

# Human RECQL5 $\beta$ stimulates flap endonuclease 1

Elzbieta Speina<sup>1,2</sup>, Lale Dawut<sup>1</sup>, Mohammad Hedayati<sup>1</sup>, ZhengMing Wang<sup>1</sup>, Alfred May<sup>1</sup>, Sybille Schwendener<sup>3</sup>, Pavel Janscak<sup>3</sup>, Deborah L. Croteau<sup>1</sup> and Vilhelm A. Bohr<sup>1,\*</sup>

<sup>1</sup>National Institute on Aging, National Institutes of Health, 251 Bayview Blvd, Baltimore, MD 21224, USA,

<sup>2</sup>Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warszawa; Poland and <sup>3</sup>Institute of Molecular Cancer Research of the University of Zurich, Switzerland

Received February 27, 2009; Revised December 16, 2009; Accepted December 17, 2009

## ABSTRACT

**Human RECQL5 is a member of the RecQ helicase family which is implicated in genome maintenance. Five human members of the family have been identified; three of them, BLM, WRN and RECQL4 are associated with elevated cancer risk. RECQL1 and RECQL5 have not been linked to any human disorder yet; cells devoid of RECQL1 and RECQL5 display increased chromosomal instability. Here, we report the physical and functional interaction of the large isomer of RECQL5, RECQL5 $\beta$ , with the human flap endonuclease 1, FEN1, which plays a critical role in DNA replication, recombination and repair. RECQL5 $\beta$  dramatically stimulates the rate of FEN1 cleavage of flap DNA substrates. Moreover, we show that RECQL5 $\beta$  and FEN1 interact physically and co-localize in the nucleus in response to DNA damage. Our findings, together with the previous literature on WRN, BLM and RECQL4's stimulation of FEN1, suggests that the ability of RecQ helicases to stimulate FEN1 may be a general feature of this class of enzymes. This could indicate a common role for the RecQ helicases in the processing of oxidative DNA damage.**

## INTRODUCTION

Helicases play important roles in the maintenance of genomic stability. They act in many DNA metabolic processes, including DNA replication, recombination, base excision repair and transcription (1–4). DNA helicases of the RecQ family have a broad amino acid sequence homology to the *Escherichia coli* RecQ helicase. These 3'–5' DNA helicases unwind a wide variety of potentially recombinogenic DNA structures, including four-way junctions, D-loops and G-quadruplex DNA.

Humans have five RecQ homologs: RECQL/RECQL1, BLM/RECQL2, WRN/RECQL3, RECQL4 and RECQL5 (5). Defective RecQ helicase function causes

genomic instability, which is manifested as an increase in the frequency of inappropriate recombination events. Mutations in *BLM*, *WRN* and *RECQL4* give rise to the hereditary disorders Bloom, Werner and Rothmund–Thomson syndromes, respectively (6–9). These diseases are associated with chromosomal instability, premature aging and cancer predisposition. The RECQL1 and RECQL5 proteins have not been genetically linked to any diseases yet; however, mutations in *RECQL1* and *RECQL5* might predispose individuals to cancer. In contrast to the other human RecQ helicases, RECQL5 exists in at least three different isoforms (10). These isoforms are generated by alternative mRNA splicing from the *RECQL5* gene that contains 19 exons. Two transcripts code for two small proteins, RECQL5 $\alpha$  (with 410 amino acids) and RECQL5 $\gamma$  (435 amino acids) that contain the core helicase motifs. The third transcript encodes the larger RECQL5 $\beta$  isoform (991 amino acids). RECQL5 $\beta$  localizes to the nucleus, whereas the two smaller isoforms are cytoplasmic (10).

RecQ helicases possess the so-called DExH helicase and RecQ-Ct (RecQ C-terminal) regions, which form the catalytic core of the enzyme (6,11). In addition to these conserved regions, RECQL5 $\beta$  contains a long C-terminal region that displays no homology to the other family members. In contrast to BLM and WRN that form oligomeric structures, the RECQL5 $\beta$  is a monomeric protein (12). It contains two separate functional domains. The N-terminal half of the protein contains the conserved DExH and Zn<sup>2+</sup>-binding domains that functions as a DNA-dependent ATPase and ATP-dependent 3'–5' DNA helicase. The unique C-terminal portion possesses an efficient DNA strand-annealing activity (12). However, strand annealing activity was also seen with the helicase domain only, RECQL5 $\alpha$  (13). Interestingly, RECQL5 has been found to interact with PCNA, Topoisomerase III  $\alpha/\beta$ , Rad51 and RNA polymerase II (10,12,14–18). The functional significance of all these interactions has not been fully explored.

While the precise role of the human RECQL5 protein in genomic stability has yet to be determined, it has been found that inactivation of *Recql5* in mouse embryonic

\*To whom correspondence should be addressed. Tel: +1 410 558 8162; Fax: +1 410 558 8157; Email: vbohr@nih.gov

stem cells and embryonic fibroblasts results in a significant increase in the frequency of sister chromatid exchanges, increased cancer susceptibility and a profound reduction in DNA replication after the treatment with a topoisomerase I inhibitor, camptothecin (19,20). It has been suggested that RECQL5 $\beta$  is an important tumor suppressor that acts by preventing inappropriate homologous recombination (HR) events via Rad51 presynaptic filament disruption (15), thus implicating RECQL5 in the regulation of homologous recombination.

FEN1 is a multifunctional endo/exonuclease that specifically recognizes 5'-flap single-stranded DNA (ssDNA) and regions of ssDNA at single strand-double strand junctions (21–25). FEN1 is a 5' to 3' exonuclease that acts at nicks in duplex DNA and also catalyzes the removal of 5' terminal RNA mononucleotide (1). FEN1 was initially identified as an essential enzyme involved in Okazaki fragment processing (26). Later, FEN1 was implicated in maintaining genomic stability (24,27) and described as a tumor suppressor protein (28). FEN1 haploinsufficiency in mice can also lead to tumor progression (29). Surprisingly, homozygous mutant (*FEN1*<sup>-/-</sup>) cells were viable; however, compared with wild-type cells, *FEN1*<sup>-/-</sup> cells exhibited a slow growth phenotype, probably due to a high rate of cell death (30). More recently, FEN1 has been shown to have a role in telomere maintenance (31).

FEN1 is associated with long-patch base excision repair (LP-BER) by virtue of its ability to cleave DNA flap structures (32–34). Through both affinity column chromatography and photoaffinity labeling, FEN1 has been found in a BER protein complex with uracil DNA glycosylase, AP endonuclease 1 (APE1), polymerase  $\beta$  (pol  $\beta$ ), DNA ligase 1 and PARP-1 (35–37). Moreover, it has been shown that the endonuclease activity of FEN1 is stimulated by APE1 (38,39). Recently, physical and functional interactions of FEN1 with WRN and BLM have been characterized (40–43). Both RecQ proteins stimulated FEN1 cleavage activity. We report here that purified RECQL5 $\beta$  protein stimulates FEN1 cleavage activity on 5'-ssDNA flap, nicked duplex DNA and 5' double-stranded flap substrates. We also show that both proteins physically interact and co-localize in the nucleus in response to oxidative stress and DNA damage. We propose that the mammalian RecQ proteins possess a generalized ability to stimulate FEN1 and thus have the potential to modulate the efficiency of reactions in which FEN1 participates.

## MATERIALS AND METHODS

### Recombinant proteins

Recombinant human RECQL5 $\beta$  helicase was over-produced as a fusion protein with an intein-chitin-binding domain (CBD) self-cleaving affinity tag in the *E. coli* BL21-codon-Plus-(DE3)-RIL strain (Stratagene), and purified as previously described (44). FEN1 was a generous gift from Dr David Wilson III (National Institute on Aging, National Institutes of Health, Baltimore, MD, USA). Both proteins were judged to

be >95% pure by protein gel electrophoresis and Simply Blue protein stain (Invitrogen, Figure 1A and B).

The helicase activity of the purified RECQL5 $\beta$  was verified by a standard helicase assay using M13mp18-based partial duplex assay (Figure 1C). The enzymatic activity of FEN1 was tested using oligonucleotide duplex with 1 nt 5'-flap (Figure 1D).

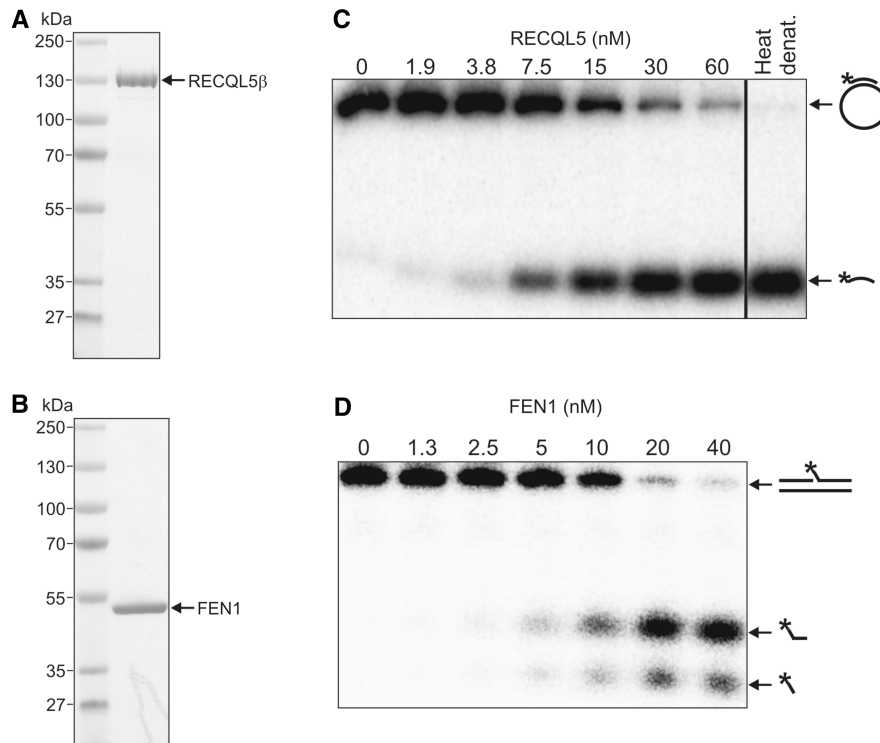
### DNA substrates

DNA oligonucleotides were synthesized and PAGE purified by Midland Certified Reagent Co. (Midland, TX, USA) or by Integrated DNA Technologies (San Diego, CA, USA). The sequences of the oligonucleotides are shown in Table 1. Template TEMP and upstream U25 primers together with downstream primers FLAP00, FLAP01, FLAP30 and FLAP60 were designed to form duplex substrates with a nick, 1 nt 5'-flap, 30 nt 5'-ssDNA flap and 60 nt 5' hairpin flap, respectively. Downstream primers were labeled at the 5'-end (FLAP00, FLAP01, FLAP30) with T4 polynucleotide kinase (Optikinase, USB) and [ $\gamma$ <sup>32</sup>P] ATP (GE Health Sciences) or at the 3'-end (FLAP60) with terminal transferase (NEB) and [ $\alpha$ <sup>32</sup>P]dATP (GE Health Sciences) followed by annealing as described previously (45). GEN1, GEN2, GEN3 and GEN4 oligonucleotides were used for creating duplex substrates with double-stranded flaps. The bubble substrate was designed from oligonucleotides GEN5, GEN6 and GEN7. GEN1, GEN3 and GEN7 were <sup>32</sup>P-labeled at the 3'-end and annealed to form duplex with GEN2, GEN3 and GEN4 (for GEN1) or GEN1, GEN2 and GEN4 (for GEN3) or GEN5 and GEN6 (for GEN7). The marker substrate for GEN activity of FEN1 against the bubble substrate was created from 3'-end radiolabeled GEN9 annealed to GEN5, GEN6 and GEN8.

The M13mp18-based partial-duplex substrate was prepared by annealing the HEL43 43-mer to circular M13mp18 ssDNA (NEB) and by extension of this molecule by 1 nt using the Klenow fragment (NEB) and [ $\alpha$ <sup>32</sup>P]dATP as described previously (44). The oligonucleotide-based partial duplex was designed by annealing 3'-end labeled HAIR49 to HAIR24.

### RECQL5 $\beta$ helicase assay

Reaction mixtures (10  $\mu$ l) contained 0.5 nM M13mp18-based or 1 nt 49 nt-based partial duplexes, 2 mM ATP and indicated concentrations of RECQL5 $\beta$ . Where indicated, replication protein A (RPA) was added at indicated concentrations. Helicase reactions with M13mp18-based partial duplex were carried out in buffer HA (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 5 mM MgCl<sub>2</sub>, 50  $\mu$ g/ml BSA, 2 mM ATP, 1 mM DTT) and helicase reactions with 49 nt-based partial duplex were carried out in HB (30 mM HEPES pH 7.6, 40 mM KCl, 5% glycerol, 10 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA). Mixtures were incubated at 37°C for 20 min, and terminated with the addition of 5  $\mu$ l of SDS stop solution (2% SDS, 50 mM EDTA, 30% glycerol, 0.1% bromophenol blue, 0.1% xylene cyanol). Products were separated on 12% nondenaturing polyacrylamide gel



**Figure 1.** Enzymatic activities of purified RECQL5 $\beta$  and FEN1. (A and B) Four to twelve percent of Bis-Tris PAGE showing purified RECQL5 $\beta$  protein and purified FEN1 protein, respectively. (C) DNA helicase assay of RECQL5 $\beta$  using a 44 nt M13mp18-based duplex radiolabeled at 3'-end. Reactions were carried out at 37°C for 30 min and contained 0.5 nM DNA, varying concentrations of RECQL5 $\beta$  (as indicated) and 2 mM ATP. The reaction products were analyzed by 10% nondenaturing PAGE. Radiolabeled species were visualized by autoradiography. The last lane contains heat-denatured substrate. (D) Flap endonuclease activity of FEN1. Reactions were carried out at 37°C for 15 min and contained 1 nM 43 nt duplex with 1 nt 5'-flap radiolabeled at 5'-end, and varying concentrations of FEN1 (as indicated). The reaction products were analyzed by 20% denaturing PAGE.

(acrylamide to bis-acrylamide, 37.5:1). Radiolabeled DNA was visualized using a Typhoon phosphorImager, (Typhoon 9400, GE Health Sciences).

#### FEN1 incision assays

Reactions (10  $\mu$ l) contained 1 nM DNA substrate and indicated concentrations of RECQL5 $\beta$  (or BSA), RPA and/or FEN1 in 30 mM HEPES pH 7.6, 40 mM KCl, 5% glycerol, 10 mM MgCl<sub>2</sub>, 100  $\mu$ g/ml BSA. Where indicated, ATP was added to a final concentration of 2 mM. RECQL5 $\beta$  (or BSA) was mixed with the substrate and buffer on ice, and pre-incubated for 10 min prior to the addition of FEN1. Where indicated, RPA was added and pre-incubated with RECQL5 $\beta$  before adding FEN1. Reactions were incubated at 37°C for 15 min (unless indicated otherwise), then terminated with the addition of 10  $\mu$ l of formamide stop solution (90% formamide, 20 mM EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), and heated to 95°C for 5 min. For kinetic experiments, reaction mixtures (50  $\mu$ l) contained 1 nM 1 nt 5'-flap duplex, 5 nM RECQL5 $\beta$  and 5 nM FEN1. Aliquots (5  $\mu$ l) were removed at different reaction time points and mixed with the equal amounts of formamide stop solution. Products were resolved on 20% polyacrylamide, 8 M urea denaturing gels (acrylamide to bis-acrylamide, 19:1). A Typhoon phosphorImager was used for detection and ImageQuant software (GE Health Sciences) was used for quantification

of the reaction products. Percent incision was calculated from the equation: percent incision =  $[P/(S + P)] \times 100$ , where  $P$  is the sum of the intensity of the bands representing incision products and  $S$  the intensity of the band representing the intact oligonucleotide. Data represent the mean of three independent experiments with standard deviations shown by error bars.

#### Cell culture

Human HeLa and HEK293T cell lines used in this study were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cells were grown in a humidified 5% CO<sub>2</sub> atmosphere at 37°C.

#### Pull-down assays

CBD-tagged RECQL5 $\beta$  was produced in *E. coli* BL21-CodonPlus(DE3)-RIL cells (Stratagene) as previously described (12). Cells harvested from a 10 ml culture were re-suspended in 1 ml of NET-150 buffer [10 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, 10% (v/v) glycerol and 0.1% (v/v) Triton X-100] supplemented with a protease inhibitor cocktail (Complete, Mini; Roche) and disrupted by sonication followed by centrifugation at 20 000g for 45 min. Clarified cell extract (typically 50–100  $\mu$ l) was incubated with 20  $\mu$ l of chitin beads (NEB) in a total volume of 400  $\mu$ l of NET-150 buffer for 2 h at 4°C. After extensive washing with

**Table 1.** Oligonucleotide sequences for DNA substrates (5' to 3')

Oligonucleotide, size	Sequence
TEMP, 44 nt	GCACTGGCCGTCGTTTTACGGTTCGTGACTGGGAAAACCCTGGCG
Downstream primers	
FLAP00, 19 nt	GTAAAACGACGGCCAGTGC
FLAP01, 20nt	AGTAAAACGACGGCCAGTGC
FLAP30, 49 nt	TTTTTTTTTTTTTTTTTTTTTTTTTTTCCAAGTAAAACGACGGCCAGTGC
FLAP60, 79 nt	AGGTCTCGAGGCTGCTCCTGCTCTATTATGAGCAGGAGCAGGCCTCGAGACCTTTCCAAGTAAAACGA CGGCCAGTGC
Upstream primer	
U25, 25 nt	CGCCAGGGTTTTCCAGTCACGACC
GEN primers	
GEN1, 34 nt	GATGTCAAGCAGTCCTAACTTTGAGGCAGAGTCC
GEN2, 18 nt	TTAGGACTGCTTGACATC
GEN3, 30 nt	GGACTCTGCCTCAAGACGGTAGTCAACGTG
GEN4, 14 nt	CACGTTGACTACCG
GEN5, 80 nt	GTTAAGATAGGTCTGCTTGGGATGTCAAGCAGTCCTAACTGGAAATCTAGCTCTGTGGAGTTGAGGCAG AGTCCTTAAGC
GEN6, 40 nt	CTCCACAGAGCTAGATTTCCAGTTAGGACTGCTTGACATC
GEN7, 80 nt	GCTTAAGGACTCTGCCTCAAATCGTCAGGGTTTCTAAAGAAGCCGACGGTAGTCAACGTGCCAAGCAGA CCTATCTTAAC
GEN8, 20 nt	GCTTTAGGACTCTGCCTCAA
GEN9, 20 nt	CCAAGCAGACCTATCTTAAC
Helicase primers	
HEL43, 43 nt	CTTGCATGCCTGCAGGTCTGACTCTAGAGGATCCCCGGGTACCG
HAIR49, 49 nt	GAGCAGGAGCAGGCCTCGAGACCTTTCCAAGTAAAACGACGGCCAGTGCCAGTGC
HAIR24, 24 nt	AGGTCTCGAGGCTGCTCCTGCTC

Duplex DNA substrates containing flap were constructed by annealing appropriate FLAP oligonucleotide and upstream primer to 44-mer template oligonucleotide. FLAP00, FLAP01, FLAP30 and FLAP60 were used to construct the nicked duplex, 1 nt 5' flap duplex, 30 nt 5' flap duplex and 60 nt 5' flap duplex substrates, respectively. Double-stranded flap and the bubble substrates were created by annealing together GEN1/GEN2/GEN3/GEN4 or GEN5/GEN6/GEN7, respectively. The M13mp18-based or oligonucleotide-based partial duplexes were prepared by annealing the HEL43 43-mer to circular M13mp18 ssDNA or HAIR49 to HAIR24, respectively.

NET-150 buffer, beads were incubated with extract of 293T cells (800 mg of protein) or recombinant FEN1 protein or GST-FEN1 fragments (200 ng) in 400  $\mu$ l of NET-150 buffer supplemented with ethidium bromide (50  $\mu$ g/ml) for 2 h at 4°C. Bound proteins were analyzed by western blotting using rabbit polyclonal anti-FEN1 antibody (28).

### Immunofluorescence

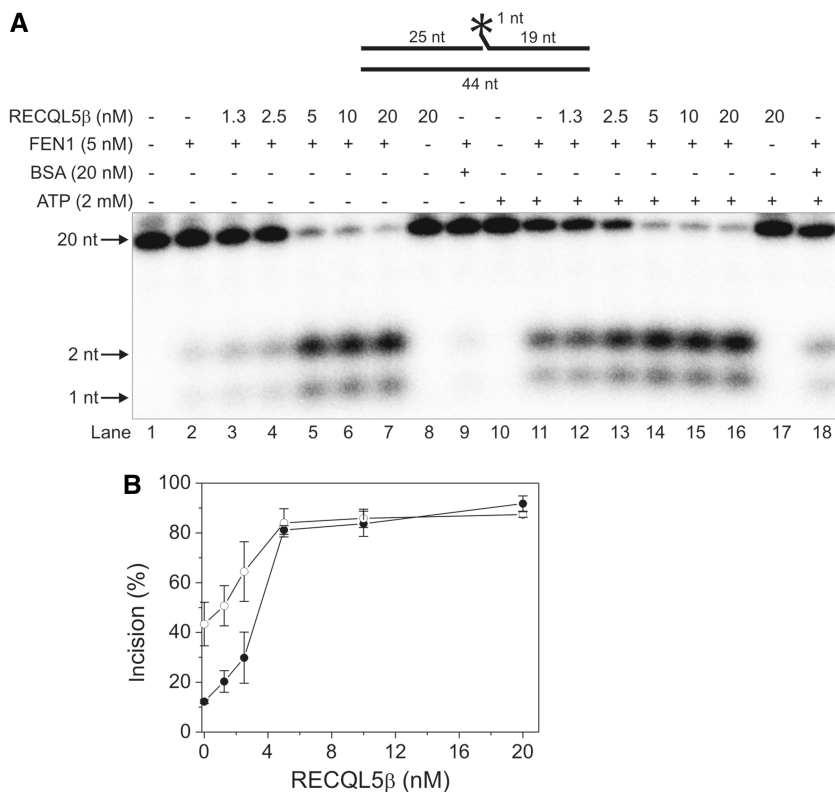
HeLa cells, grown on four-chambered slides (Lab-Tek), were transfected with the p3XFLAG-myc-CMV-24 plasmid (Sigma) containing cDNA encoding the full-length RECQL5 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. Twenty-four hours posttransfection, the cells were exposed to 100  $\mu$ M hydrogen peroxide (in plain DMEM) for 30 min at 37°C. Cells were fixed with 3.7% paraformaldehyde in PBS for 15 min at room temperature and washed five times with PBS, then permeabilized with 0.2% Triton X-100 in PBS for 10 min on ice. After blocking in PBS containing 5% BSA for 1 h at 37°C or overnight at 4°C, slides were incubated 1 h at 37°C with primary antibodies, rabbit polyclonal anti-FLAG (Sigma, 1:200) and mouse monoclonal anti-FEN1 (Abcam ab462; 1:1000); all antibodies were diluted in blocking solution. Cells were then rinsed five times with PBS and incubated with Alexa Fluor 488-conjugated donkey anti-rabbit and Alexa Fluor 647-conjugated goat anti-mouse secondary antibodies (Invitrogen A21240 and A21206, respectively; 1:1000) for 30 minutes at room temperature. After

washing five times with PBS, cells were mounted with Vectashield Hard Set mounting medium with DAPI (Vector Labs). Images were captured by a Nikon Eclipse TE2000 confocal microscope and analyzed using Volocity-5 software (Perkin Elmer).

## RESULTS

### RECQL5 $\beta$ stimulates the FEN1 cleavage reaction

RECQL5 $\beta$  was tested for its ability to stimulate FEN1 because WRN, BLM and RECQL4 have been shown to potently stimulate FEN1 (40–42, 46). The DNA substrate used in these studies consisted of a 19 bp duplex with a single unannealed 5' nucleotide adjacent to an upstream 25 bp duplex (1 nt 5'-flap). The 1 nt 5'-flap substrate was susceptible to FEN1 cleavage and 2 nt and 1 nt products were produced (Figure 2A, lane 2), as previously reported (47). These products result from FEN1 cleavage at the junction between the flap and the downstream double-stranded DNA (dsDNA) and endonucleolytic cleavage of the first nucleotide in the downstream duplex DNA. In the presence of 5 nM FEN1, 12% of 1 nM substrate was incised (Figure 2A, lane 2, and B). Under these conditions, we analyzed FEN1 cleavage as a function of RECQL5 $\beta$  concentration. FEN1 cleavage was stimulated up to 7-fold (Figure 2A, lanes 3–7 and B). As expected, RECQL5 $\beta$  alone did not incise the substrate (Figure 2A, lane 8). To confirm that the presence of RECQL5 $\beta$  caused true stimulation rather than this being an effect of RECQL5 $\beta$  stabilization of



**Figure 2.** RECQL5 $\beta$  stimulates FEN1 cleavage of 1 nt 5'-flap DNA. Reactions (10  $\mu$ l) containing 1 nM 1 nt 5'-flap DNA substrate, 5 nM FEN1 and the indicated amounts of RECQL5 $\beta$ , or 20 nM BSA, were incubated at 37°C for 15 min under conditions described in the 'Materials and Methods' section. The presence of 2 mM ATP in reaction mixtures is indicated (lanes 10-18). (A) A phosphorimage of a typical gel. (B) Percent incision from the data shown in (A), data points are the mean of three independent experiments with SDs indicated by error bars. Open circles, plus ATP; filled circles, minus ATP.

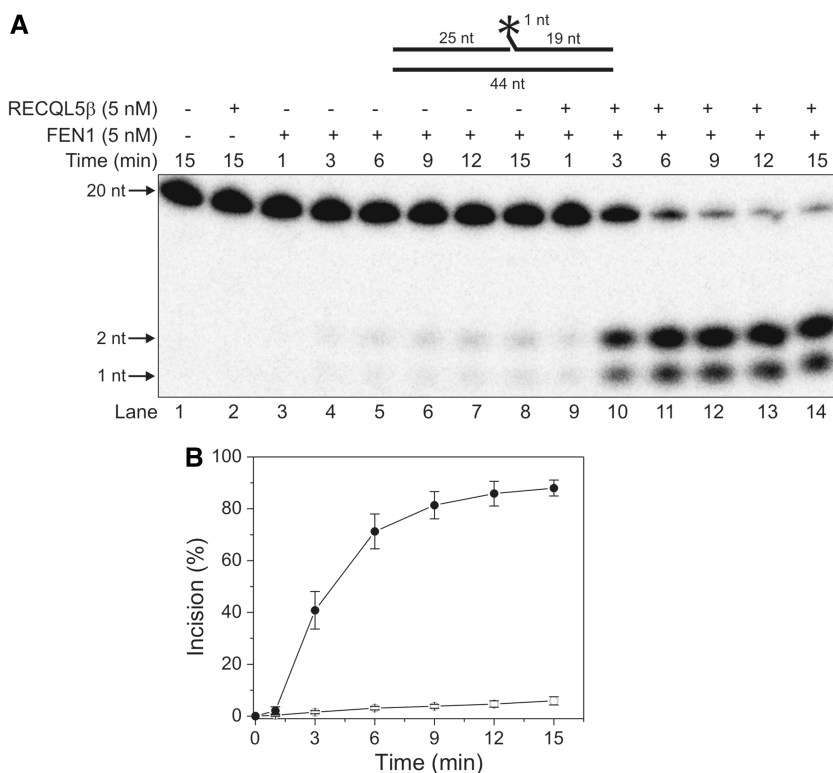
FEN1 in the reaction, we did a control reaction with BSA and found no stimulation of FEN1. In the presence of 5 nM FEN1 and 20 nM BSA only 8% of 1 nM substrate was incised (Figure 2A, lane 9).

RECQL5 $\beta$ , like other helicases, requires ATP for its helicase activity. To examine what effect helicase activity might have on the observed stimulation of FEN1, we added ATP to reactions and repeated the analysis (Figure 2A, lanes 10-18, and B). In the presence of 5 nM FEN1 and 2 mM ATP, 43% of the 1 nM substrate was incised (Figure 2A, lane 11, and B). This increase in FEN1 activity was about 30% more than FEN1 cleavage in the absence of ATP (Figure 2A, lane 2, and B). After addition of ATP, FEN1 cleavage increased by about 30% at RECQL5 $\beta$  concentrations of 1.3 nM and 2.5 nM (Figure 2A, lane 12 and 13, respectively, and B) in comparison to FEN1 cleavage without ATP (Figure 2A, lane 3 and 4, respectively, and B). These results indicate that the observed increase in cleavage products was the result of a higher initial rate of FEN1 incision activity in the presence of ATP. The extent of the reactions was similar plus or minus ATP, 87-84% respectively. RECQL5 $\beta$  alone did not cleave the substrate and BSA did not stimulate FEN1 incision (Figure 2A, lane 17 and 18, respectively). Based on these results, we conclude that helicase activity of RECQL5 $\beta$  is not required for FEN1 stimulation.

Kinetic analysis of the FEN1 catalyzed cleavage reaction on the 1 nt 5'-flap substrate showed a strong influence of RECQL5 $\beta$  on the rate of FEN1 incision (Figure 3A). In these experiments, equimolar concentrations of FEN1 and RECQL5 $\beta$  (5 nM) were used. The FEN1 concentration used resulted in a reproducible incision of 6% of the 1 nM DNA substrate after 15 min reaction incubated at 37°C (Figure 3B). Stimulation of FEN1 incision by RECQL5 $\beta$  was detected at time points from 1 to 15 min. After 3 min, FEN1 cleavage in the absence of RECQL5 $\beta$  was 1.5%; however, in the presence of RECQL5 $\beta$ , FEN1 cleaved 44% of the DNA substrate (Figure 3B). FEN1 cleavage in the absence and presence of RECQL5 $\beta$  was linear with respect to time from 1 to 6 min ( $R^2 = 1.0$  and 0.97, respectively). Linear regression analyses yielded reaction rates of 9.51 and 0.35 fmol product/min for the RECQL5 $\beta$  + FEN1 and FEN1 only, respectively. This represents a 27.2-fold rate increase in the presence of RECQL5 $\beta$ . At 12 and 15 min, the FEN1 cleavage reaction, in the presence of RECQL5 $\beta$ , achieved a plateau of 86-88% substrate incised.

#### RECQL5 $\beta$ stimulates FEN1 cleavage of duplex DNA substrates containing a long 5'-ssDNA flap or a nick

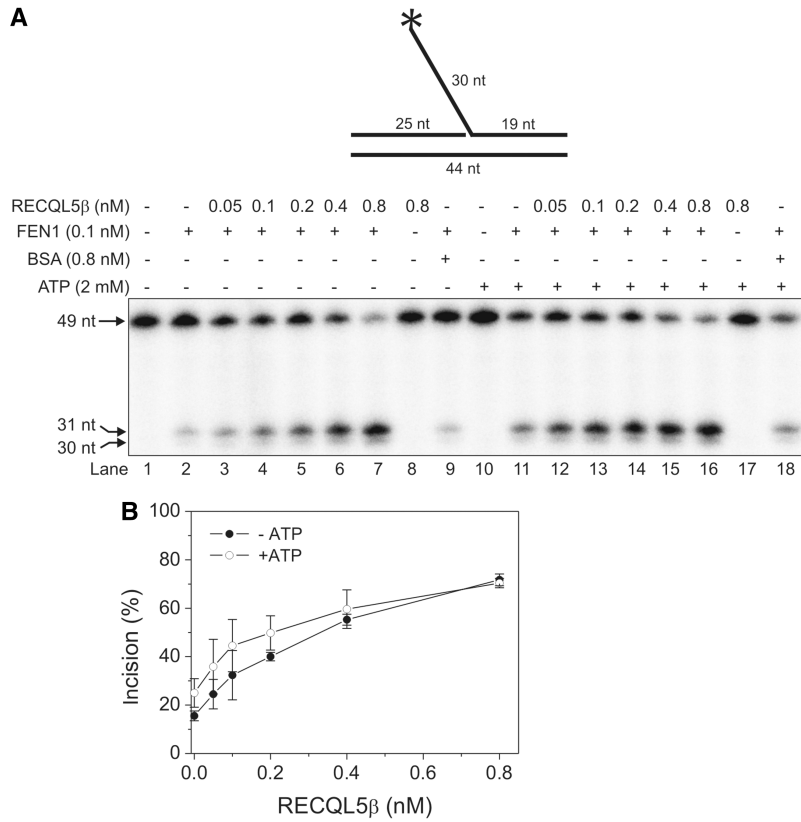
The ability of RECQL5 $\beta$  to stimulate FEN1 cleavage of the 1 nt 5'-flap substrate prompted us to examine whether RECQL5 $\beta$  can also affect the activity of FEN1 on



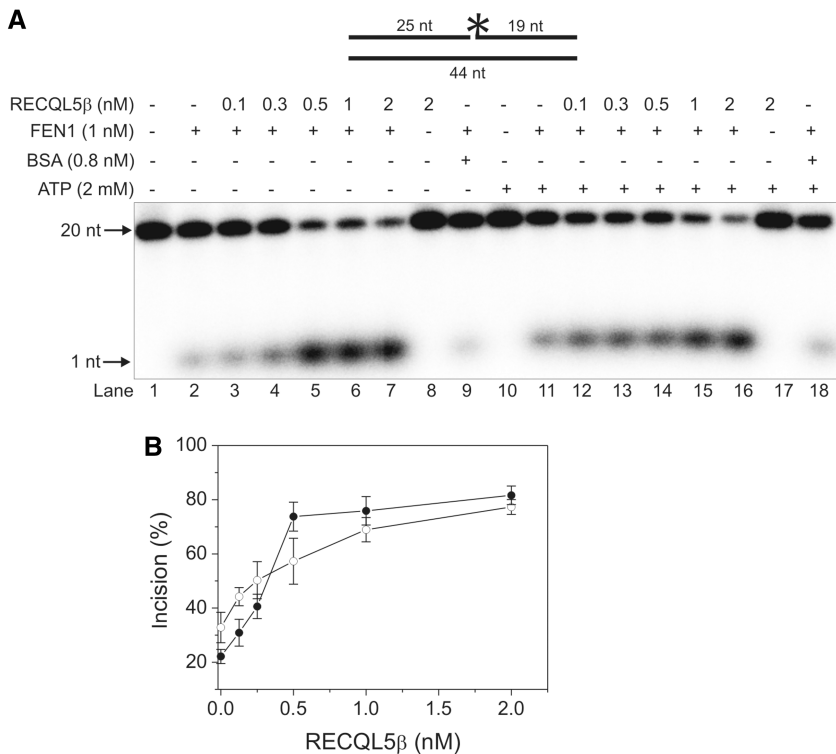
**Figure 3.** Kinetics of FEN1 cleavage of the 1 nt 5'-flap substrate in the presence or absence of RECQL5β. Reactions (70 μl) containing 1 nM 1 nt 5'-flap DNA substrate and 5 nM FEN1 were incubated at 37°C, and aliquots were removed at 1, 3, 6, 9, 12 and 15 min. The reactions conducted in the presence of RECQL5β contained 5 nM RECQL5β. (A) Phosphorimage of a typical gel from a kinetic experiment. Increasing times of incubation (1–15 min) for the FEN1 cleavage reactions conducted in the absence of RECQL5β (lanes 3–8) or the presence of RECQL5β (lanes 9–14) are indicated. Fifteen minute incubations conducted in the absence of RECQL5β + FEN1 or FEN1 are shown in lanes 1 and 2, respectively. (B) Percent incision from the data shown in (A), data points are the mean of three independent experiments with SDs indicated by error bars. Open circles, FEN1 alone; filled circles, FEN1 + RECQL5β.

oligonucleotide substrate with longer 5'-ssDNA flaps. ssDNA flap structures may have important biological functions as they are reaction intermediates during replication or during LP-BER. Flaps may also appear via strand displacement synthesis during unscheduled DNA synthesis by a DNA polymerase. RECQL5β's ability to stimulate the FEN1 cleavage reaction on a 30 nt 5'-ssDNA flap structure was tested. FEN1 alone (0.1 nM) incised 15% of the 1 nM substrate or 25% in the presence of 2 mM ATP (Figure 4A, lane 2 and 11, and B), yielding two products, the 31 nt and to a lesser extent, the 30 nt product. At equimolar concentrations of RECQL5β and FEN1 (0.1 nM), 32% or 45% of the 5'-flap substrate molecules, in the absence or presence of ATP, respectively, was incised, a 2.1- or 1.8-fold stimulation (Figure 4A, lane 4 and 13, and B). In the absence of ATP, increasing RECQL5β's concentration yielded 3.6- to 4.8-fold stimulation above FEN1's activity alone (Figure 4A, lanes 5–7 and B). Likewise, in the presence of ATP, FEN1's incision activity was stimulated 2.4- to 2.8-fold upon increasing the concentration of RECQL5β (Figure 4A, lanes 14–16 and B). RECQL5β alone did not catalyze cleavage of the 30 nt flap DNA substrate (Figure 4A, lane 8 and 17). BSA had no influence on the FEN1 incision (Figure 4A, lane 9 and 18).

FEN1 has been shown to possess 5'-3' exonuclease activity on nicked duplex DNA (48). A nick in the DNA duplex may arise as a consequence of the concerted action of DNA repair enzymes such as DNA glycosylase and AP endonuclease at the sites of damaged bases. We tested the effect of RECQL5β on FEN1 cleavage activity at the site of the nick. FEN1 (1 nM) incised 22% or 33% of the nicked DNA duplex substrate in the absence or presence of 2 mM ATP, respectively (Figure 5A, lane 2 and 11, and B). RECQL5β (0.3 nM) stimulated FEN1 to incise 41% or 50%, depending on the presence of ATP, of the nicked DNA duplex, a 1.9- or 1.5-fold stimulation of FEN1 exonuclease activity (Figure 5A, lane 4 and 13, and B). At equimolar concentrations of RECQL5β and FEN1 (1 nM), FEN1 cleavage increased to 76% or 69% incision in the absence or presence of ATP, respectively, a 3.5- or 2.1-fold stimulation above FEN1 alone (Figure 5A, lane 6 and 15, and B). RECQL5β has no endonuclease activity (Figure 5A, lane 8 and 17) and BSA did not stimulate FEN1's activity (Figure 5A, lane 9 and 18). These results indicate that RECQL5β can activate FEN1's incision of substrates containing a long 5'-ssDNA tail and FEN1's 5'-3' exonuclease activity on nicked duplex DNA.



**Figure 4.** RECQL5β stimulates FEN1 cleavage of duplex DNA containing 30 nt 5'-flap. Reactions (10 μl) containing 1 nM 30 nt 5'-flap substrate, 0.1 nM FEN1 and the indicated amounts of RECQL5β, or 0.8 nM BSA, were incubated at 37°C for 15 min. The presence of 2 mM ATP in reaction mixtures is indicated (lanes 10–18). (A) A phosphorimage of a typical gel. (B) Percent incision from the data shown in (A), data points are the mean of three independent experiments with SDs indicated by error bars.



**Figure 5.** RECQL5β stimulates FEN1 cleavage of duplex DNA containing a nick. Reactions (10 μl) containing 1 nM nicked duplex substrate, 1 nM FEN1 and the indicated amounts of RECQL5β, or 2 nM BSA, were incubated at 37°C for 15 min. The presence of 2 mM ATP in reaction mixtures is indicated (lanes 10–18). (A) A phosphorimage of a typical gel. (B) Percent incision from the data shown in (A), data points are the mean of three independent experiments with SDs indicated by error bars. Open circles, plus ATP; filled circles, minus ATP.

### RECQL5 $\beta$ does not stimulate FEN1 cleavage of a 5' hairpin flap substrate

It has been shown that flap substrates with secondary structures within the 5'-flap effectively inhibit FEN1 cleavage (41,49). Therefore, we tested RECQL5 $\beta$ 's ability to activate FEN1 cleavage on a DNA substrate containing a 5'-flap with a hairpin structure of 24 nt in the annealed region (Supplementary Figure 1). In the presence of 2 nM FEN1, about 45% of the 1 nM substrate was incised, and the addition of RECQL5 $\beta$  did not activate FEN1 (Supplementary Figure 1C, lanes 2–10). Addition of 2 mM ATP and RECQL5 $\beta$  clearly inhibited incision by FEN1 (Supplementary Figure 1C, lanes 14–22).

To investigate if the RECQL5 $\beta$  helicase could unwind an annealed region within the 5'-flap and facilitate its cleavage by FEN1, a partial duplex substrate (1 nM), mimicking a 5' hairpin flap substrate was constructed and used to screen for RECQL5 $\beta$  helicase activity. In the presence of RPA, RECQL5 $\beta$  helicase displayed weak unwinding of the annealed region within the 5'-flap (Supplementary Figure 2A). Subsequently, we investigated FEN1 cleavage of the 5' hairpin flap substrate in reactions supplemented with RECQL5 $\beta$ , 2 mM ATP and RPA. We tested two RECQL5 $\beta$  concentrations and neither showed FEN1 stimulation (Supplementary Figure 2B). These results indicate that RECQL5 $\beta$  does not stimulate FEN1 incision of a 5'-flap substrate with a secondary structure, and the helicase activity of the RECQL5 $\beta$  cannot facilitate this reaction.

### RECQL5 $\beta$ effects on GEN activity of FEN1 on double-stranded flaps and bubble substrates

The gap endonuclease (GEN) activity of FEN1 is critical for resolving stalled replication forks. It is responsible for cleavage of ssDNA regions that accumulate extensively in response to replication fork arrest (25). GEN activity specifically incises DNA replication-fork-like structures at the ssDNA region on either the lagging or the leading strand template. Therefore, DNA substrates were prepared with 3' and 5' double-stranded flap structures, which resemble DNA replication forks, and RECQL5 $\beta$  was tested for its ability to enhance FEN1 cleavage on such structures. FEN1 produced a GEN cleavage activity of 15% (2 nM FEN1) on the leading strand substrate (Figure 6A) and 24% (0.5 nM FEN1) on the lagging strand structure (Figure 6B, and C). There was also a strong 5' exonuclease activity of FEN1 on the leading strand (Figure 6A). RECQL5 $\beta$  did not increase GEN cleavage on the leading strand in the concentration range of 0.5–8 nM and addition of ATP slightly decreased the rate of GEN cleavage (Figure 6A, lanes 3–7, and lanes 12–16). However, RECQL5 $\beta$  in the absence of ATP significantly increased GEN cleavage on the lagging strand, ~2- to 2.7-fold (Figure 6B, lanes 5–7, and C). Addition of ATP resulted in less efficient stimulation of lagging strand GEN activity by RECQL5 $\beta$  (Figure 6B, lanes 12–16, and C). RECQL5 $\beta$  alone had no incision activity (Figure 6A and B, lanes 8 and 17).

Next, a bubble substrate was constructed which mimics a stalled replication fork structures (Supplementary Figure 3B). FEN1 (0.5 nM) cleaved the ssDNA–dsDNA junctions of the bubble substrate, denoted as P<sub>GENa</sub> and P<sub>GENb</sub> (Supplementary Figure 3C). The exonuclease activity of FEN1 was also slightly active on the 5'-end of bubble structure generating P<sub>5'EXO</sub> (Supplementary Figure 3C). The cleavage of the bubble structure by FEN1 alone (0.5 nM) resulted in 34% of P<sub>GENa</sub> and 5% of P<sub>GENb</sub> (Supplementary Figure 3C, lane 2). RECQL5 $\beta$  failed to stimulate FEN1's GEN cleavage in the concentration range of 0.1–2 nM and addition of ATP slightly decreased the rate of the cleavage (Supplementary Figure 3C, lanes 3–7, and lanes 12–16).

### RECQL5 $\beta$ and FEN1 interact physically

To determine whether RECQL5 $\beta$  and FEN1 interact physically, we performed affinity pull-down assays. RECQL5 $\beta$  was expressed in bacteria as a fusion with a CBD tag and bound to chitin beads. The beads were subsequently incubated with either total extract of exponentially growing human embryonic kidney cells HEK293T or with purified FEN1 protein. We found that endogenous FEN1 was bound to RECQL5 $\beta$  beads, but not to control beads coated with CBD (Figure 7A). Recombinant FEN1 was found to be bound to both RECQL5 $\beta$  beads and beads coated with CBD only. However, the affinity of FEN1 for the RECQL5 $\beta$  beads was higher than that for the control, indicating a specific interaction (Figure 7B).

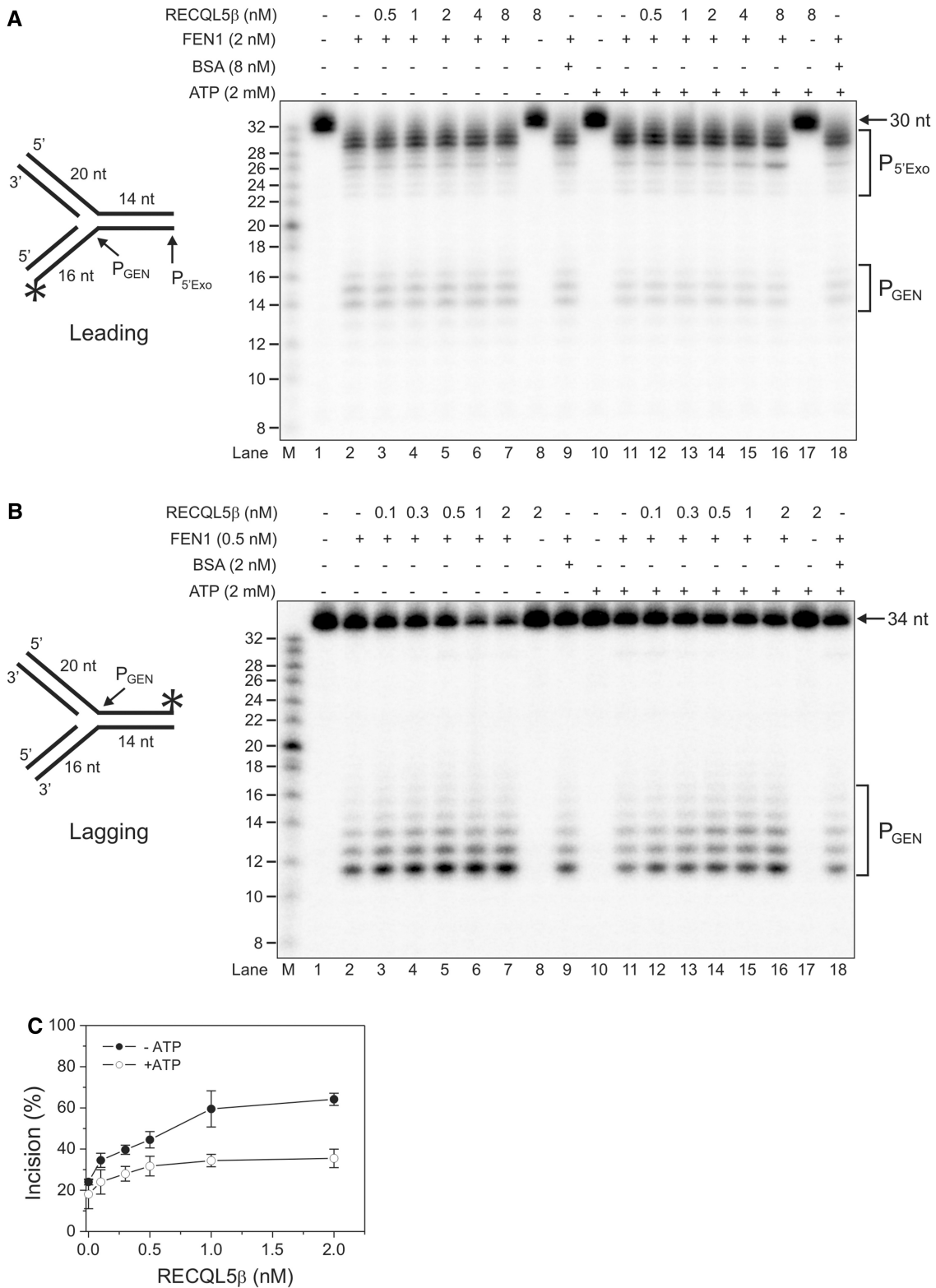
Affinity pull-down assays were also used to map the region on FEN1 that is important for the RECQL5 $\beta$ :FEN1 interaction. A graphic diagram of FEN1 and the GST fragments are shown in Figure 7C. As above, RECQL5 $\beta$  was bound to chitin beads then incubated with the purified GST-FEN1 fragments. As can be seen in Figure 7D, the fragment of FEN1 encoding the largest C-terminal fragment of FEN1 (#54) was preferentially bound by RECQL5 $\beta$ .

To test whether RECQL5 $\beta$  and FEN1 interact *in vivo*, RECQL5 $\beta$  was immunoprecipitated from the HEK293T extract and the resulting immunoprecipitate was subjected to western blot analysis. We could not detect FEN1 in this immunoprecipitate, indicating that RECQL5 $\beta$  and FEN1 do not form a stable complex *in vivo* (data not shown).

### RECQL5 $\beta$ co-localizes with FEN1 in human cells

We investigated the subcellular localization of 3XFlag-RECQL5 $\beta$ -Myc and endogenous FEN1 by confocal microscopy to explore whether 3XFlag-RECQL5 $\beta$ -Myc co-localizes with FEN1 before and after DNA damage. We transiently transfected HeLa cells with plasmid encoding a full-length copy of 3XFlag-RECQL5-Myc encoding sequence. Twenty-four hours post transfection, the cells were treated with 100  $\mu$ M H<sub>2</sub>O<sub>2</sub> for 30 min to introduce oxidative stress and DNA damage. In asynchronous untreated HeLa cells, FEN1 and RECQL5 $\beta$  displayed diffused nuclear staining with some



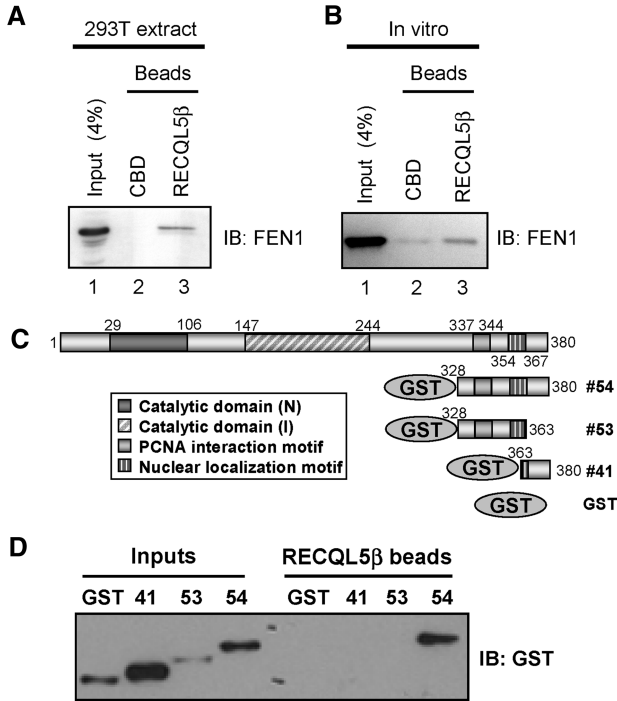


**Figure 6.** RECQL5 $\beta$  effects on FEN1's GEN activity on double-stranded flap substrates. Reactions (10  $\mu$ l) containing 1 nM 3' double-stranded flap substrates (A, Leading) or 5' double-stranded flap substrate (B, Lagging), the indicated amounts of FEN1 and RECQL5 $\beta$ , or BSA, were incubated at 37°C for 15 min. The presence of 2 mM ATP in reaction mixtures is indicated (lanes 10–18). (C) Percent incision from the data shown in (B), data points are the mean of three independent experiments with SDs indicated by error bars.

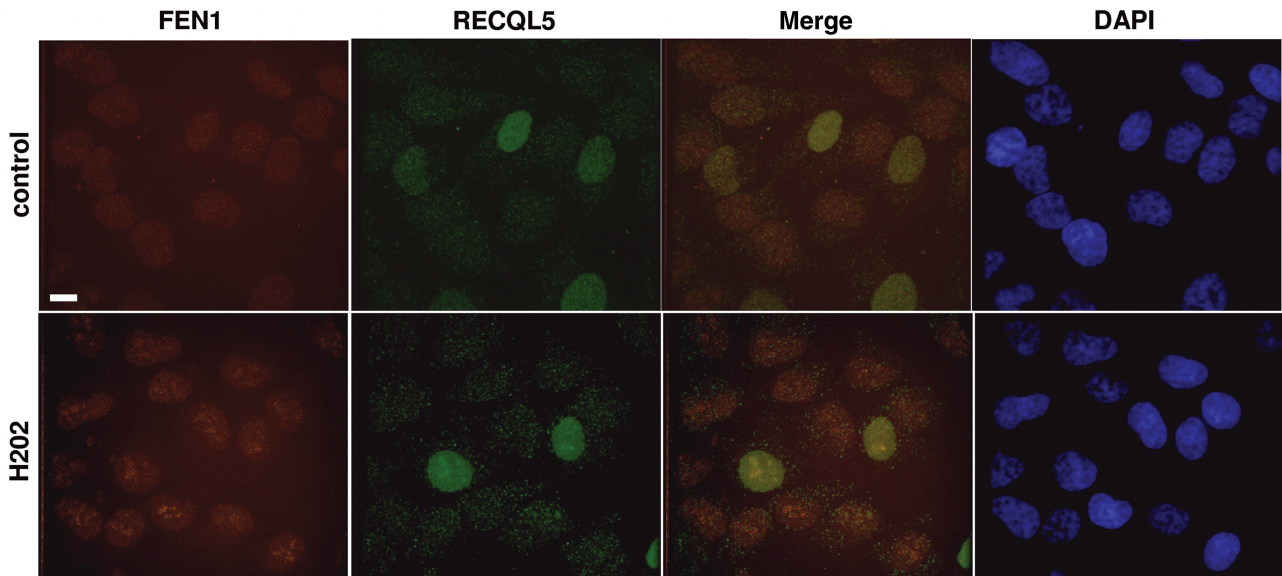
concentrated foci (Figure 8). In H<sub>2</sub>O<sub>2</sub> treated cells, we observed that both FEN1 and RECQL5β formed distinct nucleolar foci and these foci co-localized (Figure 8).

**DISCUSSION**

FEN1 participates in many processes within the cell. Specifically, it has been shown to play a role in base excision repair, homologous recombination, lagging strand DNA replication, re-initiation of stalled replications forks and telomere stability (24,31,50). Here, we report that human RECQL5β physically interacts with FEN1 and efficiently stimulates FEN1 cleavage activity on a variety of DNA substrates that are proposed intermediates in DNA replication and repair. It has been reported recently that mouse RECQL5β plays an important role in maintaining active DNA replication to prevent the collapse of replication forks and the accumulation of DSBs (20). Additionally, RECQL5β is associated with the DNA replication machinery and is present at the sites of stalled replication forks (14). The identification of RECQL5β in a replication complex suggests that RECQL5β may play a direct role in replication. It has also been suggested that FEN1 participates in lagging strand synthesis *in vivo* (26) through the processing of Okazaki fragments. RECQL5β may facilitate FEN1 cleavage of the remaining ribonucleotide (5'-flap substrates), a step required prior to the ligation of Okazaki fragments (1). Our results revealing the ability of RECQL5β to potently stimulate cleavage of the 1 nt, as well as longer, 30 nt 5'-flap, of a DNA duplex (Figures 2–4) strongly support this idea. Moreover, we show that RECQL5β is capable of stimulating FEN1 cleavage on a substrate that mimics the DNA replication fork; however, only on the lagging strand. RECQL5β failed to stimulate GEN activity on the leading strand (Figure 6). This finding might have important implications during DNA replication of the lagging stand by facilitating processing of Okazaki fragments. The 3'–5'



**Figure 7.** RECQL5β and FEN1 interact directly. Binding of FEN1 from 293T extracts (A) or purified FEN1 (B) to chitin beads coated with CBD or RECQL5β-CBD. (C) Diagram of GST-FEN1 fragments used to define which domain of FEN1 RECQL5β interacts with. (D) Binding of GST fused to various C-terminal fragments of FEN1 to chitin beads coated with RECQL5β-CBD.



**Figure 8.** Co-localization of endogenous FEN1 and overproduced 3xFlag-RECQL5β-Myc in HeLa cells treated with H<sub>2</sub>O<sub>2</sub>. Twenty-four hours after transfection of plasmid encoding 3xFlag-RECQL5β-Myc, non-synchronized cells were incubated in the presence or absence of 100 μM H<sub>2</sub>O<sub>2</sub> for 30 min and then fixed. Cells were triply stained for FEN1 (red), 3xFlag-RECQL5β-Myc (green) and DNA (blue) as described in the ‘Materials and Methods’ section, and analyzed by confocal microscopy. Yellow color in superimposed images (merge) indicates co-localization of FEN1 and 3xFlag-RECQL5β-Myc. White bar represents 50 μm.

helicase activity of RECQL5 $\beta$  could function during replication restart, RECQL5 $\beta$  may translocate to the stalled fork and ensure efficient removal of the new lagging strand by its abilities to displace the nascent Okazaki fragment and stimulate the nuclease activities of FEN1, similar to a model proposed for the coordinate action of *Escherichia coli* RecQ and RecJ (51). This would prevent deleterious DNA strand breakage and elevated recombination.

Previously, human WRN, BLM and RECQL4 have been shown to stimulate FEN1 catalytic activity (25,40–42,46). Thus it seems that there is a conserved interaction between the human RecQ helicases with FEN1 which is likely to be important for the efficient processing of DNA. In contrast to RECQL5 $\beta$ , WRN has the ability to stimulate FEN1 cleavage on both strands, lagging and leading, within the replication-like-structure as well as in collapsed replication fork substrate (25). The stimulation of FEN1 activity by WRN is independent of WRN catalytic activities and is mediated by a C-terminal region of the WRN protein (40). We also demonstrate that helicase activity is not required for stimulation of FEN1 cleavage by RECQL5 $\beta$  (Figure 2). Interestingly, it has been reported recently that inhibition of RNA polymerase II transcription by RECQL5 $\beta$  is also independent of its helicase activity (52).

FEN1 is a multifunctional protein and more than 20 proteins are known to interact with it. Recently, deletion and alanine substitution mutagenesis of FEN1 was used to map sites where PCNA, WRN, APE1, EndoG and the Rad9–Rad1–Hus1 complex interact with FEN1 (53). All the proteins tested interacted with the very C-terminus of FEN1. Consistent with how other proteins interact with FEN1, we show that RECQL5 $\beta$ 's interaction with FEN1 is mediated by the C-terminus of FEN1 (Figure 7).

There are growing pieces of evidence suggesting that the RecQ DNA helicases operate in various DNA repair processes induced by DNA replication defects, BER and nonhomologous end-joining of double-strand break repair. A role for FEN1 in BER is well-documented both *in vitro* and *in vivo* (32,54). After the damaged base is replaced by the correct one through the concerted action of DNA glycosylases, APE and Pol  $\beta$ , the repair is directed into different sub-pathways, single-nucleotide and LP-BER, depending on whether the sugar residue in the flap has been further modified. The LP-BER pathway requires FEN1 to effectively remove the modified 5'-dRP-containing flap. Pol  $\beta$  has been previously proposed to independently displace the downstream DNA strand and create a 2–11 nt flap for FEN1 cleavage (32,55). However, Pol  $\beta$  DNA synthesis on DNA containing a flap or nicked DNA is poor indicating that generation of a nicked flap intermediate would be a rate-limiting step of LP-BER DNA synthesis. A recent study demonstrated that FEN1 cleavage activity is critical in Pol  $\beta$ -mediated LP-BER DNA synthesis because it removes the blocking one nucleotide flaps (56). According to the proposed hypothesis of a 'Run and Hit' mechanism, Pol  $\beta$  relies on FEN1 cleavage to remove these barriers and proceed with LP-BER.

DNA substrates containing flaps, as used in our study (Table 1), can mimic a LP-BER intermediate because the

single-nucleotide gap has already been filled. The fact that RECQL5 $\beta$  cooperates with FEN1 on such intermediates poses a question of whether RECQL5 $\beta$  may be engaged in LP-BER *in vivo* by stimulating FEN1 to cleave the one nucleotide or longer flap. We show that in HeLa cells treated with hydrogen peroxide, which introduces oxidative damage to DNA, there is mobilization of RECQL5 $\beta$  and FEN1 and the foci of both proteins co-localize (Figure 8). Future work will focus on elucidating the potential role of RECQL5 $\beta$  in LP-BER *in vivo*.

Previously, WRN has been shown to interact and stimulate strand displacement DNA synthesis by Pol  $\beta$  (57,58). This prompted us to check whether RECQL5 $\beta$  could also cooperate with Pol  $\beta$  as well as with another important BER enzyme, AP endonuclease, APE1. However, our results showed that RECQL5 $\beta$  does not stimulate Pol  $\beta$  or APE1 (not shown).

To summarize, our results suggest that RECQL5 $\beta$  can modulate FEN1's activity and since FEN1 participates in many DNA metabolic pathways, the expression of RECQL5 $\beta$  has the potential to influence all of these FEN1-dependent processes as well. Further research is needed to evaluate the true set of pathways in which RECQL5 $\beta$  participates.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

## ACKNOWLEDGEMENTS

We would like to thank Dr Marie Rossi and Dr Takashi Tadokoro for critically reading this article. We would like to thank Dr Tomasz Kulikowicz for construction of the p3XFlag-RECQ5L $\beta$ -Myc CMV 24 plasmid. We would like to thank Dr David Wilson III (NIA) for the FEN1 protein.

## FUNDING

Intramural Research Program of the National Institutes of Health; National Institute on Aging, annual report Z01 AG000726-16; Swiss National Science Foundation (to S.S., P.J.). Funding for open access charge: National Institutes of Health.

*Conflict of interest statement.* None declared.

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