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Staphylococcus aureus DinG, a helicase that has evolved into a nuclease

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DinG (damage inducible gene G) is a bacterial superfamily 2 helicase with 5′→3′ polarity. DinG is related to the XPD (xeroderma pigmentosum complementation group D) helicase family, and they have in common an FeS (iron–sulfur)-binding domain that is essential for the helicase activity. In the bacilli and clostridia, the DinG helicase has become fused with an N-terminal domain that is predicted to be an exonuclease. In the present paper we show that the DinG protein from *Staphylococcus aureus* lacks an FeS domain and is not a DNA helicase, although it retains DNA-dependent ATP hydrolysis activity. Instead, the enzyme is an active 3′→5′ exonuclease acting on single-stranded DNA and RNA substrates. The nuclease activity can be modulated by mutation of the ATP-binding cleft of the helicase domain, and is inhibited by ATP or ADP, suggesting a modified role for the inactive helicase domain in the control of the nuclease activity. By degrading rather than displacing RNA or DNA strands, the *S. aureus* DinG nuclease may accomplish the same function as the canonical DinG helicase.

Key words: damage inducible gene G (DinG), DNA repair, helicase, iron–sulfur, nuclease, xeroderma pigmentosum complementation group D (XPD).

INTRODUCTION

Helicases unwind nucleic acids and play many essential roles in diverse pathways involving the manipulation of DNA and RNA species, including DNA replication, recombination, repair, transcription, translation and RNA splicing. Helicases have been classified into a number of superfamilies on the basis of conserved sequence motifs and the polarity of translocation during strand displacement [1,2]. Superfamily 2 includes the 5′→3′ helicases of the XPD (xeroderma pigmentosum complementation group D; Rad3) family that are essential for nucleotide excision repair in eukarya [3,4]. An unusual feature shared by this family is the presence of an FeS (iron–sulfur) cluster located in a domain that is found near the N-terminus of the protein between the Walker A and B boxes of each protein [5–7]. The family member present in bacteria is known as DinG (damage inducible gene G) [8]. The *dinG* gene was first identified in *Escherichia coli* during a genetic screen for loci up-regulated in response to DNA damage [9]. Neither deletion nor overexpression of *E. coli dinG* produced a strong phenotype [10], although a mild effect on cell survival following UV irradiation and treatment with mitomycin C and nalidixic acid was observed on deletion [10,11].

In vitro, *E. coli* DinG has been shown to function as a 5′→3′ helicase [6,10,12,13]. Biochemical studies have confirmed that *E. coli* DinG has a FeS cluster-binding domain that is essential for the helicase activity [7]. In addition to unwinding simple DNA substrates with a 5′ single-stranded tail, DinG is also active in the dissolution of D-loops, which mimic intermediates of homologous recombination, suggesting that the protein could play a role in recombinational repair [14]. The same study showed that DinG, like XPD, could unwind DNA–RNA hybrids. More recently, an elegant genetics study revealed a potential physiological role of *E. coli* DinG in the dissolution of R-loops that are formed when the replication machinery collides with the transcription machinery. This raised the possibility that the primary role of *E. coli* DinG might be the removal of RNA transcripts from the lagging strand at stalled replication forks [15].

In contrast with the situation in *E. coli*, many Gram-positive bacterial DinG sequences lack the cysteine residues essential for the FeS cluster-binding domain [16]. Given the dependence of the helicase activity on the presence of a stable FeS cluster, this raises the question of whether DinG enzymes lacking the FeS cluster are active as helicases. Furthermore, some DinG proteins from Gram-positive species, including sarDinG (*Staphylococcus aureus* DinG), have acquired an extra N-terminal exonuclease-like domain homologous with the ε proofreading subunit of DNA polymerase III (Figure 1A). This subunit is the archetypal member of the DnaQ family of 3′→5′ exonucleases. Other family members include the bacterial RNaseT and RNaseD proteins, exonuclease I and perhaps most pertinently the WRN (Werner syndrome protein) [17], which, like sarDinG, has helicase and nuclease domains [18].

In the present study, we cloned and expressed *dinG* from *S. aureus* and characterized its activity *in vitro*. Although the protein sequence includes conserved helicase motifs, sarDinG displays ssDNA (single-stranded DNA)-stimulated ATPase activity, but lacks helicase activity. The predicted exonuclease domain of *S. aureus* DinG is confirmed as a functional 3′→5′ exonuclease active against DNA and RNA *in vitro*. The exonuclease activity is modulated by mutation of the helicase ATP-binding cleft and by ATP or ADP binding, suggesting a modified role for the helicase domain in the regulation of nuclease activity in these enzymes.

EXPERIMENTAL

Preparation of DNA and RNA substrates

Oligonucleotides were purchased from IDT, purified by denaturing acrylamide gel electrophoresis and end-labelled with [^{32}P]ATP where necessary, as described previously [19]. For

Abbreviations used: CRISPR, clustered regularly interspaced short palindromic repeats; DinG, damage inducible gene G; DTT, dithiothreitol; FAM, 6-carboxyfluorescein; FeS, iron–sulfur; sarDinG, *Staphylococcus aureus* DinG; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; TBE, Tris/borate/EDTA; TEV, tobacco etch virus; XPD, xeroderma pigmentosum complementation group D; XPF, xeroderma pigmentosum complementation group F; WT, wild-type.

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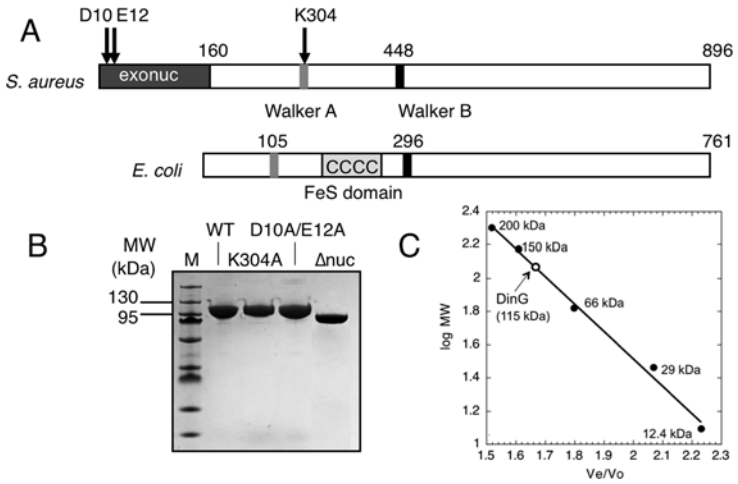

Figure 1 Comparison and purification of sarDinG proteins

(**A**) Comparison of the domain organization of DinG from *S. aureus* and *E. coli*, showing the exonuclease (exonuc) domain in the former and the FeS-binding domain in the latter. The positions of the Walker A and Walker B motifs in both proteins are indicated by grey and black boxes respectively. The positions of the three point mutations made in sarDinG are indicated by arrows. (**B**) SDS/PAGE showing purified WT and mutant sarDinG proteins. The molecular mass in kDa is indicated on the left-hand side. (**C**) Elution profile of WT sarDinG on a calibrated Superdex 200 gel-filtration column. The standard curve shown was calculated using the elution volumes of a set of standard proteins of known molecular mass, allowing the estimation of the native molecular mass of DinG, which was consistent with a monomeric composition.

partially duplex species, oligonucleotides were annealed by slow cooling from 95 °C and substrates were subsequently purified by native acrylamide gel electrophoresis as described previously [20]. Annealed substrates were assembled as follows: splayed duplex, DNA50 + DNA50Y; 5′ overhang, DNA50 + DNA25H; 3′ overhang, DNA50 + DNA25X; duplex with bubble, DNA50 + Bubble7; RNA–DNA hybrid, DNA40 + RNA25. Sequences used were: DNA50, 5′-CCTCGAGGGATCCGTCCTAGCAAGCCGCTGCTACCGGAAGCTTCTGGACC-3′; DNA50Y, 5′-GCTCGAGTCTAGACTGCAGTTGAGAGCTTGCTAGGACGGATCCCTCGAGG-3′; Bubble7, 5′-GGTCCAGAAGCTTCCGGTAGCCTACCGCTGCTAGGACGGATCCCTCGAGG-3′; DNA25X, 5′-GCTTGCTAGGACGGATCCCTCGAGG-3′; DNA25H, 5′-GGTCCAGAAGCTTCCGGTAGCAGCG-3′; DNA40, 5′-TTTTTTTTTTTTTTTGATTAATCCCAAAAGGAATTGAAAG-3′; RNA25, 5′-FAM-CUUUCAAUUCCUUUUGGGAUUAAUC-3′ (FAM is 6-carboxyfluorescein); and poly-U, 5′-FAM-UUUUUUUUUUUUUUUUUUU-3′.

Cloning and purification

The *dinG* gene from *S. aureus* (SAR1466; gene ID 2860529) was amplified by PCR from genomic DNA (strain MRSA252, A.T.C.C. strain collection) using the following primers: 5′-CCGAAAACCTGTATTTTCAGGGCATGGCAACCTATGCCGTTGTGGATTT-3′ and 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCCTACTTTTTCTTTTTTTGAATTTGTC-3′.

The amplified gene was cloned into a pDEST14 destination vector using the Gateway® cloning system (Invitrogen) for expression in *E. coli* with an N-terminal TEV (tobacco etch virus)-cleavable polyhistidine tag [21]. Site-directed mutants were designed following standard protocols. Oligonucleotide sequences for the mutants are available from the corresponding author on request.

Recombinant sarDinG was expressed in *E. coli* C43 cells, which were grown in LB (Luria–Bertani) medium with 100 μg/ml ampicillin at 37 °C until a D_{600} of 0.6–0.8 was achieved. Protein expression was induced by the addition of 0.4 mM IPTG (isopropyl β-D-thiogalactopyranoside) and continued cell growth at 37 °C for 4 h. Cells were harvested, lysed by sonication in lysis buffer [20 mM Tris/HCl (pH 7.5), 200 mM NaCl, 1 mg/ml lysozyme and 1 mM benzamidine] and centrifuged at 50 000 ***g*** for 30 min at 4 °C to clear the lysate of precipitated and insoluble proteins. Protein was bound to a nickel-chelating column (HiTrap 5 ml Chelating HP, GE Healthcare) equilibrated with column buffer [20 mM Tris/HCl (pH 7.5), 200 mM NaCl and 10 mM imidazole] and eluted with a linear imidazole gradient (500 mM). Protein-containing fractions were identified by SDS/PAGE, pooled and further purified on a HiLoad 26/60 Superdex 200 size-exclusion column (GE Healthcare) equilibrated with gel-filtration buffer [20 mM Tris/HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA and 1 mM DTT (dithiothreitol)]. Pure DinG was incubated for ~14 h at 22 °C with 200 ng/mg TEV protease to remove the N-terminal polyhistidine tag and then loaded on to a second nickel-chelating column. Pure protein collected from the flow-through was analysed by MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS to confirm the identity and integrity of the protein sequence. The Walker A box mutant (K304A) and nuclease domain mutants (D10A/E12A-DinG and Δnuc) were expressed and purified as described for the WT (wild-type) protein.

ATPase activity

ATPase assays were performed in a final volume of 240 μl containing 20 mM Mes (pH 6.5), 1 mM DTT, 0.1 mg/ml BSA, 0.2 μM protein and 10 nM DNA (ΦX174 virion DNA, New England Biolabs). Reactions were incubated at 37 °C for 1 min and initiated by the addition of 1 mM ATP/$MgCl_2$. At the indicated time points, 40 μl samples were taken and immediately added to 40 μl of 0.3 M chilled perchloric acid on a 96-well plate. Samples were equilibrated to room temperature (20 °C) prior to the addition of Malachite Green (20 μl) and, following a 12 min incubation at room temperature, the absorbance at 650 nm was measured on a SpectraMAX 250 Microplate Reader (Molecular Devices). For each reaction, a blank without protein was quantified and subtracted as background from sample reactions. All experiments were carried out in triplicate and S.E.M. values were calculated.

DNA helicase assays

Time-dependent DNA unwinding by DinG was monitored using a helicase assay. Reactions contained 20 mM Mes (pH 7.0), 1 mM DTT, 100 mM NaCl, 0.1 mg/ml BSA and 1 mM ATP/$MgCl_2$, and the relevant DNA species at 10 nM, and were incubated at 37 °C for 1 min prior to initiation by the addition of 1 μM DinG. At the indicated time points, 10 μl aliquots of the reaction mixture was added to chilled STOP buffer [10 mM Tris/HCl (pH 8), 5 mM EDTA, 300 mM NaCl, 0.5 % SDS and 1 mg/ml proteinase K] together with 5 μM competitor DNA (sequence complementary to the radiolabelled displaced strand to prevent re-annealing). Samples were incubated on ice for 15 min to allow sample deproteinization before separation on native 12 % acrylamide/TBE [Tris/borate/EDTA (1×TBE = 45 mM Tris/borate and 1 mM EDTA)] gels at 130 V for 3 h.

DNA nuclease assays

Time-dependent cleavage of DNA by DinG was determined using a nuclease assay comprising 20 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 mg/ml BSA and 5 mM $MgCl_2$ (and 1 mM ATP for WT-DinG or 5 mM ATP for K304A-DinG where indicated), together with 10 nM ^{32}P-labelled DNA. Samples were incubated at 37 °C for 1 min prior to initiation by the addition of 500 nM DinG. At the indicated time points, 10 μl aliquots were taken from the reaction mixture and added to 10 μl of formamide loading dye [95 % deionized formamide, 0.025 % Bromophenol Blue, 0.025 % Xylene Cyanol FF, 5 mM EDTA (pH 8.0) and 0.025 % SDS] and incubated at 95 °C for 5 min. Reactions were separated on denaturing 20 % acrylamide/TBE gels at 95W for 1.5 h prior to visualization by phosphorimaging and analysis using ImageGauge software to calculate the percentage of substrate cleaved.

To analyse the ATP-dependence of DinG nuclease activity, 10 nM ^{32}P-labelled DNA was incubated in 20 mM Tris/HCl (pH 7.5), 50 mM NaCl, 0.1 mg/ml BSA and 5 mM $MgCl_2$, and the indicated concentrations of ATP. Reactions were initiated by the addition of DinG and incubated at 37 °C for 60 min. Reactions were stopped by the addition of formamide loading dye, and separated and processed as described above.

RESULTS

Gene cloning and protein expression

The *dinG* gene from *S. aureus* was amplified by PCR and cloned into the expression vector pDEST14 for expression in *E. coli*. The recombinant protein was purified to homogeneity by immobilized metal affinity and gel-filtration chromatography as described in the Experimental section and analysed by SDS/PAGE (Figure 1B). The N-terminal polyhistidine tag was cleaved by incubation with the TEV protease during protein purification. A mutant unable to hydrolyse ATP (K304A in the Walker A box) and a mutant designed to abrogate the nuclease activity (D10A/E12A) were generated by directed mutagenesis. A third mutant lacking the entire nuclease domain (Δnuc) was also designed and constructed. The expression and purification of all three mutants were carried out as described for the WT protein (Figure 1B). MS confirmed successful purification of WT and mutant DinG proteins. Consistent with the lack of an FeS cluster-binding domain, no absorbance in the 350–450 nm range was detected for the pure proteins, which were colourless even at high concentrations. As observed for *E. coli* DinG [10], *S. aureus* DinG exists as a monomer in solution and the size estimated from calibrated analytical gel filtration was 115 kDa, close to the calculated size of 104 kDa for a monomer (Figure 1C).

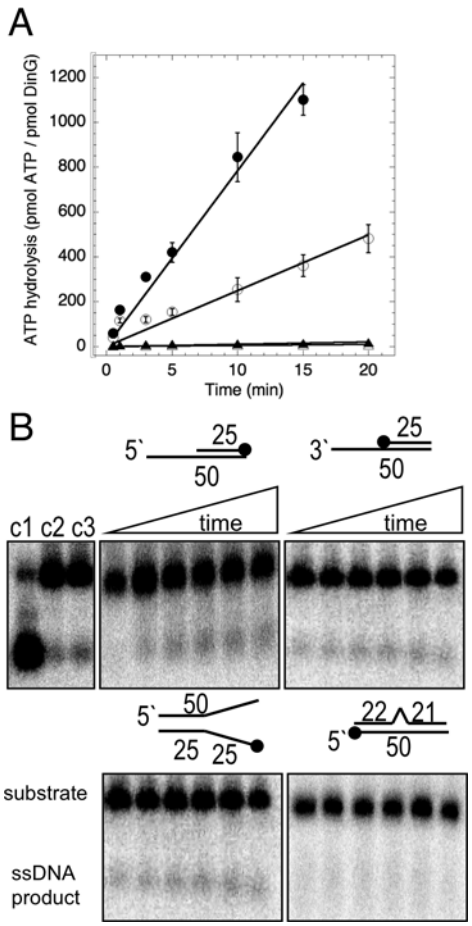

Figure 2 ATPase and helicase activity of sarDinG

(**A**) The rate of ATP hydrolysis by WT and K304A mutant DinG in the presence and absence of ssDNA. WT DinG displayed a modest ATPase activity ($k_{cat} = 25\ min^{-1}$) that was stimulated by ssDNA ($k_{cat} = 78\ min^{-1}$). The ATPase activity of the K304A mutant was negligible, as expected. Data points represent the mean of triplicate measurements, with S.E.M.s indicated. ● and ○, WT DinG; ▲ and △, K304A mutant. Closed symbols indicate the presence of ssDNA, open symboles indicate the the absence of ssDNA. (**B**) Helicase assays to determine the ability of 1 μM sarDinG to unwind DNA molecules consisting of a 5′-overhang, 3′-overhang, splayed duplex and duplex with an internal 7 nt bubble. The black dots indicate the site of the 5′-^{32}P radioactive label. Protein (1 μM) was incubated with 10 nM radiolabelled DNA at 37 °C in helicase buffer and reactions were stopped after 1, 10, 20, 30, 40 and 60 min and analysed by acrylamide gel electrophoresis. No appreciable helicase activity was detected for any substrate. Controls: c1, boiled substrate; c2, no protein control at 60 min; c3, absence of ATP/Mg at 60 min.

ATP hydrolysis by sarDinG

Helicases utilize conformational changes linked to the cyclical binding and hydrolysis of ATP to translocate along and unwind nucleic acids. sarDinG hydrolysed ATP with a rate (k_{cat}) of 25 ± 3 pmol of ATP · min^{-1} · pmol of DinG in the absence of DNA. This increased to a k_{cat} of 78 ± 9 min^{-1} in the presence of ssDNA, a 3-fold stimulation (Figure 2A). The Walker A box mutant K304A had very low residual ATPase activity, confirming the role of the helicase active site in ATP hydrolysis. By way of contrast, the equivalent rates for *E. coli* DinG have been reported as k_{cat} values of 96 min^{-1} and 1440 min^{-1} in the absence and presence of ssDNA respectively, a 15-fold stimulation [10]. Thus the enzyme from *S. aureus* appears to turn over ATP significantly more slowly in the presence of ssDNA than *E. coli* DinG.

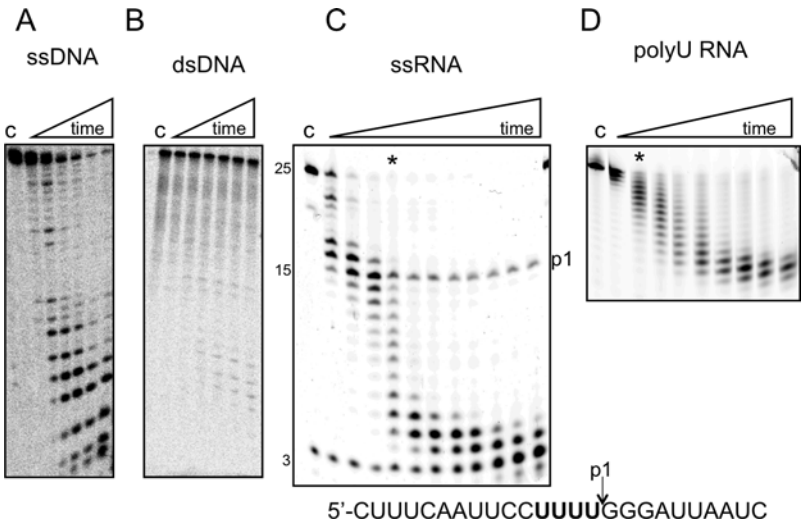

Figure 3 Nuclease activity of sarDinG on ssDNA and ssRNA substrates

(**A**) sarDinG (500 nM) was incubated with 10 nM ^{32}P-labelled oligonucleotide (DNA25X) and 5 mM $MgCl_2$ at 37 °C. Progressive cleavage in a 3′→5′ direction was observed. Reactions were stopped after 1, 10, 20, 30, 40 and 60 min and analysed by denaturing acrylamide gel electrophoresis. c, control reaction incubated at 37 °C for 60 min in the absence of protein. (**B**) The activity of sarDinG on a DNA duplex with a central 7 nt bubble. Reaction conditions were identical with those described for (**A**). No cleavage activity was observed. (**C**) Cleavage of a 25 nt ssRNA oligonucleotide (RNA25) with a 5′ fluorescein label by sarDinG. Progressive cleavage in a 3′→5′ direction was observed as for ssDNA. A pronounced pause site (p1), which persisted to the end of the reaction time, was observed to correspond to a run of four uracil residues in the RNA sequence (shown in bold below). The reaction conditions were the same as for (**A**) with time points of 0.5, 1, 2, 5, 10, 15, 20, 30, 60, 90 and 120 min. The 5 min time point is indicated with an asterisk. (**D**) Cleavage of a 15 nt poly-uracil RNA oligonucleotide (polyU) was carried out as described for (**A**). Cleavage kinetics were more uniform and considerably lower than those observed in (**C**), confirming the sequence-dependence of the nuclease reaction. The asterisk indicates the 5 min time point. Time points correspond to incubation for 1, 5, 10, 15, 20, 30, 40, 60 and 90 min.

sarDinG does not unwind DNA helicase substrates *in vitro*

The DinG protein from *E. coli* is an active 5′→3′ helicase, with 100 nM enzyme unwinding a range of substrates to completion within 2.5 min at 30 °C [10]. Bifurcated DNA substrates (splayed duplexes) are unwound more efficiently than 5′ overhangs [14]. In addition, the archaeal XPD helicase has been shown to unwind DNA substrates with an internal unpaired region (bubbles) [22]. We therefore tested sarDinG for the ability to unwind a range of DNA helicase substrates. No appreciable helicase activity was detected using 5′ and 3′ overhangs, splayed duplexes and bubble structures, even after incubation of 1 μM sarDinG with the DNA for 1 h at 37 °C (Figure 2B). Similar results were obtained using the D10A/E12A and Δnuc mutants (results not shown). The lack of helicase activity of sarDinG correlates with the lack of an FeS cluster in this enzyme; previous studies of *E. coli* DinG and archaeal XPD have demonstrated that disruption of the FeS-binding domain results in loss of helicase activity [6,7].

sarDinG functions as a 3′→5′ exonuclease *in vitro*

As sarDinG has a predicted N-terminal exonuclease domain (Figure 1A), we tested the nuclease activity of sarDinG against a variety of substrates. SarDinG displayed a robust 3′→5′ exonuclease activity when presented with a 5′ end-labelled ssDNA oligonucleotide (Figure 3A), with progressive cleavage towards the 5′ end over time. The cleavage reaction was dependent on the presence of magnesium, and mutation of the nuclease domain (D10A/E12A mutant) abolished nuclease activity (Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/bj4420077add.htm). A DNA duplex with a central unpaired bubble was not a substrate (Figure 3B). These data suggest that the exonuclease domain of sarDinG is, as predicted, a metal-dependent 3′→5′ exonuclease and requires access to the 3′ end of a DNA strand in ssDNA form.

Previous reports have suggested that *E. coli* DinG could function in the processing of RNA–DNA hybrids caused by the collision of replication forks with RNA polymerase [15], and the enzyme has been shown to unwind DNA–RNA hybrids *in vitro* [14]. Accordingly, we tested the ability of sarDinG to degrade RNA. As was observed for DNA, sarDinG degraded ssRNA (single-stranded RNA) *in vitro* with a 3′→5′ exonuclease activity. An RNA oligonucleotide of mixed sequence labelled with a 5′ fluorescein was degraded in a 3′→5′ direction (Figure 3C). A pronounced pause site was observed that coincided with a run of four uracil residues (p1 in Figure 3C), suggesting some sequence-dependence for the nuclease activity. Accordingly, we tested the enzyme against a 15U RNA oligonucleotide (Figure 3D). This was degraded with kinetics that were much lower and more uniform than for the mixed sequence substrate (compare reaction product amounts at 5 min, labelled with an asterisk in Figures 3C and 3D). Thus the exonuclease domain of sarDinG can degrade RNA in addition to DNA substrates, but is inhibited by runs of uracil residues in substrates.

sarDinG degrades recessed DNA and RNA strands in 5′ overhang substrates

Although the nuclease activity of sarDinG was not active against substrates with blunt ends, we were interested in determining whether the enzyme could process the overhang structures that are classical substrates for helicases both *in vitro* and *in vivo*. We therefore tested the ability of sarDinG to degrade oligonucleotides in fully duplex form where a 3′ or 5′ ssDNA overhang was present. A 5′ overhang substrate, the classical minimal substrate for XPD and DinG family helicases, was degraded by sarDinG with comparable reaction kinetics with those observed for fully ssDNA (Figure 4A). By contrast, a 3′ overhang was not cleaved appreciably in the same time period (Figure 4B). An RNA–DNA hybrid molecule with a 5′ DNA overhang was also a substrate

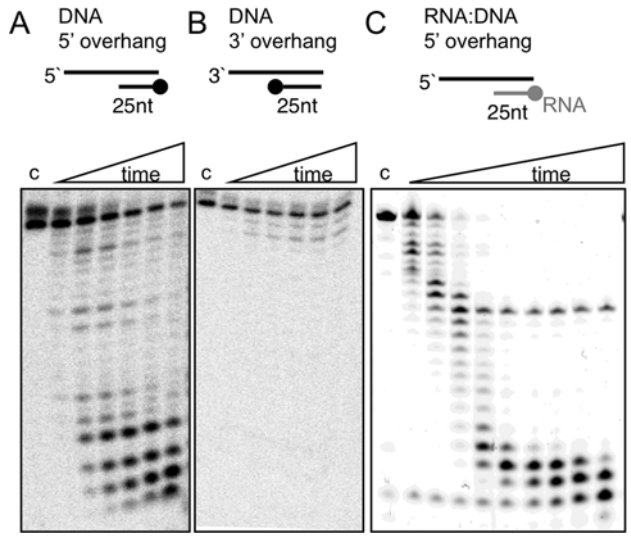

Figure 4 DinG helicase substrates are degraded by sarDinG

(**A**) sarDinG (500 nM) was incubated with a DNA duplex with a 5′ overhang, consisting of an unlabelled 50 nt oligonucleotide (DNA50) annealed to a 5′-end labelled oligonucleotide (DNA25H) and 5 mM $MgCl_2$ at 37 °C. Progressive cleavage in a 3′→5′ direction was observed. Reactions were stopped after 1, 10, 20, 30, 40 and 60 min and analysed by denaturing acrylamide gel electrophoresis. c, control reaction incubated at 37 °C for 60 min in the absence of protein. (**B**) Reaction as in (**A**) but with a DNA duplex with a 3′ overhang, consisting of an unlabelled 50 nt oligonucleotide (DNA50) annealed to a 5′-end labelled oligonucleotide (DNA25X). No appreciable cleavage was observed. (**C**) sarDinG (500 nM) was incubated with an RNA–DNA hybrid molecule consisting of an unlabelled 40 nt oligonucleotide (DNA40) annealed to a 25 nt RNA oligonucleotide with a 5′-fluorecein label (RNA25). Reaction conditions were as for (**A**) with time points 0.5, 1, 2, 5, 10, 15, 20, 30 and 60 min. The same pause site observed in Figure 3(C) was observed.

for the exonuclease activity of sarDinG (Figure 4C). These data are consistent with a role for the helicase domain of sarDinG in binding to 5′ overhangs and destabilizing the duplex at the interface between double-stranded and single-stranded regions sufficiently to allow processing by the exonuclease domain. In this way, sarDinG could achieve the same end result as *E. coli* DinG by degrading rather than displacing DNA or RNA strands.

The helicase domain of sarDinG controls the activity of the exonuclease domain

Given that the exonuclease domain of sarDinG is fused to a helicase domain that hydrolyses ATP in the presence of ssDNA, we next tested whether the exonuclease domain functioned independently or in concert with the helicase domain. Comparison of the exonuclease activity of WT and K304A mutant sarDinG on a 5′ overhang substrate suggested that the mutant was a significantly more active exonuclease (Figure 5A). To confirm this observation, the degree of cleavage of a variety of DNA substrates was calculated for the WT and K304A mutant enzymes in triplicate experiments (Figure 5B). The mutant cleaved the ssDNA, 5′ overhang and splayed duplex substrates significantly more quickly than the WT enzyme, whereas 3′ overhangs and DNA duplexes with a bubble were cut poorly by both. This suggests that changes in the ATP-binding site of the helicase domain can affect the activity of the nuclease domain, possibly due to conformational changes transmitted through the protein domains. To investigate this further, we examined the effect of ATP on DinG nuclease activity. The ability of WT sarDinG to cleave ssDNA was inhibited markedly in the presence of ATP (Figure 5C). This effect was dependent on the ATP concentration, with 1 mM ATP sufficient to inhibit cleavage completely in a 60 min incubation time. By contrast, the K304A mutant was only partially inactivated by 2 mM ATP under the same assay conditions, with full inactivation occurring at 5 mM ATP (Figure 5C).

The WT enzyme can turnover ATP in the presence of ssDNA, whereas the K304A mutant cannot. It was therefore possible that the differential inhibition of the two enzymes was due to inhibition by ADP rather than ATP. We tested this possibility by assaying the nuclease activities of both the WT and K304A mutant in the presence of ATP or ADP (Supplementary Figure S2 http://www.BiochemJ.org/bj/442/bj4420077add.htm). Both enzymes were inhibited by ADP as well as ATP, with slightly weaker inhibition by ADP apparent. The data suggest that either ATP or ADP binding at the nucleotide-binding cleft of the helicase can inhibit the exonuclease activity of sarDinG, presumably due to conformational changes transmitted from the helicase to the nuclease domain. The mutant lacks a highly conserved Walker A box lysine residue, which binds the β- and γ-phosphates of ATP. Therefore the K304A mutant would bind ATP less tightly than WT DinG, as well as lacking the ability to hydrolyse ATP. This could explain the differences observed in the concentration of ATP required to inhibit the WT and mutant enzymes.

DISCUSSION

As a 5′→3′ superfamily 2 DNA helicase with an essential FeS-binding domain, *E. coli* DinG is clearly related to the XPD/Rad3 helicase found in archaea and eukarya. The eukaryal enzyme has a well-understood role in the nucleotide excision repair pathway, whereas the functions of the archaeal and bacterial proteins are still a matter for speculation [15,16]. At some point in the evolution of the bacteria, possibly in a common ancestor of the clostridia and bacilli, the *dinG* gene fused with a gene encoding a 3′→5′ DNA exonuclease. In the bacilli, the FeS-binding domain was subsequently lost. Our own results from the *S. aureus* protein suggest that this class of DinG proteins are no longer active as helicases. This is consistent with the known requirement for an FeS-binding domain in XPD family proteins for helicase activity, and probably reflects an essential role of this domain in DNA unwinding [5,6]. It is not possible to state with certainty whether the loss of the FeS domain in the bacilli DinG family caused the loss of helicase activity or was a result of neutral evolution following a change in function for DinG due to the fusion with an exonuclease domain. In this regard it would be interesting to investigate the helicase activity of one of the enzymes that has an exonuclease domain and has preserved an FeS-binding domain [16].

In *S. aureus* DinG, the 'helicase' domain can still catalyse ssDNA-stimulated hydrolysis of ATP. The conservation of this activity suggests a continuing role for this part of the protein, albeit not as a canonical helicase. There are many other examples of fusions between helicases and nucleases, which often work together to process DNA during repair and recombination pathways. One relevant example is the XPF (xeroderma pigmentosum complementation group F) family found in archaea and eukarya. XPF is a PCNA (proliferating-cell nuclear antigen)-dependent 5′-flap endonuclease in crenarchaea [23], but is fused to an active helicase in the euryarchaea [24]. In the eukarya, two different proteins have evolved: XPF, where the helicase has become inactivated and is thought to play a role in binding DNA substrates, and FancM from the Fanconi anaemia DNA repair pathway, which has an active helicase, but a degenerate nuclease [25]. A further pertinent example is the AddAB complex which catalyses DNA 3′ end resection in

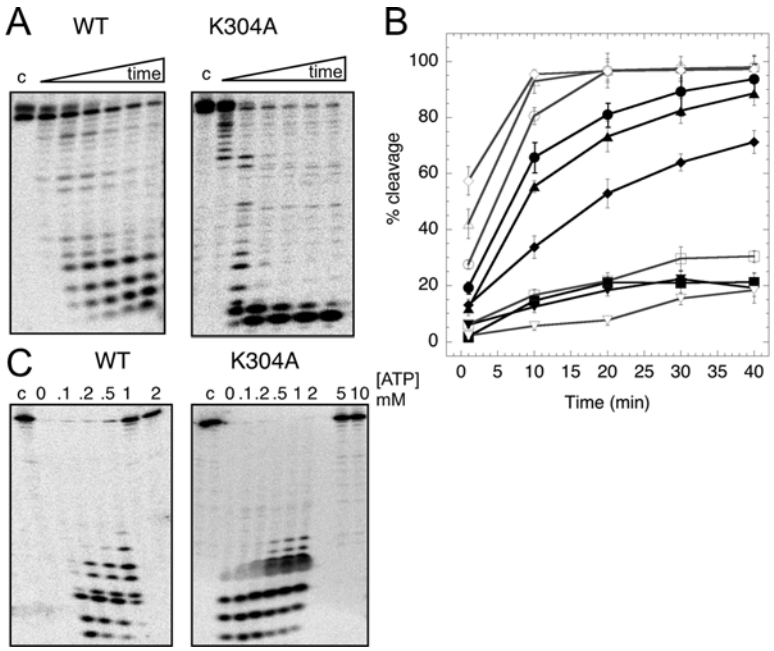

Figure 5 Modulation of nuclease activity by the helicase domain and nucleotides

(**A**) Comparison of the cleavage activity of WT and K304A sarDinG with a ^{32}P-radiolabelled 5′ overhang substrate and 5 mM $MgCl_2$ at 37°C. The K304A mutant displayed significantly higher nuclease activity than the WT enzyme. Reactions were stopped after 1, 10, 20, 30, 40 and 60 min and analysed by denaturing acrylamide gel electrophoresis. c, control reaction incubated at 37°C for 60 min in the absence of protein. (**B**) Comparison of the activities of the WT and K304A mutant proteins on a range of DNA substrates as a function of time. Substrates tested were ssDNA (● and ○), 5′ overhang (▲ and △), 3′ overhang (■ and □), splayed duplex (◆ and ◇) and bubble (▼ and ▽). WT data points have closed symbols, and K304A data points have open symbols. Each data point represents the mean of triplicate experiments with S.E.M.s indicated. (**C**) Comparison of the effect of ATP on the nuclease activity of WT and K304A sarDinG. Reactions were carried out under standard conditions with 5 mM $MgCl_2$ and a varying concentration of ATP for 60 min using the DNA25X oligonucleotide, and analysed as described previously. WT DinG was inhibited fully by 1 mM ATP, but the K304A mutant was still active in the presence of 2 mM ATP and only inhibited fully by 5 mM ATP.

Bacillus subtilis. AddAB consists of two subunits that each encode a helicase-like domain fused to an FeS-dependent nuclease. Previous studies suggest that only one of the motor domains is active as a helicase and the other has evolved to function as a sequence-dependent DNA-binding moiety, essential for the recognition of Chi-sites during strand resection [26–28].

Our observation that ATP or ADP binding can inhibit sarDinG nuclease activity, and that a mutation targeted to the ATP-binding site increases the exonuclease activity, suggests that the ‘helicase’ domain of sarDinG functions to modulate the activity of the nuclease, perhaps targeting it to suitable nucleic acid substrates. The control of DNA repair nucleases by accessory proteins or domains is a common observation, as unchecked nuclease activity is potentially dangerous. A cartoon representation of a possible mechanism for sarDinG is shown in Figure 6. In this model, binding of ATP at the cleft between the two helicase motor domains locks the exonuclease domain in an inactive state, perhaps by limiting its ability to engage 3′ DNA ends. Hydrolysis of the ATP, which is stimulated by ssDNA, results in the formation of ADP, which is bound more weakly by helicases and is therefore more likely to dissociate from the enzyme. This allows a conformational change related to those characterized for canonical helicases, resulting in the activation of the nuclease domain. Binding of another ATP molecule returns the enzyme to the inactive conformation.

Although speculative, the model fits with the data we have presented for sarDinG. For canonical helicase substrates, the helicase domain could track along the uncleaved strand in a 5′→3′ direction, whereas the nuclease degraded the other strand, as shown on the right-hand side of Figure 6. For ssDNA substrates, the exonuclease could act alone (as shown on the left-hand side of Figure 6), or potentially with another DNA strand bound *in trans* by the helicase domain. In the K304A mutant, ATP binding is very likely to be significantly weaker, consistent with the observation that higher levels of ATP are required to inhibit the nuclease. A similar control mechanism has been observed for the T4 ‘headful’ DNA-packaging nuclease gp16, which has separate nuclease and ATPase domains [29]. In this case, binding of ATP at the ATPase domain is proposed to result in a conformational change that results in activation of the nuclease. Similarly, ATP binding and hydrolysis in the Rad50 subunit of the DNA repair complex MRN (Mre11-Rad50-Nbs1) has been shown to drive conformational changes that regulate the activity of the nuclease subunit Mre11 [30]. These recent findings suggest an emerging paradigm for interdomain nuclease control by ATP-mediated conformational changes.

The role of DinG in any bacterial species is still not clear. In most bacteria, including *E. coli*, DinG is a 5′→3′ helicase like XPD, but is almost certainly not involved in the nucleotide excision repair pathway as another helicase, UvrB, fulfils this role [31]. Recent genetic data suggest a role for DinG in the dissolution of R-loops (RNA–DNA hybrids) during replication restart following the collision of replication forks with transcription units [15]. *E. coli* DinG can unwind RNA–DNA hybrids *in vitro*, and we have shown in the present study that the *S. aureus* DinG can accomplish the same thing by degrading, rather than displacing, the RNA strand. This is an attractive hypothesis for sarDinG function as, intuitively, it seems preferable to degrade RNA, which can be replaced easily, rather than DNA, which may be required for replication restart or other DNA repair pathways. Recently, DinG-like proteins have been implicated in the CRISPR (clustered regularly interspaced short

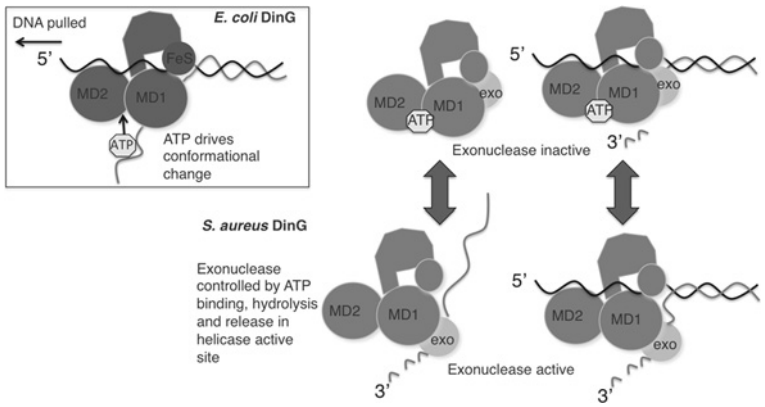

Figure 6 Cartoon representation of a possible reaction mechanism for sarDinG

E. coli DinG (boxed) has an intact FeS cluster and acts as a 5′→3′ helicase, displacing DNA or RNA strands. *S. aureus* DinG lacks an FeS cluster and displays no helicase activity, but has an additional N-terminal exonuclease domain. The model postulates a situation where the inactive helicase domain of sarDinG binds and hydrolyses ATP, potentially tracking along ssDNA in a 5′→3′ direction. The activity of the nuclease domain is controlled allosterically by conformational changes in the helicase domain driven by ATP binding, hydrolysis and release.

palindromic repeats) pathway for antiviral defence [32], where they appear in some bacterial lineages to have replaced Cas3, the helicase–nuclease complex that unwinds and degrades RNA–DNA hybrids in the final stage of viral DNA degradation [33]. These CRISPR-associated DinG-like proteins lack the cysteine residues characteristic of the FeS domain and have an N-terminal extension that could constitute a nuclease domain, although it is not recognizably related to exonuclease III. It will be interesting to determine whether these proteins have indeed been co-opted in an antiviral defence pathway, and whether their properties correlate with those of sarDinG.

AUTHOR CONTRIBUTION

Anne-Marie McRobbie, Bjoern Meyer, Christophe Rouillon, Biljana Petrovic-Stojanovska and Huanting Liu carried out the work. Anne-Marie McRobbie, Christophe Rouillon and Malcolm White analysed the data and wrote the paper.

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online data

SUPPLEMENTARY ONLINE DATA

Staphylococcus aureus DinG, a helicase that has evolved into a nuclease

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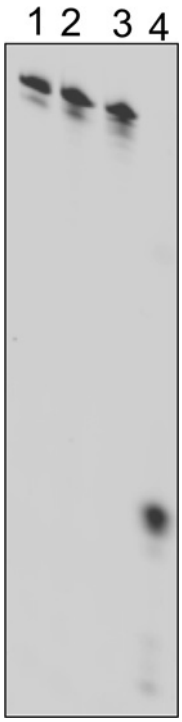

Figure S1 Comparison of the activities of wild-type and mutant D10A-E12A DinG

A 50 nt ssDNA oligonucleotide (DNA50) with a 3′ fluorescein label was incubated at 37 °C in reaction buffer as follows: (1) no protein; (2) WT DinG (100 nM) without $MgCl_2$; (3) mutant D10A-E12A DinG (100 nM) with 5 mM $MgCl_2$; and (4) WT DinG (100 nM) with 5 mM $MgCl_2$. Cleavage at the 3′ end of the DNA substrate was only observed in the presence of $MgCl_2$ and the WT enzyme, confirming the role of the N-terminal exonuclease domain in magnesium-dependent DNA degradation.

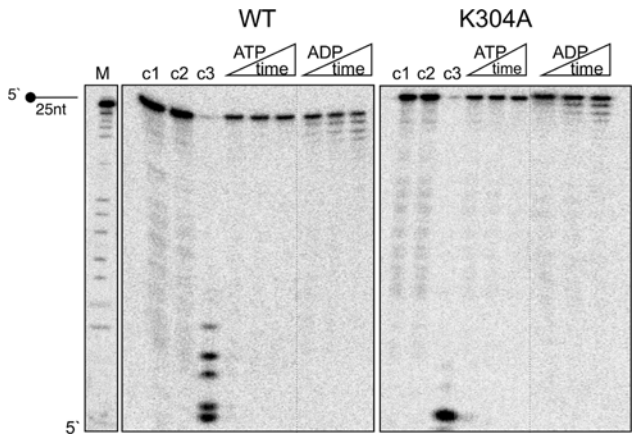

Figure S2 Comparison of the inhibitory effect of ATP and ADP on the WT and K304A mutant DinG proteins

Representative denaturing 20 % polyacrylamide/TBE gels showing the nuclease acitivty of 500 nM WT or K304A mutant DinG at 37 °C in the presence of substrate DNA25X, 5 mM $MgCl_2$ and ADP or ATP (for the WT enzyme, 1 mM; for K304A, 5 mM). Time points are 10, 30 and 60 min. M, Maxam–Gilbert A + G sequence ladder of the substrate; control c1, incubation of substrate in absence of protein for 60 min; control c2, reaction in the absence of $MgCl_2$; control c3, reaction in the presence of $MgCl_2$ and protein without any added ATP or ADP. Blank lanes separating the reactions with ADP from the other lanes have been removed for clarity.

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