Complementary Roles for Exonuclease 1 and Flap Endonuclease 1 in Maintenance of Triplet Repeats* [5]

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Trinucleotide repeats can form stable secondary structures that promote genomic instability. To determine how such structures are resolved, we have defined biochemical activities of the related RAD2 family nuclease, FEN1 (Flap endonuclease 1) and EXO1 (exonuclease 1), on substrates that recapitulate intermediates in DNA replication. Here, we show that, consistent with its function in lagging strand replication, human (h) FEN1 could cleave 5′-flaps bearing structures formed by CTG or CGG repeats, although less efficiently than unstructured flaps. hEXO1 did not exhibit endonuclease activity on 5′-flaps bearing structures formed by CTG or CGG repeats, although it could excise these substrates. Neither hFEN1 nor hEXO1 was affected by the stem-loops formed by CTG repeats interrupting duplex regions adjacent to 5′-flaps, but both enzymes were inhibited by G4 structures formed by CGG repeats in analogous positions. Hydroxyl radical footprinting showed that hFEN1 binding caused hypersensitivity near the flap/duplex junction, whereas hEXO1 binding caused hypersensitivity very close to the 5′-end, correlating with the predominance of hFEN1 endonucleolytic activity versus hEXO1 exonucleolytic activity on 5′-flap substrates. These results show that FEN1 and EXO1 can eliminate structures formed by trinucleotide repeats in the course of replication, relying on endonucleolytic and exonucleolytic activities, respectively. These results also suggest that unresolved G4 DNA may prevent key steps in normal post-replicative DNA processing.

Short tandem nucleotide repeats are widespread in mammalian genomes (1) and prone to instability because of their potential to form alternative structures during replication, transcription, or recombination. Trinucleotide repeats are of particular interest because they are associated with at least 20 human neurodegenerative disorders (2). Fragile X syndrome, the second most common cause of mental retardation, is caused by CGG repeat expansion; myotonic dystrophy by CTG repeat expansion; and Huntington chorea by CAG repeat expansions (3). All three repeats can form stable secondary structures containing stem-loops or, in the case of the CGG repeat, G4 DNA (4–6). Formation of secondary structures can affect DNA replication, and factors that promote Okazaki fragment maturation and lagging strand synthesis have been identified as critical to maintenance of repeat stability (7–9). Secondary structure formation can also impair transcription or lead to transcription-associated instability (10–13).

FEN1 (Flap endonuclease 1) is a highly conserved RAD2 family nuclease active in DNA repair and a component of the replisome in eukaryotic cells (14). The canonical activity of FEN1 is endonucleolytic excision of a 5′-flap from a duplex substrate, which represents the intermediate in processing Okazaki fragments during lagging strand replication (15). FEN1 can also function as an exonuclease and gap endonuclease. FEN1 is essential in mammalian cells (16). Yeast deficient in Rad27, the FEN1 homolog, exhibits growth defects; mutagenesis; and increased instability of CGG, CTG, and CAG repeats (8, 9, 17, 18), consistent with a role for FEN1 in stabilizing regions containing triplet repeats. Like FEN1, EXO1 is a conserved RAD2 family nuclease that possesses both 5′-flap endonucleolytic and exonucleolytic activities and has important functions in replication, repair, and recombination (14, 19, 20). Genetic analysis suggests that the activities of EXO1 and FEN1 might be partially redundant, as overexpression of Exo1 is able to rescue the mutator phenotype of yeast rad27 (21), whereas rad27Δ is synthetic lethal when combined with exo1Δ in yeast (22).

We showed recently that purified human (h)2 EXO1 acts on flap substrates and transcribed substrates containing G4 DNA (23, 24). This prompted us to compare the activities of purified hEXO1 and hFEN1 on substrates recapitulating replication intermediates bearing CTG or CGG repeats. We found that hFEN1 cleaved 5′-flaps bearing structures formed by CTG or CGG repeats, consistent with its function in lagging strand replication. hEXO1 excised these flaps but did not cleave them endonucleolytically. Cleavage by both hFEN1 and hEXO1 was reduced on flap substrates bearing CGG repeats, which form G4 DNA, relative to CTG repeats, which form stem-loops. The hydroxyl radical footprinting patterns of hFEN1 and hEXO1 correlated with the relative predominance of flap cleavage and excision: hFEN1 binding caused hypersensitivity at the flap/duplex junction, whereas hEXO1 binding caused hypersensitivity at the very 5′-end of the flap. These results identify a novel and potentially important role for EXO1 in maintenance of stability of triplet repeats during replication and extend the repertoire of structures on which EXO1 can function to include stem-loops in addition to G4 DNA.

**EXPERIMENTAL PROCEDURES**

*DNA Substrates—Substrates were generated by heating oligonucleotides at equimolar concentrations at 90 °C, followed...*
by slow cooling and overnight incubation at room temperature. Oligonucleotides were 5’-end-labeled with \([\gamma^{32}\text{P}]\text{dATP}\) using T4 polynucleotide kinase (New England Biolabs), and free nucleotides were removed using a Sephadex G-50 spin column (GE Healthcare). Flap substrates harboring CGG repeats were annealed in the presence of 40 mM KCl to stabilize possible G4 DNA structures. Structure formation by substrates containing CTG repeats was confirmed by electrophoresis on a 10% nondenaturing acrylamide gel (supplemental Fig. S1, A and B). Structure formation by substrates containing CGG repeats was confirmed by electrophoresis on an 8% nondenaturing acrylamide gel containing 40 mM KCl (Ge et al., 2015). Flap substrates harboring CGG repeats were annealed in the presence of 40 mM KCl to stabilize possible G4 DNA structures. Activity assays were carried out using hFEN1-D173A (26), a hFEN1 N-terminal construct with an inactivating mutation in the catalytic domain (a kind gift of David M. Wilson III, NIA, National Institutes of Health, Baltimore); and hFEN1 footprinting was carried out in the manufacturer’s buffer lacking Mn2+ , which is essential for hFEN1 activity.

RESULTS

hFEN1 5’-Flap Cleavage Is Impaired by CTG or CGG Repeats—We assayed the 5’-flap cleavage and excision activities of both hFEN1 and hEXO1 on substrates bearing a 38-mer unstructured flap or a (CTG)n or (CGG)n repeat in the center of that flap (Fig. 1A, upper). hFEN1 was active on substrates containing either an unstructured 5’-flap or a CTG repeat in the 5’-flap (Fig. 1A). Others have reported previously that hFEN1 is somewhat less active on flaps containing longer CTG repeats (11 or more repeats), consistent with the possibility that activity depends upon repeat length (27). Its flap endonuclease activity predominated on the unstructured flap substrate, whereas 5’-excision predominated on the substrate containing a (CTG)n repeat within the flap (Fig. 1A). Predominance of excision activity has similarly been documented in assays of yeast Rad27 (FEN1) on structured substrates bearing CTG repeats, bubbles, and loops (28, 29). hFEN1 exhibited limited flap endonuclease or 5’-excision activity on substrates bearing a 5’-flap containing a (CGG)n repeat (Fig. 1, A and B). Thus, hFEN1 distinguishes between structured and unstructured substrates and between stem-loops and G4 DNA.

hEXO1 Excises 5’-Flap Substrates, but Excision Is Inhibited by CGG Repeats—hEXO1 exhibited only exonucleolytic activity and no endonucleolytic activity on substrates bearing a 38-nt unstructured 5’-flap or CTG or CGG repeats in the 5’-flap (Fig. 2A). Excision of the substrate containing the CGG repeat was considerably reduced relative to the other substrates (Fig. 2, A and B). Similarly, we showed previously that telomeric sequences in a 5’-flap inhibit both hEXO1 exonucleolytic and 5’-flap endonucleolytic activities (24). Thus, like hFEN1, hEXO1 distinguishes between stem-loop and G4 DNA structures.
within adjacent duplex regions. To investigate whether such structures affect processing of proximal 5'-flaps by hFEN1 or hEXO1, we compared activities on 5'-flap substrates bearing an unstructured duplex region or a duplex region interrupted by a (CTG)₈ or (CGG)₆ repeat (Fig. 3A, upper). hFEN1 exhibited 5'-flap endonuclease activity on all substrates, although the substrate bearing a (CGG)₆ repeat was less efficiently cleaved than the other substrates (Fig. 3A, upper). hFEN1 carried out only limited excision of any of these substrates (Fig. 3A). The same substrates were tested for cleavage by hEXO1 (Fig. 4A). hEXO1 exhibited both flap endonucleolytic cleavage activity and exonuclease activity on the substrates carrying a duplex region or a duplex region interrupted by a (CTG)₈ repeat, but not on the substrate in which a (CGG)₆ repeat interrupted the duplex (Fig. 4B). Thus, G4 DNA (but not a stem-loop) within the duplex region inhibits both excision and flap endonucleolytic activities of both enzymes. This suggests that the presence of unresolved G4 DNA may impair post-replicative DNA processing, leading to genomic instability.

**hFEN1 and hEXO1 Bind Differently to Flap Substrates**—To investigate how hFEN1 and hEXO1 recognize their DNA substrates, we carried out hydroxyl radical footprinting, which enables comparison of subtle differences in binding on small DNA substrates. hFEN1 binding induced clear hypersensitivity near the flap/duplex junction, whereas hEXO1 binding caused modest hypersensitivity very near the 5'-end of the flap (Fig. 4A). Thus, G4 DNA (but not a stem-loop) within the duplex region inhibits both excision and flap endonucleolytic activities of both enzymes. This suggests that the presence of unresolved G4 DNA may impair post-replicative DNA processing, leading to genomic instability.
the flap (Fig. 5A, hypersensitive sites indicated by arrowheads). The differences in footprints were consistent with the distinct cleavage patterns of these two enzymes at unstructured 5′-flaps.

Footprint analysis of hFEN1 bound to substrates bearing a (CTG)₈ repeat either within the 5′-flap or interrupting the duplex region adjacent to a 5′-flap further validated the notion that the mode of binding correlates with the predominant cleavage activity. hFEN1 predominantly excised the former substrate (Fig. 1), and binding created strong hypersensitivity near the 5′-end of the DNA (Fig. 5B, left). hFEN1 endonucleolytic activity predominated on the latter substrate (Fig. 3), and binding induced hypersensitivity at the flap/duplex junction, along with a modest amount of hypersensitivity near the 5′-end at the highest enzyme concentration (Fig. 5B, right). hEXO1 did not produce a clear footprint on either substrate (data not shown).

**DISCUSSION**

We have defined the activities of hFEN1 and hEXO1 that may contribute to stabilizing structures formed by triplet repeats in the course of replication. We showed that both hFEN1 and hEXO1 can digest 5′-flap substrates that recapitulate intermediates in lagging strand DNA replication. Substrate structure determines whether digestion proceeds by an endonucleolytic or exonucleolytic pathway. The presence of a stem-loop (formed by a CTG repeat) in the flap diminished but did not prevent digestion and caused excision to predominate relative to endonucleolytic cleavage. The presence of G4 DNA (formed by a CGG repeat) in the flap further impaired digestion by hFEN1 and almost completely inhibited digestion by hEXO1. Flap digestion by hEXO1 (but not hFEN1) was also inhibited by G4 DNA interrupting the duplex region 3′ of the flap. Thus, whereas EXO1 may be redundant with FEN1 for some functions in replication, FEN1 may be especially critical for stability of regions bearing the G4 motif.

Hydroxyl radical footprinting defined distinct modes of enzyme/substrate interaction for hEXO1 and hFEN1, which correlated with the predominance of endonucleolytic cleavage or excision. hEXO1 excised a substrate bearing an unstructured 5′-flap and induced hypersensitivity near the 5′-end upon binding. hEXO1 has been reported to protect a duplex region near...
the base of a flap upon binding to a dumbbell-shaped 41-mer substrate with a very short (5 nt) 5’-flap (26), but we did not observe protection within the duplex region; differences in substrate structure probably explain these different footprints. hFEN1 cleaved a substrate bearing an unstructured 5’-flap at the flap/duplex junction and induced hypersensitivity at this junction upon binding. Similar results have been observed by micrococcal nuclease footprinting of the bovine FEN1 homolog, RTH1, on a similar substrate (30).

hFEN1 binding to a substrate bearing a stem-loop interrupting the duplex region adjacent to a flap similarly caused hypersensitivity at the flap/duplex junction. In contrast, hFEN1 binding to a substrate containing a stem-loop within the 5’-flap induced hypersensitivity very near the 5’-end. These different footprints correlate with the relative predominance of endonucleolytic cleavage or excision on these two substrates.

FEN1 has been postulated to recognize a free 5’-end of a flap and then track along the flap to find the junction with duplex DNA, where it cleaves (31, 32). This model derived in part from evidence that the activity of the bovine FEN1 homolog, RTH1, is inhibited by bulky adducts such as streptavidin-bound biotin and double-stranded DNA in the 5’-flap, although not by small adducts such as biotin (32). If tracking does occur, then the evidence that hFEN1 predominantly excised a substrate bearing a stem-loop would suggest that this structure may impede block post-replicative processing and thereby contribute to genomic fragility.

tracking. However, hFEN1 flap cleavage was only modestly affected by a \((\text{CGG})_6\) repeat within the 5’-flap and was unaffected by a G4 DNA structure formed by telomeric repeats at the very 5’-end of a flap (24). Thus, if tracking does occur, then not all 5’-adducts or 5’-structures inhibit FEN1 tracking. In particular, FEN1 might process substrates bearing G4 DNA differently from substrates bearing other structures.

G4 DNA May Impair Post-replicative DNA Processing—Strikingly, G4 DNA formed by CGG repeats inhibited both hFEN1 and hEXO1. Factors other than FEN1 and EXO1 are therefore likely to be required to resolve G4 DNA \textit{in vivo}, possibly including G4 DNA helicases such as human BLM, WRN, and FANCJ (33–35) or yeast Sgs1 and Pif1 (36, 37). Although both CTG and CGG repeats exhibit length- and orientation-dependent expansion, CGG repeats are less stable CTG repeats (38, 39), which may correlate with the difficulty in processing these structures. As repeat length increases, CGG repeats would be predicted to form increasingly intrinsically G4 DNA structures, which

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