Enzymatic properties of the *Caenorhabditis elegans* Dna2 endonuclease/helicase and a species-specific interaction between RPA and Dna2

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ABSTRACT

In both budding and fission yeasts, a null mutation of the *DNA2* gene is lethal. In contrast, a null mutation of *Caenorhabditis elegans dna2*$^+$ causes a delayed lethality, allowing survival of some mutant *C.elegans* adults to F2 generation. In order to understand reasons for this difference in requirement of Dna2 between these organisms, we examined the enzymatic properties of the recombinant *C.elegans* Dna2 (CeDna2) and its interaction with replication-protein A (RPA) from various sources. Like budding yeast Dna2, CeDna2 possesses DNA-dependent ATPase, helicase and endonuclease activities. The specific activities of both ATPase and endonuclease activities of the CeDna2 were considerably higher than the yeast Dna2 ($\sim$10- and 20-fold, respectively). CeDna2 endonuclease efficiently degraded a short 5′ single-stranded DNA tail (<10 nt) that was hardly cleaved by ScDna2. Both endonuclease and helicase activities of CeDna2 were stimulated by CeRPA, but not by human or yeast RPA, demonstrating a species-specific interaction between Dna2 and RPA. These and other enzymatic properties of CeDna2 described in this paper may shed light on the observation that *C.elegans* is less stringently dependent on Dna2 for its viability than *Saccharomyces cerevisiae*. We propose that flaps generated by DNA polymerase δ-mediated displacement DNA synthesis are mostly short in *C.elegans* eukaryotes, and hence less dependent on Dna2 for viability.

INTRODUCTION

Okazaki fragments are generated during lagging strand DNA synthesis at replication forks and processed through a complex series of enzymatic events prior to being joined to form a contiguous DNA strand (1–6). In eukaryotes, Okazaki fragment synthesis is initiated with the synthesis of a short RNA–DNA primer by the polymerase (pol) α-primase complex, followed by its elongation by pol δ to produce full-length Okazaki fragments (7,8). The RNA–DNA primers at the 5′ end of each Okazaki fragment are then processed by nucleases, leading to the generation of ligatable nicks that are sealed by DNA ligase I. Nucleases involved in this process include RNase H, Fen1 and Dna2 (3,6,9,10). RNase H and Fen1 were first implicated in the removal of the RNA primers in *in vitro* reconstitution experiments using a plasmid containing the simian virus 40 replication origin or well-defined synthetic DNA substrates (3,6,10). The *DNA2* gene was first identified through a screen for DNA replication mutants of *Saccharomyces cerevisiae* (11). It encodes an essential 172 kDa protein that possesses single-stranded (ss) DNA-dependent ATPase, DNA helicase and ssDNA-specific endonuclease activities (11–16). The endonuclease activity of *S.cerevisiae* Dna2 (ScDna2) has a strong preference for a 5′-free ssDNA tail and is stimulated by the presence of a 5′-RNA segment in the flap (13,17). This and other properties of ScDna2 suggest that it is well suited to process Okazaki fragments during lagging strand synthesis (18). ScDna2 helicase activity displaces duplex DNA in the 5′ to 3′ direction, does not require preexisting fork structures and acts in a highly distributive fashion. It is able to resolve a secondary structure in the flap, thereby facilitating the endonucleolytic cleavage of the flap by its intrinsic endonuclease activity (14). Flap structures of the

primer RNA–DNA ends of Okazaki fragments are generated by a displacement DNA synthesis reaction catalyzed by pol δ (17,19,20). Since ScDna2 is unable to completely remove the flap structure, its action results in the shortening of the flap (∼6 nt). In contrast, Fen1 cleaves at the junction of single-stranded flap and double-stranded DNAs, creating nicks that can be readily joined by DNA ligase I. *In vitro* experiments showed that both endonuclease and helicase activities of ScDna2 were stimulated by replication-protein A (RPA) at physiological salt concentrations (19). However, the cleavage of flaps by Fen1 alone was markedly inhibited by RPA. This inhibition was relieved by the addition of Dna2, suggesting that flaps long enough to bind RPA stably are processed by Dna2, and the remaining shortened flaps are processed by Fen1 (19). In the presence of RPA, the sequential action of the two endonucleases Dna2 and Fen1 ultimately leads to the formation of ligatable nicks. The sealing of the nicks by DNA ligase I completes the processing of Okazaki fragments.

Dna2 proteins are well conserved throughout eukaryotes that include humans, plants, fish, fission yeasts, frogs and nematodes (18,21–23), and share the catalytic domains for endonuclease and helicase of Dna2 (24,25). Though the motifs for endonuclease and helicase activities are well conserved, the enzymatic activities associated with each protein vary. For example, ScDna2 contains both endonuclease and helicase activities. In contrast, *Xenopus laevis* Dna2 possesses endonuclease activity but lacks helicase activity (21). *Schizosaccharomyces pombe* Dna2 is also devoid of helicase activity (H.Y. Kang, unpublished data). In addition, there is a difference in the primary structures of various eukaryotic Dna2 proteins. Most notably, no significant homology is found in the N-terminal regions of Dna2 proteins and they also vary in size (21). *S.cerevisiae* or *S.pombe* Dna2 has an extra 350–400 amino acid region at their N-termini, which is much shorter or absent in the metazoan Dna2 enzymes. The N-terminal 405 amino acid region of yeast Dna2 is dispensable for endonuclease and helicase activities (26). However, mutant yeast cells containing Dna2 devoid of the N-terminal 405 amino acid region show a temperature-dependent growth defect that can be suppressed by the multicopy expression of RPA (19,26). However, the N-terminal domain is required for maximal stimulation of ScDna2 by RPA (27).

We found that metazoan and single cell organisms differ in their Dna2 requirement for viability. Deletion of Dna2 in *Caenorhabditis elegans* did not cause complete embryonic lethality since some of the mutants survived into the F2 generation (28), whereas it is absolutely essential in *S.cerevisiae* and *S.pombe* (11,18). This situation is reversed regarding the requirement for Fen1 (28–30); deletion of Fen1 resulted in embryonic lethality in mice and *C.elegans*, whereas it was not essential in the yeasts. These observations suggest that the strategy of processing for the processing of Okazaki fragments in lower and higher eukaryotes may differ. In order to understand the significance of the differences in the Dna2-deletion phenotypes described above and the mechanisms of Okazaki fragment processing in multicellular organisms, we have isolated and characterized the Dna2 enzyme of *C.elegans*. For this purpose, we have purified the wild-type and two mutant *C.elegans* Dna2 (CeDna2) proteins and *C.elegans* RPA (CeRPA) and examined their biochemical properties. Based on their enzymatic properties described here, we suggest that the pol δ-mediated displacement DNA synthesis varies in eukaryotes, which in turn affects the Dna2 requirement for the processing of Okazaki fragments.

MATERIALS AND METHODS

Oligonucleotides, nucleoside triphosphates and enzymes

All oligonucleotides used to construct DNA substrates (listed in Table 1) were synthesized commercially (Genotech, Daejeon, Korea) and gel-purified prior to use. Nucleoside triphosphates were obtained from Boehringer Mannheim, and radioisotopes such as [γ-^{32}P]ATP (>3000 Ci/mmol) and [α-^{32}P]ddATP (>3000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. The following proteins were obtained commercially: restriction endonucleases, terminal transferase, T4 DNA ligase and T4 polynucleotide kinase were from New England Biolabs.

Table 1. Oligonucleotides used in this study

Oligo	Sequence	Length of oligonucleotide
1	5′-TTT TTT TTT TTT TTT TTT TTT TTT TCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3′	55
2	5′-TGA AAA CAT TAT TAA TGG CGT CGA GCG TCC GTT TTT TTT TTT TTT TTT TTT TTT TT-3′	56
3	5′-TGG GCT CAC GTG GTC GAC GCT GGA GGT GAT CAC CAG ATG ATT GCT AGG CAT GCT TTC CGC AAG AGA ACG GGC GTC TGC GTA CCC GTG CAG-3′	90
4	5′-ATC ACC TCC AGC GTC GAC CAC GTG AGC CCA-3′	30
5	5′-CTG CAC GGG TAC GCA GAC GCC CGT TCT CTT-3′	30
6	5′-<u>CGG ACG CTC GAC GCC ATT AAT AAT</u>GTT TTC TTT TTT TTT TTT TTT TTT TTT TTT T-3′ᵃ	55
7	5′-<u>CGG ACG CTC GAC GCC ATT AAT AAT GTT TTC</u>-3′ᵃ	30
8	5′-(dT)$_n$ CGG ACG CTC GAC GCC ATT AAT AAT GTT TTC-3′	30 + n
9	5′-TGA AAA CAT TAT TAA TGG CGT CGA GCG TCC G-3′	31
10	5′-CCG TTA GCA GTT CGC CTT GTG CCT A-3′	25
11	5′-TGA AAA CAT TAT TAA TGG CGT CGA GCG TCC GTA GGC ACA AGG CGA ACT GCT AAC GG-3′	56
12	5′-**UUU UUU UUU UUU** TTT TTT TTT TTT TCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3′ᵇ	55
13	5′-GCG CAT GTG CGT TCC ATT TGC CGA ACA ATT CAG CGG CTT TAA CCG GAC GCT CGA CGC CAT TAA TAA TGT TTT C-3′	73

ᵃUnderlined sequences are complimentary to ΦX174 ssc DNA.
ᵇBoldface, ribonucleotides.

Construction of recombinant baculoviruses to overproduce CeDna2

The plasmid pBS-*Cedna2* that contained a complete cDNA encoding CeDna2 (28) was used to amplify the full-length cDNA fragment encoding CeDna2 using the primers 5′-AAA ACT AGT ATG GAG TCT CGC AAA AGA CAG CTC CAA AAT GTC-3′ and 5′-AAA GGT ACC TTA GAG TTG AGT AGG AAT AGA AAT ATG ATT TTC-3′. The amplified fragments were digested with SpeI and KpnI and cloned into the pFastBacHTb vector (Gibco-BRL) cleaved with the same restriction enzymes to generate pFastBacHTb-*Cedna2*. The two synthetic oligonucleotides (5′-AAA ACT AGT GAT TAT AAA GAT GAC GAT GAC AAG GAT TAT AAA GAT GAC GAT GAC AAG ACT AGT TTT-3′; 5′-AAA ACT AGT CTT GTC ATC GTC ATC TTT ATA ATC CTT GTC ATC GTC ATC TTT ATA ATC ACT AGT TTT-3′) were annealed to form a duplex DNA that encodes two tandem FLAG epitopes. The duplex DNA was cleaved with SpeI, followed by insertion into the unique SpeI site in pFastBacHTb-F*Cedna2*. The orientation was confirmed by nucleotide sequence determination. We transformed the pFasBacHTb-F*Cedna2* plasmid into DH10Bac *Escherichia coli* cell (Gibco BRL), and generated recombinant baculoviruses overexpressing CeDna2 according to the manufacturer's procedures. The recombinant CeDna2 protein produced this way contained a hexahistidine tag (from the vector) and two tandem FLAG epitopes.

A specific point mutation that abolishes ATPase or endonuclease activity of CeDna2 was introduced into CeDna2 in pFastBacHTb-F*Cedna2* using the QuikChange™ site-directed mutagenesis system (Stratagene). Mutagenic primers used were as follows: (the mutagenic nucleotide is underlined) 5′-GAA GGG GAA GAT CGC TGT GAC GAT GCG AAC A-3′ (D320A, endonuclease-negative mutation) and 5′-CTG CCA GGA TCT GGA GAA ACG ACT CTA ATC T-3′ (K678E, ATPase-negative mutation). We carried out site-directed mutagenesis according to the manufacturer's procedure, and isolated pFastBacHTb-F*Cedna2* containing the desired mutations (verified by nucleotide sequence analysis). These plasmids were used to construct recombinant baculoviruses that express mutant CeDna2 enzymes as described above for wild-type CeDNA.

Cloning and overexpression of CeRPA

The two cDNAs encoding the large subunit (accession no. AAB63407; 655 amino acids) and the middle subunit (accession no. AAK71380; 288 amino acids) of CeRPA were amplified from *C.elegans* cDNA library (Clontech) by PCRs using two sets of primers: 5′-TTA AGC TTA TGG CGG CAA TTC ACA TCA ATC AC-3′ and 5′-TTA AGC TTT TAG TAG TCA CTC ATT TGT TGC AT-3′ (the large subunit); and 5′-AAG CGG CCG CGA AGG AGA GAA TTC ATG TGG AAC GAG ACT GTC GAG CAC GAG-3′ and 5′-AAG CGG CCG CGA ATT CTT AAA TAG CGG AAA CAG TTT CCT CGT C-3′ (the middle subunit). The cDNA for the small subunit (accession no. AAB37031; 143 amino acids) of CeRPA was reconstituted using synthetic oligonucleotides (sequences of which are available upon request). Briefly, five template oligonucleotides (75–99 nt long) encompassing the entire open reading frame of the small subunit were annealed with four other oligonucleotides (30 nt long) that are complementary to the 3′ and 5′ end 15 nt of the template oligonucleotides. The nicks produced upon annealing were sealed by T4 DNA ligase. The resulting linear single-stranded template was then amplified by PCR using the two oligonucleotides 5′-AAG GAT CCA ATG TTA CAG CAG GAA TCG TTC GCC CCA TCT TTT CCT GCT TC-3′ and 5′-TTA AGC TTT CAT ATT CCA GCA ACT TCC AGA TGT TGT CCT CCG ATA ACT GG-3′. The amplified PCR fragments cleaved by HindIII (large subunit) and NotI (middle subunit) were cloned into pET-Duet vector (Novagen) stepwise to obtain pET-Duet-CeRPA1&2. The orientation of the inserts and their nucleotide sequence were confirmed by DNA sequencing. The expression vector thus constructed produced the large RPA subunit as a fusion protein with additional amino acids (MGSS**HHHHHH**SQD; in boldface, a hexahistidine tag) at its N-terminus, while the middle subunit as wild type. The amplified PCR fragments of the small RPA subunit were digested by NdeI and then ligated into pACYC-Duet vector (Novagen) to obtain pACYC-Duet-CeRPA3. The small subunit expressed from this vector contained no additional amino acids.

Purification of recombinant CeDna2 and RPA proteins

The recombinant baculovirus expressing CeDna2 was infected into Sf-9 insect cells for 48 h at a multiplicity of infection of 10. Infected cells (2×10^6 cells/ml, 1 liter) were harvested, resuspended in 80 ml of buffer T (25 mM Tris–HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 mM phenyl-methylsulfonyl fluoride, 0.15 µg/ml leupeptin and antipain) containing 100 mM NaCl and 1% Nonidet P-40, and disrupted by sonication (10 cycles of a 30 s pulse and a 30 s cooling interval). The extracts were cleared by centrifuging at 37 000 r.p.m. for 1 h in a Beckman 45Ti rotor, and the supernatant (0.9 mg/ml, 80 ml) was applied directly to a 10 ml Q-Sepharose (Amersham Pharmacia Biotech) column equilibrated with buffer T plus 100 mM NaCl (buffer T_{100}, hereafter, the number indicates the concentration of NaCl added to buffer T). The column was washed with 10-column volumes of the same buffer containing neither DTT nor EDTA and eluted with buffer T_{500} (−DTT and −EDTA) plus 5 mM imidazole. The peak fractions (0.68 mg/ml, 25 ml) were pooled and loaded onto a Ni$^+$-NTA agarose (Qiagen) column (2 ml) equilibrated with buffer T_{500} (−DTT and −EDTA) plus 5 mM imidazole. After extensive washing with buffer T_{500} (−DTT and −EDTA) plus 20 mM imidazole, the column was eluted with buffer T_{500} plus 400 mM imidazole. The eluted fractions (1.4 mg/ml, 5 ml) were pooled, mixed with 400 µl of anti-FLAG M2 Ab-agarose beads (Sigma) equilibrated with buffer T_{500} and incubated at 4°C for 4 h on a rocking platform. After washing three times with 4 ml of buffer T_{500} and three times with 4 ml of buffer T_{200}, bound protein was eluted three times by incubation at 4°C for 30 min with 400 µl of buffer T_{200} containing 0.2 mg/ml of the FLAG peptide. The first eluted fraction (0.19 mg/ml, 0.4 ml) contained >70% of total protein recovered and was >95% pure. This fraction was stored at −80°C.

Recombinant yeast and human RPAs were purified from *E.coli* as described previously (27,31). Recombinant CeRPA from *E.coli* was purified similarly as for human RPA, but with modifications; Blue-Sepharose column chromatography was

omitted and a glycerol gradient sedimentation step was included. The two expression vectors, pET-Duet-CeRPA1&2 and pACYC-Duet-CeRPA3, were co-transformed into BL21 (DE3), and the expression of three RPA subunits were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside. The cells (1.5 liter culture) were harvested, suspended in 40 ml of T_{500} without EDTA and DTT, and disrupted as described previously (27). Crude extracts obtained were cleared by centrifuging at 37 000 r.p.m. for 1 h in a Beckman 45Ti rotor, and the supernatant (4.5 mg/ml, 40 ml) was applied directly to a 5 ml Ni$^+$-NTA agarose (Qiagen) column equilibrated with buffer T_{500} (−DTT and −EDTA) plus 5 mM imidazole. After washing the column with 25 ml of T_{100} (−DTT and −EDTA) plus 30 mM imidazole, proteins were eluted with T_{100} (−DTT and −EDTA) plus 400 mM imidazole. The peak protein fractions (1.0 mg/ml, 16ml) were pooled and applied to a 1 ml Hitrap Q-Sepharose (Amersham Pharmacia Biotech) column. This column was washed with 10 ml of T_{100}, followed by elution with a 15 ml linear gradient (100–600 mM) of NaCl in T buffer. The peak protein fractions eluting at ∼350 mM NaCl were pooled and concentrated using a Centricon centrifugal filter device (Millipore) according to the manufacturer's recommendations. The concentrated CeRPA (1.2 mg/ml, 0.5 ml) was loaded onto a glycerol gradient (5 ml, 15–35% glycerol in T_{250}) and subjected to centrifugation at 45 000 r.p.m. for 24 h in a SW 55Ti rotor (Beckman) at 4°C. Fractions were collected from the bottom of the gradient and analyzed by SDS–PAGE. The peak protein fractions (>95% in purity) lacked any detectable nuclease and ATPase activities and were stored at −80°C.

Preparation of substrates for the helicase and nuclease activity assay

The DNA substrates used to measure helicase activity of CeDna2 were constructed by hybridizing the oligonucleotides listed in Table 1 to ΦX174 single-stranded circular (ssc) DNA and purified as described previously (13). These partial duplex substrates used to characterizing the helicase and endonuclease activities of CeDna2 were prepared as described previously (17) using the synthetic oligonucleotides listed in Table 1. Labeling of the 5′ or 3′ ends of the substrates was as described previously (17). The structure of DNA substrates and position of radioisotope labels are indicated in each figure.

Enzyme assays

The DNA-dependent ATPase activity was measured in a reaction mixture (20 μl) containing 50 mM Tris–HCl, pH 7.8, 2 mM DTT, 2 mM MgCl$_2$, 0.25 mg/ml BSA, 250 μM cold ATP, 8.25 nM [γ-^{32}P]ATP (>3000 Ci/mmol) and 50 ng of M13 ssDNA when necessary. After incubation at 37°C for 10 min, an aliquot (2 μl) was spotted onto a polyethyleneiminecellulose plate (J. T. Baker) and developed in a 0.5 M LiCl/1.0 M formic acid. The products were analyzed using a PhosphorImager (Fuji).

Helicase assays were performed with the endonucleasedeficient mutant enzyme, HF-CeDna2D320A. The standard reaction mixtures (20 μl) contained 50 mM Tris–HCl, pH 7.8, 2 mM DTT, 2 mM MgCl$_2$, 2 mM ATP, 0.25 mg/ml BSA and the 5′-^{32}P-labeled partial duplex DNA substrate

(15 fmol). After incubation at 37°C for 10 min, reactions were stopped with 4 μl of 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]. The reaction products were subjected to electrophoresis for 40 min at 180 V through a 10% polyacrylamide gel containing 0.1% SDS in 0.5× TBE (45 mM Tris base, 45 mM boric acid and 1 mM EDTA). The gel was dried on DEAE-cellulose paper and autoradiographed. Labeled DNA products were quantitated with the use of a PhosphorImager.

To measure endonuclease activity of CeDna2, we used the standard reaction mixtures (20 μl) containing 50 mM Tris–HCl, pH 7.8, 5 mM MgCl$_2$, 2 mM DTT, 0.25 mg/ml BSA and the 5′- or 3′-^{32}P-labeled DNA substrate (15 fmol). After incubation at 37°C for 10 min, reactions were stopped with 6× stop solution. The cleavage products were resolved on a 10% polyacrylamide gel containing 0.1% SDS in 0.5× TBE (electrophoresis for 40 min at 150 V) or on a 20% sequencing gel for high resolution. The gel was dried on DEAE-cellulose paper and autoradiographed. The amount of products formed was measured using a PhosphorImager. Enzymes were diluted to 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.

RESULTS

Purification of recombinant CeDna2 enzymes

To facilitate the detection and purification of recombinant wild-type CeDna2 enzyme, hexahistidine and two tandem FLAG epitopes were fused to the N-terminus of CeDna2 (HF-CeDna2). HF-CeDna2 was purified from cell-free extracts prepared from insect cells infected with recombinant baculoviruses expressing HF-CeDna2 using Q-Sepharose, Ni$^+$-NTA agarose and anti-FLAG M2 Ab-agarose column chromatography as described in 'Materials and Methods.' As shown in Figure 1, crude extracts, fractions from each purification step and purified HF-CeDna2 were subjected to 8% SDS–PAGE. The gels in duplicate were Coomassie stained (Figure 1, lanes 1–7) and analyzed by western blotting (Figure 1, lanes 8–14). Expression of wild-type CeDna2 was detected in the crude extracts prepared from infected, but not from uninfected insect cells with monoclonal antibodies against the hexahistidine epitope (Figure 1, compare lanes 8 and 9). Each purification step progressively enriched HF-CeDna2 (Figure 1, lanes 2–5 and 9–12), resulting in the preparation of CeDna2 of purity >95% after the final anti-FLAG M2 Ab-agarose chromatographic step (Figure 1, lanes 5 and 12). To prepare endonuclease- and ATPasedeficient mutant enzymes, conserved amino acid in CeDna2 (corresponding to those critical for endonuclease and ATPase activities of *S.cerevisiae* Dna2) was changed (14,24). Alteration of Asp320 to Ala and Lys678 to Glu resulted in the formation of the endonuclease-deficient (HF-CeDna2D320A) and ATPase/helicase-deficient (HF-CeDna2K678E) CeDna2, respectively. The two mutant enzymes were purified using the same procedure described for the wild-type enzyme. The purity and yield of the mutant enzymes were comparable with those of wild-type CeDna2 (data not shown) (Figure 1A, lanes 6 and 7; lanes 13 and 14).
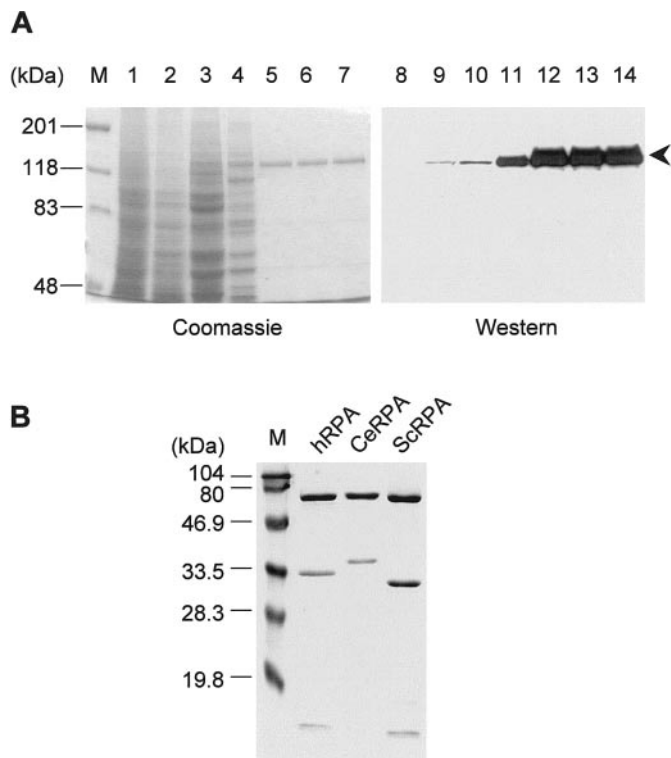
Figure 1. SDS–PAGE and western blot analysis of recombinant CeDna2 and CeRPA proteins. (**A**) Crude extracts, fractions obtained during each purification step, and purified wild-type and mutant CeDna2 enzymes were subjected to 8% SDS–PAGE. The gel was stained with Coomassie blue (left) and analyzed in western blot analysis (right) using monoclonal antibodies specific for the hexahistidine epitope. Lanes 1 and 8, crude extracts (50 μg for SDS–PAGE; 10 μg for western blot analysis) from uninfected insect cells; lanes 2 and 9, crude extracts (50 μg; 10 μg) from insect cells infected with baculovirus overproducing wild-type CeDna2; lanes 3 and 10, fractions (50 μg; 10 μg) obtained from Q-Sepharose column chromatography; lanes 4 and 11, active fractions (20 μg; 5 μg) from the Ni²⁺-NTA column; lanes 5 and 12 (1 μg; 200 ng), fractions from the anti-FLAG affinity column. Lanes 2–5 and 9–12, wild-type HF-CeDna2. Lanes 6 and 13, purified HF-CeDna2D320A (endonuclease-deficient; 1 μg and 200 ng, respectively); lanes 7 and 14, HF-CeDna2K678E (helicase-deficient; 1 μg and 200 ng, respectively). The numbers at the left of the Figure indicate the molecular size (in kDa) of marker proteins (indicated as M). The position of HF-CeDna2 is also indicated. (**B**) Purified recombinant RPA proteins (1 μg each) isolated from *E.coli* overproducing strains were subjected to 12% SDS–PAGE and the gel was stained with Coomassie blue. hRPA, human RPA; CeRPA, *C.elegans* RPA; and ScRPA, *S.cerevisiae* RPA. The numbers at the left of the Figure indicate the molecular size (in kDa) of marker proteins (indicated as M).

In order to examine the influence of RPA on CeDna2, we isolated the CeRPA. A database search for protein sequences similar to *S.pombe* RPA1, RPA2 and RPA3 yielded hypothetical proteins homologous to each subunit from *C.elegans* (accession no. AAB63407, 655 amino acids; accession no. AKK71380, 288 amino acids; and accession no. AAB37031, 143 amino acids, respectively). cDNAs encoding these three CeRPA subunits cloned from *C.elegans* cDNA libraries were coexpressed in *E.coli* and purified as described in 'Materials and Methods'. Although all three proteins were expressed relatively efficiently in soluble forms (data not shown), the small subunit of CeRPA was not present in the final preparation of CeRPA unlike the human and yeast RPA preparations (Figure 1B). Densitometric analysis of the final preparation indicated that the large and middle subunits were stoichiometric (data not shown). This is an unexpected finding since the large subunit of RPA can associate with a preassembled sub-complex containing the middle and small subunits in human RPA (31,32). AAB37031 appears to encode a CeRPA3 subunit that contains an additional 23 amino acid peptide at its N-terminus unlike other RPA3 proteins. If this region is excluded in alignment analysis, AAB37031 showed a weak, but significant sequence homology (identity 20–29%) to RPA3 from human, *S.cerevisiae* and *S.pombe* (data not shown). This level of homology is slightly lower than those (31–38%) found among human, *S.cerevisiae* and *S.pombe* RPA3. At present, however, we cannot rule out the possibility that the putative CeRPA3 subunit we have cloned is not the authentic CeRPA3 subunit.

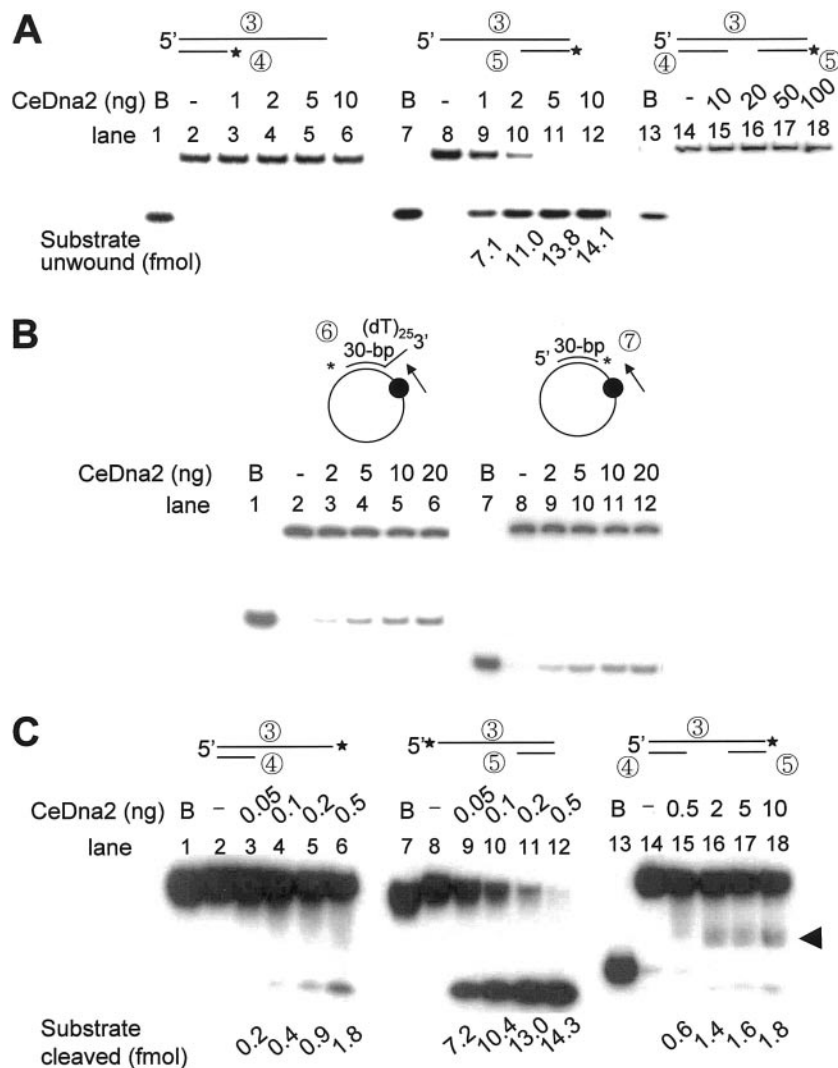## Enzymatic activities of recombinant CeDna2 enzymes

The CeDna2 preparations described above were examined for the presence of DNA-dependent ATPase and endonuclease activities (Figure 2). The ATPase-deficient mutant (HF-CeDna2K678E) enzyme did not hydrolyze ATP at any concentrations of protein used (Figure 2A), while the wild-type and endonuclease-deficient mutant (HF-CeDna2D320A) proteins exhibited comparable ATPase activities (Figure 2A). Wild-type CeDna2 was dependent on the presence of ssDNA for the hydrolysis of ATP (Figure 2A). HF-CeDna2 and HF-CeDna2K678E cleaved a Y-structured 5′ end labeled substrate with indistinguishable efficiency (Figure 2B, compare lanes 1–4 and 11–14), whereas HF-CeDna2D320A was completely inactive (Figure 2B, lanes 6–9). These results indicate that each point mutation inactivated the enzymatic activity of interest as intended.

Both helicase and endonuclease activities of CeDna2 act preferentially upon 5′ ssDNA tails

Since the endonuclease activity of CeDna2 cleaved substrate DNAs, HF-CeDna2D320A was used to characterize the DNA helicase activity of CeDna2. We first determined the polarity of duplex unwinding by this CeDna2 mutant enzyme. As shown in Figure 3A, HF-CeDna2D320A displaced a partial duplex DNA substrate containing a 5′ ssDNA tail (Figure 3A, lanes 7–12), but not a 3′-tailed ssDNA (Figure 3A, lanes 1–6), indicating that this enzyme translocates and unwinds duplex DNA in the 5′ to 3′ direction. The DNA substrate with ssDNA flanked by duplex DNA at both ends was hardly unwound by the CeDna2 helicase (Figure 3A, lanes 13–18). We also examined whether a forked DNA structure influenced the CeDna2 helicase activity. For this purpose, two DNA substrates were prepared, one containing a 25 nt 3′ ssDNA tail and the other without any tail (Figure 3B). Unwinding of the two substrates by CeDna2 was comparable when the same amounts of enzyme were used (Figure 3B), suggesting that a forked DNA structure does not stimulate the helicase activity of CeDna2.

We then examined the endonuclease activity of CeDna2 using the same three substrates that were used to determine the polarity of CeDna2 helicase activity (Figure 3A). These three substrates were labeled at either the 5′ or 3′ end of the template DNA (Figure 3C). HF-CeDna2 degraded the 5′ overhang substrate most efficiently (Figure 3C, lanes 9–12), and hardly degraded the 3′ overhang substrate. The amount of the

Figure 2. Analysis of endonuclease and ATPase activities of wild-type and mutant CeDna2 proteins. (**A**) ssDNA-dependent ATPase activity of the wild-type and mutant CeDna2 proteins is measured. Reaction mixtures (20 µl) contained 2 mM $MgCl_2$, 250 µM ATP, 8.25 nM [γ-^{32}P]ATP (>3000 Ci/mmol) and the indicated amounts of enzyme. The mixtures were incubated at 37°C for 10 min, and the hydrolysis of ATP was measured as described previously (14). Closed and open symbols in the plot denote that ATPase activity of each enzyme used was measured in the presence and absence of M13 sscDNA, respectively. The experiment was repeated four times, and the average of the four experiments is shown with error bars as indicated. (**B**) Endonuclease activity of purified recombinant wild-type and mutant CeDna2 enzymes was measured with the Y-structured substrate as indicated at the top of the Figure. The position of the 5′ ^{32}P-labeled end (denoted as asterisks) is as shown. Increasing levels (0.02–0.2 ng) of wild-type or mutant CeDna2 enzyme were incubated with the substrate DNA (15 fmol) at 37°C for 10 min in standard reaction mixtures as described in 'Materials and Methods'. Cleavage products formed were analyzed in 10% acrylamide gel as described previously (13). The amount (fmol) of cleavage product formed is indicated at the bottom of the gel. For the reactions shown in lanes 5, 10 and 15, Mg^{2+} was omitted.

60 nt 5′ tail cleaved with 0.05 ng (0.6 fmol) of CeDna2 was 7.2 fmol (Figure 3C, lane 9), while the amount of the 60 nt 3′ tail cleaved with 10-fold more enzyme was only 1.8 fmol (Figure 3C, lane 6). Thus, the cleavage efficiency of the 5′ ssDNA tail by CeDna2 was at least 40-fold greater than that of the 3′ ssDNA tail. These results also indicate that CeDna2 acts in a highly catalytic fashion in cleaving a 5′ ssDNA tail. Consistent with the result that a DNA substrate with ssDNA flanked by partial duplexes at both ends was hardly unwound by CeDna2 helicase, it was poorly degraded even in the presence of considerably higher levels (0.5–10 ng) of CeDna2 (Figure 3C, lanes 15–18). Our results above demonstrate that CeDna2 requires a 5′ free ssDNA end for endonucleolytic cleavage as well as for duplex unwinding.

CeDna2 can utilize short 5′ ssDNA tails for endonuclease and helicase activity

In our preliminary studies, we found that a 5′ ssDNA tailed (>10 nt) DNA substrate was efficiently degraded to 2–3 nt sized products by CeDna2 endonuclease activity (data not shown and see below). In contrast, the 5′ ssDNA tails left after cleavage by ScDna2 were highly heterogeneous and longer (>6 nt) than those generated by CeDna2 (17). This result indicates that CeDna2 can cleave very short 5′ ssDNA tails. In order to determine the minimal length of the 5′ tail ssDNA cleaved by CeDna2, several substrates with varying 5′ ssDNA tail lengths (Figure 4A, 5–10 nt and 25 nt) were examined. In this experiment, we used two different concentrations of Mg^{2+} (0.5 and 5 mM), since the size of cleavage products was significantly affected by Mg^{2+} concentrations used (data not shown). In the presence of 0.5 mM $MgCl_2$, CeDna2 released substantial levels of 10–11 nt long products from the 5′ oligo (dT)$_{25}$ tail substrate (Figure 4A, lane 7), whereas substrates with short 5′ oligo (dT)$_{5–8}$ tails were poorly cleaved by CeDna2 (Figure 4A, lanes 1–4). When the oligo (dT) is >8 nt, cleavage occurred efficiently (Figure 4A, lanes 5 and 6). Although the oligo (dT)$_{25}$ tail gave rise to 10–12 nt cleavage products, the most prominent products from 5′ oligo (dT)$_{6–10}$ were 5 nt. Products longer than the size of the

Figure 3. DNA helicase activity of CeDna2. The structures of the partial duplex DNA substrates used are shown at the top of each figure. The asterisks indicate ^{32}P-labeled ends. (**A**) HF-CeDna2D320A, the endonuclease-deficient mutant enzyme, was used to determine the helicase properties of CeDna2. Reaction mixtures (20 µl) containing indicated amounts of HF-CeDna2D320A and 15 fmol of each substrate were incubated at 37°C for 10 min and the products were analyzed on a 10% polyacrylamide gel. *B* denotes boiled substrate controls. The amount (fmol) of unwound substrates is presented at the bottom of the figure. (**B**) Reaction mixtures (20 µl) containing either the flush-ended or 25 nt 3′-tailed substrate (15 fmol) and indicated amounts of HF-CeDna2D320A were incubated at 37°C for 10 min. The products were analyzed on 10% polyacrylamide gel. (**C**) The partial duplex DNA substrates used and the positions of ^{32}P-labeled end (indicated as asterisks) are as indicated at the top of the Figure. Reaction mixtures (20 µl) containing the indicated levels of wild-type CeDna2 and 15 fmol of each substrate were incubated at 37°C for 10 min, and the reaction products were analyzed as in Figure 2B. *B* denotes the boiled substrate controls. The amount (fmol) of substrate cleaved is presented at the bottom of the figure. Note that increased levels (10–20-fold) of wild-type HF-CeDna2 were used for the cleavage of the substrate with internal ssDNA flanked by partial duplexes at both ends. The arrowhead indicates the migration position of cleavage products from this substrate.

5′ ssDNA tail were also produced. These products were formed most likely due to the melting of the duplex region at low Mg^{2+} concentrations since they were not observed in higher Mg^{2+} concentrations (see below). CeDna2 catalyzed cleavage of the 5′ oligo $(dT)_{6-10}$ tails more efficiently at 5 mM than at 0.5 mM Mg^{2+}. When the length of the 5′ oligo (dT) tail was varied from 7 to 25 nt, the major cleavage product was 5 nt long (Figure 4A, lanes 10–14). In the presence of 5 mM Mg^{2+}, cleavage products longer than the length of 5′ tail of the substrate were hardly observed unlike reactions carried out with 0.5 mM Mg^{2+}. Since the CeDna2 endonuclease reaction is more specific and efficient with 5 mM of Mg^{2+}, all of the subsequent endonuclease assays in this report contained 5 mM

of Mg^{2+}. We also examined the influence of levels of HF-CeDna2 on the cleavage of 5′ oligo (dT)-tailed substrates of various lengths. In keeping with the results described above, the efficiency of nuclease activity increased as the 5′ ssDNA tail length was increased (Figure 4B). We also examined the influence of the length of the 5′ oligo (dT) tails on the helicase activity of CeDna2D320A. Unwinding of the substrate was observed when it contained a 5′ oligo (dT) tail longer than 8 nt (Figure 4C).

A comparison of the cleavage activity of ScDna2 and CeDna2 was carried out with two substrates containing a 5′ oligo $(dT)_7$ or $(dT)_{10}$ tail (Figure 5). Both 5′ oligo $(dT)_7$ and $(dT)_{10}$ tails were much more efficiently (30–40-fold)

degraded by CeDna2 than by ScDna2 (Figure 5). CeDna2 (0.2 ng, 1.7 fmol) cleaved 2.6 fmol of the 5′ oligo $(dT)_7$ tail and 10.8 fmol of the 5′ oligo $(dT)_{10}$ tail (Figure 5, lanes 2 and 9, respectively), indicating that CeDna2 can act catalytically in cleavage of the short 5′ ssDNA tail. In contrast, ScDna2 hardly cleaved the 5′ oligo $(dT)_7$ tail (Figure 5). Consistent with this result, ScDna2 helicase activity unwound the DNA substrates with the 5′ oligo $(dT)_7$ or $(dT)_{10}$ tail poorly (data not shown).

These results confirm that CeDna2 can utilize short 5′ ssDNA tails for cleavage or unwinding, whereas ScDna2 cannot.

The influence of an RNA segment in a chimeric RNA–DNA flap on endonuclease activity of CeDna2

We investigated the effect of a terminal RNA segment in a chimeric RNA–DNA on the endonuclease activity of CeDna2. Such RNA–DNA structures are likely to be present in a flap generated by displacement DNA synthesis catalyzed by pol δ during Okazaki fragment extension. For this purpose, the cleavage of two different 25 nt flap substrates by CeDna2 were examined; one of the substrates contained a chimeric 12 nt oligo (U) and 13 nt oligo (dT) flap and the other a 25 nt oligo (dT) flap (Figure 6A). The presence of the 5′ terminal RNA segment in the flap substrate slightly inhibited endonucleolytic cleavage by CeDna2 at all concentrations of Mg^{2+} tested (Figure 6A and B). The reduction of the cleavage efficiency with the 5′ terminal RNA flap was very low and might as well be within the experimental error. This contrasts with the observation that endonuclease activity of ScDna2 is stimulated by the presence of a terminal RNA segment in a chimeric RNA–DNA flap (17). The cleavage of the RNA–DNA chimeric flap resulted in the generation of cleavage products of 14 nt or longer in length (Figure 6A, lanes 6–8), indicating that CeDna2 does not degrade the oligoribonucleotide. Thus, the endonuclease activity of CeDna2 is specific for ssDNA, like ScDna2 (17).

Influence of CeRPA on enzymatic activities of CeDna2

To determine whether RPA can stimulate CeDna2, recombinant CeRPA was prepared as described above. Although our preparations of CeRPA lacked the small subunit, we found that it binds to ssDNA one-third as efficiently as hRPA or ScRPA (data not shown). Despite this, we used these preparations to examine the effect of CeRPA on the enzymatic activities associated with CeDna2. We first checked whether CeRPA stimulates DNA unwinding activity of HF-CeDna2D320A. In preliminary experiments, we found that CeRPA neither stimulated nor inhibited unwinding of duplex DNA by CeDna2 in the absence of exogenous salt (data not shown),
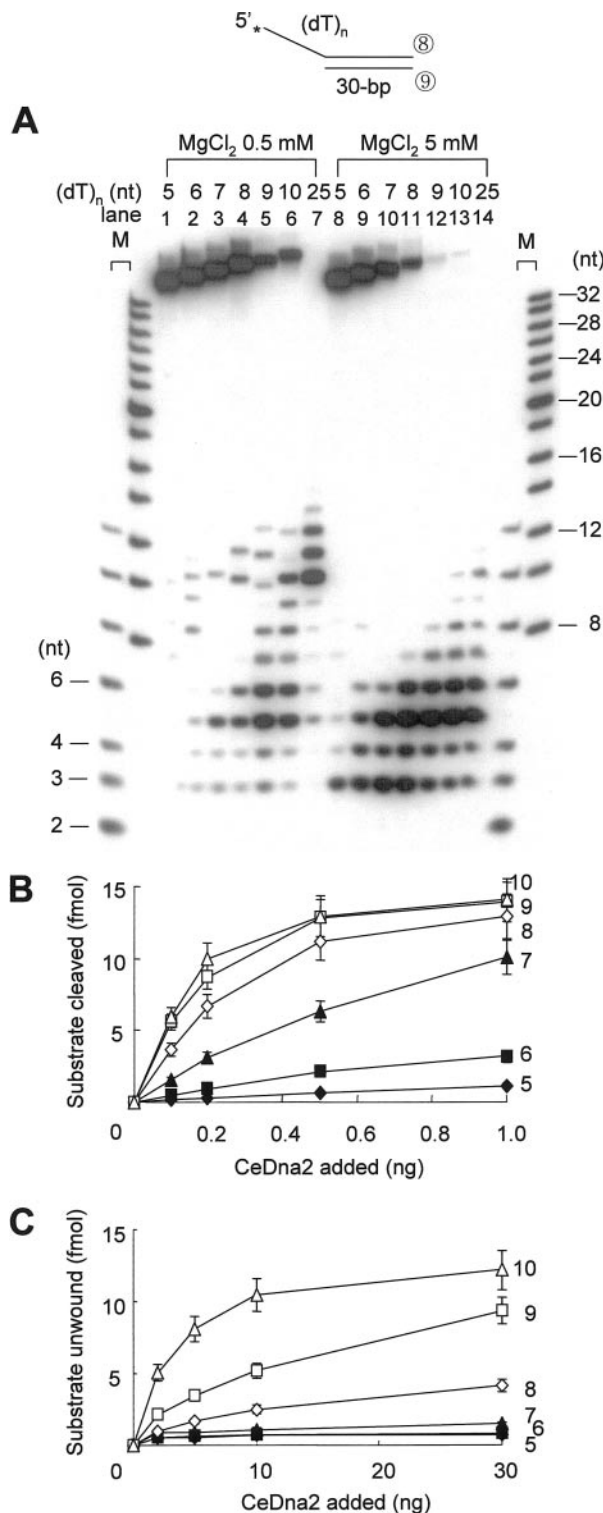
Figure 4. CeDna2 cleaves very short 5′ free ssDNA. The partial duplex DNA substrates containing varying lengths of the 5′-tail were as shown at the top of the Figure. The asterisk indicates the ^{32}P-labeled end. (**A**) Reaction mixtures (20 µl) containing 15 fmol of the substrate and 0.2 ng of HF-CeDna2 were incubated at 37°C for 10 min in the presence of two different levels (0.5 or 5 mM) of $MgCl_2$. The length (nt) of the 5′ tail $(dT)_n$ in the substrate used in each lane is as indicated in the Figure. The cleavage products were then analyzed on a 20% polyacrylamide gel, and M denotes the molecular size markers. The numbers (in nt) shown on the left and right of the Figure indicate the size of the markers. (**B**) Reaction mixtures (20 µl) containing 15 fmol of each substrate with a differing length (5–10 nt) of the 5′ (dT) tail and 5 mM $MgCl_2$ were incubated at 37°C for 10 min in the presence of indicated amounts of CeDna2. The products were analyzed on a low-resolution polyacrylamide gel (10%). The amount of substrate cleaved was measured with the use of a PhosphorImager and plotted against the amounts of enzyme used. The symbols for the length of 5′ dT tail are as shown. (**C**) The same experiment as (B) was repeated with HF-CeDna2D320A in place of wild-type HF-CeDna2, and 2 mM of ATP was added to support unwinding of the substrate. The unwound products were analyzed on a 10% polyacrylamide gel and quantitated as described in Figure 3A. For (B and C), the experiments were repeated three times, and the average of the three experiments is shown with error bars as indicated.
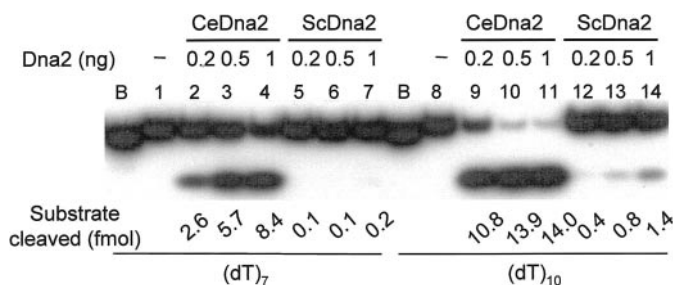
Figure 5. Comparison of the cleavage of short 5′ free ssDNAs by CeDna2 and ScDna2. The partial duplex substrates used were the same as depicted in Figure 4A. The two 5′ overhang substrates with 7 and 10 nt dT tails [(dT)$_7$ and (dT)$_{10}$, respectively] were used in this experiment and are as indicated. Reaction mixtures (20 µl) containing 15 fmol of the indicated substrates and 0.2–1 ng of HF-CeDna2 or ScDna2 were incubated at 37°C for 10 min in the presence of 5 mM MgCl$_2$. The cleavage products were then analyzed on a low-resolution 10% polyacrylamide gel.

but stimulated the helicase activity of CeDna2 in the presence of a physiological salt concentration (125 mM NaCl). In the presence of this salt condition, the rates of CeDna2-catalyzed unwinding reaction and cleavage of a flap-structured substrate were examined in the presence of increasing levels (0–50 ng) of CeRPA (Figure 7). Both the unwinding (Figure 7A and B) and the endonuclease (Figure 7C and D) activities were stimulated by increasing levels of CeRPA.

Stimulation of Dna2 by RPA is species-specific

Preliminary studies indicated that ScDna2 was stimulated by ScRPA, but inhibited by human RPA, suggesting that there may be a species-specific interaction between RPA and Dna2. In order to examine this possibility, RPAs from human and yeast were also isolated as shown in Figure 1B. The influence of the three different RPA complexes (from human, yeast and *C.elegans*) on endonuclease activity of ScDna2 (Figure 8A) was examined initially. In the absence of RPA, ScDna2 (20 ng) cleaved 1.8 fmol of the 5′ ssDNA tail substrate (Figure 8A, lane 2) in the presence of 125 mM NaCl in the reaction. It was noted that ScDna2 endonuclease activity was severely inhibited in the presence of 125 mM NaCl (data not shown). For this reason, an increased level (20 ng) of ScDna2 enzyme was used to measure the cleavage. The addition of ScRPA (10 and 50 ng) markedly stimulated the endonuclease activity of ScDna2 (to a saturation level) (Figure 8A, lanes 9 and 10). With lower levels (2 ng) of ScDna2, the addition of 2 and 10 ng of ScRPA increased substrate cleavage 10- and 40-fold, respectively (Figure 8A, lanes 12–15). The addition of CeRPA (10 or 50 ng) marginally stimulated (1.5-fold) the ScDna2 endonuclease activity (Figure 8A, lanes 3 and 4). ScDna2 endonuclease activity was slightly enhanced at lower levels (10 ng), but inhibited at higher levels (50 ng) of human RPA (Figure 8A, lanes 6 and 7). RPA from all three species did not contain detectable nuclease activity (Figure 8A, lanes 5, 8 and 11). These results demonstrate that ScDna2 endonuclease activity is stimulated specifically by ScRPA. Additional experiments were carried out to further establish that the species-specific effects of RPA applied to CeDna2 as well. As shown in Figure 8B and C, neither ScRPA nor hRPA stimulated the
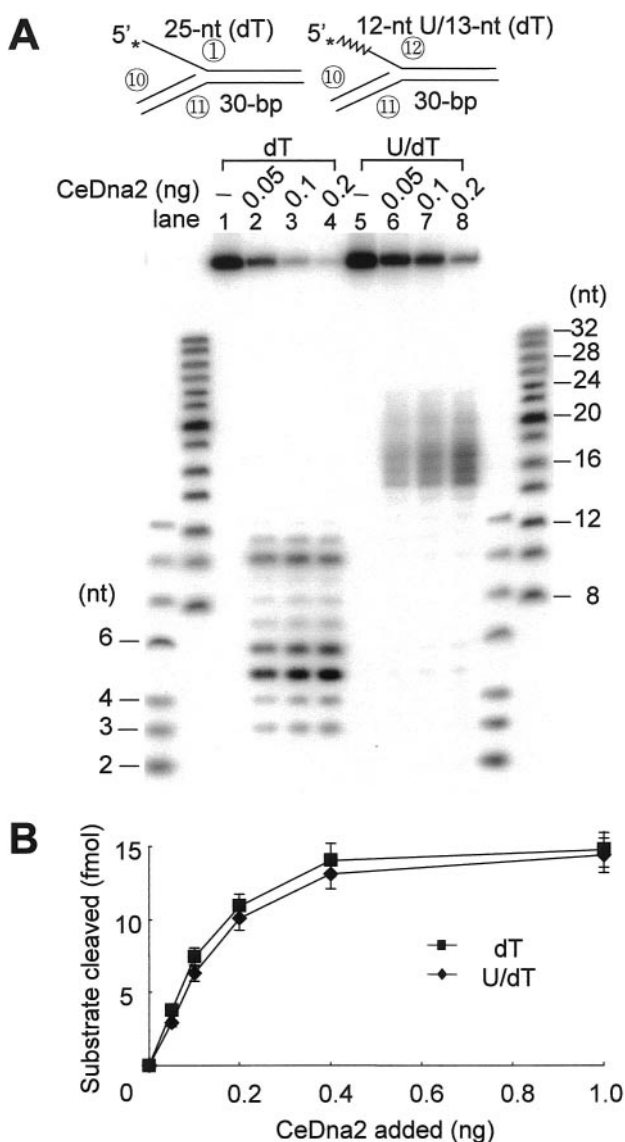
Figure 6. Influence of the 5′ terminal RNA segment on CeDna2 endonuclease activity. The two flap substrates used are shown at the top of the Figure (asterisk, ^{32}P-labeled ends). The ssDNA 5′ tail (25 nt in length) consists of a homopolymeric 12 nt oligonucleotide (U) segment [(U)$_{12}$, wave line], a 13 nt oligo(dT) segment [(dT)$_{13}$, thin line]. (**A**) The two substrates were incubated with the indicated amount of HF-CeDna2 at 37°C for 10 min in the presence of 5 mM MgCl$_2$ and the reaction products formed were analyzed on a 20% denaturing polyacrylamide gel. M denotes molecular size markers. The numbers shown on the left and right of the Figure indicate the size of the markers. dT, the flap with the 5′ DNA only tail; U/dT, the flap with an RNA–DNA chimeric tail. (**B**) The reaction mixtures (20 µl) containing 0.5 mM Mg^{2+} and 15 fmol of the RNA–DNA chimeric (filled squares) or DNA only substrate (filled diamonds) were incubated at 37°C for 10 min in the presence of increasing amounts (0.05, 0.1, 0.2, 0.4 or 1.0 ng) of enzyme. The amount of substrate cleaved is presented in the Figure. dT and U/dT are the same as indicated above. The average values of four experiments are shown with error bars as indicated.
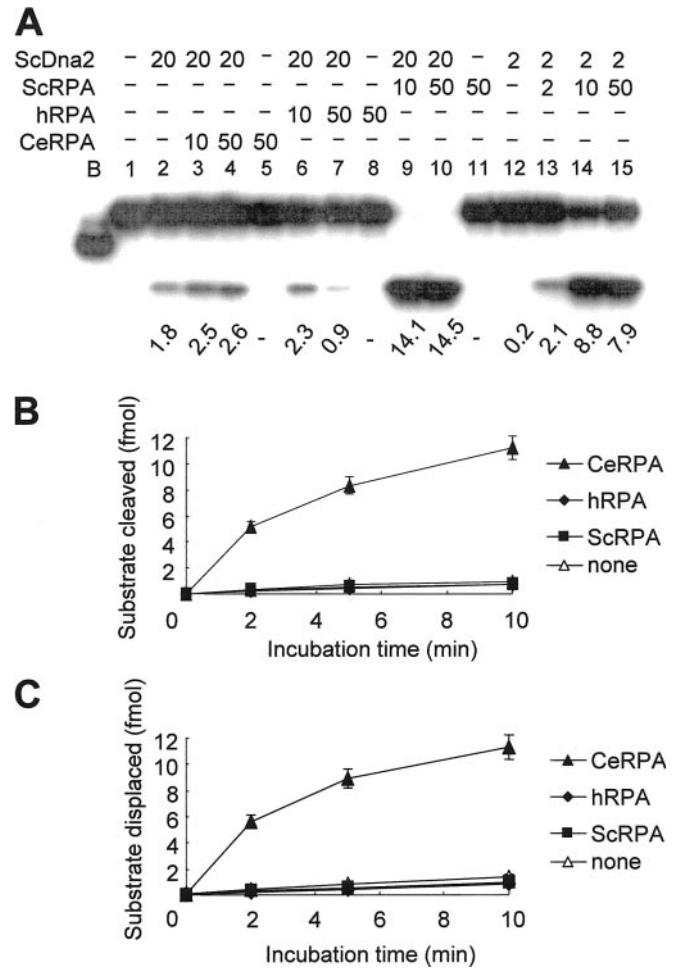
CeDna2 endonuclease or helicase activity, whereas CeRPA markedly stimulated both. From these results, we conclude that a species-specific protein–protein interaction between Dna2 and RPA is essential for the stimulation of Dna2 enzymatic activities.
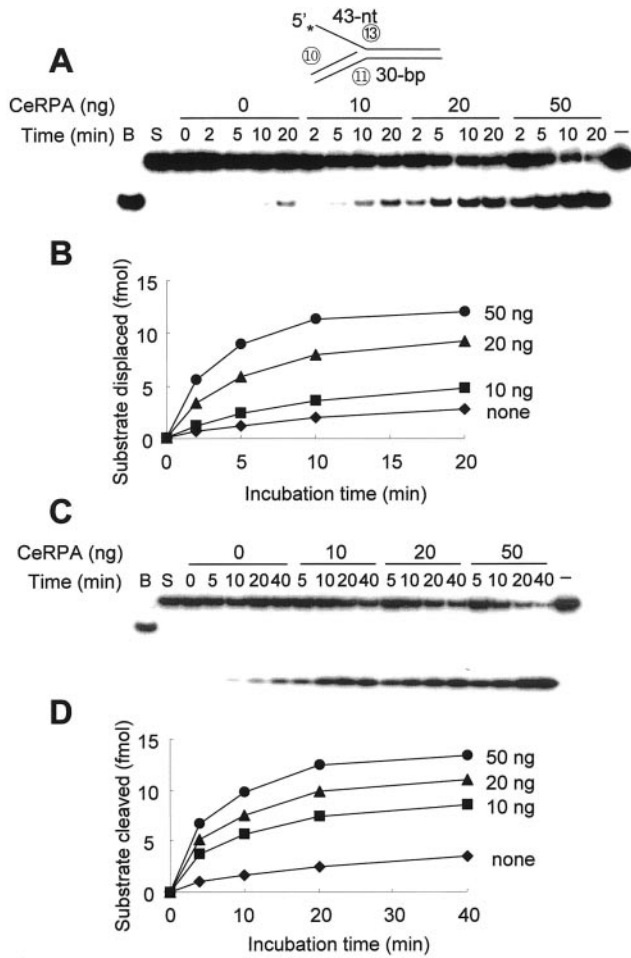
Figure 7. Stimulation of CeDna2 helicase and endonuclease activities by CeRPA. The flap-structured substrate used in this experiment and the position of the ^{32}P-labeled end is shown at the top of the Figure. (**A**) Reaction mixtures (20 μl) containing 15 fmol of the substrate, 2 mM ATP and 125 mM NaCl were preincubated on ice for 10 min in the presence of the indicated amounts (0–50 ng) of CeRPA, followed by incubation at 37°C for 10 min with HF-CeDna2D320A (10 ng). The products formed were then analyzed on a 10% polyacrylamide gel and quantitated as described in 'Materials and Methods'. (**B**) The amount of substrate unwound by enzyme in (A) is presented in the Figure. The levels of CeRPA added are as indicated. (**C**) The same reaction was repeated as in Figure 7A, but in the absence of ATP with 2 ng of HF-CeDna2. (**D**) The amounts of substrate cleaved by enzyme in (C) and the level of CeRPA used were as indicated. The experiment was repeated three times, and one representative result is shown.

Figure 8. The RPA stimulation of the enzymatic activities of Dna2 is species-specific. The substrate used and reactions carried out in this experiment were the same as described in Figure 7. (**A**) Effects of RPA from different species on the endonuclease activity of ScDna2. Reaction mixtures (20 μl) containing 15 fmol of the substrate were incubated at 37°C for 10 min in the presence of different RPA (Sc, *S.cerevisiae*; h, human; Ce, *C.elegans*). The amounts of RPA and ScDna2 added are as indicated in the figure. (**B**) Influence of incubation time on the endonuclease activity of CeDna2. Reaction mixtures (20 μl) containing 2 ng of HF-CeDna2 and 15 fmol of the DNA substrate were incubated at 37°C for the indicated times in the presence of different RPA as indicated. The amount of RPA used in each reaction was 50 ng. (**C**) A time-course experiment for helicase activity of CeDna2. Reaction mixtures (20 μl) containing 10 ng of HF-CeDna2D320A and 15 fmol of the DNA substrate were incubated at 37°C for the indicated times in the presence of different RPAs as indicated. The amount of RPA used for each reaction was 50 ng. For (B and C), the experiments were repeated three times, and the average of the three experiments is shown with error bars as indicated.

DISCUSSION

In this paper, we expressed and purified wild-type and mutant recombinant CeDna2 and CeRPA proteins and extensively characterized the enzymatic properties of CeDna2 in an effort to gain insights into Okazaki fragment maturation of a multi-cellular organism. The open reading frame (1069 amino acids) of CeDna2 deduced from the full-length cDNA sequence is significantly shorter than ScDna2 (1522 amino acids) because it lacks the 405 amino acid domain present at the N-terminus of ScDna2. Nevertheless, the essential ATPase (11,12) and nuclease (13) motifs in ScDna2 are well conserved in CeDna2 and recombinant CeDna2 possessed robust endonuclease and ATPase activities, like ScDna2 (Figures 2 and 3).

CeDna2 and ScDna2 share many essential features of their enzymatic properties. Both are ssDNA-specific endonuclease and ATPase/helicase that act preferentially upon a 5′ ssDNA tail. The helicase activity of both enzymes translocates in the 5′ to 3′ direction, and does not require the fork-like structure for unwinding. In contrast, Dna2 from fission yeast, humans (J.H. Kim, unpublished data) and frogs (21) lacks or contains very low levels of ssDNA-dependent ATPase activity.

Both endonuclease and helicase activities of Dna2 were stimulated by RPA derived from the same species. This may account for our earlier observation that expression of *S.pombe dna2+* failed to rescue the null mutation of *S.cerevisiae DNA2*

(data not shown). In contrast, other reports showed that the overproduction of Dna2 from human and *X.laevis* complemented the temperature-sensitive growth defect caused by a *dna2-1* mutant allele (21,22). This is most likely due to the fact that human or *X.laevis* Dna2 enzymes, when overproduced in yeast, are sufficiently functional without RPA. It is noteworthy that ScRPA did not inhibit CeDna2 activity and vice versa.

Despite many enzymatic similarities between CeDna2 and ScDna2, several crucial differences were noted that may suggest subtle differences in the processing of Okazaki fragments between yeasts and *C.elegans* (see below).

(i) The specific activities of endonuclease and helicase activities of CeDna2 were significantly higher ($\sim$10- and 20-fold, respectively) than those of ScDna2. The extent of stimulation of endonuclease and helicase activities of CeDna2 by CeRPA is far less than that observed with ScDna2 and ScRPA. This may be due to the fact that CeDna2 lacks the N-terminal 45 kDa domain, which is present in ScDna2; this domain was shown previously to be required for maximal stimulation of ScDna2 by ScRPA (27). Alternatively, the small subunit of CeRPA, which is not present in our preparation, is required for the optimal stimulation of CeDna2.

(ii) The $5'$ ssDNA preference to the $3'$ ssDNA tail was more prominent with CeDna2 than with ScDna2 (40-fold versus 2-fold) (Figure 3C) (17). CeDna2 acted in a highly catalytic manner in the absence of RPA, while ScDna2 requires RPA for its catalytic activity (19).

(iii) The levels of Mg^{2+} affected the substrate cleavage pattern of CeDna2, but hardly affected that of ScDna2. It is not clear at present how Mg^{2+} affected the cleavage pattern of CeDna2. One possibility is that CeDna2 undergoes conformations changes depending upon the amounts of Mg^{2+} bound to the enzyme, which affects the positioning of its active site with respect to the $5'$ end of an ssDNA tail.

(iv) The presence of a terminal RNA segment present in a flap substrate slightly inhibited the endonuclease activity of CeDna2, unlike ScDna2, which is markedly stimulated by the terminal RNA. It is worthwhile to mention that the endonuclease activity of the Archaea Dna2 from the hyperthermophile *Pyrococcus horikoshii* is completely inhibited by the presence of a terminal RNA segment at the $5'$ end of flaps (33). This observation raises the possibility that in *C.elegans*, primer RNAs are mostly removed (probably by RNase H) before CeDna2 acts upon the flap.

(v) CeDna2 efficiently degraded very short (<10 nt) ssDNA tail, whereas ScDna2 did not (Figure 5). If this property is critical *in vivo*, it may reflect that displacement DNA synthesis by pol δ generates mostly short flaps in *C.elegans*, thus allowing both Fen1 and Dna2 to process the short flaps. According to our findings, the $5'$ flaps processed by CeDna2 still have 2–3 nt residues and thus a subsequent action of Fen1 or other enzymes (such as exoI or the $3'$ to $5'$ proofreading function of pol δ) is required to further process the remaining flaps into ligatable nicks (34,35).

It is well accepted that ssDNA flaps, if they are not removed, leads to double-stranded DNA breaks (34,36). Accumulation of double-strand DNA breaks over a threshold level (>30/yeast cell) results in cell death (37,38). If this were the case, viability would depend on the efficient removal of the long flaps. In budding and fission yeasts, Dna2 is absolutely required for viability (11). In contrast, CeDna2 is not as essential as ScDna2 for cell viability. The homozygous *dna2* deletion affected the viability of F1 embryos rather mildly, although lethal effects were observed in the following generation. Almost all F1 embryos (*dna2* is absent both maternally and zygotically) hatched and $\sim$90% matured to adults. However, most of the F2 embryos either did not hatch or died at the L1 to L2 stage (28). Since *dna2*-deleted adults survive for a certain period of time, it suggests that the formation of long flaps is rare in *C.elegans*. When rare long flaps can not be processed appropriately due to the absence of functional Dna2, they are eventually repaired most probably via a double-strand break repair pathway. This process is likely to introduce deletions into some essential genes that cause lethality. Alternatively, the delay in lethality observed with *dna2*-deleted *C.elegans* mutants may be due to the gradual loss of chromosomes that occurs in the absence of a functional Dna2. This notion would be in keeping with the finding that the synthesis of telomeres in *dna2* mutants of *S.cerevisiae* is less efficient than in wild type (39).

Unlike budding and fission yeasts, Fen1 appears to play an essential role in metazoan organisms, such as *C.elegans* and mice, since its absence leads to embryonic lethality (28–30). This could be explained if Fen1 is responsible for the processing of most flap structures, which is possible only when short flaps are predominantly formed. The difference in dependence of yeasts and metazoans on Dna2 and Fen1 for viability is not incompatible with our previous model in which processing of Okazaki fragments requires the combined action of two endonucleases (19). Our *in vitro* data and *in vivo* observations raise the possibility that the processing of Okazaki fragments in different eukaryotes can vary. We propose that the degree of importance of Dna2 and Fen1 in each eukaryote is determined by the extent of displacement DNA synthesis carried out by pol δ during Okazaki fragment synthesis; as the length of the flap generated by displacement DNA synthesis increases, viability becomes more dependent on Dna2. We and others have shown that ScDna2 is required for the processing of long flaps (19) but not for the processing of short flaps of Okazaki fragments (36,40). Therefore, it is crucial to determine the frequency with which long flaps occur and their average size during Okazaki fragment synthesis in order to evaluate the *in vivo* role of Dna2 in eukaryotes.

In order to address this issue, it is essential that an *in vitro* DNA replication system is reconstituted. Currently, we have succeeded in isolating human Dna2 in a soluble form and plan to investigate its role in supporting the processing of lagging strand in the reconstituted SV40 DNA replication system.

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