J. Microbiol. Biotechnol. (2015), 25(10), 1768–1771
http://dx.doi.org/10.4014/jmb.1503.03092

jmb

Cohesion Establishment Factors Stimulate Endonuclease Activity of hFen1 Independently and Cooperatively

Do-Hyung Kim[1], Jeong-Hoon Kim[2], Byoung Chul Park[3], Sayeon Cho[4*], and Sung Goo Park[3*]

[1]*Bio Research Team, LG Household & Health Care Research Park, Daejeon 305-343, Republic of Korea*
[2]*Epigenomics Research Center, Korea Research Institute of Bioscience & Biotechnology, Daejeon 305-806, Republic of Korea*
[3]*Functional Proteomics Research Center, Korea Research Institute of Bioscience & Biotechnology, Daejeon 305-806, Republic of Korea*
[4]*College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea*

Received: March 25, 2015
Revised: May 6, 2015
Accepted: May 28, 2015

First published online
June 2, 2015

*Corresponding authors
S.G.P.
Phone: +82-42-860-4262;
Fax: +82-42-860-4269;
E-mail: sgpark@kribb.re.kr
S.C.
Phone: +82-2-820-5595;
Fax: +82-2-816-7338;
E-mail: sycho@cau.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Human Fen1 protein (hFen1) plays an important role in Okazaki fragment processing by cleaving the flap structure at the junction between single-stranded (ss) DNA and double-stranded (ds) DNA, an intermediate formed during Okazaki fragment processing, resulting in ligatable nicked dsDNA. It was reported that hChlR1, a member of the cohesion establishment factor family, stimulates hFen1 nuclease activity regardless of its ATPase activity. In this study, we found that cohesion establishment factors cooperatively stimulate endonuclease activity of hFen1 in *in vivo* mimic condition, including replication protein-A-coated DNA and high salt. Our findings are helpful to explain how a DNA replication machinery larger than the cohesion complex goes through the cohesin ring structure on DNA during S phase in the cell cycle.

Keywords: Cohesion establishment factors, Fen1, Okazaki fragment processing

Within eukaryotes, DNA replication should be completed in a short time period of the cell cycle. Therefore, DNA replication is initiated at multiple replication origins and proceeds bidirectionally from each origin to flanking DNA. For simultaneous synthesis of both strands, the leading strand is continuously replicated whereas the lagging strand is synthesized as short Okazaki fragments and ligated into one long DNA strand. Efficient processing of the Okazaki fragment is essential for DNA replication and cell cycle progression. Human Fen1 protein (hFen1) plays an important role in Okazaki fragment processing by cleaving the flap structure at the junction between single-stranded (ss) DNA and double-stranded (ds) DNA, an intermediate formed during Okazaki fragment processing, resulting in ligatable nicked dsDNA. Two sister chromatids replicated during the S phase are held together by cohesion mediated by the cohesin ring and separated into daughter cells during anaphase, which is critical for the control of genome integrity. The cohesin ring is composed of Smc1, Smc3, Scc1, and Scc3 [4, 6]. Cohesion is constructed during the G1 phase by cohesion establishment factors, including ChlR1, Ctf7, Ctf4, Ctf18, Dcc1, and Ctf8. Ctf18, together with Dcc1 and Ctf8, forms a complex with the four small subunits of RFC, which is involved in loading of PCNA onto DNA near cohesion and required for achieving sister chromatid cohesion. Mutations in these proteins give rise to chromosome loss during mitosis [3, 4]. Human ChlR1 protein (hChlR1) is an ATP-dependent DNA helicase interacting with the human Ctf18 complex and hFen1. The helicase activity of hChlR1 is enforced by the Ctf18 complex, and the nuclease activity of Fen1 is stimulated by hChlR1 [5]. Ctf7/eco1-dependent acetylation of the cohesin subunit Smc3 is required for establishment of cohesion. It was reported that Ctf7/eco1 genetically and biochemically interacts with the Okazaki fragment flap endonuclease Fen1 [6]. In addition, Ctf4 couples the replicative helicase

complex to the DNA replication machinery [7].

We previously reported that hChlR1, a member of the cohesion establishment factor family, stimulates hFen1 nuclease activity regardless of its ATPase activity [9]. In this study, the effects of hCtf18, hCft4, and hCtf7 proteins, other members of the cohesion establishment factor family, on the *in vitro* nuclease activity of hFen1 were examined to further elucidate the relationship between other cohesion establishment factor family members and Okazaki fragment processing.

Since hChlR1 stimulates hFen1 endonuclease activity regardless of its ATPase activity [9] and is reported to interact with other cohesion establishment factors (*e.g.*, Ctf18) that generate an alternative RFC complex with RFC2-5, Dcc1, and Ctf8, we investigated whether other cohesion establishment factors also promote hFen1 activity by nuclease assays. The oligonucleotides used to construct DNA substrates (listed in Table 1) were commercially synthesized (Genotech, Daejeon, Korea) and gel-purified prior to use [1]. Nucleoside triphosphates were obtained from Boehringer Mannheim and [γ-^{32}P]ATP (>3,000 Ci/mmol) was purchased from Amersham Pharmacia Biotech. Enzymes used in this study, including restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase, were purchased from Enzynomics (Daejeon, Korea). Oligonucleotide-based partial duplex substrates, except for the equilibrating flap substrates, were prepared as previously described [2] using the synthetic oligonucleotides listed in Table 1. Equilibrating flap substrates were prepared as described previously [9]. Briefly, an upstream oligonucleotide was labeled at the 5′-end with [γ-^{32}P] ATP and T4 polynucleotide kinase. The 5′-labeled oligonucleotide was annealed to a template oligonucleotide and a downstream oligonucleotide (molar ratio of 1:3:10, respectively). The annealed substrates were purified by polyacrylamide gel electrophoresis [2] and their specific activities (2,500 cpm/fmol) were determined by liquid scintillation counting (Beckman). The pET-23d(+)-hFen1 plasmid was constructed as described previously [11, 12]. The plasmid carrying human Fen1 with a C-terminal 6× His-tag was introduced into *E. coli* BL21 (DE3) CodonPlus-RIL (Stratagene) and the recombinant protein was induced at $A_{600} = 0.4$ by adding 0.4 mM isopropyl β-D-thiogalactopyranoside at 30°C for 3 h. Human Fen1 was purified according to a previous procedure [11]. Peak fractions from Mono S column chromatography were subjected to glycerol gradient sedimentation, as described elsewhere [2]. Active peak fractions from the glycerol gradient were pooled, aliquoted, and stored at −80°C until further use. Cohesion establishment factors were kindly provided by Dr. Jerard Hurwitz.

We carried out nuclease assays in the presence of hCtf18, hCtf7, and hCft4. To measure the endonuclease activities of hFen1, a reaction mixture (20 μl) containing 50 mM Tris-HCl (pH 7.8), 50 mM or 150 mM NaCl, 8 mM $MgCl_2$, 2 mM DTT, 0.25 mg/ml BSA, and 5′-[^{32}P]-labeled DNA substrate (15 fmol) was used. After standing on ice for 5 min, the reaction mixtures were incubated with enzymes at 37°C for 10 min and the reaction was then terminated by adding 6× stop solution (60 mM EDTA, pH 8.0, 40% (w/v) sucrose, 0.6% SDS, 0.25% bromophenol blue, and 0.25% xylene cyanol). Cleavage products were resolved on a 10% polyacrylamide gel containing 0.1% SDS in 0.5× TBE (electrophoresis at 150 V for 40 min) or a 20% sequencing gel for the higher resolution. Gels were dried on DEAE-cellulose paper and subjected to autoradiography. The amount of product formed was quantitated using a PhosphorImager.

First, we performed nuclease assays with the hCtf18 complex that contained hCtf18, Dcc1, Ctf8, and RFC2-5. We found that the hCtf18 complex also stimulated hFen1 activity in a dose-dependent manner, similar to hChlR1, suggesting the role of hCtf18 on hFen1 endonuclease activity (Fig. 1). We also tested if hPCNA had additional effect on hFen1 activity in the presence of various amounts of hCtf18. hPCNA has been known to interact with hFen1 through the PIP domain and stimulates its nuclease activity [13]. Our previous study showed that addition of hPCNA exerts no synergistic or additive effects on hChlR1-mediated hFen1 activity [6]. When hPCNA was added to the reaction that contained 32 fmol of hCtf18 complex, hPCNA synergistically stimulated hFen1 activity (Fig. 1, compare lane 3 with lane 7). These synergistic effects of hPCNA on hCtf18-mediated hFen1 activity might be due to interaction between hPCNA and the hCtf18 complex.

Table 1. Oligonucleotides used in this study.

Primer	Size (nt)	Sequence
729	52	5′-CGA ACA ATT CAG CGG CTT TAA CCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3′
5TY-1	50	3′-GGC AAT CGT CAA GCG GAA CAC GGA TCG AGC TGC GGT AAT TAT TAC AAA AG-5′
5TBG	26	5′-CCG TTA GCA GTT CGC CTT GTG CCT AG-3′

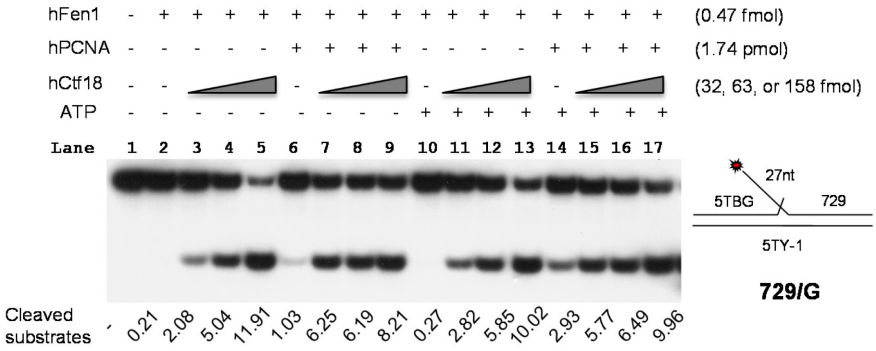

Fig. 1. Cohesion establishment factor hCtf18 stimulates hFen1 nuclease activity.

Endonuclease activity of hFen1 was measured with the indicated amounts of hFen1 and hCtf18 as described in Materials and Methods. hFen1 endonuclease activity was determined in the presence of the hCtf18 complex and hPCNA using the 729/G fixed flap substrate with 125 mM NaCl in the reaction. The structure of the 729/G fixed flap substrate is shown at the top of the gel. The amounts of cleavage products are indicated at the bottom of the gel.

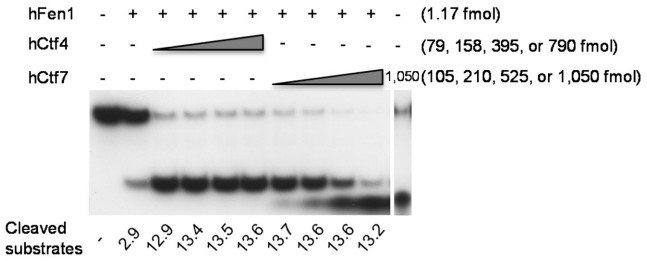

Fig. 2. Cohesion establishment factors hCtf4 and hCtf7 stimulate hFen1 nuclease activity.

Endonuclease activity of hFen1 was measured in the presence of hCtf4 or hCtf7 under the same experimental conditions as described in Fig. 1, using the 729/G fixed flap substrate with 125 mM NaCl in the reaction. The amounts of added hCtf4 or hCtf7 are indicated at the right of the gel. The far right lane shows that 100 ng of hCtf7 has no endonuclease activity.

Interestingly, in the presence of a large amount of hCtf18 complex, hFen1 endonuclease activity was not enhanced in the presence of hPCNA (Fig. 1, compare lane 5 with line 9), which suggests that hCtf18 somehow disrupts the association between hPCNA and hFen1 and thus its activity towards hFen1 activity. In addition, ATP gave no effect on hFen1 activity in the presence of hPCNA or hCtf18.

To assess the effects of other cohesion establishment factors, hCtf7 and hCtf4, on the endonuclease activity of hFen1, we also measured the endonuclease activity of hFen1 with hCtf7 and hCtf4. Like other cohesion establishment factors, hCtf4 stimulated the endonuclease activity of hFen1 separately (Fig. 2, left). Exonuclease contamination was detected when hCtf7 was added to the reaction (Fig. 2, right). The contaminants, however, had no endonuclease activity (Fig. 2, far right lane). Even if it was contaminated with exonuclease, we conclude that hCtf7 stimulated the endonuclease activity of hFen1.

Finally, we added hChlR1, hCft18 complex, and hCft4 to the reaction mixtures for hFen1 nuclease activity assays in the presence or absence of replication protein A (RPA) that binds to ssDNA to prevent ssDNA from winding back on itself or from forming secondary structures. As shown in Fig. 3, hChlR1, hCft18 complex, and hCft4 stimulated hFen1 nuclease activity in a reaction using the fixed-flap structured DNA substrate in the absence of hRPA. However, hFen1 activity measured in the presence of hRPA resembled *in vivo* activity, since ssDNA is covered with RPA in cells, even though hFen1 activity is inhibited by high salt concentration and RPA proteins. Since hFen1 activity was stimulated most strongly when all cohesion establishment factors were present in *in vivo* mimic condition (Fig. 3, lane 17), the results suggest that these factors stimulate enzymatic activity of hFen1 cooperatively, leading to the acceleration of Okazaki fragment processing near cohesion.

In this study, we found that cohesion establishment factors cooperatively stimulate the endonuclease activity of hFen1, thus allowing efficient cleavage of model substrates even if DNA is covered with RPA protein. Our findings that the cohesion establishment factors could stimulate the activity of hFen1 suggest that the acceleration of Okazaki fragment processing near cohesions may aid in reducing the size of the replication machinery, thereby facilitating its entry through the cohesin ring. Further studies will be needed to clarify the precise relationship between cohesion establishment factors and the machinery for lagging strand DNA synthesis [10].

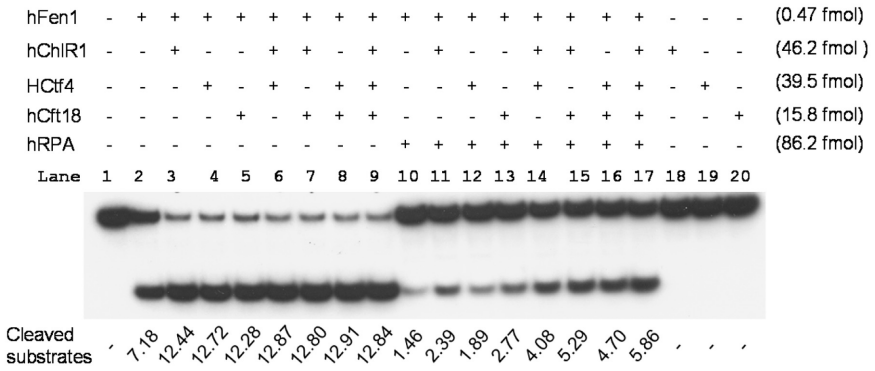

Fig. 3. Cohesion establishment factors cooperatively stimulate hFen1 activity.
Effects of the diverse cohesion establishment factors (hChlR1, hCtf4, and hCtf18) on the endonuclease activity of hFen1 were examined using the 729/G substrate with 50 mM NaCl in the reaction (in the presence or absence of hRPA). The amounts of cleavage products are indicated at the bottom of the gel. The data are representative of three independent experiments with similar results.

Acknowledgments

We thank Dr. Jerard Hurwitz for providing the proteins. We are also grateful to Drs. Kwang-Hee Bae and Sang-Chul Lee for helpful advice and careful reading of the manuscript. This work was supported by a grant from the National Research Foundation (NRF-2011-0028172).

References

1. Bae SH, Choi E, Lee KH, Park JS, Lee SH, Seo YS. 1998. Dna2 of *Saccharomyces cerevisiae* possesses a single-stranded DNA-specific endonuclease activity that is able to act on double-stranded DNA in the presence of ATP. *J. Biol. Chem.* **273:** 26880-26890.
2. Bae SH, Seo YS. 2000. Characterization of the enzymatic properties of the yeast Dna2 helicase/endonuclease suggests a new model for Okazaki fragment processing. *J. Biol. Chem.* **27:** 38022-38031.
3. Farina A, Shin, JH, Kim DH, Bermudez VP, Kelman Z, Seo YS, Hurwitz J. 2008. Studies with the human cohesin establishment factor, ChlR1. Association of ChlR1 with Ctf18-RFC and Fen1. *J. Biol. Chem.* **283:** 20925-20936.
4. Guacci V, Koshland D, Strunnikov A. 1997. A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in *S. cerevisiae*. *Cell* **91:** 47-57.
5. Haber JE. 1974. Bisexual mating behavior in a diploid of *Saccharomyces cerevisiae*: evidence for genetically controlled non-random chromosome loss during vegetative growth. *Genetics* **78:** 843-858.
6. Haering CH, Lowe J, Hochwagen A, Nasmyth K. 2002. Molecular architecture of SMC proteins and the yeast cohesin complex. *Mol. Cell* **9:** 773-788.
7. Kang YH, Farina A, Bermudez VP, Tappin I, Du F, Galal WC, Hurwitz J. 2013. Interaction between human Ctf4 and the Cdc45/Mcm2-7/GINS (CMG) replicative helicase. *Proc. Natl. Acad. Sci. USA* **110:** 19760-19765.
8. Kao HI, Henricksen LA, Liu Y, Bambara RA. 2002. Cleavage specificity of *Saccharomyces cerevisiae* flap endonuclease 1 suggests a double-flap structure as the cellular substrate. *J. Biol. Chem.* **277:** 14379-14389.
9. Kim DH, Kim JH, Park BC, Lee DH, Cho S, Park SG. 2014 Human ChlR1 stimulates endonuclease activity of hFen1 independently of ATPase activity. *Bull. Kor. Chem. Soc.* **35:** 3005-3008.
10. Rudra S, Skibbens RV. 2012. Sister chromatid cohesion establishment occurs in concert with lagging strand synthesis. *Cell Cycle* **11:** 2114-2121.
11. Storici F, Henneke G, Ferrari E, Gordenin DA, Hubscher U, Resnick MA. 2002. The flexible loop of human FEN1 endonuclease is required for flap cleavage during DNA replication and repair. *EMBO J.* **21:** 5930-5942.
12. Stucki M, Jonsson ZO, Hubscher U. 2001. In eukaryotic flap endonuclease 1, the C terminus is essential for substrate binding. *J. Biol. Chem.* **276:** 7843-5849.
13. Warbrick E, Lane DP, Glover DM, Cox LS. 1997. Homologous regions of Fen1 and p21Cip1 compete for binding to the same site on PCNA: a potential mechanism to co-ordinate DNA replication and repair. *Oncogene* **14:** 2313-2321.