The Nuclease Domain of the *Escherichia Coli* RecBCD Enzyme Catalyzes Degradation of Linear and Circular Single-Stranded and Double-Stranded DNA†

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ABSTRACT: The 30 kDa C-terminal domain of the RecB protein (RecB30) has nuclease activity and is believed to be responsible for the nucleolytic activities of the RecBCD enzyme. However, the RecB30 protein, studied as a histidine-tagged fusion protein, appeared to have very low nucleolytic activity on single-stranded (ss) DNA [Zhang, X. J., and Julin, D. A. (1999) *Nucleic Acids Res. 27,* 4200−4207], which raised the question of whether RecB30 was indeed the sole nuclease domain of RecBCD. Here, we have purified the RecB30 protein without a fusion tag. We report that RecB30 efficiently degrades both linear and circular single- and double-stranded (ds) DNA. The endonucleolytic cleavage of circular dsDNA is consistent with the fact that RecB30 has amino acid sequence similarity to some restriction endonucleases. However, endonuclease activity on dsDNA had never been seen before for RecBCD or any fragments of RecBCD. Kinetic analysis indicates that RecB30 is at least as active as RecBCD on the ssDNA substrates. These results provide direct evidence that RecB30 is the universal nuclease domain of RecBCD. The fact that the RecB30 nuclease domain alone has high intrinsic nuclease activity and can cleave dsDNA endonucleolytically suggests that the nuclease activity of RecB30 is modulated when it is part of the RecBCD holoenzyme. A new model has been proposed to explain the regulation of the RecB30 nuclease in RecBCD.

The RecBCD holoenzyme from *Escherichia coli*, also known as exonuclease V, is a heterotrimeric enzyme composed of the RecB, RecC, and RecD protein subunits. The enzyme is a DNA helicase, a single-stranded (ss)DNA[1] nuclease, and a double-stranded (ds)DNA nuclease. As a multifunctional enzyme in *E. coli*, RecBCD has two conflicting roles: degrading invading foreign DNA and repairing double-strand breaks in the *E. coli* genome (*1*−*3*). These paradoxical roles are resolved by a specific 8-nucleotide (nt) DNA sequence, the recombination hot spot, Chi (5′-GCTGGTGG) (*4*, *5*). Chi appears at high frequency in the *E. coli* genome and is designed by nature to protect the genome from degradation by RecBCD. This allows RecBCD to operate in chromosomal maintenance, rescuing the *E. coli* genome from the deleterious effects of double-strand breaks via recombinational repair (*6*, *7*).

The RecBCD holoenzyme was classified as an ATP-dependent ssDNA and dsDNA exonuclease, and an ATP-stimulated ssDNA endonuclease, based upon the observations that the enzyme digests linear ssDNA and dsDNA and circular ssDNA, but not circular dsDNA (*8*−*12*). The nucleolytic activities of the RecBCD enzyme play important roles in the initiation of recombination and repair of dsDNA breaks in *E. coli* (*1*, *2*, *6*, *13*, *14*). The enzyme initiates recombinational repair from a dsDNA end. After binding to an end, the enzyme processively unwinds the linear dsDNA with two helicase motors of opposite polarities, RecB and RecD (*15*−*17*). The ssDNA produced by the helicase(s) is the substrate for the nuclease activity of the enzyme (*10*, *11*, *18*). Degradation of the DNA duplex is thought to be asymmetric, with the 3′-terminal strand (3′ → 5′ direction) being degraded more vigorously than the 5′-terminal strand (5′ → 3′ direction) (*19*, *20*), suggesting that RecBCD has both 3′ → 5′ and 5′ → 3′ ssDNA exonucleolytic activities (*7*, *21*). When a translocating RecBCD molecule recognizes a Chi site, the enzyme pauses, and the 3′ → 5′ exonucleolytic activity is attenuated (*19*, *20*). The enzyme then continues unwinding the DNA at approximately one-half the initial rate (*22*), but now the 5′ → 3′ exonucleolytic activity of RecBCD is activated (*21*, *23*). Binding of RecA to the 3′-terminated single strand, produced after the recognition of Chi, initiates homologous recombination with an unbroken chromosome (*24*, *25*). The biochemical basis for the effect of Chi on RecBCD is not well-understood.

Mutagenesis studies in RecBCD suggest that RecB30, the 30 kDa C-terminal domain of RecB, is required for all nucleolytic activities, namely, ssDNA exo-/endonucleolytic and dsDNA exonucleolytic activities, of the RecBCD holoenzyme (*26*−*28*). The nucleolytic activity of RecB is lost when RecB30 is removed by limited proteolysis (*26*). In addition, the isolated hexahistidine-tagged RecB30 domain has very low but measurable nuclease activity. A weak nonspecific nuclease activity on circular ssDNA could be observed only when the RecB30 domain was fused to a

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[1] Abbreviations: dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; BSA, bovine serum albumin; nt, nucleotide residue; RecB30, the 30 kDa C-terminal domain of *E. coli* RecB protein; DTT, dithiothreitol; bp, base pair(s).

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Table 1: The Oligonucleotides Used in This Study

Substrates and markers:
34A-mer: 5′-d(CGG CTG GTG GCA CTC GGA CCT GAC CCT GTG AAT G)-3′
34B-mer: 5′-d(CAT TCA CAG GGT CAG GTC CGA GTG CCA CCA GCC G)-3′
34K-mer: 5′-d(CGC ACT CGC AGG TGG GGA CCT GAC CCT GTG AAT G)-3′
34L-mer: 5′-d(CAT TCA CAG GGT CAG GTC CCC ACC TGC GAG TGC G)-3′
6A-mer: 5′-d(CGG CTG)-3′
4A-mer: 5′-d(CGG C)-3′
3A-mer: 5′-d(CGG)-3′
2A-mer: 5′-d(CG)-3′
phagemid M13 mp18(+)
plasmid pET15b

RecB30^{D1067A} site-directed mutagenesis primers:
5′-d(GGC ATG TTA AAA GGC TTT ATC GCC CTG GTG TTC CGC CAC GAA GGG)-3′
5′-d(CCC TTC GTG GCG GAA CAC CAG GGC GAT AAA GCC TTT TAA CAT GCC)-3′

RecB30^{D1080A} site-directed mutagenesis primers:
5′-d(GGG CGT TAT TAC CTG CTC GCC TAT AAA TCC AAC TGG TTG GG)-3′
5′-d(CC CAA CCA GTT GGA TTT ATA GGC GAG CAG GTA ATA ACG CCC)-3′

heterologous ssDNA binding protein, the gp32 protein of phage of T4 (*26*), or at high protein concentration in the presence of poly(ethylene glycol) (*29*). This raises the question of whether RecB30 is indeed the sole nuclease domain of RecBCD or whether other domains or subunits of RecBCD are required for full nucleolytic activities of RecB30.

Sequence analysis shows that this domain is present in several bacterial, archaeal, eukaryotic, and phage proteins and forms a new nuclease family, the "RecB nuclease family" (*30*). Asp^{1067}, Asp^{1080}, and Lys^{1082} residues of RecB30 compose a motif that is highly conserved in the family (*30*). Single point mutations in this motif create mutant RecBCD holoenzymes that are processive helicases with no measurable nuclease activity (*26*, *28*). As noted previously (*28*), this motif is quite similar to the active sites of other nucleases, including type II restriction endonucleases such as *Eco*RI, *Eco*RV, *Pvu*II, *Bgl*I, and *Fok*I (*31*, *32*), the *E. coli* MutH protein (*33*), and the bacteriophage λ exonuclease (*34*). Therefore, we predict that RecB30 would cleave dsDNA endonucleolytically, as can the restriction enzymes. However, this had never been seen for RecBCD or any pieces of RecBCD.

To address these questions, we have characterized the nucleolytic activities of the isolated RecB30 domain, free of any fusion peptide, on ssDNA and dsDNA in both linear and circular forms. Here, we report that RecB30 efficiently degrades linear ssDNA and dsDNA. Interestingly, RecB30 can also efficiently cleave circular ssDNA and dsDNA, indicating that RecB30 has endonucleolytic activity on dsDNA as well as on ssDNA. However, the putative Mg^{2+}-binding site mutants, RecB30^{D1067A} and RecB30^{D1080A}, do not degrade the same substrates. Degradation kinetics of RecB30 on the ssDNA and dsDNA substrates have been determined, and quantitative measurements show that, for a linear ssDNA oligonucleotide, RecB30 is at least as active as RecBCD. These results provide direct evidence that RecB30 is the sole nuclease domain of the RecBCD enzyme (*28*). On the basis of these results, a new model has been proposed to explain the regulation of the nucleolytic activities of RecB30 in the RecBCD holoenzyme.

MATERIALS AND METHODS

Materials. ATP was purchased from Sigma Co. (St. Louis, MO). [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]ddCTP (3000 Ci/mmol), [α-^{32}P]AMP (3000 Ci/mmol), and circular single-stranded M13mp18 (+) phage DNA were purchased from Amersham (Piscataway, NJ). Circular double-stranded pET15b plasmid DNA was purchased from Novagen (Madison, WI). Terminal deoxynucleotidyl transferase, polynucleotide kinase, bovine serum albumin, and *E. coli* ExoI nuclease were purchased from New England Biolabs (Beverly, MA). Oligodeoxynucleotides listed in Table 1 for the nuclease reactions and for molecular weight markers were purchased from MWG-Biotech (High Point, NC) or were synthesized at the National Institutes of Health (Bethesda, MD). The DNA concentrations were determined from A_{260} measurements using absorption coefficients calculated from the nucleotide sequence for the oligomeric substrates (*35*). The RecBCD holoenzyme was purified as previously described (*26*, *36*). Chelex resin was purchased from Bio-Rad (Hercules, CA). Puratronic $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$ were purchased from Alfa Aesar (Ward Hill, MA).

Construction of a Plasmid Encoding the RecB30 Protein and Site-Directed Mutagenesis. The plasmid pET15b-RecB30 encoding the His_6-tagged C-terminal domain of RecB (amino acids 928−1180) was constructed as previously described (*29*). The expression cassette encoded an N-terminal histidine affinity tag, an intervening six amino acid thrombin recognition site, and the residues 928−1180 of RecB. This system produces a 269-residue soluble fusion protein, which upon thrombin cleavage yields a homogeneous 257-residue RecB30 protein, which includes an N-terminal GSHM tetrapeptide resulting from the C-terminal part of the thrombin cleavage site (GS) and an HM sequence encoded by the *Nde*I site at which the RecB30 gene was inserted. The pET15b-RecB30 plasmid was used as the template for site-directed mutagenesis to produce two single point mutants of RecB30 by changing Asp^{1067} or Asp^{1080} to Ala using the QuickChangeTM site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotides used in the mutagenesis experiment (MWG Biotech) are listed in Table 1.

Protein Expression and Purification of Wild-type and Mutant RecB30 Proteins. The plasmids encoding RecB30 protein were transformed into the *E. coli* strain BL21(DE3) (Novagen), and protein overexpression was induced in log phase by 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 10 h at 25 °C. The cells were lysed by adding lysozyme to 0.2 mg/mL in lysis buffer containing 20 mM

Tris-HCl (pH 7.4), 100 mM NaCl, and 1 mg/mL benzamidine, followed by sonication. The lysate was centrifuged, and the supernatant was applied to a pre-equilibrated 5 mL Ni^{2+}-charged HiTrap-Sepharose Chelating column (Amersham, Piscataway, NJ). The column was extensively washed with the wash buffer (20 mM Tris-HCl, pH 7.4, and 500 mM NaCl), followed by three step gradients of imidazole in the wash buffer (50, 200, and 500 mM, respectively). The histidine-tagged RecB30 fusion protein was eluted at an imidazole concentration of 200 mM. The RecB30 fusion protein was extensively dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.4) and 100 mM NaCl at 4 °C, and then was cleaved with thrombin (Sigma, St. Louis, MO) to remove the His-tag (1 mg fusion protein/1 U thrombin) for 1 h at 25 °C. The digested mixture was applied to a 1 mL HiTrap Benzamidine FF agarose column (Amersham, Piscataway, NJ) and subsequently applied to a 5 mL Ni^{2+}-charged HiTrap Chelating column. The thrombin-cleaved RecB30 protein was eluted at 30 mM imidazole, and was then loaded onto a 320 mL Sephacryl 100 gel filtration column (Amersham, Piscataway, NJ) equilibrated with a buffer containing 20 mM Tris-HCl (pH 7.4) and 50 mM NaCl for further purification. This three-step purification procedure yielded 80−120 mg of protein from 1 L of LB culture with purity of about 97% as determined by SDS−PAGE (see Supporting Information).

For the nuclease activity assays, the RecB30 protein was further purified by two additional chromatographic methods, ion exchange and hydroxylapatite chromatography, to eliminate any minor contaminants. The RecB30 protein was first purified using a 5 mL HiTrap Q ion exchange column (Amersham, Piscataway, NJ), with a linear NaCl gradient from 0 to 500 mM NaCl in 20 mM Tris-HCl (pH 7.4) buffer, and RecB30 was eluted in fractions containing 120−200 mM NaCl. Then, RecB30 was purified at 4 °C using a 5 mL hydroxylapatite column (Bio-Rad, Hercules, CA) equilibrated with a buffer of 20 mM K_2HPO_4 (pH 6.8) and 2 mM DTT (4.0 mg of protein/mL of resin). RecB30 was eluted with a linear gradient consisting of 50 mL of 20 mM K_2HPO_4 (pH 6.8) and 50 mL of 500 mM K_2HPO_4 (pH 6.8). RecB30 was eluted from 60 to 100 mM K_2HPO_4. The final purity of the protein was more than 98%. The purification of RecB30 mutants was performed using the same procedures as described for wild-type RecB30. The protein concentrations were determined from absorption measurements using an absorption coefficient of $A_{280} = 39\,400\ M^{-1}\ cm^{-1}$. The nuclease activities of RecB30 remained stable for at least 3 months at 4 °C when the protein was stored in the buffer containing 20 mM Tris-HCl (pH 7.4), 50 mM KCl, and 2 mM DTT.

DNA Substrate Preparation. Oligonucleotides used in ssDNA and dsDNA nuclease activity assays are listed in Table 1. The commercial oligonucleotides were further purified by isolation from a 15% denaturing polyacrylamide gel, followed by ethanol precipitation. The oligonucleotides were labeled with ^{32}P at their 5′-ends using [γ-^{32}P]ATP and T4 polynucleotide kinase, following the protocols in ref *37*, and purified by gel filtration using a Micro Bio-Spin P-30 column (Bio-Rad, Hercules, CA). The oligonucleotides were labeled with ^{32}P at the 3′-ends by addition of [α-^{32}P]ddCTP catalyzed by 10 units of terminal transferase in a 50 μL reaction containing 20 mM Tris-HOAc (pH 8.0), 10 mM $MgCl_2$, 50 mM KOAc, 1 mM DTT, 2.5 mM $CoCl_2$, 10 pmol oligonucleotide, 100 μCi of [α-^{32}P]ddCTP, and 100 μg/mL of bovine serum albumin (BSA). The reaction was carried out at 37 °C for 30 min before termination by heating the mixture to 70 °C for 10 min. The labeled oligonucleotides were separated from free [α-^{32}P]ddCTP using the Micro Bio-Spin P-30 columns. All of the molecular weight marker oligonucleotides used in this study were labeled at the 5′-end with [γ-^{32}P]ATP and T4 polynucleotide kinase.

The substrates used for nuclease activity assays were 34-mer ssDNA (34A and 34K) and dsDNA (34A/34B, and 34K/34L) oligonucleotides (Table 1). Oligonucleotide 34A was 5′-labeled or 3′-labeled to make the substrate used in Figure 1 for ssDNA nucleolytic activity assay. Oligonucleotide 34A was 5′-labeled or 3′-labeled and hybridized to 34B to make the substrate used in Figure 3 for dsDNA nuclease activity assay. Oligonucleotide 34K was 5′-labeled and used in Figure 7 for ssDNA cleavage kinetics. The labeled oligonucleotide (34K) was hybridized to 34L to make the substrate for the dsDNA incision kinetic study shown in Figure 7. Hybridization was carried out in 10 mM Tris-HCl, pH 8.0, and 5 mM $MgCl_2$ using 1:3 molar ratios of labeled/unlabeled oligonucleotides for preparation of dsDNA substrates. Substrates were purified from 6 to 10% nondenaturing polyacrylamide gels and ethanol precipitation. The specific activities were 6×10^6 cpm/pmol for 5′-labeled substrates and 2.5×10^6 cpm/pmol for 3′-labeled substrates.

Nuclease Reaction Assays with 5′- and 3′- ^{32}P-End-Labeled ssDNA and dsDNA Substrates. The nuclease reaction was carried out with the indicated amount of RecB30 or mutant enzyme and the designated amount of ^{32}P-labeled ssDNA or dsDNA in 10 μL of *nuclease reaction buffer* [40 mM Tris-HCl (pH 7.4), 5.0 mM $MgCl_2$, 5.0 mM DTT, 150.0 mM NaCl, and 0.1 mg/mL of BSA]. The reaction was incubated at a specified temperature for a defined time and terminated by adding 2.0 μL of the *quenching* solution (2.0% SDS, 40 mM EDTA, 3.0% bromphenol blue, 3.0% xylene cyanol, and 40% glycerol). An aliquot of each terminated reaction mixture was mixed with an equal volume of *sample buffer* (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol) and run on a 15% denaturing (7 M urea) polyacrylamide gel at 1500 V for 2.5 h using TBE running buffer (89 mM Tris base, 89 mM boric acid, pH 8.0, and 2.0 mM EDTA). The gel was dried, and the bands were visualized and quantified by PhosphorImager (Molecular Dynamics, Amersham, Piscataway, NJ). The intensity of each band in the phosphorimage was determined using the volume integration function of the PhosphorImager. The concentration of each labeled fragment i in lane j was calculated using the equation: $\text{Fragment}_{ij} = [(\text{intensity}_{ij}/(\text{total})_j] \times \text{DNA}_o$, where intensity_{ij} is the integrated intensity of band i in lane j, $(\text{total})_j$ is the sum of all integrated bands in lane j, and DNA_o is the initial DNA concentration.

Nuclease Reaction Assay with Single-Stranded and Double-Stranded Circular DNA. The endonuclease activities of wild-type and mutant RecB30 proteins were assayed using circular single-stranded M13mp18 and circular double-stranded pET15b DNA as the substrates under standard conditions in the nuclease reaction buffer as described above, in a volume of 10 μL. The reactions were quenched by adding 2.0 μL of the quenching solution as specified above and were moni-

tored on a 1% (w/v) agarose gel in TBE buffer with ethidium bromide staining (0.5 μg/mL).

Kinetic Analysis of Linear ssDNA and dsDNA Degradation by RecB30. The incision kinetics of linear ssDNA and dsDNA by RecB30 at 25 °C were performed under the nuclease assay conditions using 1 nM RecB30, but varying concentrations of DNA substrates. The reactions were incubated for 10, 30, 60, 120, 300, and 600 s, respectively, and the initial reaction velocities were determined from the slope of a plot of the substrate concentration versus time, using the $t = 0$ point and at least the first two time-points of the reaction. V_{max} and K_m values were calculated by directly fitting the data to the Michaelis−Menten equation using SigmaPlot software (SigmaPlot, Chicago, IL).

The Effects of Divalent Metal Ions and ATP on Nuclease Activities of RecB30. RecB30 (20 nM) was incubated with 3 nM ssDNA (34A) or 3 nM dsDNA (34A/34B) in 10 μL of nuclease reaction buffer without Mg^{2+} and DTT. To determine the effect of different metal ions on the catalytic capacity of RecB30, 5 mM $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, or $ZnCl_2$ was included in the reaction. To determine the effect of ATP on the catalytic capacity of RecB30, various concentrations of ATP were added to the reactions and the concentration of Mg^{2+} was maintained at 5 mM. The reaction was incubated at 25 °C for 10 min and manipulated as described for the standard nuclease assays.

RESULTS

Construction and Purification of Wild-type and Mutant RecB30 Nucleases. The putative nuclease domain of the RecBCD enzyme was previously mapped to the C-terminal 253-residue fragment (amino acids 928−1180) of RecB, and biochemical studies were mainly carried out using a histidine-tagged fusion protein (*26*, *27*, *29*). In this study, the isolated RecB30 domain instead of a fusion protein was used in all nucleolytic activity assays in order to eliminate any possible contribution to or interference with the nucleolytic activity of RecB30 from a fusion tag. We also used multidimensional nuclear magnetic resonance spectroscopy (NMR) to ensure that the isolated RecB30 domain and the RecB30 mutants have folded structures.

Analysis of structural and evolutionary relationships indicates that the RecB30 nuclease is structurally similar to some restriction endonucleases, suggesting that Asp^{1067} and Asp^{1080} residues of RecB30 form a Mg^{2+}-binding site (*38*). To characterize the roles of Asp^{1067} and Asp^{1080} residues in the nuclease activities of RecB30, they were separately changed to alanine by site-directed mutagenesis to create two single point mutants, $RecB30^{D1067A}$ and $RecB30^{D1080A}$. These two RecB30 mutants were also expressed as histidine-tagged fusion proteins and cleaved by thrombin to remove the fusion tag and purified by the same methods as described for the wild-type protein. They were shown to have essentially identical structures as the wild-type protein by NMR (unpublished results). For the nucleolytic activity assays, the wild-type and mutant RecB30 proteins were purified by the consecutive steps of Ni^{2+}-affinity (one column for the fusion protein and one for the cleaved protein), gel filtration, ion exchange, and hydroxylapatite chromatography to eliminate any minor impurities resulting from cellular nucleases (see Supporting Information).

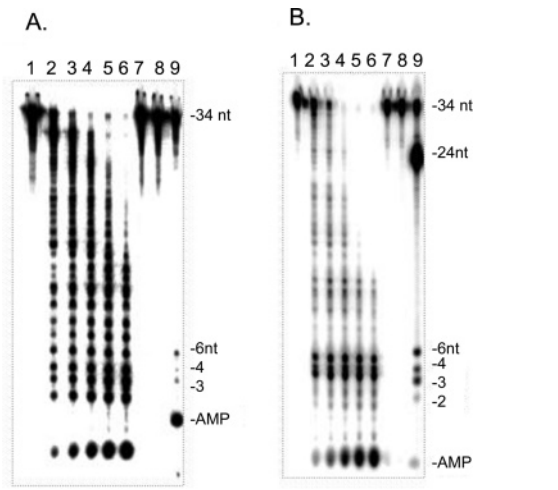

FIGURE 1: Time-course nuclease reactions of wild-type and mutant RecB30 proteins with a 5′- or 3′-labeled linear ssDNA substrate. Wild-type RecB30 (20 nM) or a mutant (20 nM) was incubated with the ^{32}P-labeled 34A oligonucleotide (10 nM) (Table 1) in the nuclease reaction buffer at 37 °C. The reactions were analyzed on 15% denaturing PAGE and visualized by autoradiography as described in Materials and Methods. (A) Pictorial representation of nuclease activities of wild-type RecB30 (lanes 1−6), $RecB30^{D1067A}$ (lane 7), and $RecB30^{D1080A}$ (lane 8) mutants on the 5′-^{32}P-labeled ssDNA. Samples for the time course experiment (lanes 1−6) were taken at 0, 1, 5, 10, 30, and 60 min, respectively. Oligonucleotide markers (lane 9) were [α-^{32}P]AMP, 3-nt, 4-nt, 6-nt and 34A, respectively. (B) Pictorial representation of nuclease activities of RecB30 (lanes 1−6), $RecB30^{D1067A}$ (lane 7), and $RecB30^{D1080A}$ (lane 8) mutants on the 3′-^{32}P-labeled ssDNA. Samples for the time course experiment (lanes 1−6) were taken at 0, 1, 5, 10, 30, and 60 min, respectively. Oligonucleotide markers (lane 9) were [α-^{32}P]AMP, 2-nt, 3-nt, 4-nt, 6-nt, 24-nt, and 34A, respectively.

Degradation of ^{32}P-Labeled Linear ssDNA by RecB30. When the highly purified wild-type RecB30 was incubated with a 5′- or 3′-^{32}P-labeled 34-nt ssDNA substrate, RecB30 degraded the substrate to a series of smaller labeled products in the presence of ATP and Mg^{2+} (Figure 1). However, unlike Mg^{2+}, ATP had no effect on the nucleolytic activities of RecB30 on the different DNA substrates, including ssDNA, dsDNA, and circular DNA (Supporting Information). Like other nucleolytic activity assays for RecBCD, BSA was included in our nucleolytic activity assay buffer. However, BSA was shown to have no effect on the ssDNA activity of RecB30 (data not shown). The putative Mg^{2+}-binding site mutants, $RecB30^{D1067A}$ and $RecB30^{D1080A}$, did not digest the same substrate (Figure 1), further supporting that the observed nucleolytic activity is intrinsic to RecB30. The time-course experiment showed that RecB30 makes a broad distribution of 5′- or 3′-labeled products, consistent with endonucleolytic cleavage of the substrate. RecB30 also made mononucleotide and dinucleotide products (Figure 1), which were not observed before for RecBCD using either small ssDNA oligonucleotides (*39*) or denatured dsDNA substrates (*9*, *10*).

Degradation of Circular ssDNA by RecB30. To determine whether RecB30 can act as a ssDNA endonuclease, RecB30 was assayed for its activity on a circular ssDNA substrate. RecB30 digested the circular ssDNA, M13mp18 phage DNA, to a series of smaller, 100−500 nt ssDNA fragments (Figure 2), indicating that the RecB30 not only linearizes the circular ssDNA but also further digests it into smaller ssDNA oligonucleotides. However, the $RecB30^{D1067A}$ and $RecB30^{D1080A}$

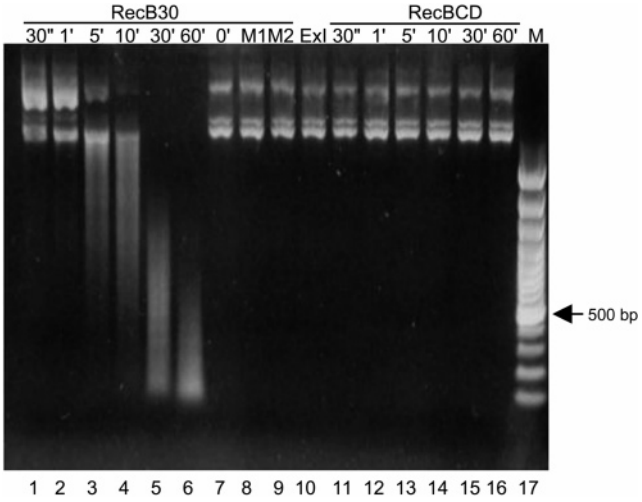

FIGURE 2: Nuclease reactions of wild-type and mutant RecB30 proteins with a circular ssDNA substrate. The reactions were carried out at 25 °C by incubating 0.4 μg of M13mp18 ssDNA (25 nM) with 20 nM of the wild-type RecB30 or mutant RecB30, or 20 units of *E. coli* Exonuclease I in the nuclease reaction buffer as described in Materials and Methods, and the samples were analyzed on a 1% agarose gel. Lane 1 shows the reaction with *E. coli* ExoI nuclease for 60 min. Lanes 2−9 are the reactions with wild-type RecB30 for 0, 0.5, 1, 2, 5, 10, 30, and 60 min, respectively. Lanes 10 and 11 are the reactions with RecB30^{D1067A} (Mut1) and RecB30^{D1080A} (Mut2) mutant proteins for 60 min, respectively. Lane 12 (M) contained the GeneRuler 100bp DNA Ladder Plus marker (Fermentas, Inc.).

mutants did not degrade the same substrate, and neither did *E. coli* Exonuclease I, which only acts upon linear ssDNA (Figure 2). The reaction with RecB30 is much faster than that observed previously with the His_6-tagged RecB nuclease domain (*29*). Under the optimal conditions in that study, 1.8 μM of the His_6-tagged protein was required to cleave the circular ssDNA once (to make a linear ssDNA product) in a 1-h reaction. However, as shown in Figure 2, the same circular ssDNA is cleaved multiple times by about 100-fold lower protein concentration (20 nM) of the untagged RecB30 in less than 5 min.

Nucleolytic Activity of RecB30 on Linear dsDNA. The nuclease activity of RecB30 or RecB on dsDNA has not been measured previously. To determine whether the RecBCD nuclease domain can act on dsDNA independently of its associated DNA helicase and ATPase subunits, the nucleolytic activity of RecB30 on ^{32}P-labeled dsDNA oligonucleotides was determined. When the wild-type RecB30 was incubated with a 5′- or 3′-end ^{32}P-labeled dsDNA substrate, RecB30 degraded the substrate to a series of smaller, labeled products (Figure 3). In addition to 4−6-mer oligonucleotide products, the major products were mononucleotides and dinucleotides, which were not observed with RecBCD holoenzyme using linearized dsDNA (*9*, *10*). The observed dsDNA nuclease activity is intrinsic to RecB30, since the RecB30^{D1067A} and RecB30^{D1080A} mutants did not degrade the same substrate (Figure 3). The fact that the RecB30^{D1067A} and RecB30^{D1080A} mutants abolish both the ssDNA and dsDNA nucleolytic activities of RecB30 indicates that a single active site is responsible for the cleavage of both ssDNA and dsDNA.

RecB30 Cleaves dsDNA Endonucleolytically. To determine whether RecB30 can cleave dsDNA endonucleolytically, RecB30 was assayed for its activity on a circular dsDNA

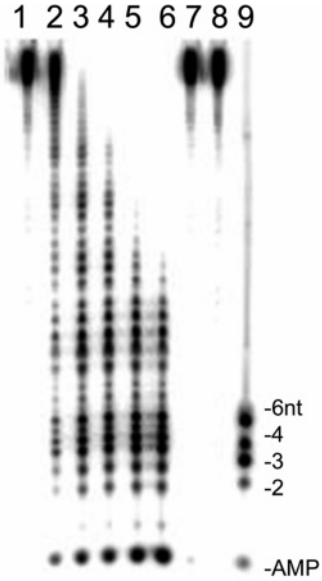

FIGURE 3: Time-course nuclease reactions of wild-type and mutant RecB30 proteins with a blunt-ended dsDNA substrate. Wild-type RecB30 (20 nM) or a mutant (20 nM) was incubated with the 3′-^{32}P-labeled dsDNA (10 nM) (34A/34B, Table 1) at 25 °C in the nuclease reaction buffer. The reactions were analyzed on 15% denaturing PAGE and visualized by autoradiography as described in Materials and Methods. Reactions contained RecB30 (lanes 1−6), RecB30^{D1067A} (lane 7), and RecB30^{D1080A} (lane 8) mutants. Samples for the time-course experiment (lanes 1−6) were taken at 0, 1, 5, 10, 30, and 60 min, respectively. Oligonucleotide markers (lane 9) were [α-^{32}P]AMP, 2-nt, 3-nt, 4-nt, and 6-nt, respectively.

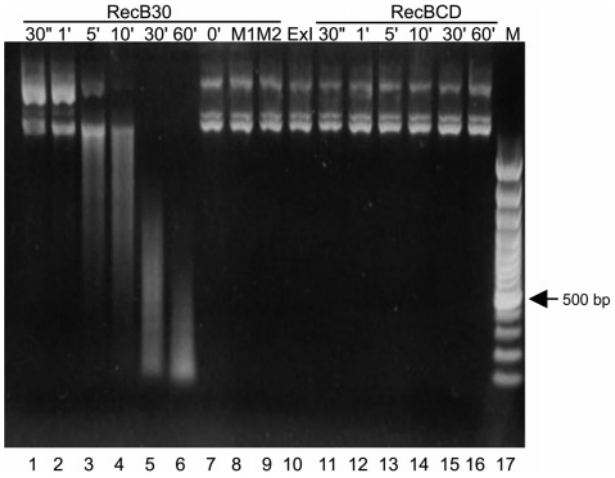

FIGURE 4: Nuclease reactions on circular dsDNA with RecB30 and RecBCD holoenzyme. The reactions were carried out at 25 °C by incubating 0.4 μg of pET15b plasmid dsDNA (25 nM) with 20 nM of the wild-type RecB30 or mutant RecB30, or 20 units of *E. coli* Exonuclease I in the nuclease reaction buffer as described in Materials and Methods, and the samples were analyzed on a 1% agarose gel. Lanes 1−7 are the reactions with wild-type RecB30 for the indicated times. Lanes 8 and 9 are the reactions with RecB30^{D1067A} (M1) and RecB30^{D1080A} (M2) mutant proteins for 60 min, respectively. Lane 10 shows the reaction with *E. coli* ExoI nuclease for 60 min. Lanes 11−16 show the reaction of RecBCD on the dsDNA for the indicated times. Lane 17 (M) contained the GeneRuler 100bp DNA Ladder Plus marker (Fermentas, Inc.).

substrate. As shown in Figure 4, RecB30 digested the circular pET15b dsDNA plasmid to a series of small oligonucleotides, indicating that the RecB30 not only linearizes the circular dsDNA but also further digests it into smaller oligonucleotides. However, the RecB30^{D1067A} and RecB30^{D1080A} mutants

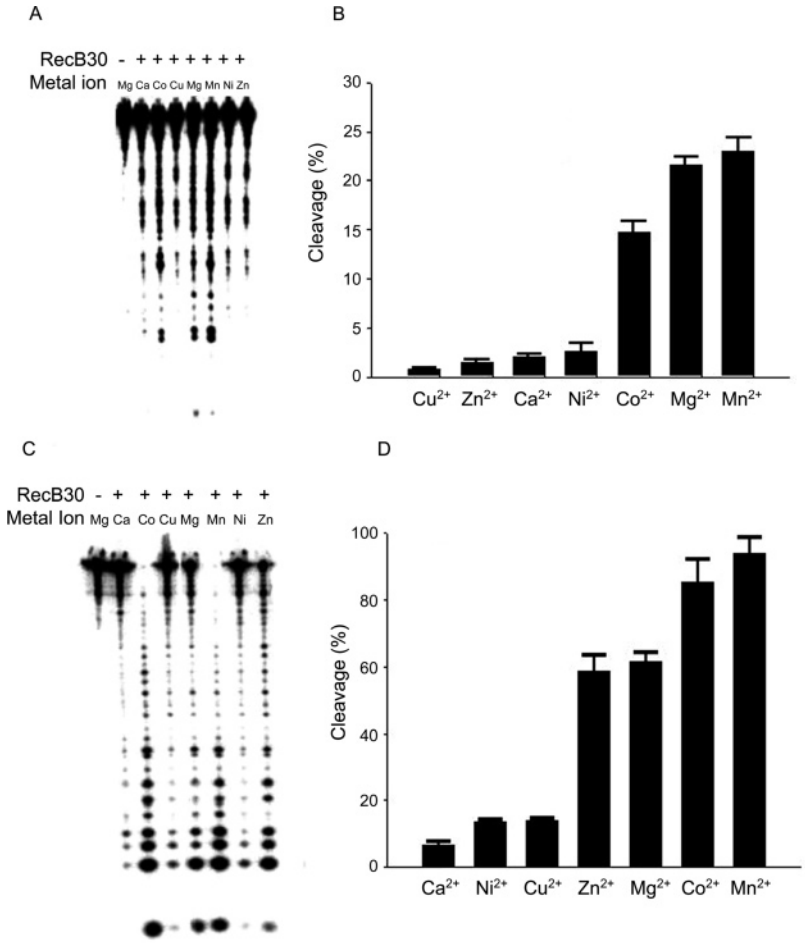

FIGURE 5: Divalent metal ion dependence of RecB30 nuclease activities. The nuclease activity of RecB30 was assayed in the presence of different divalent metal ions. The reactions were carried out by incubation of 2 nM of RecB30 with 3 nM of ^{32}P-labeled ssDNA (34A) or dsDNA (34A/34B) labeled at the 5′- end, in the nuclease assay buffer at 25 °C for 10 min. (A) Pictorial representation of nuclease activity of RecB30 on the 5′-labeled ssDNA. Lanes 2−8 represent 5 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+}, respectively. (B) Divalent metal ion-dependent ssDNA nuclease activities of RecB30 quantified by PhosphorImager. (C) Pictorial representation of nuclease activity of RecB30 on the 5′-labeled dsDNA. Lanes 2−8 represent 5 mM Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Mg^{2+}, Ni^{2+}, and Zn^{2+}, respectively. (D) Divalent metal ion-dependent nuclease activities on dsDNA of RecB30 quantified by PhosphorImager.

did not digest the same substrate, nor did *E. coli* Exonuclease I, which only acts upon linear ssDNA (Figure 4), indicating that the observed dsDNA nucleolytic activity is intrinsic to RecB30. This result is particularly interesting, since RecBCD was shown to be inactive on circular dsDNA (*9*, *10*). This prompted us to reexamine the endonuclease activity of the RecBCD holoenzyme on the circular dsDNA. As shown in Figure 4, RecBCD did not degrade the circular pET15b plasmid dsDNA under reaction conditions (5 mM Mg^{2+} and 1 mM ATP) where it has substantial nuclease activity on linear dsDNA (*19*, *40*, *41*).

Effect of Divalent Metal Ions on the Nucleolytic Activities of RecB30. Mg^{2+} is known to be important not only for the nucleolytic reactions but also for the ATPase and helicase activities of RecBCD (*8*, *9*, *42*). RecB30 makes it possible for us to focus on the effects of Mg^{2+} and other divalent metal ions on the nucleolytic activities of RecBCD without the complication due to the effects of the holoenzyme-associated ATPase and helicase activities. No activity was detected for our purified RecB30 unless Mg^{2+} ion was added to the nuclease assay buffer (data not shown). This result indicates that exogenous Mg^{2+} ion is required for RecB30 nucleolytic activity.

To characterize the effect of other divalent metal ions on the ssDNA nuclease activity of RecB30, Ca^{2+}, Co^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, and Zn^{2+} were assayed for their abilities to activate the RecB30-catalyzed degradation of linear ssDNA (Figure 5A). The results show that the divalent metal ions modulate the ssDNA nuclease activity of RecB30. For the 5′-labeled ssDNA substrate, Mg^{2+}- or Mn^{2+}-bound RecB30 is an active ssDNA nuclease; Co^{2+} also shows considerable activation, while Ca^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+} ions stimulate the nuclease reactions of RecB30 only slightly (Figure 5B). Similar results were observed for both the 3′-labeled ssDNA substrate and M13mp18 phage DNA substrate (data not shown). The effect of these divalent metal ions on the dsDNA nuclease activity of RecB30 was determined with a 5′-labeled blunt-ended dsDNA fragment (Figure 5C). The results indicate that Mg^{2+} exhibits significant stimulation of the dsDNA nuclease activity of RecB30 and other divalent metal ions also modulate the dsDNA nuclease activity of RecB30. Interestingly, Mn^{2+}- or Co^{2+}-bound RecB30 is a more active dsDNA nuclease than Mg^{2+}-ligated RecB30; Zn^{2+} shows comparable activation as Mg^{2+}, while Ca^{2+}, Cu^{2+}, and Ni^{2+} ions stimulate the nuclease reactions of RecB30 slightly (Figure 5D). Similar results were observed for both the 3′-

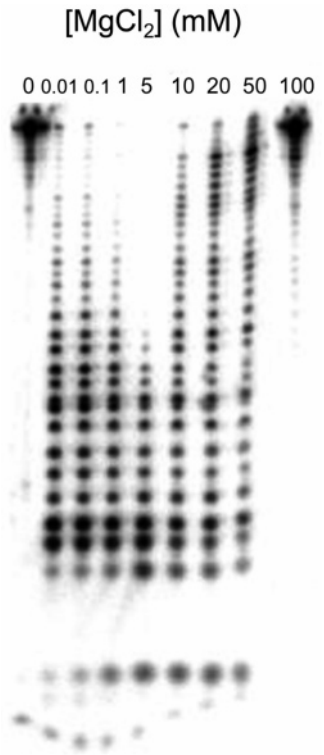

FIGURE 6: Mg^{2+} ion concentration dependence of RecB30 nuclease activity. RecB30 (20 nM) was incubated with 3 nM of 5′ ^{32}P-labeled ssDNA (34A, Table 1) in the nuclease assay buffer at 25 °C for 10 min, as described in Materials and Methods.

labeled dsDNA substrate and circular dsDNA substrate (data not shown). Moreover, extending the reaction time also increases the cleavage for each ion (data not shown).

The Mg^{2+}-concentration dependency of the RecB30 activity was determined under the identical (about 205 mM) ionic strength (Figure 6). The results indicated that, for both ssDNA (Figure 6) and dsDNA (data not shown), nucleolytic reactions were stimulated by a broad range of Mg^{2+} concentration in a dose-dependent manner but were inhibited by high concentration of the ion. Optimal nuclease activity occurred in the presence of about 5 mM Mg^{2+} and was completely suppressed when the Mg^{2+} concentration reached 100 mM at an ionic strength of about 340 mM (Figure 6).

Kinetic Analysis of RecB30-Catalyzed Degradation of Linear ssDNA and dsDNA. As a nonspecific and nonprocessive ssDNA endonuclease, RecB30 digests each ssDNA substrate to produce two products, which can be used as the substrates for the subsequent nuclease reaction, and the final products are mononucleotides, dinucleotides, and other slightly longer oligomers (Figure 1). To avoid problems resulting from the increase of substrate concentration during the degradation, the initial reaction rates were determined from the disappearance of the initial substrate, instead of from the increase of the resulting multiple products. The initial reaction rates were determined from the slope of a plot of the substrate concentration versus the reaction time, using the $t = 0$ point and at least the first two time-points of the reaction. V_{max} and K_m values were determined by fitting the data to the Michaelis−Menten equation (Figure 7). The reaction rate constant, k_{cat}, and the K_m for a 34-mer ssDNA substrate were determined at 25 °C to be 1.4 ± 0.1 s^{-1} and 134 ± 22 nM, respectively. The specific activity of RecBCD for the ssDNA was previously measured, and the activity

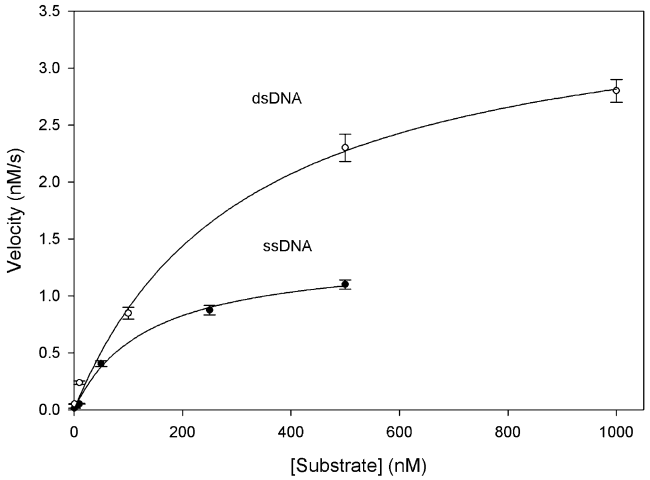

FIGURE 7: Kinetic analysis of RecB30 nuclease on the linear ssDNA and dsDNA substrates. Kinetic analysis of RecB30 was performed using various concentrations of ssDNA (34K; open circles) or dsDNA (34K/34L; filled circles) substrate (Table 1) (1−1000 nM) and constant concentration of RecB30 (1 nM) at 25 °C following the procedure described in Materials and Methods. After incubation at 25 °C for 10, 30, 60, 120, 300, and 600 s, respectively, the reactions were terminated and analyzed by 15% denaturing PAGE. The initial reaction velocity for each substrate concentration was determined from the slope of a plot of the substrate concentration versus the reaction time. The data were then fitted to the Michaelis−Menten equation to determine the V_{max} and K_m values. The k_{cat} value was calculated from V_{max} /[E_o]. The solid lines show the fitted curves, with $K_m = 134 \pm 22$ nM and $k_{cat} = 1.4 \pm 0.1$ s^{-1} (ssDNA substrate), and $K_m = 315 \pm 53$ nM and $k_{cat} = 3.7 \pm 0.2$ s^{-1} (dsDNA substrate).

was found to be about 2.5 mol oligomer cleaved/s per mol RecBCD for a 26-mer nucleotide at 37° C in the presence of ATP (*39*). This result indicates that RecB30 and RecBCD have essentially identical activities for ssDNA (RecB30 for a 34-mer and RecBCD for a 26-mer). The Mg^{2+}-binding site mutants of RecB30 were inactive. As a consequence, we could not establish unambiguously their kinetic parameters under V_{max} conditions.

We also measured the nuclease activity of RecB30 for the same oligonucleotide in the dsDNA (Figure 7). The reaction rate constant, k_{cat}, and the K_m for the dsDNA substrate were determined at 25 °C to be 3.7 ± 0.2 s^{-1} and 315 ± 53 nM, respectively, compared to 1.4 ± 0.1 s^{-1} and 134 ± 22 nM for the ssDNA substrate. The results indicate that RecB30 binds more tightly on ssDNA than on dsDNA but is more active on dsDNA than on ssDNA.

DISCUSSION

The nuclease active site of RecBCD had been mapped by limited proteolysis and site-directed mutagenesis to the 30 kDa C-terminal domain of the RecB subunit (*26*, *27*, *29*). Nuclease activity was detectable when the isolated 30 kDa domain was tested, as well as with the RecB subunit alone and with RecBC, lacking the RecD subunit. The nuclease activity of each of these polypeptides was much lower than that of RecBCD under all conditions, suggesting that the nuclease activity of the 30 kDa domain was stimulated in some way by other subunits when in the context of the RecBCD holoenzyme.

In this report, we have isolated a form of the RecB nuclease domain that has no extraneous histidine-tag peptide.

The results of nuclease assays with this version of the RecB nuclease domain show that it in fact has high intrinsic activity and that it is capable of cleaving dsDNA as an endonuclease. Thus, the RecBCD holoenzyme must be constructed so as to modulate the activity of this intrinsically powerful nuclease domain.

The Nuclease Domain of RecBCD Has an Intrinsic dsDNA Endonucleolytic Activity. The RecB30 nuclease and other members of the "RecB nuclease domain" family (*30*) were postulated previously (*28*) to share similar active sites with several restriction endonucleases, including *Eco*RI, *Eco*RV, *Pvu*II, *Bgl*I, and *Fok*I (*31*, *32*), the *E. coli* MutH protein (*33*), and the exonuclease produced by bacteriophage λ (*34*). These nucleases share a common structural core comprising four β-strands and one α-helix. The restriction endonucleases cleave the two strands of dsDNA in a concerted fashion with an inversion of configuration at the phosphorus, to produce DNA fragments with a 3′-OH and a 5′-phosphate (*32*). This analysis suggested that the RecB30 nuclease might also function as an endonuclease on dsDNA. However, endonuclease activity on dsDNA had never been seen for RecBCD or any pieces of RecBCD. In this study, we have shown that RecB30 degrades a duplex DNA circle to small oligonucleotides. Three key residues of RecB30 (Asp^{1067}, Asp^{1080}, and Lys^{1082}) compose a nuclease catalytic center (with consensus [**G/V**]hh**D**...[**D/E**]h**K** (h indicates a hydrophobic residue)) that is also found in the restriction enzymes. Replacement of the conserved Asp residues rendered RecB30 or RecBCD inactive in cleaving ssDNA and dsDNA substrates, as shown in this and previous studies (*14*, *26*, *27*, *29*). We therefore believe that the observed dsDNA endonucleolytic activity of RecB30 is intrinsic to the RecBCD holoenzyme.

The structure of the RecB30 nuclease domain in the RecBCD holoenzyme has become available with the recent publication of the three-dimensional structure of RecBCD (*43*). The domain is described as having a structure similar to that of the λ exonuclease (*43*), suggesting that it is also similar to the restriction endonucleases, in agreement with the inference based on the active site sequence comparisons (*28*, *38*). The structure also shows that other subunits could prevent the nuclease active site in RecB30 from getting access to the duplex circles, explaining why RecB30 efficiently degrades duplex circles endonucleolytically, while RecBCD does not.

Divalent Metal Ions Modulate the Nucleolytic Activities of RecB30. Metal ion substitution has proven to be a useful strategy for understanding the important properties of metal ion cofactors in metalloenzymes. On the basis of our preliminary characterization, it is possible to clarify some features important to the nuclease reaction catalyzed by RecB30. Our results indicate that divalent metal ions modulate the nuclease activities of RecB30. RecB30 appears to be sensitive to the size and coordination preferences of seven divalent metal ions used in this study (*44*−*46*). Mn^{2+}-, Mg^{2+}-, or Co^{2+}-bound RecB30 has the highest activity for either ssDNA or dsDNA. However, Cu^{2+} and Ni^{2+} are smaller, while Ca^{2+} is bigger than those three ions, and show less activation effects for both substrates (Figure 5). The coordination number, geometry, and ligand preferences of Cu^{2+} and Zn^{2+} are different from the rest of ions, and therefore contribute to the poorer stimulation effects (Figure 5). Interestingly, a profound difference has been seen for the Zn^{2+} ion. Zn^{2+}-bound RecB30 is quite active on dsDNA (Figure 5C,D) but significantly less active on ssDNA (Figure 5A,B). Moreover, the binding affinity of a metal ion also plays an important role in the catalyzing nuclease reaction, since extending the reaction time also increases the cleavage for each ion (data not shown). Taken together, the divalent metal ions modulate the nuclease activities of RecB30 by the size, ion chemistry, and binding affinity. It is possible that RecBCD is more selective than RecB30 when binding to a divalent metal ion. Previously, it was shown that the nucleolytic activities of RecBCD enzyme could be suppressed by the addition of Ca^{2+} ion to the reaction mixture (*42*), and our results with RecB30 nuclease domain support this observation.

Because of the abundance of Mg^{2+} in vivo (*44*−*46*), Mg^{2+} is most likely the in vivo nuclease cofactor for RecB30 and RecBCD. Mg^{2+} is essential for the nucleolytic activities of RecB30. However, when the Mg^{2+} ion levels are higher than 20 mM, nuclease activities of RecB30 are inhibited. This inhibition may result from protein conformational changes or a decrease in DNA-binding ability as a result of nonspecific binding of the cation by the enzyme or by a general electrostatic effect. NMR relaxation studies indicate that RecB30 forms aggregates in the presence of high concentration of Mg^{2+} ions (>20 mM), and a substrate binding assay indicates that high levels of Mg^{2+} inhibit substrate binding (unpublished results). High levels of Mg^{2+} may disrupt the specific interaction between RecB30 and its DNA substrate. Taken together, it suggests that Mg^{2+} regulates RecB30 activities, and therefore also RecBCD activities, by modulating the protein's conformation and its substrate binding capability.

Replacement of the putative Mg^{2+}-binding acidic residues, Asp^{1067} and Asp^{1080}, rendered RecB30 inactive in cleaving DNA substrates. These results suggest that the RecB30 nuclease follows a similar reaction mechanism to many restriction nucleases. The Asp^{1067} and Asp^{1080} residues are likely involved in coordinating Mg^{2+} ion, which then polarizes the phosphate to enhance its susceptibility to nucleophilic attack by a water molecule, during phosphodiester bond hydrolysis. The Lys^{1082} residue perhaps stabilizes the pentacovalent phosphorus in the transition state by compensating its extra negative charge. Mechanistic similarity between RecBCD and the restriction endonucleases is further supported by the observation that RecBCD digests linear duplex DNA to give 3′-hydroxyl-, and 5′-phosphate-terminated oligonucleotides (*9*, *10*), and these cleavage products are similar to those observed for many restriction endonuclease (*32*).

High Intrinsic Nucleolytic Activities of RecB30 Are Regulated in RecBCD Holoenzyme. As pointed out by Delagoutte and von Hippel, the biological function of a DNA helicase subunit of a larger enzyme complex is to unwind a dsDNA substrate and then to transfer the resulting ssDNA products to the next reaction step (*47*). In the case of RecBCD, the helicase domains of the enzyme unwind dsDNA and make the ssDNA strands available for cleavage by the RecB30 nuclease domain. RecB30 alone efficiently catalyzes dsDNA degradation, indicating that RecB30 can function independently of its associated RecBCD helicases. The high intrinsic nucleolytic activities of RecB30 argue that

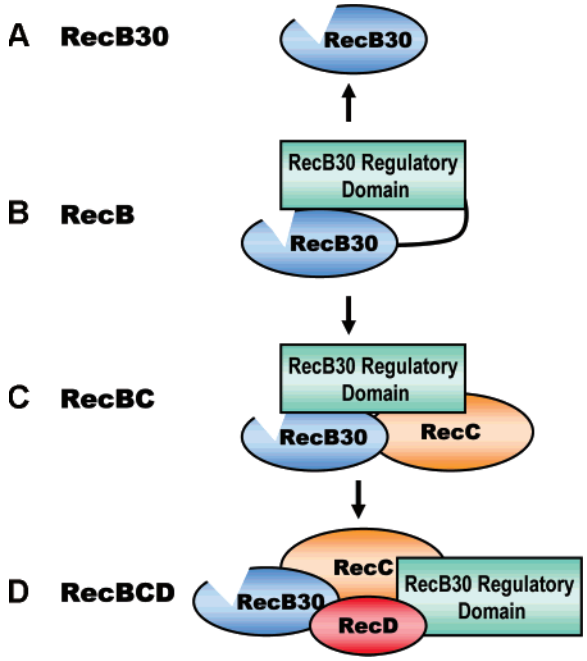

FIGURE 8: Model for the regulation of RecBCD nuclease activity through protein−protein interaction. (A) The RecB30 nuclease domain, independent of its regulatory domain, has the highest nuclease activity. (B) RecB is a weak nuclease due to the interaction between the RecB30 nuclease domain and its regulatory domain. (C) RecBC is also a weak nuclease. RecC could interact with both the RecB30 nuclease domain and its regulatory domain, but this interaction does not significantly activate the nuclease activity of RecB30. (D) RecBCD is an active nuclease. RecD stimulates the nuclease activity of RecB by interacting with the RecB30 regulatory domain and RecC to greatly expose the RecB30 nuclease domain.

the other subunits or domains in RecBCD modulate the nuclease activity of RecB30. A new model (Figure 8) has been proposed to interpret regulation of the nucleolytic activities of RecB30.

The RecB30 nuclease activity is at least as high as that of RecBCD. The full-length RecB protein, consisting of an amino-terminal helicase/ATPase domain and the C-terminal RecB30 nuclease domain, is a weak nuclease (*26*), suggesting that the N-terminal part of RecB serves as a regulatory domain that suppresses the intrinsic nuclease activity of RecB30 (Figure 8). Similarly, the amino-terminal peptides of the histidine-tagged or gp32-fused RecB30 could also interfere with the active site of RecB30, and therefore, RecB30 appeared to have very low nuclease activity in those constructs (*26*, *29*). We note that analysis of the RecBCD structure indicates that amino acid residues at the very end of the linker region that connects the nuclease domain to the rest of RecB, but N-terminal to the nuclease domain itself (residues 909−930), form an α-helix that sits above the nuclease active site (*43*). This part of RecB (which, notably, is not present in the RecB30 domain studied here) was proposed to control the nuclease activity of RecBCD by blocking access of ssDNA to the active site. This short peptide might comprise part or all of the regulatory domain that we propose herein. Other regulators, including ATP, the Chi sequence, and proteins such as RecC and RecD, could also modulate the interaction between RecB30 nuclease and its regulatory domain and therefore regulate the nuclease activities and specificities of RecB30 (Figure 8).

RecBC is a weak nuclease (*26*, *39*, *48*), suggesting that RecC does not substantially perturb the interaction between RecB30 and its regulatory domain. The addition of RecD to RecBC produces RecBCD, a potent DNA nuclease in the presence of ATP. This suggests that RecD greatly changes the interaction between the regulatory domain and RecB30 to relieve the inhibition by that domain, by greatly exposing the RecB30 nuclease domain (Figure 8). RecD is situated close to RecB30 in RecBCD (*43*), although it is not clear that it interacts directly with RecB30. The binding of ATP or DNA in the helicase/ATPase domain of RecB might also change the conformation of this regulatory domain and its interaction with the nuclease domain and therefore enhance the nuclease activities of RecBC and RecBCD (*8*−*10*). A conformational change induced by the binding of ATP in the RecD helicase/ATPase could also stimulate the nuclease activity of RecBCD (*39*).

CONCLUSION

In summary, RecB30 provides a simple model system for characterization of the nucleolytic reactions of the RecBCD holoenzyme. Our studies show that the isolated RecB30 nuclease domain can function independently of its associated helicase/ATPase and can efficiently degrade ssDNA and dsDNA. In particular, RecB30 can cleave dsDNA endonucleolytically, as can the restriction endonucleases. This had never been observed before for RecBCD or any pieces of RecBCD. The fact that RecB30 has high intrinsic nuclease activities not only provides direct evidence that RecB30 is the sole nuclease domain of RecBCD but also suggests that the Chi DNA sequence and other domains or subunits down-regulate the nucleolytic activities of the RecB30 nuclease, and could also prevent the nuclease from getting access to the dsDNA, especially duplex circles. The comprehensive characterization of RecB30 in complexes with other domains or subunits of the holoenzyme will likely provide additional information on the regulation of the RecB30 nuclease in the holoenzyme.

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SUPPORTING INFORMATION AVAILABLE

Three figures illustrating the purity of recombinant RecB30 and mutants, Mg^{2+} ion and ATP concentration-dependences of RecB30 nuclease activities on ssDNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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