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Characteristics of nuclease activity of the SbcCD complex from *Deinococcus radiodurans*

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The bacterium *Deinococcus radiodurans* is extremely resistant to the intense ionizing irradiation which causes extensive DNA double-strand breaks (DSBs). The *deinococcal* SbcCD complex (drSbcCD) is required for DSB repair. The *drSbcC* and *drSbcD* genes were cloned and overexpressed in *Escherichia coli* cells, respectively. The nearly homogeneous drSbcC and drSbcD proteins were purified and reconstituted to form a stable complex *in vitro*. The drSbcCD complex has an ATP-independent 3′→5′ exonuclease activity to cleave both dsDNA and ssDNA substrates in the presence of either Mn^{2+} or Mg^{2+} ion. The drSbcCD complex also has an ATP-independent endonuclease activity. It can cleave the circular ssDNA, nick the supercoiled circular dsDNA, cleave the 3′ flap DNA substrate at the site of the single-strand branch adjacent to duplex DNA, and cleave the hairpin DNA taking no account of the DNA end free or not. It is a kind of secondary structure-specific endonuclease. The drSbcCD complex still has a 3′→5′ exonuclease activity when the DNA termini are blocked by the proteins. These results suggest that the drSbcCD complex takes part in the metabolism of DNA, and its nuclease activities may play important roles in DNA repair process.

Keywords: *Deinococcus radiodurans*/nuclease activity/SbcCD.

Abbreviations: DSB, double-strand break; FITC, fluoresceinisothiocynate; PAGE, polyacrylamide gel electrophoresis; drSbcCD, the *deinococcal* SbcCD complex.

DNA double-strand break (DSB) is a kind of potentially and highly cytotoxic DNA lesion as it disrupts the genomic integrity. It is caused by extra- and intracellular factors such as ionizing irradiation, chemical modification, abortive DNA replication and active oxygen radicals. Improper repair or failure to repair DSB may be lethal or lead to genomic aberrations.

In wild type *Escherichia coli*, rejoining of the chromosomal DNA fragmented by gamma irradiation can be completely blocked by only three mutations: RecA, RecB or RecC (*1*). RecB and RecC combine with RecD to work as a single enzyme-the RecBCD recombinase. RecBCD can produce a 3′-terminated ssDNA end to initiate the recombination reaction mediated by RecA. Therefore, DSB in *E. coli* is generally repaired by RecABCD-dependent homologous recombination (*2–4*).

In eukaryotes, DSB repair is mediated primarily by two distinct but interrelated pathways: homologous recombination and non-homologous end joining (*5*, *6*). The Mre11–Rad50 complex (MR) is required in both pathways (*7*). In *Saccharomyces cerevisiae*, Mre11, Rad50 and Xrs2 are the members of the Rad52 epistasis group and they can form the scMR complex. The scMR complex is required for the repair of ionizing irradiation-induced damage in mitotic cells and the Spo11-introduced meiotic DSBs (*8–10*). Mice deficient in any one of the *Mre11*, *Rad50* or *Nbs1* genes are embryonic lethal. Human mutation in *Nbs1* gene causes Nijmegen breakage syndrome (NBS), and mutation in *Mre11* gene causes ataxia telangiectasia-like disease (ATLD). Cells derived from these patients exhibit chromosome instability, increased sensitivity to ionizing irradiation, defective induction of stress-activated signal transduction pathways and radio-resistant DNA synthesis. However, no mutation in *Rad50* gene has been linked to genetic diseases in humans (*11*). Based on amino acid sequence analysis, homologs of Mre11 and Rad50 have been identified in all kingdoms of life, while the Nbs1 protein is only found in eukaryotes (*12*). SbcD and SbcC, forming the SbcCD complex, are the bacterial orthologs of Mre11 and Rad50, respectively (*13*).

Deinococcus radiodurans was characterized because of its incredible resistance to the intense ionizing irradiation, which can cause extensive DSBs (*14*, *15*). Based on the genome sequence of *D. radiodurans*, essentially all of the DNA repair genes identified in *D. radiodurans* strain R1 have functional homologs in other prokaryotic species (*16*). Compared to *E. coli*, surprisingly, the *recBC* genes are missing, while the *sbcCD* genes are preserved in *D. radiodurans*. We previously observed that the *D. radiodurans* mutant devoid of the SbcD protein displayed a reduced survival and a delay in the kinetics of DSB repair and cell division following ionizing irradiation (*17*). This result was also demonstrated by Bentchikou

et al. (*18*). Thus, the drSbcCD complex is a significant protein compound in the ionizing irradiation-induced DSB repair. In *E. coli*, the SbcCD complex is also required for the successful repair of *Eco*KI-generated DSBs (*19*). In *Bacillus subtilis*, the SbcC protein appears to play a role in repair of DNA interstrand crosslinks (*20*).

In this study, we cloned, overexpressed and purified the drSbcC and drSbcD proteins, reconstituted the drSbcCD complex and tested its activities *in vitro*. We demonstrated that the drSbcCD complex has both endo- and exonuclease activities. These results implied that the drSbcCD complex is important for recombination, DNA repair and genomic stability in this organism.

Materials and methods

Plasmid construction

Deinococcus radiodurans strain R1 was obtained from the ATCC, Manassas, VA. *Deinococcus radiodurans* genomic DNA was isolated as described (*17*). A 2744-bp fragment included the full-length of drSbcC (dr1922) coding region was amplified from the genomic DNA by PCR using primers 5′-AAAACATATGAAGCCGCTGC ACCTCACC-3′ and 5′-AAAACTCGAGTCCGTCCACCCGAAT CACGT-3′. The amplified DNA product was cloned into pMD18-T (Takara), then digested with Nde1 and Xho1 and finally cloned into pET29b (Novagen) to create pET29b–drSbcC. Because the putative full-length drSbcD protein (dr1921) overexpressed in pET plasmids was insoluble, the truncated *drSbcD* gene beginning from the second methionine next to the N′-terminal initiative methionine was cloned (Fig. 1A). A 1196-bp fragment was amplified from the genomic DNA by PCR using primers 5′-AAAACATATG CGCGTACTTCATACCGCC-3′ and 5′-AAAAC TCGAGCTGCG CGCCCTCCCG-3′. The amplified DNA product was cloned into pMD18-T (Takara), then digested with Nde1 and Xho1 and cloned into pET29b (Novagen) to create pET29b–drSbcD. Each of these plasmids encodes the drSbcC or drSbcD protein with the peptide –Leu–Glu–$(his)_6$– appended to its C-terminus. The DNA sequencing was used to verify that the cloned DNAs were the *drSbcC* and *drSbcD* genes, respectively.

drSbcC and drSbcD proteins expression

The drSbcC protein was expressed in *E. coli* strain BL21 (DE3) pLysS transformed with pET29b–drSbcC. The drSbcD protein was expressed in *E. coli* strain BL21 (DE3) transformed with pET29b–drSbcD. Cells were grown to $OD_{600} = 0.6$ in 6 l of Luria–Bertani broth containing 30 μg/ml kanamycin sulphate at 37 and 30°C with vigorous shaking, respectively. Protein expression was induced by the addition of isopropyl-D-thiogalatopyranoside (IPTG) to the final concentration of 0.8 mM, and the culture was further incubated for 5 h. All induced cells were harvested by centrifugation and shock frozen in liquid nitrogen, then stored at −80°C.

drSbcC and drSbcD proteins purification

The *deinococcal* SbcC and SbcD proteins were purified through three chromatographic steps. Cells were thawed on ice and then resuspended in 100 ml of lysis buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 10 mM imidazole) with one tablet of complete EDTA-free protease inhibitor mix (Roche Molecular Biochemicals). Cells were lysed by sonication, then insoluble matter was removed by centrifugation at 15,000 × *g* at 4°C for 30 min. The supernatant was saved and applied on 10 ml of Ni^{2+}-nitrilotriacetic acid (NTA) superflow resin (Qiagen) pre-equilibrated with lysis buffer, then the column was washed with 100 ml of lysis buffer followed by 100 ml of wash buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 20 mM imidazole, 10% glycerol). Finally, the bound proteins were eluted with a linear gradient of 10–300 mM imidazole by elution buffer (50 mM sodium phosphate pH 8.0, 500 mM NaCl, 300 mM imidazole, 10% glycerol) at a flow rate of 1 ml/min. Fractions were assayed by SDS–polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining. The drSbcC- or drSbcD-containing fractions were pooled and dialysed against A buffer (25 mM Tris–HCl pH 8.0, 10% glycerol), and then applied on 25 ml Q Sepharose Fast Flow column (Amersham Biosciences) pre-equilibrated with A buffer. The column was washed with 125 ml of A buffer, finally the protein was eluted with a linear gradient from 0 to 500 mM NaCl in A buffer at a flow rate of 2 ml/min. Fractions from Q Sepharose Fast Flow column containing drSbcC or drSbcD protein were detected, and then concentrated by ultrafiltration using a Centricon 30 microconcentrator (Amicon). The concentrated solution was injected onto a Sephacryl S-300 column (Amersham Biosciences) and separated in B buffer (50 mM Tris–HCl pH 7.5, 300 mM NaCl, 10% glycerol) at a flow rate of 0.5 ml/min. Fractions containing nearly homogeneous protein were collected, concentrated and stored at −80°C. The protein concentration was determined by using the protein assay reagent kit (BioRad) with BSA as standard.

Reconstitution of the drSbcCD complex

The nearly homogeneous drSbcC and drSbcD proteins ([drSbcD] > [drSbcC]) were mixed and incubated on ice overnight to allow for complex formation. The drSbcCD complex was finally separated from free drSbcC and drSbcD by gel filtration on a Sephacryl S-300 column pre-equilibrated in buffer B. Fractions containing the drSbcCD complex were pooled, concentrated and stored at −80°C.

Oligonucleotide substrates

Oligonucleotides were synthesized and labelled by Invitrogen, Inc. The sequences of the synthetic oligonucleotides used in this study are shown in Table 1. The blunt duplex DNA substrate was composed of TP8 annealed to TP9 with TP9 labelled with fluoresceinisothiocynate (FITC) at the 5′ or 3′ end. The branched DNA substrate was composed of DAR40 annealed to DG110 with DG110 labelled with FITC at the 3′ end. The flap substrate was composed of DAR39 annealed to DG68 annealed to DAR128 with DAR128 labelled with FITC at the 3′ end. Hairpin DNA substrates were prepared by HP2 and HP30 labelled with FITC at the 3′ end. The labelled HP2 and HP30 were held at 70°C for 10 min and then allowed to be cooled slowly to anneal. The covalently closed dumbbell substrate ccDB98 was prepared by ncDB98 labelled with FITC at the 5′ end. The labelled ncDB98 was boiled and snap-cooled with T4 DNA ligase (Takara) at 16°C overnight. The duplex DNA substrate 79Tbio was prepared by 79T labelled with FITC at the 3′ end annealed to 79B labelled with biotin at the 5′ end. The duplex DNA substrate 79Bbio was prepared by 79T annealed to 79B labelled with biotin at the 5′ end and FITC at the 3′ end. The streptavidin binding substrates were prepared by 79Tbio or 79Bbio incubated with streptavidin (Amresco) on ice for 30 min. All above substrates were finally purified or verified by using 12% native-PAGE. The single-strand standard DNA marker labelled with FITC at 3′ end was also synthesized and labelled by Invitrogen, Inc. The sequences of DNA maker are A, $(AG)_7A$, $(AG)_{15}$, $(AG)_{22}A$, $(AG)_{30}$ and $(AG)_{37}A$.

Bacteriophage substrates

ΦX174 virion and RFI DNAs (New England Biolabs) were used for endonuclease assays.

Nuclease reaction

One microgram of various DNA substrates were incubated with 5 μg of the purified drSbcCD complex in reaction buffer (25 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM DTT, 5 mM $MnCl_2$, 1 mM ATP) at 30 or 20°C. Reactions were terminated by adding an equal volume of 6 × DNA loading buffer (30 mM EDTA, 36% glycerol, 0.05% xylene cyanol FF, 0.05% bromophenol blue, pH 7.0). The reaction products were boiled for 4 min before loading onto 12% denaturing polyacrylamide gels with 7 M urea. Otherwise the reaction products were loaded directly onto 12% native polyacrylamide gels. The electrophoresed gels were visualized using Fujifilm LAS-300 system.

Table 1. Oligonucleotide sequences.

Name	Sequence	Reference
TP8	GACCTGGCACGTAGGACAGCATGGGATCTGGCCTGTGTTACACAGTGCTACAGACTGGAACAAA AACCCTGCAG	(*21*)
TP9	CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGACAGGCCAGATCCCATGCTGTCCTACG TGCCAGGTC	(*21*)
DAR40	CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGTAAGACAGGCCAGATC	(*21*)
DG110	GCTTCCGCAGATCCTGCACAGTGCTACAGACTGGAACAAAAACCCTGCAGTACTCTACTCATCTC	(*21*)
DAR39	GATCTGGCCTGTCTTACACAGTGCTACAGACTGGAACAAAAACCCTGCAG	(*21*)
DG68	CGACTAGACCGGACAGAAT	(*21*)
DAR128	CTGCAGGGTTTTTGTTCCAGTCTGTAGCACTGTGCAGGATCTGCGGAAGCTACTCTACTCATCTC	(*21*)
HP2	AAAAAAGACCTGGCACGTAGGACAGCAGCTGCTGTCCTACGTGCCAGGTCAAAAAA	(*22*)
HP30	AAAAAAGACCTGGCACGTAGGACAGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAATGCTGT CCTACGTGCCAGGTCAAAAAA	(*22*)
ncDB98	GCTGAATAGAAACCTATCTGTGTGCCCGGGTTTTCCCGGGCACACAGATAGGTTTCTATTCAGCC CTTCCAGCCCCGGGTTTTCCCGGGGCTGGAAGG	(*23*)
79T	GACGACCGTGGTAGACATTCGTCACAGACTTAGTGGATCTCAATTCACGCGGAATAATATGACG AGGGACTCACTAGCT	(*24*)
79B	AGCTAGTGAGTCCCTCGTCATATTATTCCGCGTGAATTGAGATCCACTAAGTCTGTGACGAATG TCTACCACGGTCGTC	(*24*)

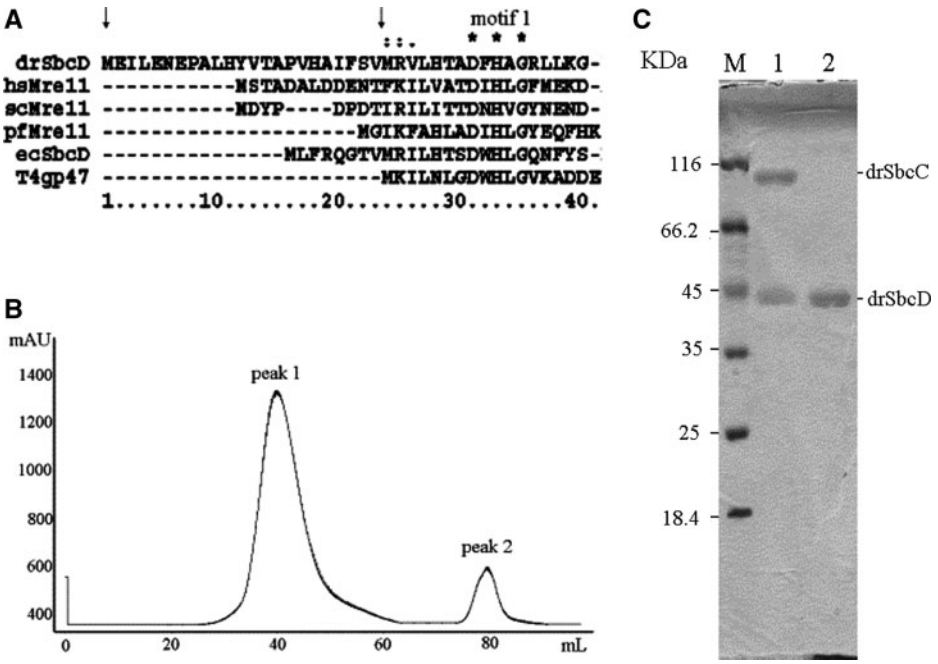

Fig. 1 Purification of drSbcC and drSbcD proteins. (A) Alignment of drSbcD with homologs from humans (hsMre11), *S. cerevisiae* (scMre11), *P. furiosus* (pfMer11), *E. coli* (ecSbcD) and bacteriophage T4 (T4gp47). Numbers indicate amino acid positions. The arrows indicate the positions of methionine. *drSbcD* gene was cloned at the beginning of the second methionine. (B) Gel filtration analysis of drSbcCD. Peak 1 that eluted from the gel filtration column is the coelution of the drSbcCD complex, while peak 2 corresponds to the elution of excess drSbcD. (C) SDS–PAGE of drSbcCD (peak 1) and drSbcD (peak 2) stained with Coomassie blue.

Results

drSbcC and drSbcC can form a stable complex in vitro

The drSbcC and drSbcD proteins overexpressed in and recovered from *E. coli* cells were near homogeneity after three column steps. The global drSbcD protein was eluted from the Sephacryl S-300 column in the range of 45 kDa. The drSbcC protein was eluted in the void volume of the Sephacryl S-300 column with an exclusion limit of ~1,500 kDa. After two proteins were mixed, the resulting mixture was again to be separated by the Sephacryl S-300 column. The mixture gave rise to two peaks (Fig. 1B), one contains the drSbcC and drSbcD proteins co-eluted in the void volume, and the other contains only the excess drSbcD (Fig. 1C). Thus, the drSbcD profile was shifted to co-elute with drSbcC as a higher molecular weight species, suggesting that drSbcC and drSbcD can form a stable, higher-order structural complex *in vitro*.

The drSbcCD complex is an ATP-independent 3′→5′ exonuclease

To detect the exonuclease activity of drSbcCD, drSbcCD was incubated with 3′ FITC-labelled

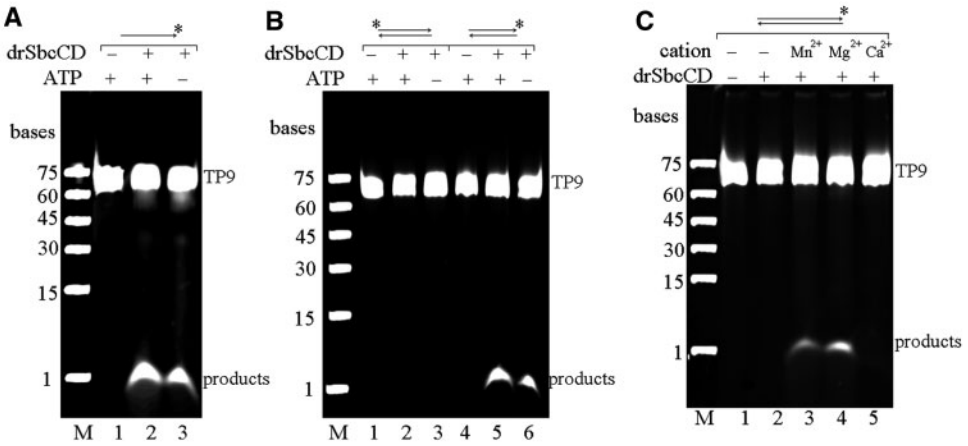

Fig. 2 The ATP-independence of 3′→5′ exonuclease activity of the drSbcCD complex. (A) The 3′ end-labelled TP9 was used to examine ssDNA exonuclease activity. In lanes 2 and 3, whether ATP is present or not, the drSbcCD complex has the ssDNA exonuclease activity, and the products of mononucleotide were released. (B) The 5′ or 3′ end-labelled duplex DNA (TP9 annealed to TP8) was used to examine dsDNA exonuclease activity. In lanes 2 and 5, the drSbcCD complex has the 3′–5′ dsDNA exonuclease activity. In lanes 5 and 6, whether ATP is present or not, the drSbcCD complex has the dsDNA exonuclease activity, and the products of mononucleotide were released. (C) The effect of various cations on the exonuclease activity of the drSbcCD complex. In lane 2, without metal ion, no exonuclease activity was detected. In lanes 3 and 4, in the presence of Mn^{2+} and Mg^{2+} ions, the drSbcCD complex has the exonuclease activity. In lane 5, Ca^{2+} ion is not the cofactor of the drSbcCD complex. *, indicates the position of FITC-label. Each reaction was incubated at 30°C for 1 h. The reaction mixtures were resolved on the 12% denaturing polyacrrylamide gel.

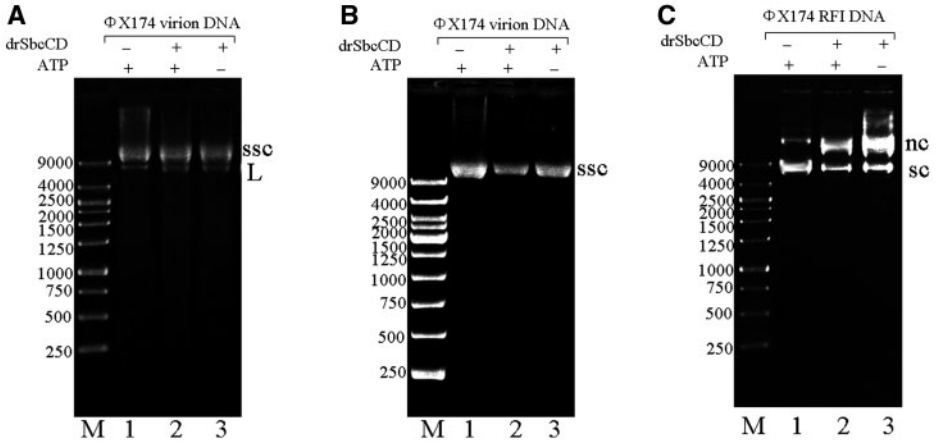

Fig. 3 ATP-independent ssDNA endonuclease and nicking nuclease activity of the drSbcCD complex. (A) ΦX174 virion DNA was used to examine the ssDNA endonuclease activity. In lanes 2 and 3, no ssDNA endonuclease activity of the drSbcCD complex was detected. (B) Purified circular ΦX174 virion DNA was used to examine the ssDNA endonuclease activity. In lanes 2 and 3, drSbcCD had an ATP-independent ssDNA endonuclease activity. ssc, circular ΦX174 ssDNA. L, linear ΦX174 ssDNA. (C) ΦX174 RFI DNA was used to examine the dsDNA endonuclease activity. In lanes 2 and 3, the drSbcCD complex has ATP-independent nicking nuclease activity. sc, supercoiled circular dsDNA; nc, nicked circular dsDNA. Each reaction was incubated at 30°C for 1 h. The reaction mixtures were run in the 0.8% agarose gel and stained with EB.

single-strand oligonucleotide and 5′ or 3′ FITC-labelled double-strand oligonucleotide substrates at 30°C for 1 h. As shown in Fig. 2A and B, the drSbcCD complex was figured to have both single- and double-strand exonuclease activities. In Fig. 2B, the different digesting fashions of 5′ and 3′ labelled substrates indicated that the drSbcCD complex degrades the labelled DNA strand from the 3′ end. The degraded products showed that single nucleotides were released. The drSbcCD complex also digested the substrates in the absence of ATP. Thus, the drSbcCD complex is an ATP-independent 3′→5′ exonuclease.

The exonuclease activity of the drSbcCD complex requires the presence of either Mn^{2+} or Mg^{2+} ion

To determine the effect of different bivalent cations on the drSbcCD exonuclease activity, Mn^{2+}, Mg^{2+} or Ca^{2+} ion was added into the reaction mixtures, respectively. As shown in Fig. 2C, the exonuclease activity was detected in the presence of either Mn^{2+} or Mg^{2+} ion, suggesting that the exonuclease activity of the drSbcCD complex requires the presence of bivalent cations, but it is not absolutely Mn^{2+}-dependent.

The drSbcCD complex has ATP-independent endonuclease and nicking nuclease activity

To examine whether the drSbcCD nuclease would act on the circular ssDNA, ΦX174 virion DNA, which was composed of both circular and linear ssDNA, was incubated with the purified drSbcCD complex at 30°C for 1 h. The reaction mixtures were run in 0.8% agarose gel and stained with ethidium bromide (EB). As shown in Fig. 3A, no circular ΦX174 ssDNA was detected to be decomposed, but when the purified

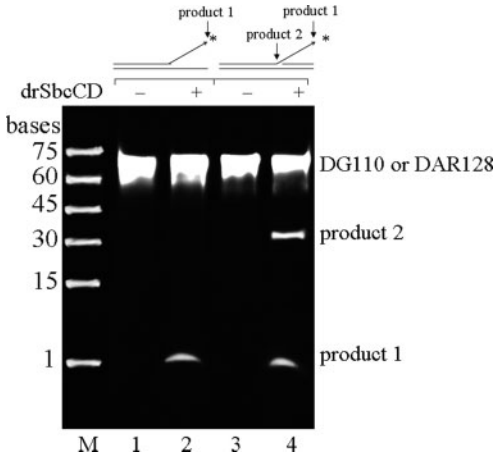

Fig. 4 3′ flap nuclease activity of the drSbcCD complex. The drSbcCD complex was incubated with either a 3′-labelled branched DNA substrate (lanes 1 and 2) or a 3′-labelled flap substrate (lanes 3 and 4). The drSbcCD complex can degrade both substrates at the tip of 3′-terminus as a 3′→5′ exonuclease (product 1). The drSbcCD complex also can cleave the 3′ flap substrate at the site of the single-strand branch adjacent to duplex DNA (product 2). Asterisk indicates the position of FITC-labelled. Each reaction was incubated at 30°C for 1 h. The reaction mixtures were resolved on the 12% denaturing polyacrylamide gel.

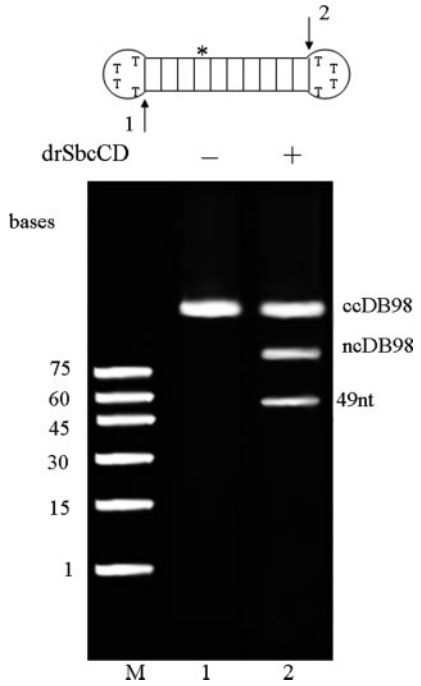

Fig. 5 Structure-specificity of endonuclease of the drSbcCD complex. The drSbcCD complex can cleave the covalently closed dumbbell substrate ccDB98. The products of 49- and 98-nt fragments are indicated in lane 2 (49-nt and ncDB98). Forty-nine-nucleotide fragment was cleaved at the sites of 1 and 2 simultaneously. Ninety-eight-nucleotide fragment was cleaved at the sites of 1 or 2, respectively. Asterisk, the position of FITC-labelled. Each reaction was incubated at 30°C for 1 h. The reaction mixtures were resolved on the 12% denaturing polyacrylamide gel.

circular ΦX174 ssDNA was used as an exclusive substrate, as shown in Fig. 3B, the drSbcCD complex has an obvious endonuclease activity to digest the circular ssDNA.

To examine whether the drSbcCD nuclease would act on the circular dsDNA, the nuclease reaction was repeated using the ΦX174 RFI DNA as a substitute for the ΦX174 virion DNA. As shown in Fig. 3C, the drSbcCD complex could incise one-strand DNA of the supercoiled ΦX174 to generate a nicked circular dsDNA. We also found that drSbcCD was able to efficiently nick the supercoiled dsDNA in the absence of ATP. Thus, the drSbcCD complex has an ATP-independent nicking nuclease activity.

The drSbcCD complex is a 3′ flap nuclease

Both branched and 3′ flap DNA substrates were used to detect the nuclease activity of the drSbcCD complex. As shown in Fig. 4, the drSbcCD complex was active on the 3′ end of the substrates. It can degrade substrates as a 3′→5′ exonuclease to release mononucleotide at the tip of 3′-terminus. Interestingly, the drSbcCD complex also has endonucleolytic activity to cleave the 3′ flap DNA substrate at the site of the single-strand branch adjacent to duplex DNA. Nevertheless, the drSbcCD complex has no endonucleolytic activity on the single-strand region of the branched DNA substrate.

The drSbcCD complex is a structure-specific endonuclease

Because the drSbcCD complex has the endonuclease activity to digest the circular plasmid-length ssDNA and cleave 3′ flap ssDNA, which suggested that the drSbcCD complex might act on secondary structures of ssDNA substrate. ccDB98 is a dumbbell structure substrate with a 45-bp duplex linked by two loop regions. After the drSbcCD complex was incubated with ccDB98, two major products of ~98 and 49 nt were observed (Fig. 5). The size of products revealed that the drSbcCD complex could cleave the covalently closed substrates on one or both junctions. Thus, the drSbcCD complex is a secondary structure-specific endonuclease.

The drSbcCD complex can recognize and nick hairpins with either fully paired or mismatch loops

To test whether the drSbcCD complex is active on hairpin substrates, a fully paired hairpin (HP2) and a hairpin with a 30-base homopolymeric loop (HP30) were used. The reactions were performed at 20°C for 2 h. After incubation, two products were observed (Fig. 6). One was mononucleotides, the other was the result of an incision at the distal end of the hairpin relative to the 3′ labelled extremity. However, the product resulting from incision at the junction of the duplex with the 3′ ssDNA poly (dA) overhang was not seen. This result showed that the drSbcCD complex can not cleave the single-strand region of the branched DNA substrate.

The drSbcCD complex has 3′→5′ exonuclease activity at the protein-blocked DNA end

The substrate 79Tbio was labelled with both 3′ FITC and 5′ biotin at the same end of duplex DNA. Biotin has high affinity interaction with the protein avidin. When streptavidin, the avidin obtained from

streptomyces avidinii, was added into the substrate, the labelled terminus could be blocked completely. The substrate 79Tbio, blocked by streptavidin or not, was incubated with the drSbcCD complex at 20°C for 2 h. The products were analysed by denaturing- or native-PAGE. As shown in Fig. 7A, the sizes of products generated by the drSbcCD complex in the presence or absence of streptavidin were detected by the denaturing-PAGE. Although the streptavidin binding to the 5′-end of the substrate blocked the access to the 3′-end of the complementary strand at the same terminus, the teriminal mononucleotide was released. It was also detected by native-PAGE (Fig. 7B).

To gain the information on the cleavage events at the non-blocked terminus of the substrate, we also constructed the substrate 79Bbio labelled with both 3′ FITC and 5′ biotin at the termini of oligonucleotide 79B. Under the same condition to the substrate 79Tbio, the products were resolved on the native-PAGE. As shown in Fig. 7C, the teriminal mononucleotide was released. Compared the products generated from 79Tbio with those from 79Bbio, we found that no DSB was introduced by the drSbcCD complex at the protein-bound DNA end. Thus, the drSbcCD complex prefers exonuclease activity to endonuclease activity at the DNA ends.

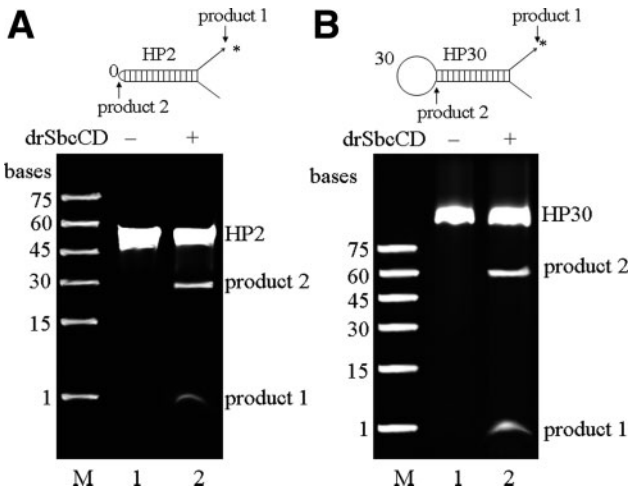

Fig. 6 Hairpin loops recognition and nicking of the drSbcCD complex. The full paired hairpin, HP2, and the hairpin with a 30-base loop, HP30, were incubated with the drSbcCD complex. The products are indicated. Asterisk indciates the position of FITC-labelled. Each reaction was incubated at 20°C for 2 h. The reaction mixtures were resolved on the 12% denaturing polyacrylamide gel.

Discussion

The *drSbcC* and *drSbcD* genes were cloned and over-expressed in *E. coli* cells. The products of the *drSbcC* and *drSbcD* genes were analysed by SDS–PAGE. Two bands, ~110 kDa (drSbcC) and 45 kDa (drSbcD), were detected. The drSbcD protein was eluted by gel filtration in the range of 45 kDa, indicating that drSbcD is a globular monomer under the native condition. The drSbcC and the reconstituted drSbcCD complex were eluted in the void volume of a Sephacryl S-300 column with an exclusion limit of ~1,500 kDa. The separation mechanism of gel filtration involves both shape and mass of the molecule. Proteins with filamentous

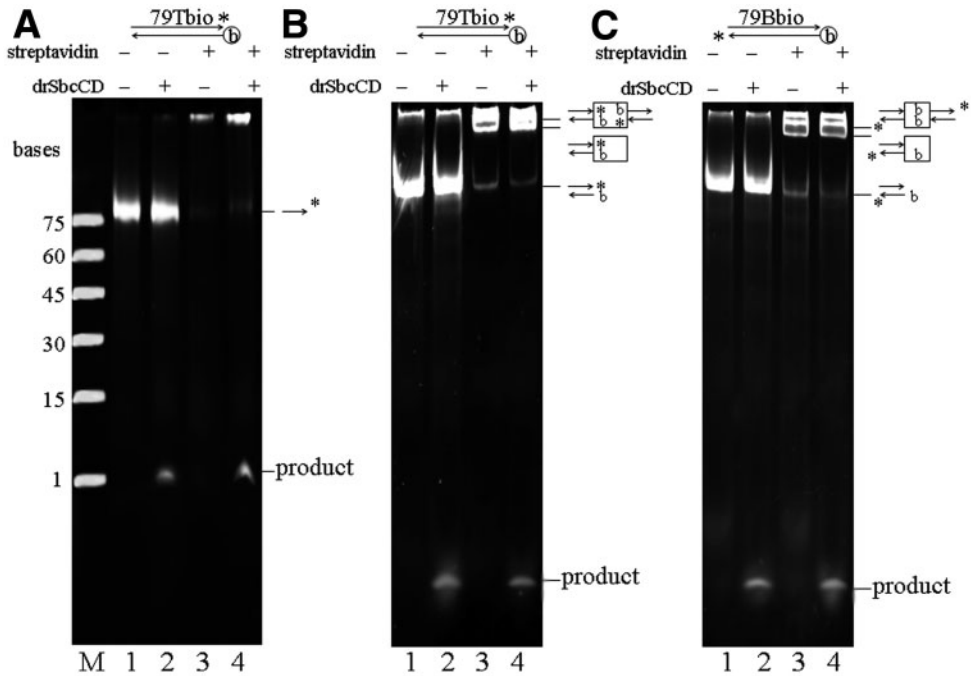

Fig. 7 Exonuclease activity (3′→5′) of the drSbcCD complex at the protein-blocked DNA end. (A) The drSbcCD complex was incubated with the substrate 79Tbio. The mononucleotides were released in the presence of streptavidin (lane 4). The reaction mixtures were resolved on the 12% denaturing polyacrylamide gel. (B) The drSbcCD complex was incubated with the substrate 79Tbio. (C) The drSbcCD complex was incubated with the substrate 79Bbio. The reaction mixtures of both B and C were resolved on the 12% native polyacrylamide gel. Asterisk indicates the position of FITC-labelled. B, the position of biotin-labelled. Each reaction was incubated at 20°C for 2 h.

shape do not migrate typically upon gel filtration. According to atomic force microscopy imaging of the MR complex, Rad50-SbcC-gp46 family protein is the filamentous protein with two globular domains linked by a central coiled coil (*25*). The elution volume of drSbcC or the reconstituted drSbcCD complex was not corresponding with the real molecular weight, this result revealed that drSbcC or the drSbcCD complex would appear the same morphology of the phylogenetically conserved MR complex. According to other MR complexes, it suggested that the drSbcCD complex might interact to bridge DNA ends and facilitate DNA joining *in vivo*.

Biochemical analysis of the drSbcCD complex demonstrated that the drSbcCD complex possesses both exo- and endonuclease activities. SbcD, the nuclease subunit of the SbcCD complex, belongs to a protein family of phosphoesterase (*26*). All phosphoesterases contain the conserved sequence -DXH$(X)_{25}$GDXXD$(X)_{25}$GNHD/E, which is important for metal ion binding and catalysis (*27*). The transition metal ions are required for activation of phosphoesterase activity. In *E. coli*, either Mg^{2+} or Mn^{2+} ion can stimulate the exonuclease activity. This activity in 5 mM Mg^{2+} but not in 5 mM Mn^{2+} is inhibited by 0.5 mM EDTA, indicating that free Mg^{2+} ion is able to stimulate the reaction (*28*). Since EDTA has no effect on Mn^{2+} ion, Mn^{2+} ion might be the optimum divalent metal ion to stimulate the exonuclease activity (*28*). In *S. cerevisiae* and humans, the exonuclease activity has absolute requirement for Mn^{2+} ion, which could not be replaced by other divalent metal ions (*22*, *29*). As expected, the drSbcCD complex is a metallonuclease requiring for either Mn^{2+} or Mg^{2+} ion. However, divalent metal ions are essential for catalysis. SbcC, a kind of bipartite ATP binding cassette (ABC) ATPase, belongs to a subgroup within the SMC family that show extensive sequence similarity in or around their ATP-binding motifs (*26*). ATP can stimulate SbcC to bind DNA (*30*). However, the non-hydrolyzable analogue ATPγS partially inhibits the exonuclease activity of scMR and pfMR (*22*, *31*). It implies that the hydrolyzable form of ATP is required for protein to perfectly perform the nucleolytic action. Interestingly, the drSbcCD complex is an ATP-independent nuclease. However, the reason is unclear. The drSbcCD complex has both ssDNA and dsDNA exonuclease activity with a 3′→5′ polarity that releases mononucleotide products. This activity is similar to that of ecSbcCD, scMR, hsMR and pfMR (*22*, *28*, *29*, *31*).

The virion circular ssDNA digested by ecSbcCD, scMR, hsMR and pfMR produced higher gel mobility, indicate the activity of endonuclease (*22*, *28*, *29*, *31*). Because the substrate of ΦX174 virion DNA firstly was not homogenous, the remainder of linear ssDNA could interrupt the endonucleolytic action on the circular ssDNA, and no ssDNA endonuclease activity was detected. When the circular ssDNA was purified, we finally found that the drSbcCD complex has ssDNA endonuclease activity. This result revealed that drSbcCD complex prefers linear ssDNA. Interestingly, all MR complexes can not cleave a homopolymeric oligonucleotide endonucleolytically, suggesting that they might act on the structure-specific ssDNA substrate (*22*). Here, we synthesized a dumbbell structure substrate (ccDB98) with a 45-bp duplex linked by two loop regions (*23*). The drSbcCD complex can cleave this covalently closed substrate at one or both junctions. It demonstrated that the drSbcCD complex is a secondary structure-specific endonuclease.

When ΦX174 virion ssDNA was replaced by the double-stranded supercoiled form, the drSbcCD complex can convert the supercoiled DNA into nicked circular form. It implied that the drSbcCD complex is a dsDNA-dependent endonuclease. This result was consistent with the character of secondary structure-specific endonuclease of the drSbcCD complex. It suggested that the drSbcCD complex would act on the regions of ssDNA occurring within dsDNA.

Unlike hsMre11 (*21*), the drSbcCD complex is much more active on the 3′ flap DNA than on the 3′ branched DNA. This activity is very important for *D. radiodurans* to repair DSBs generated by ionizing irradiation. Zahradka *et al.* have recently described a novel mechanism of an 'extended synthesis dependent strand annealing' (ESDSA) to account for this repair process (*32*). According to this model, the extended 3′-single-strand overhangs anneal under the constraints of their single-stranded homology, to form the intermediates containing large numbers of 3′ flap DNA fragments. These 3′ flap DNA fragments could be incised by the drSbcCD complex.

Long DNA palindromes can form hairpin or cruciform structures by intrastand base pairing, which inhibit DNA replication and disrupt the genomic integrity. In *E. coli*, the inhibition of DNA replication is overcome significantly in *sbcC* or *sbcD* mutants. The ecSbcCD nuclease cleaves the hairpin DNA to generate DSB that then can be repaired by recombination (*33*). In humans, the fully paired hairpin intermediates made by the RAG proteins are important in lymphoid gene rearrangement. hsMR, the human homologue of ecSbcCD, is a suitable candidate to open hairpin intermediates in V(D)J recombination. hsMre11 protein has endonuclease activity on the hairpin loops, but it can not open the RAG-generated hairpins (*21*). In *S. cerevisiae*, scMre11 together with scRad50 in a protein complex can perform this activity perfectly (*22*). Thus, the efficient function of hsMre11 might require the presence of hsRad50. As expected, the drSbcCD complex can recognize and nick hairpins with either fully paired or mismatch loops. It also revealed that the drSbcCD complex is a secondary structure-specific endonuclease.

The ecSbcCD complex can remove protein from a 5′ terminal protein-bound DNA by introducing a DSB under the conditions where its exo/endonuclease activity was restricted to the dsDNA termini (*24*). It implies that ecSbcCD might perform a number of deblocking roles in cells. Unexpectedly, non-DSB was introduced by drSbcCD, but the 3′→5′ exonuclease activity at the protein blocked DNA end was observed. The drSbcCD complex prefers exonuclease to endonuclease activity at the DNA ends. Therefore, we thought that

the balance of exo- and endonuclease activities of the drSbcCD complex to exactly produce DSB at the protein blocked end would require other repair factors recruited *in vivo*.

In *E. coli*, the primary function of the SbcCD complex *in vivo* is to recognize and cleave DNA secondary structures, such as hairpin structures that the long DNA palindromes and trinucleotide repeats have the potential to adopt during DNA replication. The collapsed replication fork is reformed by RecABCD-mediated recombination (*33*, *34*). However, RecBC is missing in *D. radiodurans*. In *S. cerevisiae*, a hypothesis was speculated that the MR complex cooperates with a DNA helicase to process DSBs in the similar mode of RecABCD-mediated homologous recombination in *E. coli*. The MR complex has the nucleolytic activity preferentially on the secondary structures present in the DNA strands that harbor the 5′ termini of the DNA breaks, then the created 3′ ssDNA tails could mediate homologous recombination repair (*22*). Therefore, the characteristics of enzyme actitivity of the drSbcCD complex give the insightful understanding of the mechanism of maintaining genomic integrity.

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Conflict of interest

None declared.

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