Exonuclease 1 (Exo1) is an evolutionarily conserved eukaryotic nuclease that plays a multifaceted role in maintaining genome stability. The biochemical attributes of Exo1 have been extensively characterized via conventional assays. However, the key step governing its activation remains elusive. Extending the previous finding that Exo1 can digest a randomly selected single-stranded DNA (ssDNA) but not a poly(dT) oligonucleotide and using purified recombinant Exo1 and nuclease and electrophoretic mobility shift assays, here we determined that DNA hairpins with a stem size of 4 bp or longer are able to activate Exo1-mediated digestion of ssDNA. We further provide evidence suggesting that Exo1 uses an evolutionarily conserved residue, Lys185. This residue interacted with the phosphate group bridging the third and fourth nucleotide on the digestion strand of the substrate DNA for duplex recognition, critical for Exo1 activation on not only ssDNA but also dsDNA. Additionally, the defect of an exo1-K185A mutant in duplex digestion was partially rescued by longer overhanging DNA. However, we noted that the enhanced Exo1 nuclease activity by longer overhanging DNA is largely eliminated by replication protein A (RPA), likely because of the previously reported RPA activity that strips Exo1 off the ssDNA. We conclude that duplex DNA contact by Exo1 is a general mechanism that controls its activation and that this mechanism is particularly important for digestion of duplex DNA whose nascent ssDNA is bound by RPA.

Exo1 is an evolutionarily conserved Rad2 family nuclease that plays a multifaceted role in DNA metabolic pathways governing genome stability, including mismatch repair (1–3), homologous recombination (HR)2–mediated double-strand break (DSB) repair (4), telomere maintenance, lagging strand maturation, and recovery of stalled replication forks (5, 6). Exo1 was first isolated from fission yeast, Schizosaccharomyces pombe, undergoing meiosis and revealed to degrade dsDNA from its 5′ end, leaving a 3′ single-stranded DNA (ssDNA) tail (7). Later, Exo1 was also found to carry 5′ flap endonuclease activity. The functions of Exo1 are largely fulfilled via its 5′-to-3′ dsDNA exonuclease activity, which is capable of digesting a 5′ end embedded in a double-strand break or a gapped or nicked duplex. In mismatch repair, Exo1 removes long tracts of DNA, including the mispaired nucleotide (2, 8). The reaction presumably starts from a nick in a manner directed by MutS/L family mismatch repair proteins (2, 3, 8, 9). In both telomere maturation and HR-mediated DSB repair, the 3′ ssDNA overhang generated by Exo1 from a chromosomal end or a DSB end allows assembly of either the telomeric nuclease-protein complex or the Rad51 nucleoprotein filament. At stalled replication forks, Exo1 digests the nascent lagging-strand DNA to suppress fork reversal and prevent replication fork collapse (5, 6, 10). A major function derived from Exo1′s 5′ flap endonuclease activity is in lagging-strand maturation, where overexpressing Exo1 suppresses the temperature-sensitive phenotype of rad2Δ in budding yeast (1).

As a key repair enzyme, Exo1 is both positively and negatively regulated by protein factors and posttranslational modifications. MutSα in mismatch repair, MRX(N) and 9–1–1 complexes in DSB end resection have been reported to enhance the processivity of Exo1/hEXO1 (3, 11–13). On the other hand, the DNA damage checkpoint down-regulates Exo1, which imposes a negative feedback loop on DNA end resection (14) and stabilizes a stalled replication fork from Exo1 digestion (6, 15). In addition, the Ku complex, a key player in the nonhomologous end joining pathway, which competes with HR in DSB repair, strongly inhibits Exo1-catalyzed digestion of a DSB end (16–18). It remains puzzling that the Ku complex protects DSB ends from Exo1 digestion but allows their access by the ligase IV complex to join the broken chromosome (19, 20).

The crystal structure of the human EXO1 (hEXO1) catalytic core (1–352) in complex with its substrate DNA has been solved, which elucidated the molecular details of the Exo1 nuclease reaction. Based on the crystal structures, hEXO1 has been proposed to accommodate substrates subjected to both exonucleolytic and endonucleolytic digestion with a well-supervised conformation, catalyzing both reactions with the same catalytic center (21). Interestingly, binding of hEXO1 to the duplex region of its substrate DNA was captured as the first snapshot in a further time-resolved crystallography study (22), which suggests duplex capture as a critical step in Exo1/hEXO1 activation. However, this premise remains to be tested in functional assays.

Besides duplex and 5′ flap DNA, Exo1 is also able to degrade ssDNA (11). ssDNA digestion by Exo1, a reaction strongly inhibited by RPA (11), is less well characterized and is intriguing because a poly(dT) oligonucleotide is completely resistant...
Activation of Exo1 nuclease on single-stranded DNA

to Exo1-digestion (23). The drastic difference between a regular oligonucleotide and a poly(dT) as substrate for Exo1, however, provides a unique system to define the mechanism of Exo1 activation. Here, with this system, we determined that Exo1 is highly versatile in capturing transient hairpin structures with a stem size of 4 bp or longer, which, in turn, activates Exo1 to digest ssDNA via a combination of endonucleolytic and exonucleolytic mechanisms. The available hEXO1 crystal structures led us to further identify a highly conserved lysine 185 from hEXO1/Exo1, which interacts with the phosphate group bridging the third and fourth nucleotide on the digestion strand of the substrate DNA. Importantly, the corresponding lysine-to-alanine mutation of Lys185 not only disrupted ssDNA digestion by Exo1 but also largely impaired Exo1-catalyzed digestion of a duplex DNA and failed to fully complement the CPT sensitivity of exo1Δ in yeast. Thus, duplex contact by Exo1 is a general mechanism to control Exo1 activation.

Results

ssDNA digestion by Exo1 is sequence selective

Exo1 has been reported to digest regular ssDNA substrates (11) but not a poly(dT) oligonucleotide (23), which provides a biochemical system to determine the key factor(s) in Exo1 activation. Recombinant budding yeast Exo1 with a WT coding sequence is poorly expressed in E. coli; hence, current biochemical studies employ either insect cells or yeast as the host system to express and purify recombinant Exo1 (4, 24–27). By codon-optimizing Exo1’s primary coding sequence, we managed to express and purify the full-length Exo1 and a nuclease-dead mutant, exo1-D173A, from E. coli (Fig. S1A). Consistent with previous studies (11, 23), purified Exo1 is able to digest an 80-mer oligonucleotide (H80) randomly selected from our laboratory stock but not a (dT)80 oligonucleotide (Fig. 1A). Moreover, poly(dA), poly(dT), poly(dC), and poly(dG) oligonucleotides were all refractory to Exo1 digestion (Fig. S1B). Thus, ssDNA digestion by Exo1 is not simply due to its preference to cleave at a certain nucleotide.

Hairpin formation drives Exo1-catalyzed digestion of ssDNA

A titration of Exo1 on 3’ end–labeled H80 revealed intermediates that gradually decreased in size (Fig. 1A) and clustered between 7 and 22 nt from the 5’ end of H80. With an increasing amount of Exo1, the digestion reached as far as ~7 nt toward the 3’ end. Sequence analysis of the H80 oligonucleotide revealed two relative stable hairpins, each with a stem region of 5 bp in length (Fig. 1B). One hairpin is located at the very 3’ end of H80. The other hairpin, with a stem size of 5 bp plus a G-A mismatch within the stem, is located between 8 and 23 nt from the 5’ end (Fig. 1B), a position where the digestion intermediates proximal to the 5’ end were clustered. Importantly, deletion of 40 bases from the 3’ end of H80 or replacing them with thymidine nucleotides, which remove the 3’ hairpin and result in H40 and H40–40T oligonucleotides, respectively, limited the bulk of digestion to a position prior to the end of 5’ hairpin (Fig. 1A). Thus, in Exo1-catalyzed ssDNA degradation, an embedded hairpin is important to trigger Exo1 digestion of its 5’ upstream DNA.

Exo1 digests ssDNA via a combination of exonucleolytic and endonucleolytic mechanisms

When the digestion of 3’ end–labeled H80 by Exo1 was examined closely, the step size of intermediates showed a mixture of both endo- and exonucleolytic cleavage events (Fig. 1A). To confirm this, H80, H40–40T, and H40 were 5’ end–labeled and tested for their initial cleavage by Exo1. For H80 and H40–40T, both exonucleolytic and endonucleolytic products were detected, with the major endonucleolytic cleavage sites located in the 5’ hairpin loop of H80 and H40–40T (Fig. 1C). Interestingly, unlike H40–40T, deletion of the 3’ hairpin in H40 not only decreased the overall reaction rate but also caused a nearly 3-fold reduction in endonucleolytic product formation (Fig. 1C). Notably, the only difference between H40–40T and H40 is the length of 3’ ssDNA adjoining the 5’ hairpin, where a longer 3’ ssDNA in H40–40T stimulates hairpin-activated ssDNA cleavage by Exo1.

Hairpin formation promotes binding of Exo1 to ssDNA substrates

human Exo1 is a threading enzyme for endonucleolytic digestion, where a 5’ flap needs to thread through the binding pocket for its cleavage (22). Binding of streptavidin to a 3’-biotinylated (dT)40 oligonucleotide, which presumably traps Exo1 on ssDNA, did not activate its digestion by Exo1 (Fig. S1C). Therefore, the hairpin-activated Exo1 cleavage of ssDNA is not simply due to hairpin-mediated pausing of Exo1 during its ssDNA threading. Alternatively, we surmise that a transient duplex region donated by the embedded hairpin may facilitate Exo1 loading. Therefore, EMSAs were conducted with exo1-D173A, a nuclease-dead version of Exo1, to avoid substrate digestion. Under physiological salt conditions (150 mM KCl), tight binding of exo1-D173A to ssDNA was observed, with a low nanomolar apparent dissociation constant of 10 μM (Fig. 1D). Importantly, exo1-D173A displayed a nearly 2-fold higher affinity for the hairpin-forming H80 over (dT)40 substrate (Fig. 1D). Therefore, the hairpin structures embedded in ssDNA likely facilitate its complex formation with Exo1.

DNA hairpin with a stem size of as short as 4 bp is sufficient to activate Exo1

Hairpins in H80 have a maximum stem size of 5 bp and are likely dynamic but sufficient for Exo1 to capture. To further characterize the transient nature of DNA hairpins utilized by Exo1, ssDNA substrates with more defined hairpins were constructed, where three, four, or six adenine nucleotide runs were engineered proximal to the 3’ end of a (dT)35 oligonucleotide to allow formation of hairpin DNA but with the potential of various loop sizes (Fig. 2A). As a control, a fixed hairpin DNA (3’-6GC) based on six G-C pairs was also designed (Fig. 2A). Consistent with an early report where the presence of a hairpin strongly interrupts heteroduplex formation (28), minimal interstrand pairing was observed when the 5’ end–labeled oligonucleotides were analyzed by native PAGE (Fig. S2A). Upon their incubation with Exo1, little reaction was observed with 3’-3A (Fig. 2B). Digestion occurred on 3’-4A and 3’-6A, with both exonucleolytic and endonucleolytic products detected. The 3’-6A substrate is around 2-fold more reactive than the 3’-4A substrate (Fig. 2B). Notably, on the 3’-6GC substrate with a fixed hairpin, exonuclease digestion is considerably
Activation of Exo1 nuclease on single-stranded DNA

A

\[
\begin{array}{ccccccc}
\text{(dT)}_80 & \text{H80} & \text{H40-40T} & \text{H40} \\
\text{Exo1} & - & 20 & M & - & 2 & 20 & 100 & 160 & M (nM) \\
\end{array}
\]

\[3' - ^{32}P \text{ label}\]

B

\[
\begin{aligned}
\text{H80} & : 1 & 8 & 23 & 66 & 80 \\
\text{H40-40T} & : 1 & 8 & 23 & 40 (\text{TTT} \ldots \text{TTT}) & 80 \\
\text{H40} & : 1 & 8 & 20 & 40 & 80 \\
\end{aligned}
\]

C

\[
\begin{array}{ccccccc}
\text{(dT)}_80 & \text{H80} & \text{H40-40T} & \text{H40} \\
\text{Exo1} & - & 20 & M & - & 4 & 2 & 20 & - & 4 & 2 & 20 & - & 4 & 2 & 20 & 100 & (nM) \\
\end{array}
\]

\[5' - ^{32}P \text{ label}\]

D

(i) \[
\begin{array}{ccccccc}
\text{H80} & \text{(dT)}_80 & \text{exo1-D173A} \\
\text{H80} & - & 3 & 6 & 12 & 25 & - & 3 & 6 & 12 & 25 (nM) \\
\end{array}
\]

(ii) \[
\begin{array}{ccccccc}
\text{H80} & \text{(dT)}_80 \\
\text{exo1-D173A} & - & 3 & 6 & 12 & 25 & - & 3 & 6 & 12 & 25 (nM) \\
\end{array}
\]

\[
\begin{aligned}
\% \text{DNA bound} & : 100 \\
[\text{exo1-D173A}] / \text{nM} & : 0 & 20 & 40 & 60 & 80 & 100 \\
\end{aligned}
\]
Regulation of Exo1 catalyzed digestion of ssDNA by RPA

Exo1 is a threading enzyme (21). Binding of RPA to ssDNA may impose a barrier for threading, inhibiting ssDNA digestion by Exo1 (11, 29). Similarly, as reported, digestion of 3′-end-labeled H80 by Exo1 is inhibited by RPA but not RPA-A^-B^-, an RPA mutant defective in DNA binding (Fig. 3A). Importantly, in a similar reaction but with 5′-end-labeled H80, RPA inhibits not only endonucleolytic digestion but also exonucleolytic hairpin-mediated ssDNA digestion requires the C terminus of Exo1, we first mapped the core domain of yeast Exo1, which retains its full nuclease activity. The crystallized nuclease core domain of HExO1 contains its N-terminal 352 amino acids and is fully active in vitro (21). The yeast exo1(1–348), which spans the nuclease core region conserved between yeast and human, is, however, nearly 10-fold less active regarding its exonuclease activity against a 5′-recessed DNA end bearing a 7-nt ssDNA overhang (Fig. S3A). Extension of the C terminus to amino acid 403 fully restored its nuclease activity (Fig. S3A), which thus represents a minimal catalytic core of Exo1. Importantly, the ability of exo1(1–403) to digest ssDNA resembles that of the full-length Exo1 (Fig. S3B). We conclude that hairpin-mediated ssDNA digestion is an attribute of the Exo1 nuclease core domain.

**Figure 2. Exo1-catalyzed digestion of ssDNA substrates harboring 3′ hairpins with various stem sizes.** A, diagrams showing the sequences of the tested ssDNA substrates. B, Exo1 (1, 10, 50 nm) or exo1-D173A (50 nm) (DA) was incubated with 3′ end-labeled substrate as indicated (5 nm) for 10 min at 30 °C before analysis on a 20% denaturing polyacrylamide gel. The results are shown in i and are quantified in ii. Error bars represent the standard deviation of three independent experiments. Oligonucleotide length markers were shown in M lane.

**Figure 1. Influence of hairpin deletion on ssDNA digestion.** A, Exo1 (2, 20, and 100 nm) was incubated with 3′ end-32P-labeled H80 (5 nm), (dT)80 (5 nm), H40 – 40T (5 nm), or H40 (5 nm) for 10 min at 30 °C. The reactions were analyzed by 20% denaturing PAGE. Oligonucleotide length markers were shown in M lane. B, diagram of potential DNA secondary structures in H80, H40 – 40T, and H40. Arrows with numbers mark the position of the nucleotides starting from the 5′ end. C, Exo1 (0.4, 2, and 20 nm) was incubated with 5′ end-32P-labeled H80 (5 nm), (dT)80 (5 nm), H40 – 40T (5 nm), or H40 (5 nm) for 10 min at 30 °C. The reactions were analyzed by 20% denaturing PAGE, similar as in A. Oligonucleotide length markers were shown in M lane. D, electrophoretic mobility shift assay of (dT)80 and randomly selected ssDNA H80. i, the exo1-D173A mutant (3, 6, 12, and 25 nm) was incubated with either (dT)80 (5 nm) or H80 (5 nm) at 30 °C for 10 min. The protein-DNA complex was analyzed by 6% native PAGE. ii, quantification of the data shown in i. Error bars represent the standard deviation of three independent experiments. Statistical analysis via two-tailed Student’s t-test revealed that there is a significant difference in the percentage of ssDNA binding. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
cleavage (Fig. 3B). We speculate that binding of RPA to ssDNA also removes DNA hairpins, blocking Exo1.

The exo1-K185A mutation confers a defect in nuclease activation and causes CPT sensitivity in cells

Our study of hairpin-directed ssDNA digestion by Exo1 showed that a duplex region with a minimum length of 4 bp is necessary to support Exo1 digestion. In the crystal structure of hEXO1 bound to its substrate duplex DNA with a recessed 5’ end (22), a highly conserved lysine 185 (Fig. 4A) forms a salt bridge/hydrogen bond with the phosphate group that connects the third and fourth base from the 5’ end of the digestion strand; therefore, it may be critical in duplex recognition. Mutation of lysine 185 into alanine (Fig. S1A) resulted in nearly complete inactivation of Exo1 on degrading 3’-4A and 3’-6A substrates (Fig. 4B). Importantly, compared with WT Exo1, the exo1-K185A mutant also displayed a 3- to 4-fold reduction in its exonuclease activity against a recessed 5’ end with a 7-nt ssDNA overhang (Fig. 4C). Extension of the overhanging length to 27 nt did not affect the exonuclease activity of WT Exo1, regardless of the absence or presence of RPA (Fig. S4A). Changing the overhang length from 7 nt to 27 nt, on the other hand, increased the exonuclease activity of the exo1-K185A mutant by nearly 2-fold, which was, however, largely offset by the presence of RPA (Fig. S4B). Collectively, our results suggest that duplex recognition is critical to trigger Exo1 activation, which is particularly important when the ssDNA region of 3’-overhanging DNA is either short or RPA bound (Fig. 5). To ascertain the importance of duplex recognition for the function of Exo1 in cells, we sought to find out whether the exo1-K185A mutation renders yeast cells sensitive to DNA-damaging agents. exo1Δ is only mildly sensitive to DNA-damaging agents and has been reported to cause slow growth of yeast cells in the presence of camptothecin (CPT) (14, 30), a chemotherapeutic drug that traps the topoisomerase 1 cleavage complex on DNA and blocks DNA replication (31). Ectopic expression of WT EXO1 but not exo1-K185A from a CEN plasmid complemented the CPT sensitivity of the exo1Δ strain (Fig. 4D). Thus, duplex recognition is important for Exo1 function in cells.
**Activation of Exo1 nuclease on single-stranded DNA**

**Discussion**

Exo1, a multitasking nuclease in eukaryotes, has been characterized extensively as a 5’-3’ dsDNA exonuclease and 5’ flap endonuclease (32, 33). Exo1 prefers dsDNA bearing a 3’ ssDNA overhang (11). The 3’ adjoining ssDNA, which enhances the exonuclease activity of Exo1, is, however, not essential for Exo1 activation, as Exo1 is still capable of digesting a blunt-ended duplex DNA, albeit less actively (11, 16, 34). Here we reported that a duplex region as short as 4 bp provided by a hairpin is sufficient to trigger Exo1-catalyzed digestion of an ssDNA sub-

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### Figure A

| Protein          | Sequence          |
|------------------|-------------------|
| Exo1_Human       | KVTIKMDQFGCNGLBDQARLGM |
| Exo1_Scer        | RRLTIKNDYGECBICRDFNFK |
| Exo1_Agos        | RRLTIKNDYEGFICRDDFVH |
| Exo1_Klac        | RRLTIKNDNACECIDDRNFERP |
| Exo1_Grou        | RRLTIKNDYEGFICRDDFCF |
| Exo1_Spomb       | QTVFLRMDGFNCCTIRRNDDIAN |

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### Figure B

- **(i)**
  - Exonucleaseic products
  - Endonucleolytic products

- **(ii)**
  - Graph showing % Digestion vs [Exo1 / exo1-K185A] (nM)

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### Figure C

- **(i)**
  - Exo1
  - exo1-K185A

- **(ii)**
  - Graph showing % Digestion vs [Exo1 / exo1-K185A] (nM)

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### Figure D

- exo1Δ
- pRS316
- pRS316-EXO1
- pRS316-exo1-K185A
- Growth in YPD and CPT at different concentrations
The coding sequence of Exo1 (1–348) was redesigned through codon optimization by Genscript (sequence available upon request) to facilitate its expression in *E. coli*. The synthesized gene with an engineered C-terminal His tag was cloned into pET21a to yield pET21a-exo1(1–348). To construct expression vectors for the full-length Exo1 and exo1(1–403), the coding sequences of various Exo1 C-terminal fragments were amplified from genomic DNA (S288C) and fused in-frame with the synthesized coding sequence of exo1(1–348) using an internal BamHI restriction site starting at 627 nt, resulting in pET21a-Exo1 and pET21a-exo1(1–403), respectively. To express full-length Exo1 in *E. coli*, pET21a-Exo1 was transformed into RosettaTM(DE3)pLys cells (Novagen). Overnight culture of *E. coli* cells harboring pET21a-Exo1 was diluted into 12 liters of Luria-Bertani medium with a dilution factor of 1:50. When the A600 of the culture reached 0.6–0.8, isopropyl 1-thio-β-D-galactopyranoside was added to the culture to a final concentration of 0.5 mM. The cell culture was further incubated overnight at 16 °C to induce Exo1 expression. A cell pellet was harvested by centrifugation, resuspended in 20 μL of lysis buffer (50 mM Tris–HCl, pH 8.0, 50 mM KCl, 1 mM DTT, 1 mM EDTA, and 10 mg/mL lysozyme), incubated at 37 °C for 10 min, and stored at −80 °C.

**Experimental procedures**

Expression and purification of Exo1 from *E. coli*

Activation of Exo1 nuclease on single-stranded DNA

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**Activation of Exo1 nuclease on single-stranded DNA***

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**Figure 4. Exo1-K185A is nuclease defective and fails to complement the CPT sensitivity of the exo1Δ strain.** A, sequence alignment of the region surrounding Exo1-Lys185 amino acid 180–202 among human and different yeast species. Scer, Saccharomyces cerevisiae; Agos, Ashbya gossypii; Klac, Kluyveromyces lactis; Zrou, Zygosaccharomyces rouxii; Spomb, S. pombe. Predicted secondary structures were labeled based on the crystal structure of human EXO1. B, duplex capture is crucial for Exo1 activation on a short 3′-overhanging DNA or a RPA occupied long 3′-overhanging DNA. C, Duplex DNA with a short 3′-overhang. D, Duplex DNA with a long 3′-overhang. In the absence of RPA, the initial Exo1 loading to the ssDNA region of a long 3′-overhanging DNA facilitates its diffusion on the substrate DNA for the duplex capture. 

**Figure 5. A model depicting Exo1 activation and its regulation by RPA.**
Activation of Exo1 nuclease on single-stranded DNA

of ~30 g was harvested from 12 liters of cell culture and stored at -80 °C for future use.

All protein purification procedures were conducted at 4 °C. To lyse 30 g of cell pellets, 150 ml of lysis buffer (pH 7.4; 150 mM KCl, 0.5 mM EDTA, 10% glycerol, 40 mM KH₂PO₄, 0.01% NP-40, 1 mM β-ME (2-mercaptoethanol), 1 mM PMSF, 5 mg/ml leupeptin, 5 mg/ml chymostatin, 5 mg/ml pepstatin, and 2.5 mg/ml aprotinin) was added before homogenization by sonication. Cell lysates were centrifuged with a speed of 20,000 × g at 4 °C for 20 min. The supernatant was loaded onto a 5-ml sulfo-nitrocellulose column. The column was washed with 50 ml of wash buffer (pH 7.4) containing 150 mM KCl, 0.5 mM EDTA, 10% glycerol, 40 mM KH₂PO₄, 0.1% NP-40, and 1 mM β-ME before Exo1 protein was eluted by 50 ml of elution buffer (pH 7.4) containing 500 mM KCl, 0.5 mM EDTA, 10% glycerol, 40 mM KH₂PO₄, 0.01% NP-40, and 0.2 mM β-ME. The eluate was further incubated with 400 μl of nickel-nitrotriacetic acid resin for 1 h. After washing the nickel-nitrotriacetic acid resin with 30 ml of wash buffer (pH 7.4) containing 500 mM KCl, 0.5 mM EDTA, 10% glycerol, 40 mM KH₂PO₄, 0.1% NP-40, and 1 mM β-ME, and 15 mM imidazole, Exo1 was eluted three times with 0.4 ml of elution buffer (pH 7.4), each containing a stepped imidazole gradient of 50, 100, and 200 mM, respectively. The Exo1 was peaked in the fraction with 200 mM imidazole. The purified Exo1-His₆ (~60 μg) was stored at -80 °C as small aliquots. exo1-D173A, exo1-K185A, exo1(1–403), and exo1(1–348) were expressed and purified similarly as full-length Exo1.

DNA substrates

The sequence of non-poly(dT)-derived oligonucleotides used in this work is listed in Table S1. For 5' end labeling, the oligonucleotide was incubated with T4 polynucleotide kinase (New England Biolabs) in the presence of [γ-³²P]ATP (PerkinElmer Life Sciences) at 37 °C for 1 h. For 3' end labeling, the oligonucleotide was incubated with terminal deoxynucleotidyltransferase (New England Biolabs) in the presence of [α-³²P]cordycepin 5'-triphosphate at 37 °C for 1 h. After the labeling reaction, the remaining free mononucleotides were removed by passing the sample through a Micro Bio-Spin™ 6 column (Bio-Rad). Duplex DNA substrates were constructed by annealing the respective oligonucleotides in 1× NEBuffer 3 (50 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 100 mM NaCl, and 1 mM DTT) using a PCR machine programmed as follows: 95 °C for 5 min, followed by a 0.2 °C decrease per minute to 25 °C. Specifically, D1 or D1–27 was annealed with D2 to create a duplex DNA with a 7-nr or 27-nt 3’ overhang, respectively.

Nuclease assays

Exo1 nuclease assays were conducted in a 10-μl reaction system where 5 nM DNA substrate was incubated with the indicated amount of Exo1 in reaction buffer containing 20 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 150 mM KCl, 1 mM DTT, and 200 μg/ml BSA at 30 °C for 10 min. To stop and deproteinize the reaction, the mixture was further incubated with 0.2% SDS and 0.5 mg/ml proteinase K at 37 °C for 5 min before heat treatment at 95 °C for 5 min in loading buffer (95% formamide, 0.025% (w/v) bromphenol blue, 0.025% (w/v) orange G, and 5 mM EDTA). The sample was chilled on ice for 5 min and then fractionated in 20% polyacrylamide denaturing gel electrophoresis running in 0.5X Tris borate-EDTA buffer before phosphorimaging analysis.

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays of the exo1-D173A nuclease dead mutant were carried out in a 10-μl reaction system that contains 40 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 150 mM KCl, 1 mM DTT, 200 μg/ml BSA, and 5 nM DNA substrate and the indicated amount of exo1-D173A. The samples were incubated at 30 °C for 10 min. For each reaction, 2 μl of 6X loading dye (10 mM Tris-HCl (pH 8.0), 0.025% (w/v) orange G, and 50% glycerol) was added. The mixture was fractionated on a 6% native polyacrylamide gel running in 0.5X Tris borate-EDTA buffer before phosphorimaging analysis.

CPT sensitivity assays

The ORF of Exo1 together with the 500-bp flanking sequence upstream and downstream was amplified by PCR using yeast genomic DNA as a template and cloned into pRS316 to create pRS316-EXO1, a CEN plasmid carrying EXO1 under its native promoter. The K185A mutation was further introduced into pRS316-EXO1 via site-directed mutagenesis to create pRS316-exo1-K185A. The exo1Δ strain, yGI198 (MATa hoΔ hmlΔ ade1::ADE1 his3Δ1 ade1–100 leu2–3,112 trp1::hisG lys5 ura3–52), was a gift from Dr. Grzegorz Ira (36). Plasmids were transformed into yGI198 to obtain exo1Δ/pRS316, exo1Δ/pRS316-EXO1, and exo1Δ/pRS316-exo1-K185A, which were harvested from exponentially growing cultures at 30 °C as small aliquots. Cells were washed with sterile water and adjusted to equal cell density around 3 × 10⁷ cells/ml. 10-fold serial dilutions were performed by mixing 50 μl of cells and 450 μl of sterile water. 5 μl of individual cell mixtures was spotted onto yeast extract-peptone-dextrose (YPD) agar plates containing various amount of camptothecin (Santa Cruz Biotechnology). Plates were incubated at 30 °C for 2 days before imaging.

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