Journal of Biochemistry and Molecular Biology, Vol. 33, No. 2, March 2000, pp. 155-161

JOURNAL OF
Biochemistry
and
Molecular Biology

In vitro Evidence that Purified Yeast Rad27 and Dna2 are not Stably Associated with Each Other Suggests that an Additional Protein(s) is Required for a Complex Formation

Sung-Ho Bae and Yeon-Soo Seo*
Center for Cell Cycle Control, Samsung Biomedical Research Institute,
Sungkyunkwan University School of Medicine, Suwon 440-746, Korea

Received 2 December, 1999, Accepted 14 January 2000

The *Saccharomyces cerevisiae* Rad27, a structure-specific endonuclease for the Okazaki fragment maturation has been known to interact genetically and biochemically with Dna2, an essential enzyme for DNA replication. In an attempt to define the significance of the interaction between the two enzymes, we expressed and purified both Dna2 and Rad27 proteins. In this report, Rad27 could not form a complex with Dna2 in the three different analyses. The analyses included glycerol gradient sedimentation, protein-column chromatography, and coinfection of baculoviruses followed by affinity purification. This is in striking contrast to the previous results that used crude extracts. These results suggest that the interaction between the two proteins is not sufficiently stable or indirect, and thus requires an additional protein(s) in order for Rad27 and Dna2 to form a stable physical complex. This result is consistent with our genetic findings that *Schizosaccharomyces pombe* Dna2 is capable of interacting with several proteins that include two subunits of polymerase δ, DNA ligase I, as well as Fen-1. In addition, we found that the N-terminal modification of Rad27 abolished its enzymatic activity. Thus, as suspected, we found that on the basis of the structure determination, N-terminal methionine indeed plays an important role in the nucleolytic cleavage reaction.

Keywords: Dna2, DNA replication, Fen-1, Okazaki fragments, Rad27.

Introduction

In mammalian cells, Fen-1, a 5' to 3' exonuclease, is known to play a crucial role in the Okazaki fragment maturation. It removes RNA segments on the Okazaki fragments with the assistance of RNase HI (Ishimi *et al.*, 1988; Goulian *et al.*, 1990; Murante *et al.*, 1994; Waga and Stillman, 1994; reviewed in Lieber, 1997 and Bambara *et al.*, 1997). RNase HI first hydrolyzes the initiator RNA of the primer DNA, leaving a single ribonucleotide at the RNA-DNA junction, which is subsequently removed by the 5' to 3' exonuclease activity of Fen-1. According to this current model, DNA polymerase (pol) δ, replication protein-A (RPA), proliferating cell nuclear antigen (PCNA), replication factor-C (RFC), RNase HI, Fen-1, and DNA ligase I are necessary and sufficient to reconstitute the lagging strand synthesis *in vitro* in mammalian cells (Waga and Stillman, 1998). This result established that Fen-1 and RNase HI are essential enzymes in the Okazaki fragment maturation *in vitro*. In addition to the 5' to 3' exonuclease activity, however, Fen-1 was recently found to possess a structure-specific endonuclease activity that cleaves the 5' unannealed single-stranded (ss) DNA or RNA at the duplex junction (Harrington and Lieber, 1994; Murante *et al.*, 1994; Murante *et al.*, 1995; Murante *et al.*, 1996). Moreover, it was reported that mammalian RNase HI is able to cleave 5' of the last ribonucleotide of the fully unannealed RNA-DNA hybrid molecule (Murante *et al.*, 1998). These findings raise the intriguing possibility that the Okazaki fragment maturation could occur through a more complex process than previously inferred. For example, the Okazaki fragment maturation is likely to require the formation of a 5'-tail prior to cleavage by Fen-1 and/or RNase HI.

Despite the critical role of Fen-1 in an *in vitro* reconstituted system, deletion of the *RAD27* gene encoding yeast homolog of mammalian Fen-1 produced a viable yeast strain. This observation has remained puzzling, since deletion of the DNA ligase I gene, required for the joining of Okazaki fragments processed by Rad27/RNase HI, renders cells inviable (Johnston and Nasmyth, 1978). Thus, the viability of the yeast cells lacking Rad27 argues strongly for the existence of an alternative pathway for the Okazaki fragment processing *in vivo*.

*To whom correspondence should be addressed.
Tel: +82-331-299-6440; Fax: +82-331-299-6435
E-mail: ysseo@medical.skku.ac.kr

Dna2 was implicated in the Okazaki fragment maturation on the basis of the genetic and physical interaction with Rad27 (Budd and Campbell, 1997). The data suggest that Rad27 and Dna2 might act as a complex in order to carry out its essential function *in vivo* (Budd and Campbell, 1997). The significance of this interaction in DNA replication, however, has not yet been clearly demonstrated. Rather, it raised several related questions: (i) Are Rad27 and Dna2 necessary and sufficient for a complex formation? (ii) If there are other protein(s) required for the complex formation, then what are they? (iii) In the context of the complex, will the biochemical activity of each enzyme be affected? For example, will Rad27 alter the ssDNA-specific endonuclease activity intrinsically associated with Dna2 (Bae *et al.*, 1998)? As an initial attempt to address these questions, we decided to examine the ability of Rad27 to form a complex with Dna2 using purified proteins. In this paper, we present biochemical evidence that Dna2 is unable to form a complex directly with Rad27 and requires an additional protein(s) for complex formation. In addition, we found that Rad27 was inactivated by the modification of its N-terminus, suggesting that amino acid residues at the N-terminus are involved in the nucleolytic cleavage reaction. This result is in agreement with the structural prediction that the initiator methionine is likely to participate in cleavage of DNA as an active site.

Materials and Methods

Oligonucleotides, DNA, and nucleoside triphosphates (NTPs) All oligonucleotides used for the construction of flap-structured substrates (73, 52, 51, and 25 nucleotides (nt) in length) were synthesized commercially (GIBCO-BRL, Grand Island, USA) and gel-purified prior to use. Oligonucleotides used to prepare substrates, positions of radioisotopic labels in the substrates, and the substrate structures were as described in each figure. The 73- and 52-mers were described elsewhere (Park *et al.*, 1997). The 3' ends (25-nt) of 73- and 52-mers are complementary to the 5' end of 51-mer (5'-GGA AAA CAT TAT TAA TGG CGT CGA GCT AGG CAC AAG GCG AAC TGC TAA CGG-3'), and the 25-mer (5'-CCG TTA GCA GTT CGC CTT GTG CCT A-3') is complementary to 3' end of 51-mer. Nucleoside triphosphates were obtained from Boehringer Mannheim (Mannheim, Germany), and [γ-^{32}P]ATP (> 5000 Ci/mmol) and [α-^{32}P]dCTP (> 6000 Ci/mmol) were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

Cloning of the RAD27 gene and construction of recombinant baculoviruses The *RAD27* gene encoding a *Saccharomyces cerevisiae* Fen-1 homolog was cloned from YPH499 (*MAT*a, *ade2-101*, *ura3-52*, *lys2-801*, *trp1-Δ63*, *his3-Δ200*, *leu2-Δ1*) with the use of PCR. The primers, 5'-CCG GAA TTC **ATG** GGT ATT AAA GGT TTG-3 and 5'-CCG CTC GAG **TCA** TCT TCT TCC TTC TGT G-3', were used to introduce an *Eco*RI site (underlined) at the initiation codon (bold type) and a *Xho*I site (underlined) at the stop codon (bold type). The amplified gene was inserted into the plasmid pCRII (Invitrogen) and confirmed by DNA sequence analyses. The *RAD27* gene was subcloned into the *Eco*RI site of pBlueBac4 and pBlueBacHis2B (Invitrogen, San Diego, USA) to generate pRAD27 and pHX-RAD27, respectively. The recombinant baculoviruses were constructed as recommended by the manufacturer (Invitrogen, San Diego, USA). The recombinant baculovirus generated from pRAD27 produced wild type Rad27 (wtRad27), while those from pHX-Rad27 produced N-terminally modified Rad27 (hxRad27) with additional 45 amino acids residues (MPRGS**HHHHHH** **H**GMASMTGGQ QMGRDLYDDD DKDPSSRSAA GTMEF) including six histidines (bold type) and the Xpress epitope (underlined) fused to its N-terminal methionine. The N-terminal modification of Rad27 served to facilitate not only its purification, but also detection of its expression and complex formed with Dna2.

Purification of the recombinant proteins in insect cells The recombinant Dna2 protein (HX-Dna2), with six histidine residues and Xpress epitope at its amino terminus, was prepared as described previously (Bae *et al.*, 1998). In order to purify wtRad27, the recombinant baculoviruses containing *RAD27* were infected into Hi-5 insect cells for 48 h at a multiplicity of infection of 10. The infected cells (1×10^6 cells/ml, 2.4 liters) were harvested and resuspended in 40 ml of buffer T_{100} (25 mM Tris-HCl/pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 mM PMSF, 0.15 mg/ml leupeptin and antipain). The number in subscript indicates the concentration of NaCl in millimolar added to buffer T. Cells were disrupted by sonication (4 cycles of 30-s pulse and a 2-min cooling interval). The extracts (15 mg/ml, 40 ml) were cleared by centrifuging at 37,000 rpm for 1 h in a Beckmann 45 Ti rotor, and the supernatant was applied directly to a heparin-Sepharose column (2.5 cm×4 cm) equilibrated with buffer T_{100}. The column was washed with 5 volumes of the same buffer and eluted with buffer T_{500}. The peak protein (1.5 mg/ml, 30 ml) was pooled and dialyzed for 4 h against buffer T_0 to obtain a conductivity equivalent to T_{100}. The dialysate was loaded onto a ssDNA-cellulose column (2.5 cm×2 cm) equilibrated with buffer T_{100}. The column was washed with 5 column volumes of the same buffer and eluted with a 150-ml linear gradient of 100-500 mM NaCl in buffer T. The flap endonuclease activity, which peaked at 150 mM NaCl, was pooled (0.14 mg/ml, 30 ml) and diluted with 20 ml of buffer T_0. The protein solution was applied to Resource S column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with buffer T_{100}. After washing with 10 column vol of the same buffer, the column was eluted with a 15-ml linear gradient of 100-600 mM NaCl in buffer T. The active fractions eluted at 200-300 mM NaCl were pooled (0.5 mg/ml, 4 ml) and an equal volume of buffer T, containing 3 M ammonium sulfate, was added prior to injection onto the phenyl-Superose column (Amersham Pharmacia Biotech, Uppsala, Sweden). The column was then washed with a 10 column vol of buffer T plus 1.5 M ammonium sulfate and eluted with a 15-ml linear gradient of 1.5-0 M ammonium sulfate in buffer T. The active fractions eluted at 1 M ammonium sulfate were pooled (0.3 mg/ml, 2.5 ml) and dialyzed for 4 h against buffer T_{500} containing 50% glycerol. Aliquots were stored at -80°C.

The extract containing hxRad27 was prepared as described for wtRad27. The peak fractions (1.8 mg/ml, 30 ml) eluted from heparin-Sepharose column (2.5 cm×4 cm) using T_{500} (DTT and

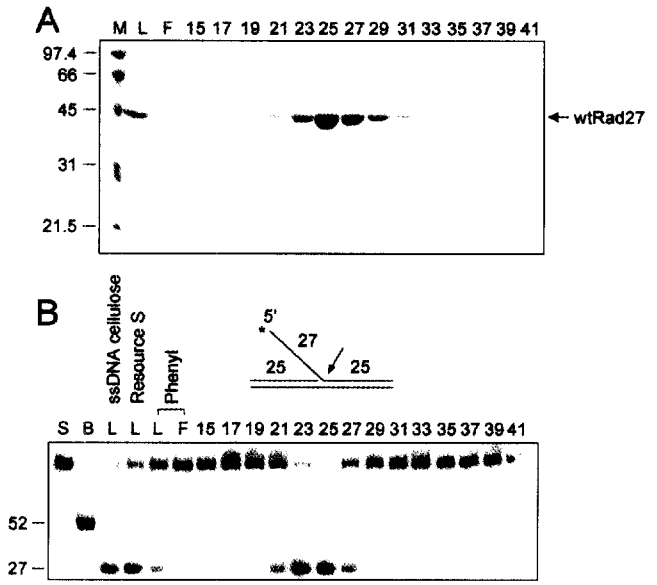

Fig. 1. Analysis of flap-endonuclease activity using phenyl-Superose column. The peak protein fraction of the Resource S column was loaded onto a phenyl-Superose column and eluted with a linear gradient, as described under "Materials and Methods". *A*, SDS-PAGE (12%) of the phenyl-Superose fractions (20 μl) was performed and the gel was Coomassie-stained. The load (*L*), flow-through (*F*), and the fractions analyzed are indicated at the top of the figure. The numbers at the *left* of the figure indicate the molecular sizes (in kDa) of marker proteins (Bio-Rad, Richmond, USA) (indicated as *M*), which include phosphorylase B (97.4 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa). *B*, autoradiograms of DNA products formed after incubation of each phenyl-Superose fraction (0.05 μl) with 20-μl reaction mixtures containing 15 fmol of the flap substrate (see "Materials and Methods"). The products were analyzed on 12% polyacrylamide gel containing 0.1% SDS in 0.5× TBE (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA). *S* and *B* denote substrate alone and boiled substrate controls, respectively. The migration positions of boiled substrate and cleavage products are indicated at the *left* of the figure. The schematic structure of the substrate is shown at the *top* of the figure. The *asterisk* indicates the 5'-^{32}P-labeled end and an *arrow* indicates the position of cleavage. The numbers denote the length of each oligonucleotide.

EDTA were omitted) plus 5 mM imidazole were directly loaded onto Ni^{2+}-NTA agarose column (1.5 cm×3 cm). The column was washed with 5-column vol of T_{500} (-DTT, -EDTA) plus 20-mM imidazole and eluted with T_{500} (-DTT, -EDTA) plus 400-mM imidazole. The peak protein was pooled (2.0 mg/ml, 10 ml) and dialyzed for 4 h against buffer T_0 in order to obtain a conductivity equivalent to T_{100}. The dialysate was loaded onto a ssDNA-cellulose column (2.5 cm×2 cm) equilibrated with buffer T_{100}. The column was washed with a 5-column volume of the same buffer and eluted with a 150-ml linear gradient of 100-500 mM NaCl in buffer T. The fractions containing hxRad27 eluted early in the gradient (120 mM NaCl) were pooled (0.2 mg/ml, 15 ml) and stored at -80°C.

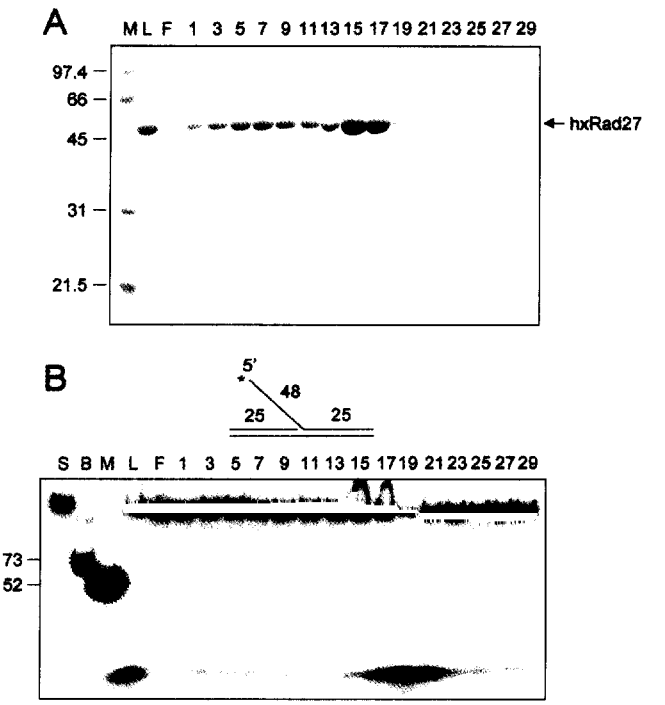

Fig. 2. N-terminal His-tag fusion inactivates the enzyme activity of Rad27. The peak protein fraction of the Ni^{2+}-NTA column was loaded onto a ssDNA-cellulose column and eluted with a linear gradient, as described under "Materials and Methods". *A*, SDS-PAGE (12%) of the phenyl-Superose fractions (20 μl) was performed and the gel was Coomassie-stained. The load (*L*), flow-through (*F*), and the fractions analyzed are indicated at the top of the figure. Protein molecular size markers (indicated as *M*) are as described in Fig. 1. *B*, autoradiograms of DNA products formed after incubation of each ssDNA-cellulose fraction (1 μl) with 20-μl reaction mixtures, as described in Fig. 1. *S* and *B* denote substrate alone and boiled substrate controls, respectively. The migration positions of boiled substrate and control size marker (*M*) are indicated at the *left* of the figure. The schematic structure of the substrate is shown at the *top* of the figure. The *asterisk* indicates the 5'-^{32}P-labeled end. The numbers denote the length of each oligonucleotide.

Preparation of nuclease substrates The flap-structured substrates were prepared as described previously (Bae *et al.*, 1998), but with some modifications. Labeling of the 5'-ends of oligonucleotides was performed by incorporating [γ-^{32}P]ATP (Amersham Pharmacia Biotech, Uppsala, Sweden) with polynucleotide kinase. The 52-mer (10 pmol) was first labeled at its 5'-end by incorporating [γ-^{32}P]ATP with polynucleotide kinase (Promega, Madison, USA). The 52-mers thus labeled were then annealed to the 51-mer (40 pmol) alone for a Y-structured substrate, or annealed to both 51-mer and 25-mer (40 pmol each) for a flap-structured substrate (Figs. 1 and 3). For some experiments, the 72-mer was used in place of the 52-mer (Fig. 2). The annealing reactions were carried out as described previously (Seo *et al.*, 1991). These substrates were gel-purified prior to use. The specific activities of both substrates were comparable and ranged from 2,000 to 3,000 cpm/fmol.

Flap-endonuclease assay The standard reaction mixtures (20 μl) for assay flap-endonuclease activity contain 25 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 2 mM DTT, 0.25 mg/ml bovine serum albumin and 15 fmol of a flap-structured substrate prepared as described above. Reactions were carried out at 30°C for 30 min and stopped by adding 4 μl of 6× stop solution (60 mM EDTA, 40% sucrose, 0.6% SDS, 0.25% bromophenol blue, 0.25% xylene cyanol). The reaction products were subjected to electrophoresis for 1.5 h at 150 V through 10% polyacrylamide gel containing 0.1% SDS in 0.5× TBE (45 mM Tris-base, 45 mM boric acid, 1 mM EDTA). The gel was dried on DEAE-cellulose paper and subjected to autoradiography. Labeled DNA products were quantitated with use of a PhosphorImager (Molecular Dynamics, Sunnyvale, USA).

Glycerol gradient sedimentation analysis Rad27 or Dna2 alone (50 μg), or a mixture containing both (50 μg each) was loaded onto a glycerol gradient (5 ml, 15 to 35%) in a buffer T plus 100 mM NaCl. After centrifugation at 45,000 rpm for 24 hr in a Beckman SW55.Ti rotor, fractions (200 μl) were collected from the bottom of the gradient and subjected to 8% SDS-polyacrylamide protein gel electrophoresis.

Results and Discussion

Expression and purification of Rad27 Crude extracts from insect cells infected with baculoviruses expressing either wild-type Rad27 (wtRad27), or His_6-tagged Rad27 (hxRad27), contained significant levels (approximately 5 to 10% of total proteins) of the target enzymes, while the control extracts obtained from uninfected cells did not (data not shown). Expression of each Rad27 enzyme was confirmed by Western blot analyses using either anti-Rad27 polyclonal antibodies, or anti-histidine monoclonal antibodies (data not shown).

Purification of wtRad27 was achieved by the use of serial column chromatographic steps that included heparin-Sepharose, ssDNA-cellulose, Resource S, and phenyl-Superose. Proteins and enzymatic activities were analyzed across the fractions obtained from the last step of purification, as shown in Fig. 1. The wtRad27 protein was efficiently retained in the phenyl-Superose column since both the protein and its activity were not observed in the flow-through fraction (Fig. 1A and B). The wild type Rad27 migrated slightly faster than the 45-kDa marker protein in agreement with its predicted molecular weight (43.3 kDa, 382 amino acids). A SDS-PAGE analysis of phenyl-Superose fractions in a 12% SDS-polyacrylamide gel yielded a polypeptide that copurified with junction cleavage activity (Fig. 1A and B). The DNA fragment produced by wtRad27 migrated at a position expected for 27-nt oligonucleotide, indicating that the cleavage occurred at the junction of ssDNA and dsDNA (Fig. 1B). In order to further confirm this result, we carried out three additional purification steps in an analytical scale using blue Sepharose, FPLC mono Q, and glycerol gradient sedimentation. This attempt resulted in no further increase in the specific activity of structure-specific endonuclease activity of Rad27 (data not shown), indicating that the enzyme preparation was maximally pure after the phenyl-Superose column chromatography.

We used a Ni^{2+}-NTA agarose to purify the hxRad27 enzyme in addition to the heparin-Sepharose and ssDNA-cellulose chromatographs, as described in "Materials and Methods". The use of Ni^{2+}-NTA affinity column chromatography removed most polypeptides present as impurities in the heparin-Sepharose column (data not shown). The proteins enriched at this step were highly pure (> 95%) and were loaded onto the ssDNA-cellulose column. The hxRad27 protein was efficiently retained in the ssDNA-cellulose column since the protein was not found in the flow-through fraction (Fig. 2A). Fractions eluting from the ssDNA-cellulose column chromatography were analyzed for proteins in 12% SDS-polyacrylamide gel (Fig. 2A). The linear salt gradient elution gave rise to an early (~120 mM), but broad peak of proteins (Fig 2A. fractions 1 to 17). This is probably due to the fact that Rad27 binds to differing sequences of ssDNA in the column with different affinities. The hxRad27 protein migrated slower than the 45-kDa marker protein in keeping with its increased molecular weight (48.7 kDa, 427 amino acids) due to the additional 45 amino acids at its N-terminus. Structure-specific endonuclease activities were also examined using a flap substrate (Fig. 2B). Unlike the wtRad27, the junction cleavage activity was undetected across the gradient fractions tested. Instead, the 5' end labels was released maximally at fraction 19. This is not coincidental with the protein peak of hxRad27 (fraction 15 and 17). This indicates that the nuclease activity observed is attributable to another protein(s) present in the fraction. Consequently, the fractions 1 to 13, which contained substantial amounts of hxRad27, displayed no endonuclease activity with a flap structure substrate. The fact that the N-terminally modified hxRad27 enzyme lacked no detectable endonuclease activity suggests that the N-terminal region of Rad27 could play an essential role for enzyme action. This confirms a recent prediction based on the structure determination that Rad27 requires an intact N-terminus for its catalytic activity (Hwang *et al.*, 1998). Although the N-terminal modification of Rad27 did result in the loss of enzymatic activity of Rad27, it may not cause a global change in the three-dimensional structure of Rad27. Since the N-terminal methionine is located in the vicinity of the active site, the addition of a polyhistidine tag to the N-terminal methionine destroyed only the enzymatic activity, leaving the domain structure(s) that is crucial for protein-protein interaction unaffected.

Yeast Rad27 did not cleave Y-structured substrate In order to confirm that the enzyme we isolated has the same substrate specificity that was demonstrated previously (Zhu *et al.*, 1997), we directly tested whether or not the enzyme is capable of cleaving the Y structure. In response to the increased amounts of wtRad27, the flap DNA was cleaved to produce ssDNA of 47 to 49-nt in size (Fig. 3, left). In contrast, the wtRad27 was unable to cleave the 5'-tail in the Y-

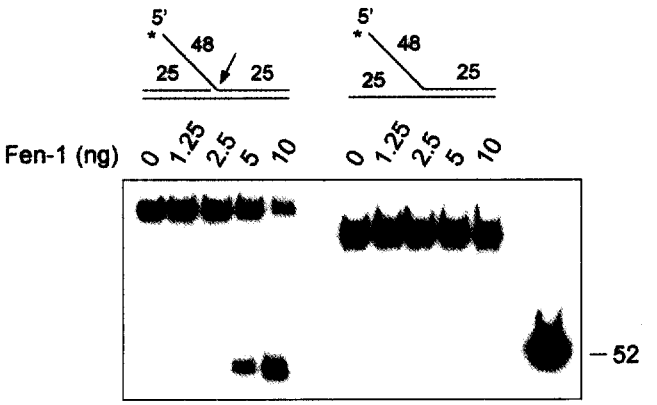

Fig. 3. Structure specificity of Rad27 endonuclease. The schematic structure of each substrate used, and position of ^{32}P-labeled ends (denoted as *asterisks*), is shown at the *top* of the figure. An arrow indicates the position of cleavage and the numbers denote the length of each oligonucleotide. The indicated amounts of wtRad27 in the 20-µl reactions (see "Materials and Methods") were incubated with 15 fmol of either flap (*left*) or Y-structured (*right*) substrates at 30°C for 30 min and the reaction products were analyzed as described in Fig. 1. The number denotes a control size marker (52-mer).

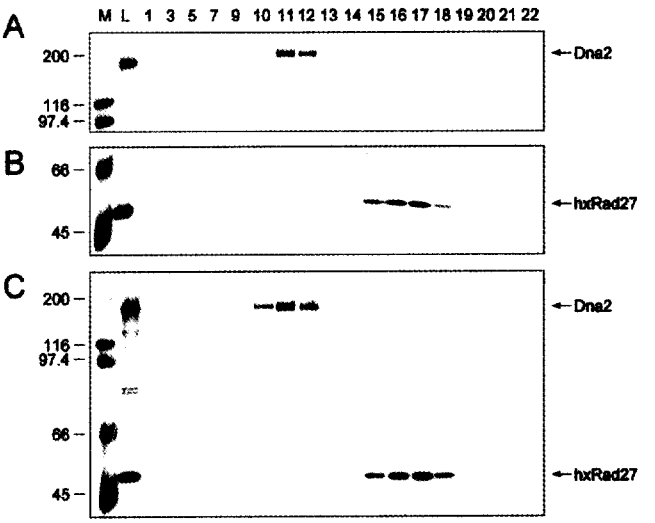

Fig. 4. Glycerol gradient sedimentation analyses of Dna2 and Rad27. The purified Dna2 (100 µg) or hxRad27 (50 µg) protein alone (*A* and *B*, respectively), or a mixture of two proteins (*C*) was loaded onto 15-35% glycerol gradient (5 ml, 100 mM NaCl) and centrifuged at 45,000 rpm for 24 h using a SW55Ti rotor (Beckmann). Fractions (0.2 ml) were collected from the bottom of the tubes. Proteins were analyzed by SDS-PAGE (8%) and the gels were silver-stained. The load (*L*) and fractions analyzed are indicated at the top of the figure. Protein molecular size markers (indicated as *M*) are as described in Fig. 1. The migration positions of Dna2 and hxRad27 are indicated at the *right* of the figure.

structured substrate (Fig. 3 right). Moreover, the wtRad27 we prepared also had 5' to 3' exonuclease activity (data not shown). This result demonstrates that the Rad27 we prepared is enzymologically active and possesses the same substrate specificity as previously demonstrated.

The DNA cleavage analysis of several different oligonucleotide structures revealed that Rad2 (Fen-1 homolog of *Schizosaccharomyces pombe*) possesses both 5'-flap endonuclease and 5'-> 3' double-stranded DNA exonuclease activities just like its mammalian counterpart. However, GST-Rad2p incised a 5'-flap and a 5'-pseudo-Y structure on base 3' of the branch point in the duplex region, and also degraded double-stranded DNA (Alleva and Doetsch, 1998). This contrasts strongly to the result obtained with yeast Rad27; the N-terminal modification of Rad2 did not result in inactivation of the enzymatic activity of Rad2. Thus, *S. pombe* Rad2 is more versatile or flexible in utilizing substrate than *S. cerevisiae* Rad27 or mammalian Fen-1. However, the potential role of the unique ability of *S. pombe* Rad2 to cleave to the Y structure still needs to be determined in the future.

Purified Rad27 and Dna2 are unable to form a complex

In order to demonstrate the direct complex formation of Rad27 with Dna2, and to define the biochemical activities of the complex formed, we attempted to reconstitute the Rad27-Dna2 complex using purified enzymes. For this purpose, we first allowed hxRad27 and Dna2 to form a complex on ice and examined the sedimentation profile in glycerol gradient centrifugation, as described in "Materials and Methods". Normally, the sedimentation coefficient of a given protein can be altered if other proteins are able to form a stable complex with it. In control experiments, Dna2 sedimented faster in a 15 to 35% glycerol gradient (peaking at fractions 10 to 11) than hxRad27 did (peaking at fractions 15 to 18) (Fig. 4A and B) when we used individual enzymes. When the two proteins were mixed, preincubated on ice for 30 min, and sedimented together, neither of the two showed any detectable alteration in their sedimentation coefficients (Fig. 4C). This result demonstrates that Rad27 is unable to form a complex with Dna2 when both enzymes are pure.

Since we used hxRad27 that may not be functionally interchangeable *in vivo* with its wild type allele, we could not rule out the possibility that the N-terminal modification (His_6 tag) also affected the ability of Rad27 to form a complex with Dna2. An alternative possibility is that the complex formation between the two enzymes may occur only while they are being expressed within the cells. In order to exclude these two possibilities, we coinfected insect cells with two recombinant baculoviruses expressing wtRad27 and HX-Dna2. The crude extracts obtained from coinfected cells contained significant levels of two proteins. The level of wtRad27 expressed was significantly higher than that of Dna2 (Fig. 5, lane 2). The fractions containing both wtRad27 and HX-Dna2 were prepared by loading the crude extracts and eluting the proteins with 0.5 M NaCl from the heparin-Sepharose column (Fig. 5, lane 4). When these fractions passed through the Ni^{2+}-column, as expected only HX-Dna2 was efficiently retained in the

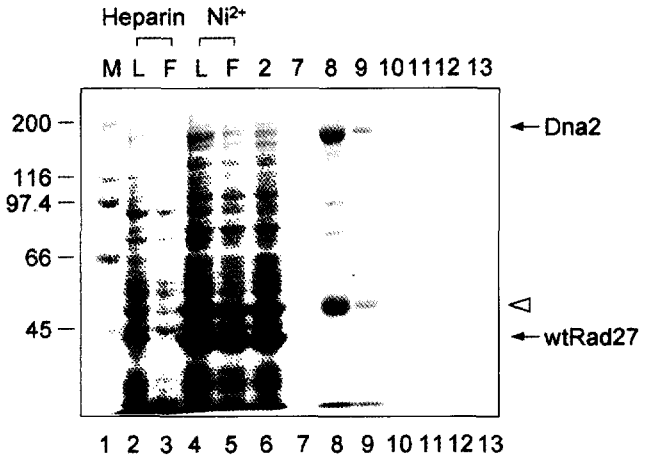

Fig. 5. Dna2 and Rad27 do not form a complex *in vivo*. The two baculoviruses expressing Dna2 and Rad27 were simultaneously coinfected into Hi-5 insect cells. The extracts prepared from the coinfected cells were precleared by the use of heparin-Sepharose (Heparin) column chromatography and then loaded into Ni^{2+}-NTA (Ni^{2+}) column. Proteins were analyzed by SDS-PAGE (8%) and the gels were Coomassie-stained. The load (*L*), flow-through (F), and fractions analyzed are indicated at the top of the figure. Protein molecular size markers (indicated as *M*) are as described in Fig. 1. The migration positions of Dna2 and wtRad27 are indicated at the *right* of the figure. An open arrowhead indicates the migration position of the porteolytic fragment of Dna2 (see text).

column (Fig. 5, lane 5). This is due to the presence of polyhistidine residues at the N-terminus of HX-Dna2. HX-Dna2 was efficiently eluted by the addition of imidazole in the washing buffer (Fig. 5, lanes 7 to 10). In strong contrast, wtRad27 was found in the flow-through and wash fractions only (Fig. 5, lanes 5 and 6). It is noteworthy that wtRad27 migrates faster than the 45-kDa marker, whereas the hxRad27 migrates slower (See Figs. 1 and 2). Thus, the coeluted 48-kDa polypeptide (indicated as an open arrowhead) is the proteolytic fragment of Dna2 containing the N-terminal region. This was confirmed by Western blot analysis (data not shown). Therefore, the attempt to purify Rad27-Dna2 complex in a Ni^{2+}-column resulted in no indication that the two proteins interacted with each other *in vivo*. This agrees with the result obtained from the glycerol gradient sedimentation. This also demonstrates that the complex formation did not occur even *in vivo* while the two proteins were being expressed within the same insect cells. Consistent with the results above *in vitro* and *in vivo*, we failed to observe the retention of wtRad27 on a protein column in which Dna2 was coupled to the matrix (data not shown).

In summary, we employed three different methods in order to support our conclusion that Rad27 and Dna2 alone are insufficient to form a complex. (1) We showed that the incubation of a N-terminally modified Rad27 did not form a complex with Dna2 upon glycerol gradient sedimentation analysis. (2) To exclude the possibility that the N-terminal modification resulted in an inefficient complex formation, we first co-expressed Rad27 (wild type) and Dna2 (His-tagged) in insect cells, and then tried to isolate a Rad27-Dna2 complex, if any, using a Ni^{2+}-column, but we failed to obtain any complex. (3) Furthermore, we used a wild-type Rad27 and different, but highly sensitive method-namely, the retention of Rad27 in a Dna2-coupled protein column. By taking all of these results together, we concluded that an additional protein(s) is required for the complex formation of Rad27 with Dna2. Currently, it is unclear what is additionally required for a stable complex formation of Dna2 with Rad27. We observed that the biochemical properties of Dna2 alone (Bae *et al.*, 1998; singly expressed and purified to homogeneity from insect cells) were not identical to those of Dna2 (Budd and Campbell, 1995 and Budd *et al.*, 1995; partially purified, and complexed with other proteins such as Rad27) prepared from the yeast cells. Currently two issues have to be resolved: (i) What kind of polypeptides is in the complex? (ii) How do they influence the biochemical activities of Dna2? This is an interesting issue that requires elucidation of the nature of the complex that probably contains Dna2, Rad27, and other yet unidentified proteins. The identification of each component polypeptide and successful reconstitution of the complex would be essential in understanding the action mechanism of the two endonucleases, as well as its precise role *in vivo*.

Acknowledgments We thank Kyung-Ae Kim for the excellent technical help. We also thank Drs. Dong Wook Kim and Hee-Dai Kim for critical reading of the manuscript. We are greatly indebted to other members of our laboratory for their helpful comments. This work was supported by a grant from the Creative Research Initiatives Program given to Y.-S. S.

References

Alleva, J. L. and Doetsch, P. W. (1998) Characterization of *Schizosaccharomyces pombe* Rad2 protein, a FEN-1 homolog. *Nucleic Acids Res.* **26**, 3645-3650.

Bae, S. H., Choi, E., Park, J. S., Lee, S. H., Lee, K. H. and Seo, Y. S. (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.

Bambara, R. A., Murante, R. S. and Hendericksen, L. A. (1997) Enzymes and reactions at the eukaryotic DNA replication forks. *J. Biol. Chem.* **272**, 4647-4650.

Budd, M. E. and Campbell, J. (1995) A yeast gene required for DNA replication encodes a protein with homology to DNA helicases. *Proc. Natl. Acad. Sci. USA* **92**, 7642-7646.

Budd, M. E., Choe, W.-C. and Campbell, J. (1995) *DNA2* encodes a DNA helicase essential for replication of eukaryotic chromosomes. *J. Biol. Chem.* **270**, 26766-26769.

Budd, M. E. and Campbell, J. (1997) A yeast replicative helicase, Dna2 helicase, interacts with yeast FEN-1 nucleases in carrying out its essential function. *Mol. Cell. Biol.* **17**, 2136-2142.

Goulian, M., Richards, S. H., Heard, C. J. and Bigsby, B. M. (1990) Discontinuous DNA synthesis by purified mammalian proteins. *J. Biol. Chem.* **265**, 18461-18471.

Harrington, J. J. and Lieber, M. R. (1994) The characterization of a mammalian DNA structure-specific endonuclease. *EMBO J.* **13**, 1235-1246.

Hwang, K. Y., Baek, K., Kim, H. Y. and Cho, Y. (1998) The crystal structure of flap endonuclease-1 from *Methanococcus jannaschii*. *Nat. Struct. Biol.* **5**, 707-713.

Ishimi, Y., Claude, A., Bullock, P. and Hurwitz, J. (1988) Complete enzymatic synthesis of DNA containing the SV40 origin of replication. *J. Biol. Chem.* **263**, 19723-19733.

Johnston, L. H. and Nasmyth, K. A. (1978) *Saccharomyces cerevisiae* cell cycle mutant cdc9 is defective in DNA ligase. *Nature* **274**, 891-893.

Lieber, M. R. (1997) The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. *Bioessays* **19**, 233-240.

Murante, R. S., Hendericksen, L. A. and Bambara, R. A. (1998) Junction ribonuclease: An activty in Okazaki fragment processing. *Proc. Natl. Acad. Sci. USA* **95**, 2244-2249.

Murante, R. S., Huang, L., Turchi, J. J. and Bambara, R. A. (1994) The calf 5'- to 3'-exonuclease is also an endonuclease with both activities dependent on primers annealed upstream of the point of cleavage. *J. Biol. Chem.* **269**, 1191-1196.

Murante, R. S., Rumbaugh, J. A., Barnes, C. J., Norton, J. R. and Bambara, R. A. (1996) Calf RTH-1 nuclease can remove the initiator RNAs of Okazaki fragments by endonuclease activity. *J. Biol. Chem.* **271**, 25888-25897.

Murante, R. S., Rust, L. and Bambara, R. A. (1995) Calf 5' to 3' exo/endonuclease must slide from a 5' end of the substrate to perform structure-specific cleavage. *J. Biol. Chem.* **270**, 30377-30383.

Park, J. S., Choi, E., Lee, S. H., Lee. C. and Seo, Y. S. (1997) A DNA helicase from *Schizosaccharomyces pombe* stimulated by single-stranded DNA-binding protein at low ATP concentration. *J. Biol. Chem.* **272**, 18910-18919.

Seo, Y. S., Lee, S. H. and Hurwitz, J. (1991) Isolation of a DNA helicase from HeLa cells requiring the multisubunit human single-stranded DNA-binding protein for activity. *J. Biol. Chem.* **266**, 13161-13170.

Stillman, B. (1994) Smart machines at the DNA replication fork. *Cell* **78**, 725-728.

Waga, S. and Stillman, B. (1994) Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication *in vitro*. *Nature* **369**, 207-212.

Waga, S. and Stillman, B. (1998) The DNA replication fork in eukaryotic cells. *Annu. Rev. Biochem.* **67**, 721-751.

Zhu, F. S., Biswas, E. E. and Biswas, S. B. (1997) Purification and characterization of the DNA polymerase a associated exonuclease: the RTH1 gene product. *Biochemistry* **36**, 5947-5954.