Human ChlR1 Stimulates Endonuclease Activity of hFen1 Independently of ATPase Activity

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Human ChlR1 protein (hChlR1), a member of the cohesion establishment factor family, plays an important role in the segregation of sister chromatids for maintenance of genome integrity. We previously reported that hChlR1 interacts with hFen1 and stimulates its nuclease activity on the flap-structured DNA substrate covered with RPA. To elucidate the relationship between hChlR1 and Okazaki fragment processing, the effect of hChlR1 on in vitro nuclease activities of hFen1 and hDna2 was examined. Independent of ATPase activity, hChlR1 stimulated endonuclease activity of hFen1 but not that of hDna2. Our findings 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.

Key Words : ChlR1, Dna2, Fen1, Okazaki fragment processing

Introduction

In eukaryotic cells, DNA replication is initiated at multiple origins and proceeds bidirectionally from each origin to flanking DNA. For simultaneous synthesis of both strands, the leading strand is continuously replicated by DNA polymerase ε whereas the lagging strand is synthesized as short Okazaki fragments by primase, DNA polymerases α and δ, and then ligated into one long DNA strand. These enzymes build up the replisome in conjunction with other components, such as Mcm2-7 helicase, PCNA, and RFC.1-4 In yeast, Okazaki fragments are processed by well-organized multiple enzymatic reactions involving Dan2, Fen1 and DNA ligase I, and subsequently ligated into a long DNA strand. To complete synthesis of lagging strand DNA, Okazaki fragments must be processed about 50 million times per cell cycle in mammalian cells. Failure of Okazaki fragment processing in yeast is associated with increasing times per cell cycle in mammalian cells. Failure of Okazaki fragment processing, resulting in ligatable nicked dsDNA. This step is crucial for cells to maintain genome integrity. Two sister chromatids replicated during S phase are linked by the cohesion complex until metaphase and separated into daughter cells during anaphase. Human ChlR1 protein (hChlR1) is an ATP-dependent DNA helicase interacting with hFen1. Depletion of hChlR1 in HeLa cells leads to abnormal separation of sister chromatids.5

In this study, to clarify the relationship between Okazaki fragment processing and hChlR1, we examined the effect of hChlR1 on in vitro endonuclease activity of hFen1. We present the evidence that hChlR1 induces acceleration of Okazaki fragment processing near cohesion, resulting from stimulation of hFen1 activity to reduce the size of replisome structure.

Experimental Section

Oligonucleotides, Nucleoside Triphosphates and Enzymes. The oligonucleotides used to construct DNA substrates (listed in Table 1) were commercially synthesized (Genotech, Daejeon, Korea) and gel-purified prior to use. Nucleoside triphosphates were obtained from Boehringer Mannheim and [γ-32P]ATP (> 3000 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 Enzymatics (Daejeon, Korea).

Preparation of Substrates. Oligonucleotide-based partial duplex substrates, except for the equilibrating flap substrates, were prepared as previously described10 using the synthetic oligonucleotides listed in Table 1. Equilibrating flap substrates were prepared as described previously.11 Briefly, a upstream oligonucleotide was labeled at 5'-end with [γ-32P]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 electrophoresis10 and their specific activities (2,500 cpm/fmol) were determined by liquid scintillation counting.
Purification of Human Fen1 and Other Proteins. The pET-23d(+)−hFen1 plasmid was constructed as described previously. The plasmid carrying human Fen1 with 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 (IPTG) at 30 °C for 3 h. Human Fen1 was purified according to the previous procedure. Peak fractions from Mono S column chromatography were subjected to glycerol gradient sedimentation, as described elsewhere. Active peak fractions from the glycerol gradient were pooled, aliquotted, and stored at $−80 °C$ until further use. hDna2 protein was kindly provided by Dr. Jerard Hurwitz.

Assay of Nuclease Activity. To measure the endonuclease activities of hDna2 and hFen1, the reaction mixture (20 µL) containing 50 mM Tris–HCl (pH 7.8), 50 mM NaCl, 8 mM MgCl$_2$, 2 mM DTT, 0.25 mg/mL BSA and 5′-[32P]-labeled DNA substrate (15 fmol) was used. Enzymes were diluted to the appropriate concentrations prior to use in buffer containing 50 mM Tris–HCl (pH 7.8), 2 mM DTT, 0.5 mg/mL BSA, 10% glycerol, 0.5 M NaCl and 0.02% Nonidet P-40. After standing on ice for 5 min, reaction mixtures were incubated with enzymes at 37 °C for 10 min and 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 0.5X 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.

Results and Discussion

hChlR1 Stimulates Nuclease Activity of hFen1 with Various Substrates. In a previous study, we reported that hChlR1 stimulates hFen1 nuclease activity in a reaction using the fixed-flap structured DNA substrate covered with RPA. To compare the stimulation of hFen1 activity by hChlR1 on substrates with different structure, we used fixed-flap and equilibrating-flap structured substrates. As shown in Figure 1(a), hChlR1 could stimulate nuclease activity of hFen1 on both the equilibrating-flap structured and fixed-flap structured substrates. However, hFen1 exhibited slightly

**Table 1. Oligonucleotides used in this study**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Size (nt)</th>
<th>Sequence</th>
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<tr>
<td>D_F</td>
<td>40</td>
<td>5′-AGG TCT CGA CTA CCA CCC GTC AAC CCG ACG CCA CCT CCG TTT CAA TC-3′</td>
</tr>
<tr>
<td>T_F</td>
<td>51</td>
<td>3′-GCT GGC ACG GTC GGA TTT AAA GTT AGG GCA GTG GGG CTG CGG TGG AGG ACG-5′</td>
</tr>
<tr>
<td>U_F</td>
<td>26</td>
<td>5′-CGA CGG TGC CAG CTT AAA TAA TTT CAA TGG ACT AAT CGT CCA CCT CCA CCG TGG AGG ACG-3′</td>
</tr>
<tr>
<td>D_E</td>
<td>58</td>
<td>5′-TAG GTC TCG ACG ACT AAC TCT AGT CGT TGT TCC ACC GGT CAA CCC GCC GAC GCC ACC TCG TG-3′</td>
</tr>
<tr>
<td>T_E</td>
<td>85</td>
<td>3′-GCT GGC ACG GTC GGA TTT AAA GTT AAT CAG AGC TGC TGA TTT AGA TCA GCA ACA AGG TGG GCA GTG GGG CTG CGG TGG AGG ACG-5′</td>
</tr>
<tr>
<td>U_E</td>
<td>39</td>
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</tr>
<tr>
<td>TTB</td>
<td>25</td>
<td>5′-CGC TTA GCA GTC CGT TGT CCA CCT CC-3′</td>
</tr>
<tr>
<td>TBG</td>
<td>26</td>
<td>5′-CGT TTA GCA GTC CGT TGT CCA CCT CC-3′</td>
</tr>
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</table>

**Figure 1.** hChlR1 stimulates the cleavage of various substrates by hFen1. (a) Endonuclease activity of hFen1 was measured with the indicated amounts of hFen1 and hChlR1 as described in Experimental Section. The fixed-flap and equilibrating-flap substrates, whose structures are shown at the top of the gels, were prepared as described. Reaction products were separated on a 10% polyacrylamide gel containing 0.1% SDS in 0.5X 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.
weaker activity with the equilibrating-flap structured substrate and cleaved only up to 65% of total substrate since only the 5’-overhang flap-structured DNA could be cleaved. To further examine the stimulatory effect of hChlR1, we then employed substrates containing a long flap that easily generate secondary structures (Fig. 1(b)). Up-regulation of hFen1 activity by hChlR1 resulted in removal of the long flap from the 729/G substrate containing a 1nt 3’-overhang. Stimulation of hFen1 activity by hChlR1 on substrate 729 was lower than that on substrate 729/G since much higher amount of hFen1 (as much as 100 times) was required to generate similar amounts of cleaved products from substrate 729. These results clearly demonstrate that hChlR1 stimulates the endonuclease activity of hFen1 on substrates with equilibrating-flap or fixed-flap structures.

**ATPase Activity of hChlR1 and hPCNA is Dispensable for Stimulation of hFen1 Activity.** hChlR1, a member of the DEAD/DEAH family of helicases, translocates single-stranded DNA in 5’ to 3’ direction in the presence of ATP. To determine whether ATPase activity is required for stimulation of hFen1 by hChlR1, hFen1 nuclease activity was measured using the wild-type protein and the helicase activity-dead mutant referred as hChlR1 (KR). Similar to the wild-type protein, hChlR1 (KR) mutant also stimulated

![Figure 2](attachment:image.png)

**Figure 2.** ATPase activity and PCNA are dispensable for hFen1 activation by hChlR1. (a) Endonuclease activity of hFen1 was measured in the presence of hChlR1 (the wild-type) or hChlR1(KR) (ATPase activity-deficient mutant) using the fixed flap substrate. The amounts of added hChlR1 are indicated at the right of the gel. (b) Graph of the data obtained in Figure 2(a). (c) Effects of hFen1 on stimulating hChlR1 and hPCNA were examined using the 729/G substrate in the presence of increasing amounts of hFen1. (d) Influence of salt concentration on hFen1 activation by hChlR1. Endonuclease activity of hFen1 was measured under different salt concentrations (50 or 125 mM) using hRPA-covered or naked 729/G substrates. Due to the inhibitory effects of high salt concentrations on hFen1 activity, varying amounts of hFen1 were used. The amounts of cleavage products are indicated at the bottom of the gel.
Dna2 is stimulated by RPA, while the activity of Rad27 is inhibited by RPA. It is plausible that the endonuclease activity of hDna2 is stimulated by ATP to a much lesser extent since this protein possesses helicase activity that aids in flap cleavage (Fig. 3(b)).

Conclusions

In this study, we found that hChlR1 cooperatively stimulates endonuclease activity of hFen1, thus allowing efficient cleavage of model substrates even if DNA is covered with RPA protein. Previously hChlR1, a cohesion establishment factor, was identified as an activator of hFen1. Here we demonstrated that the ATPase activity of hChlR1 is dispensable for stimulation of hFen1 activity, raising a possibility that the stimulatory effect of hChlR1 does not result from resolving the secondary structure of the flap via helicase activity. Further studies will be needed to clarify the precise relationship between hChlR1 and the machinery for lagging strand DNA synthesis.

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References