA double-strand break repair (DSBR), mismatch repair (MMR) and error-free post-replicative repair by template switching (1–3). Exo1 exhibits both 5' to 3' exonuclease activity and 5' flap endonuclease activity (4). The exonuclease activity of Exo1 is crucial for the resection of DNA double-strand break (DSB) ends and the removal of mispaired nucleotides to enable the rejoining of DNA ends (1, 5, 6). During MMR, Exo1 excises DNA from the nick generated by Mlh1-Pms1 in the 5' to 3' direction to create a gap for subsequent repair steps (3, 5). The resection of DSBs generates long ssDNA tails that initiate DNA repair by homologous recombination (HR) and activate the ATR-dependent cell cycle checkpoint (7, 8). The process of DNA resection is thought to be initiated by endonucleolytic cleavage near the breakpoint that is mediated by the Mre11-Rad50-NBS1 (MRN) complex (Mre11-Rad50-Xrs2 in budding yeast) together with the CtIP (Sae2 in budding yeast) protein. Following this initial endocleavage, the resulting “clean” 5' ends are further resected by Exo1 and Dna2, which act redundantly to generate long 3' ssDNA overhangs (e.g. 2–4 kb in budding yeast) required for HR and the ATR checkpoint (9, 10). The resection of dsDNA ends to create ssDNA overhangs also inhibits DSB repair by nonhomologous end-joining (NHEJ) and attenuates the ATM-dependent checkpoint pathway (11, 12). Exo1's 5' flap endonuclease activity is thought to resolve DNA intermediates formed during replication and recombination. Genetic studies in yeast suggest that the flap endonuclease activity of Exo1 plays a redundant role with Fen1 (Rad27 in budding yeast) in Okazaki fragment maturation. Consistent with this notion, overexpression of yeast EXO1 or human Exo1 proteins functionally complements the replication defects of a *rad27* mutant (13).

The regulation of Exo1 activity ensures efficient break processing while avoiding unscheduled or uncontrolled DNA digestion that could lead to cell death or genomic instability. Indeed, Exo1 function is regulated in some manner by a number of proteins that function in DSB resection, including MRN, CtIP, Ku, RPA, SOSS1, BLM, SWR1, ATM, ATR, Rad53 and CDK (14–32). We have previously shown that Exo1 activity during DSB resection is promoted by the sliding DNA clamp PCNA and inhibited by the 14-3-3 adaptor proteins through direct protein-protein interactions. PCNA binds to the PCNA-interacting protein box (PIP box) located in the C-terminus of Exo1 and supports the processive nuclease activity of Exo1 during DNA resection (33). 14-3-3 proteins contact the central domain of Exo1 and restrain its damage association and DNA resection activities (34). However, an Exo1 mutant lacking PCNA-binding activity is transiently recruited to sites of DNA damage by an unknown mechanism (33, 34). Here we have investigated the role of poly(ADP-ribose) produced in response to DNA damage as a regulator of Exo1.

A prominent early response to DNA breaks is protein PARylation by the enzyme poly(ADP-ribose) polymerase 1 (PARP1) (35, 36). PARP1's enzymatic activity is activated by binding to DNA breaks, causing a localized burst of PARylation on many proteins at sites of DNA damage such as histone H1, XRCC1 as well as PARP1 itself. This posttranslational modification is transient, and is rapidly removed by the activity of poly(ADP-ribose) glycohydrolase (PARG), resulting in a robust, but transient pulse of protein PARylation at

sites of DNA damage. The synthesis of PAR chains is an early response to DNA damage that creates docking sites for many checkpoint and repair proteins and chromatin remodeling factors (e.g. XRCC1, Ligase 4, NBS1, SSB1, BARD1, CHD4, ALC1, CHRF and APLF) with PAR-binding activity (36–44). Although the precise roles of the transient PARylation in the DNA damage response remains to be defined, deficiencies in the PAR-binding activities of these factors affect chromatin structural remodeling and the kinetics of DNA repair (35, 45).

In this study, we show that Exo1 is a PAR-binding protein and that this PAR-binding activity contributes to the timely recruitment of Exo1 to DNA damage sites. Contrarily, PAR binding inhibits both the exonuclease and 5' flap endonuclease activities of Exo1, suggesting that Exo1 activity may be held in check at damage sites until PAR is removed by the action of PARG. This delay could provide an opportunity for the cell to integrate various physiological signals before activating the long-range resection of DNA during DSB repair or nucleotide excision during MMR.

2. Materials and Methods

2.1. Plasmids, antibodies and chemicals

GFP-Exo1(WT), GFP-Exo1(1– 507), GFP-Exo1(508– 846) and GFP-Exo1(PIP) in the pEGFP-C1 vector, mCherry-Difopein(WT) and mCherry-Difopein(MUT) in the pmCherry-C1 vector, and baculovirus expression constructs encoding C-terminally His-tagged Exo1(WT) and Exo1(PIP) were described previously (33, 34). C-terminally His-tagged Exo1(1– 507) was cloned into the Gateway donor vector pDONR221 through PCR and BP recombination, and then transferred into pDEST8 through LR recombination, according to the manufacturer's protocols (Life Technologies). GST-AF1521 in pGEX4T-1 was obtained from Dr. Michael Nielsen (University of Copenhagen). GST-PARP1C in pGEX-6P1 and His-PARP1(DBD) in pET28a(+) were described before (33, 46). Rabbit antibodies against *Xenopus* Exo1, Dna2 and PCNA were described previously (33). Anti-GST antibodies were home raised in rabbits using a GST-EGFP fusion protein as antigen. Anti-GFP (Clontech, 632569), anti-mCherry (BioVison, 5993– 100), anti-PAR polymer antibodies (Trevigen, 4335-MC– 100), Olaparib (Selleckchem, S1060), ADP-HPD (EMD Biosciences, 118415), ADP-ribose (Sigma, A0752) and Poly(A) RNA (Roche, 10108626001) were purchased from the respective vendors.

2.2. Cell culture, transfection, laser microirradiation, live-cell imaging and immunofluorescence staining

Human U2OS and HEK293T cells were cultured in DMEM with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. Plasmid DNA was transfected into U2OS and HEK293T cells using TransIT-LT1 (Mirus) transfection reagent, according to the manufacturer's protocols. A customized laser microirradiation and live-cell imaging system was described before (33, 34, 47). Immunofluorescence staining to detect PAR signal at the DNA damage sites after laser irradiation was performed as previously described (33, 34). Anti-PAR primary antibodies were used at 1:100. Primary antibodies were detected with goat anti-mouse Alexa

Fluor 488-conjugated secondary antibodies (Life Technologies, A11001, used at 1:250). DNA was visualized with Hoechst 33342 staining (1 µg/ml).

2.3. *Xenopus* nuclear extract, immunodepletion, immunoblotting, chromatin binding and PARylation assays in the *Xenopus* extract

Xenopus nucleoplasmic extract derived from unfertilized eggs was prepared from synthetic nuclei assembled in a crude egg extract, as previously described (48). To deplete xExo1 from the *Xenopus* nuclear extract, 10 µl protein A agarose beads coupled with 40 µl xDna2 antiserum or both xExo1 and xDna2 anti-serum were incubated with 50 µl extract for 45 min at 4 °C. Beads were then removed from the extract by centrifugation (Beckman Microfuge E with a single fixed speed, 1 min). The extract supernatant was then subjected to two additional rounds of depletion under the same conditions. Immunoblotting was performed using DyLight 800-or DyLight 680-conjugated secondary antibodies (Pierce) and an Odyssey Infrared Imaging System (LI-COR Biosciences), as described previously (33, 34).

To assay protein PARylation in the *Xenopus* extract, NPE supplemented with an ATP regenerating system (2 mM ATP, 20 mM Phosphocreatine and 5 µg/ml Creatine Phosphokinase) was incubated with a 400 bp dsDNA fragment (generated by PCR) (25 ng/µl). Samples were withdrawn at the indicated times in Fig. 4A and analyzed by SDS-PAGE followed by far-western blotting using GST-AF1521. Briefly, after protein transfer, the PVDF membrane was incubated with 3.75 µg/ml purified GST-AF1521 recombinant protein overnight at 4 °C. The membrane was then blotted for GST using anti-GST antibodies and DyLight 680-conjugated secondary antibodies.

2.4. Recombinant protein expression and purification

N-terminally GST-tagged AF1521 was expressed in the *E. coli* strain BL21(DE3) and was affinity-purified using FPLC with a GStap FF column (GE healthcare), according to the manufacturer's protocol. N-terminally GST-tagged PARP1C containing the catalytic domain of human PARP1 (aa. 375–1014) was expressed in *E. coli* Rosetta cells, and was first affinity-purified on a glutathione Sepharose column, and then further purified on a Superdex 200 size-exclusion column (GE healthcare) in buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM DTT and 5% glycerol. N-terminally His-tagged DNA binding domain of human PARP1 (DBD; aa 1–374), and N-terminally His-tagged full length human PARP1 and PARP1C protein fragment in pET28a(+) were expressed in *E. coli* Rosetta cells and purified as described previously (46, 49). Purified proteins were dialyzed in PBS containing 10% glycerol, frozen in liquid nitrogen and stored at – 80 °C. C-terminally His-tagged Exo1(WT), Exo1(–PIP) or Exo1(1–507) was expressed in sf9 cells using the baculovirus expression system and purified using HisPur cobalt resin (Pierce) as described previously (33).

2.5. *In vitro* PARylation, PAR chain synthesis and purification, PAR binding and competition

For *in vitro* PARylation, GST-PARP1C (2 µM) was enzymatically auto-modified in a reaction containing the PARP1 DBD (2 µM), a 24 mer nicked DNA oligo (2 µM), and NAD⁺ (500 µM) for 1 hr at 37 °C, as described previously (46, 50). PAR chains were

synthesized and purified as described previously (50, 51). Briefly, PAR polymers were synthesized in a reaction containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 1 mM DTT, 12.5 ug/mL calf thymus DNA, 2 μM His-PARP1 protein, 4 μM His-PARP1C protein fragment, and 2 mM NAD⁺. PARylated PARP1 was precipitated in 10% ice-cold TCA, and the pellet was resuspended in 1 M KOH and 50 mM EDTA, and then incubated for 1 hour at 60 °C to detach PAR polymers from the denatured PARP1. The detached PAR chains were recovered from a dihydroxyboryl Bio-Rex 70 (DHBB) column and were desalted using a PD-10 desalting column (GE Healthcare). Purified PAR chains were air-dried and dissolved in water at a final concentration of 10 mM. The molar concentration of PAR (expressed as the concentration of ADP-ribose units) was estimated as follows: [PAR] = [(A₂₆₀) cm⁻¹]/[13,500 cm⁻¹M⁻¹] (51). For the PAR-binding experiments, unmodified and PARylated GST-PARP1C (2 μM) were immobilized by incubating with GSH-sepharose beads for 30 min at room temperature in a binding buffer containing 50 mM Tris-HCl pH 7.5, 250 mM NaCl, 1 mM DTT and 1% NP-40. The beads were washed extensively with the binding buffer to remove DBD and the DNA oligo. For GST pull-down in cell lysate, HEK293T cells expressing GFP, GFP-Exo1, GFP-Exo1(1–507) or GFP-Exo1(508–846) were lysed in the lysis/binding buffer (150 mM NaCl, 1 mM EDTA, 20 mM Tris, 1% NP-40, 5 mM NaF, 0.1 mM Na₃VO₄, 1 mM PMSF, 10 μM Olaparib, 3 μM ADP-HPD, pH8.0) for 1 hr at 4 °C. Bead-immobilized, modified or unmodified GST-PARP1C was then incubated with the cell lysate for 2 hr at 4 °C. Beads were then washed 3 times with the lysis/binding buffer. For GST pull-down with purified proteins, His-tagged Exo1(WT) (2.5 μg) or Exo1(1–507) (0.6 μg) was incubated with PAR-modified or unmodified GST-PARP1C for 20 min at 4 °C. Beads were then washed four times with the lysis/binding buffer above. For the PAR binding competition assay, Exo1(WT)-His recombinant protein was bound to PARylated GST-PARP1C immobilized on GSH-sepharose beads in a column. Beads were then washed extensively with the lysis/binding buffer described above. PAR polymer, ADP-ribose monomer, or polyadenylic acid was then loaded onto the columns three times for binding competition at room temperature. Bead-bound Exo1(WT)-His was then analyzed by SDS-PAGE and immunoblotting.

2.6. DNA substrate labeling, DNA end resection in *Xenopus* extracts and with purified proteins, 5' flap cleavage assay

The 3' ³²P-labeled, 6 kb DNA substrate for resection was prepared as previously described (33). A typical resection reaction in the *Xenopus* extract contained 6 μl untreated or treated NPE (immunodepletion and/or addition of recombinant proteins) supplemented with an ATP regenerating system (2 mM ATP, 20 mM Phosphocreatine and 5 μg/ml Creatine Phosphokinase) and 1.5 μl radiolabeled DNA substrate (2 ng/ul). In a typical *in vitro* resection reaction, 30 nM Exo1-His and 2 ng/μl radiolabeled DNA substrate were incubated in the reaction buffer (20 mM Hepes pH 7.5, 50 mM KCl, 0.5 mM DTT, 5 mM MgCl₂, 5% glycerol) at room temperature. Following incubation, 1.5 μl reactions were stopped at indicated times by incubating with 10 μl stop buffer (8 mM EDTA, 0.13% phosphoric acid, 10% Ficoll, 0.2% bromophenol blue, 0.5% SDS, 80 mM Tris-HCl, pH 8.0) supplemented with Proteinase K (2 mg/ml) for 2 hr at 37 °C. Samples were then resolved on 0.8% TAE-agarose gels overnight, followed by gel drying and autoradiography, as previously described (33). To assay the flap endonuclease activity of Exo1, a 5' ³²P-labeled flap DNA substrate

was generated by annealing 3 ssDNA oligos (5'- CCA GTG AAT TCG AGC TCG GTA CCC GCT AGC GGG GAT CCT CTA-3', 5'-³²P-ATT GGT TAT TTA CCG AGC TCG AAT TCA CTG G-3', 5'-TAG AGG ATC CCC GCT AGC GGG-3') as described in Lee et al (4). 5' ³²P-labeled oligos of 10, 11, 12 and 13 nucleotides in length derived from the 5' sequence of the flap oligo were used as markers. The reaction samples were treated the same way as resections with 3' ³²P-labeled dsDNA substrate but resolved in a 20% polyacrylamide gel containing 8 M urea. The gel was then incubated with a destaining buffer overnight for fixation and removal of urea before drying and autoradiography.

3. Results

3.1. The N-terminal domain of Exo1 mediates its initial recruitment to sites of DNA damage

The recruitment of Exo1 to sites of DNA damage is actively regulated. Using a laser-microirradiation method to create DNA breaks in human cells, we have shown that the interaction of PCNA with the C-terminal domain of Exo1 (751– 846) promotes Exo1 damage association, whereas the interaction of 14-3-3 proteins with the central region (508– 750) suppresses Exo1 damage recruitment (33, 34). Notably, deletion of the N-terminal domain (Exo1 residues 1–507) slowed the recruitment of Exo1 to damage sites, revealing its contribution to Exo1 damage association (Fig. 1A). The N-terminal domain of Exo1 alone is robustly recruited to DNA damage (Fig. 1A) albeit in a transient manner lasting for only 5– 10 mins compared to the full length Exo1 protein, which stably associates with the damage during the first 20 min after laser irradiation (Fig. 1A). These results suggest that the N-terminal domain of Exo1 increases its rate of association with DNA damage.

3.2. Protein PARylation promotes the early damage recruitment of Exo1

The transient association of the Exo1 N-terminal domain with DNA damage is reminiscent of the kinetics of protein PARylation at DNA damage sites. Using PAR-specific antibodies, we detected transient PARylation specifically at the sites of DNA damage for a period of 5– 10 mins after laser microirradiation (Fig. 1B). These observations raised the possibility that protein PARylation may contribute to the damage recruitment activity of the Exo1 N-terminal domain. In support of this idea, blocking PAR synthesis with the PARP inhibitor Olaparib almost completely abrogated the damage recruitment activity of Exo1(1–507) (Fig. 1C). Consistent with the role of the N-terminal domain in Exo1 damage recruitment, Olaparib treatment also partially inhibited the damage association of the full length Exo1 protein, with more effects observed within the first 5 mins after laser irradiation (Fig. 1C). Furthermore, Olaparib completely inhibited the damage recruitment of a PIP-box mutant of Exo1 that lacks PCNA-binding activity (Fig. 1C). Taken together, these results strongly suggest that protein PARylation at DNA breaks promotes the initial recruitment of Exo1 through its N-terminal domain.

3.3. Exo1 directly binds to PAR through the N-terminal domain

To determine how PARylation activity promotes Exo1 damage recruitment, we tested the possibility that Exo1 is a PAR binding protein. Since the N-terminal domain of Exo1 is recruited to damage sites in a PAR-dependent manner (Fig. 1C) we asked whether this domain binds to PAR *in vitro*. To this end, we used an *in vitro* PARylation system to

generate PAR chains on a GST-fused recombinant protein containing the C-terminal domain of PARP1 (aa. 375–1014; PARP1C) encompassing the automodification domain, WGR domain and the catalytic core of PARP1 (Fig. 2A) (46). PARylated GST-PARP1C protein (GST-PARP1C-PAR) was then immobilized on glutathione agarose beads and incubated with cell lysate containing expressed GFP, GFP-Exo1(WT), GFP-Exo1(1–507) or GFP-Exo1(508–846). As a control, bead-immobilized unPARylated GST-PARP1C was incubated with the cell lysates under the same condition. As shown in Fig. 2B, GFP-Exo1(WT) and GFP-Exo1(1–507) were associated with GST-PARP1C-PAR, whereas GFP-Exo1(508–846) or GFP alone was not. None of these proteins associated with GST-PARP1C lacking the PAR modification under the same condition (Fig. 2B). These results indicate that Exo1 associates with PARylated protein(s) and that the N-terminal domain of Exo1 is both necessary and sufficient for its PAR-binding activity.

To test whether the N-terminal domain of Exo1 directly and specifically binds to PAR, we incubated purified recombinant, C-terminally His-tagged Exo1(WT) and Exo1(1–507) proteins with bead-immobilized GST-PARP1C-PAR or GST-PARP1C (Fig. 2C). Both Exo1(WT) and Exo1(1–507) associated with PARylated GST-PARP1C, but not unmodified GST-PARP1C (Fig. 2D). Importantly, the binding of Exo1 to PARylated PARP1C was effectively competed by protein-free PAR polymers, whereas monomeric ADP-ribose or poly(A) RNA had little or no effect on Exo1's PAR-binding activity. These results demonstrate the specificity of Exo1's PAR-binding activity (Fig. 2E). We conclude that the N-terminal domain of Exo1 mediates the initial damage recruitment of Exo1 by directly binding to PAR generated at sites of DNA damage.

3.4. 14-3-3 proteins suppress the PAR-binding activity and damage recruitment of Exo1

We previously showed that 14-3-3 proteins bind to the central domain of Exo1, suppressing its interaction with PCNA (34). To determine whether 14-3-3s also regulate Exo1's PAR binding activity, we first examined the effects of deletion of the central domain of Exo1 on its PAR-binding activity. As shown in Fig. 3A, GFP-Exo1(CR) exhibited a much higher level of PAR-binding than GFP-Exo1(WT), raising the possibility that interaction with 14-3-3s also restrains the PAR-binding activity of Exo1. To further test this idea, we overexpressed a peptide antagonist Difopein (Difopein(WT)) to specifically disrupt the interaction between Exo1 and all 14-3-3 isoforms in cells. A mutant form of Difopein (Difopein(MUT)) that lacks 14-3-3-binding activity was used as a control. We previously showed that overexpression of mCherry-Difopein (WT), but not mCherry-Difopein(MUT), abolishes the interaction of Exo1 with 14-3-3s (see Fig. 2B in (34)). Overexpression of mCherry-Difopein(WT) dramatically increased the PAR binding of GFP-Exo1(WT) expressed in mammalian cells in comparison to overexpression of the mCherry-Difopein(MUT) protein (Fig. 3B). In contrast, mCherry-Difopein(WT) overexpression had no or little effect on the PAR binding of GFP-Exo1(CR) that lacks the 14-3-3-binding activity (Fig. 3B). These results, together with our recently published findings (34), reveal that 14-3-3 proteins negatively regulate the damage association of Exo1 by suppressing its binding to PAR and PCNA.

3.5. Inhibition of PARylation did not affect Exo1-mediated DNA end resection in the *Xenopus* egg extract

Although the PAR binding activity of Exo1 facilitates recruitment to sites of damage (Fig. 1), it is unknown how this interaction affects DSB resection. To examine DNA resection activity of Exo1, we used a cell-free *Xenopus* nucleoplasmic extract (NPE) system that faithfully recapitulates the DNA end resection activities observed in cells (33, 34, 52). We first determined whether protein PARylation occurs in NPE in response to DNA DSBs. PARylation was detected using the recombinant PAR binding protein AF1521 (53, 54) fused to GST (GST-AF1521). A far-western blot using GST-AF1521 to detect PARylated proteins demonstrated a robust, but transient, accumulation of PARylated proteins in the NPE in response to DSBs (Fig. 4A and Fig. S1A). This DSB-induced PARylation activity was significantly inhibited by the PARP inhibitor Olaparib, and the signal was enhanced by pretreatment of the NPE with the PARG inhibitor ADP-HPD (Fig. 4B). Together, these results indicate that DNA damage-dependent PARylation of proteins occurs efficiently and transiently in *Xenopus* NPE in response to DNA DSBs.

To determine whether protein PARylation is important for DSB resection by Exo1, we incubated NPE with Olaparib to block PAR synthesis and then added a ^{32}P -labeled, 6 kb dsDNA fragment as a substrate for DNA resection. The rate of DNA resection in the NPE was unaffected by addition of Olaparib (Fig. 4C). Because Dna2 functions redundantly with Exo1 in this DNA resection assay, we also immunodepleted Dna2 from NPE then examined the effects of Olaparib on DNA resection (Fig. 4D). After depleting Dna2, Olaparib still had no effect on DNA resection activity (Fig. 4E). The interaction of PCNA with the C-terminus of Exo1 increases the processivity of Exo1 during resection, and this interaction could mask the effect of PAR binding activity on the DNA resection reaction. We therefore immunodepleted both Exo1 and Dna2 from the NPE then added recombinant Exo1(PIP)-His protein that lacks PCNA-binding activity to the depleted extract (Fig. 4F and Fig. S1B). DNA resection activity was subsequently measured in the presence or in the absence of Olaparib. Again we did not see significant effects of PARP inhibition on DNA resection by Exo1 lacking PCNA binding activity (Fig. 4G) (33). Taken together, these results indicate that protein PARylation is dispensable for resection of DSBs with “clean” DNA ends.

3.6. PAR inhibits the exonuclease activity and 5' flap endonuclease activity of Exo1

The N-terminal PAR-binding domain of Exo1 also contains the nuclease domain (aa 1–325), raising the possibility that PAR-binding affects the nuclease activities of Exo1. To test this, we first determined whether PAR binding affects the exonuclease activity of Exo1, which is required for DSB resection as well as MMR. To this end, PAR chains were enzymatically synthesized with recombinant PARP1 and purified by column chromatography (50) for use in *in vitro* DNA resection reactions. The purified PAR polymers, monomeric ADP-ribose, poly(A) RNA, or water was added to an *in vitro* DNA resection reaction containing Exo1-His and a 3' ^{32}P -labeled, 6 kb dsDNA fragment as substrate. As shown in Fig. 5A, PAR polymers dramatically inhibited the exonuclease activity of Exo1. In contrast, addition of monomeric ADP-ribose in an amount equal to the amount of ADP-ribose in the PAR chains had no effect on Exo1 exonuclease activity. Although poly(A) RNA partially inhibited Exo1's exonuclease activity, RNA was a less potent inhibitor compared to PAR polymers

(Fig. 5A). The inhibition of Exo1's exonuclease activity by PAR chains may provide a mechanistic explanation for why blocking PAR synthesis with olaparib does not affect DSB resection in the NPE, despite its role in promoting the initial damage recruitment of Exo1 (Figs. 1 and 2). In the presence of olaparib, the recruitment of Exo1 may be slowed, but conversely, exonuclease activity is uninhibited by PAR to generate a normal level of resection activity.

To determine whether PAR-binding also affects the 5' flap endonuclease activity of Exo1, we generated a 42 bp dsDNA substrate with a ³²P labeled 5'-flap located in the center (Fig. 5B). The 5'-flap endonuclease activity of Exo1 was similarly inhibited by PAR polymers, but not by monomeric ADP-ribose. As for exonuclease activity (Fig. 5A), poly(A) RNA also partially inhibited the flap endonuclease activity of Exo1 (Fig. 5B). Cleavage of the 5' flap occurred at one nucleotide inwards in the double-strand region, consistent with published results (55). Together, these results indicate that PAR polymers specifically inhibit both the exonuclease and flap endonuclease activities of Exo1.

4. Discussion

In this study, we have identified Exo1 as a novel PAR-binding protein and that PAR-binding activity resides in the N-terminus of Exo1 containing the nuclease domain (Fig. 2). The DNA damage dependent activation of PAR synthesis in cells facilitates the initial damage recruitment of Exo1 (Fig. 1). *In vitro*, PAR polymers suppress the exonuclease and flap endonuclease activities of Exo1 (Fig. 5). This counterbalancing effect of PAR binding may explain why no overt effects on DSB end resection were observed in the *Xenopus* egg extract when protein PARylation was inhibited (Fig. 4). These data, together with our previously reported findings, suggest a model for the control of Exo1 function in DNA end resection through a multiple protein-protein interactions. In response to DNA DSBs, PARP1 quickly relocates to DNA breaks, leading to its activation and a transient wave of PARylation on multiple proteins. The resulting PAR chains provide a docking platform for the rapid damage recruitment of Exo1 through the PAR-binding activity of its N-terminus. During this initial stage of damage association, Exo1 resection activity is held inactive by PAR until its clearance by PARG. The second stage, more sustained damage association of Exo1 is mediated by the sliding DNA clamp PCNA that also loads onto DSBs. The direct interaction between PCNA and the PIP-box in the C-terminus of Exo1 tethers Exo1 to the DNA substrate and promotes its processivity in resection. PAR and PCNA increase Exo1 damage association in an apparently additive manner, whereas both pathways are antagonized by the 14-3-3 adaptor proteins, which interact with the central domain of Exo1 to suppress its interactions with PAR and PCNA. This coordinated regulation of Exo1 activity by multiple interacting partners ensures an orchestrated resection process and a proper level of ssDNA for the activation of HR and the ATR checkpoint (Fig. 6).

PARylation plays an important role in both DSBR and single-strand break repair (SSBR) and is a prominent target of cancer therapy (56–58). PAR synthesis at sites of DNA damage has been shown to recruit a number of protein factors through their PAR-binding activities, which promotes chromatin modification and remodeling, DNA repair and checkpoint activation (36, 59). Our results indicate that the initial damage recruitment of Exo1 is

mediated by its direct PAR-binding activity resided in the N-terminal domain. However, PAR-binding also inhibits the DNA resection activity of Exo1, which could serve to slow or delay DNA end resection activity and provide time for cells to integrate cues from both inside and outside of the cell before committing to long-range resection. For example, PARylation promotes microhomology-mediated end joining (MMEJ) that requires only limited end resection by MRN and CtIP, but not by Exo1 (60, 61). By temporarily suspending Exo1 nuclease activity during its initial damage recruitment, PAR may prevent premature resection at a DSB before a commitment is made between HR, MMEJ and classic NHEJ repair pathways. Due to the highly negatively charged nature of PAR chains, it is likely that PAR inhibits Exo1 nuclease activity by directly competing with the binding of the DNA substrate to the nuclease domain. Alternatively, PAR-binding may function as an allosteric negative regulator of Exo1's nuclease activities. Previously, Poirier and colleagues predicted a PAR-binding motif in Exo1 (residues 125–135; ITHAMAHKVIK) (62). However, mutations of the key residues predicted to be important for the PAR-binding activity (ITHAMAAVIA) did not affect PAR-binding of Exo1 (data not shown), suggesting that the PAR-binding motif is located elsewhere in the N-terminal domain.

Inhibition of Exo1 nuclease activities by PAR may also play a role in the progression of DNA replication forks. In budding yeast lacking either the checkpoint kinase Rad53 or 14-3-3 genes, DNA resection by EXO1 leads to fork instability and DNA damage in response to replication stress (25, 26, 28). These observations imply that unscheduled or uncontrolled Exo1 activity is normally prevented at the stalled or collapsed replication forks. PAR-mediated inhibition of Exo1 nuclease activities may contribute to this regulation. Consistent with this idea, PARP1 is activated in response to replication stress and thought to be important for the restart of stalled forks (63–65). Previous studies suggest that PARP1 protects fork structure by promoting fork reversal or by preventing Mre11-dependent degradation of stalled forks (63–65). It will be interesting to determine in the future whether inhibition of Exo1 resection function by PAR represents another mechanism for fork protection and restart in the presence of replication stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Exo1 is a novel poly(ADP-ribose)-binding protein
- Poly(ADP-ribose)-binding of the N-terminal domain of Exo1 promotes its early recruitment to sites of DNA damage
- 4-3-3 proteins restrain the damage association of Exo1 by suppressing its interactions with poly(ADP-ribose) and PCNA
- Poly(ADP-ribose) inhibits the 5'-to-3' exonuclease activity and the 5' flap endonuclease activity of Exo1
- Protein poly(ADP-ribosyl)ation is dispensable for the resection of DNA double-strand breaks

Figure 1. The N-terminal domain of Exo1 promotes its initial damage recruitment in a PARylation-dependent manner

A). Left panel: Diagram of functional domains in wild type Exo1 and truncation mutants (not to scale). Middle panel: Representative images for the damage association of GFP-Exo1, GFP-Exo1(508–846) and GFP-Exo1(1–507) shown in the left panel. Red lines indicate the sites of laser irradiation in cells. Right panel: Quantified results for the damage association of GFP-Exo1 and its mutants during the first 20 min after laser irradiation. Each

data point is the average of independent measurements of 5 cells. Error bars represent standard deviation.

B). Immunofluorescence staining of PAR at sites of DNA damage at various times after laser irradiation.

C). Left panels: Representative images for the damage association of GFP-Exo1(1–507), GFP-Exo1(WT) and GFP-Exo1(PIP) in cells treated with DMSO or Olaparib. Red lines indicate the sites of laser irradiation in cells. Right Panel: Quantified results for the damage association GFP-Exo1(1–507), GFP-Exo1(WT) and GFP-Exo1(PIP) in cells treated with DMSO or Olaparib during the first 20 min after laser irradiation. Each data point is the average of independent measurements of 5 cells. Error bars represent standard deviation.

Figure 2. Exo1 directly interacts with PAR via its N-terminal domain

A). Generation of PAR chains on PARP1C, through in vitro auto-PARYlation in the presence of DBD, NAD⁺ and DNA fragments. PARYlated PARP1C resulted in lower mobility on a polyacrylamide gel.

B). Pull-down of GFP, GFP-Exo1(WT), GFP-Exo1(1–507) or GFP-Exo1(508–846) expressed in 293T cells with bead-immobilized GST-PARP1C-PAR or GST-PARP1C. Note that GFP-Exo1(508–846) exhibited a slower gel mobility than GFP-Exo(1–507), although the latter has a larger molecular weight.

- C).** Purified recombinant Exo1(WT)-His and Exo1(1–507)-His expressed in Sf9 cells.
- D).** Pull-down of purified recombinant Exo1(WT)-His or Exo1(1–507)-His with bead-immobilized GST-PARP1C-PAR or GST-PARP1C.
- E).** Competition of PAR polymer, monomeric ADP-ribose or poly(A) RNA for binding to purified Exo1(WT)-His with bead-immobilized GST-PARP1C-PAR.

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Figure 3. The PAR-binding activity of Exo1 is antagonized by 14-3-3 proteins that interact with the central domain of Exo1

A). Pull-down of GFP-Exo1(WT) or GFP-Exo1(ΔCR) expressed in 293T cells with bead-immobilized GST-PARP1C-PAR.

B). Pull-down of GFP-Exo1(WT) or GFP-Exo1(ΔCR) in 293T cells overexpressing mCherry, mCherry-Difopein(WT) or mCherry-Difopein(MUT) with bead-immobilized GST-PARP1C-PAR.

Figure 4. PARylation is dispensable for DNA end resection in the *Xenopus* egg extract
A). Far-western using GST-AF1521 detected robust, but transient protein PARylation in *Xenopus* NPE induced by a dsDNA fragment.
B). Effects of the PARP inhibitor Olaparib and the PARG inhibitor ADP-HPD on protein PARylation in NPE incubated with a dsDNA fragment for 5 min.
C). Effects of Olaparib on end resection of a 6 kb dsDNA fragment in NPE. PARPi, Olaparib.
D). Depletion of Dna2 from the *Xenopus* NPE.

- E).** Effects of Olaparib on DNA end resection in NPE depleted of xDna2.
- F).** Depletion of both Exo1 and Dna2 from the *Xenopus* NPE.
- G).** Effects of Olaparib on DNA end resection in NPE depleted of both xDna2 and xExo1 and supplemented with Exo1(PIP)-His.

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Figure 5. PAR inhibits the nuclease activities of Exo1 *in vitro*
A). Effects of PAR, ADP-ribose and poly(A) RNA on the exonuclease activity of purified Exo1.
B). Effects of PAR, ADP-ribose and poly(A) RNA on the 5' flap endonuclease activity of purified Exo1.

Figure 6. A model for the regulation of Exo1 in DNA end resection by PAR, 14-3-3s and PCNA
 The damage association of Exo1 is regulated by three distinct domains in Exo1, which bind to PAR, 14-3-3s and PCNA, respectively. Immediately after DNA damage, protein PARylation at DNA damage sites promotes the initial damage recruitment of Exo1 through the direct interaction between PAR and the N-terminus of Exo1. At this point, the Exo1 nuclease activity is held until the clearance of PAR from the DNA damage sites by PARG. Through binding to the PIP-Box in the C-terminus of Exo1, PCNA facilitates the damage retention of Exo1 and increases its processivity in DNA resection. 14-3-3 proteins interact with the central domain of Exo1, suppress Exo1's binding to PAR and PCNA and restrain

DNA end resection. The coordinated regulation of Exo1 by multiple interacting factors ensures an orchestrated and controlled DSB resection process.

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