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ORIGINAL PAPER

The carboxyl terminal of the archaeal nuclease NurA is involved in the interaction with single-stranded DNA-binding protein and dimer formation

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Abstract The nuclease NurA is present in all known thermophilic archaea and has been implicated to facilitate efficient DNA double-strand break end processing in Mre11/Rad50-mediated homologous recombinational repair. To understand the structural and functional relationship of this enzyme, we constructed five site-directed mutants of NurA from *Sulfolobus tokodaii* (StoNurA), D56A, E114A, D131A, Y291A, and H299A, at the conserved motifs, and four terminal deletion mutants, StoNurAΔN (19–331), StoNurAΔNΔC (19–303), StoNurAΔC (1–281), and StoNurAΔC (1–303), and characterized the proteins biochemically. We found that mutation at the acidic residue, D56, E114, D131, or at the basic residue, H299, abolishes the nuclease activity, while mutation at the aromatic residue Y291 only impairs the activity. Interestingly, by chemical cross-linking assay, we found that the mutant Y291A is unable to form stable dimer. Additionally, we demonstrated that deletion of the C-terminal amino acid residues 304–331 of StoNurA results in loss of the physical and functional interaction with the single-stranded DNA-binding protein (StoSSB). These results established that the C-terminal conserved aromatic residue Y291 is involved in dimer formation and the C-terminal residues 304–331 of NurA are involved in the interaction with single-stranded DNA-binding protein.

Keywords StoNurA · StoSSB · Homologous recombination · DNA repair · *Sulfolobus tokodaii*

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Introduction

The repair of DNA double-strand breaks (DSBs) in all organisms is crucial to maintain genomic integrity and viability. DSBs are frequently generated by either external agents, such as ionizing radiation and mechanical stress, or internal errors during replication and recombination. If left unrepaired or repaired inappropriately, DSBs can cause mutagenic events such as chromosome loss, deletion, duplication, and translocation (Hopfner et al. 2002; Khanna and Jackson 2001; Karran 2000). In bacteria, DSBs are repaired mainly by homologous recombination pathways RecBCD, RecQ-RecJ (RecFOR), AddAB, and AdnAB (Niu et al. 2009). In eukaryotic organisms, there are two major pathways in DSB repair: homologous recombination (Mre11/Rad50 complex) and non-homologous end joining (Ku70/K80 complex) (Kowalczykowski et al. 1994; Paques and Haber 1999). In archaea, no homolog of bacterial recombination repair pathways has been found; however, eukaryotic Mre11 and Rad50 homologs are present and conserved in all the genomes. Thus, Mre11/Rad50-mediated recombination repair was proposed to be important in repair of the DSBs in archaea including thermophilic archaea (Haber 2000; Hopfner et al. 2000).

In the genomes of all thermophilic archaea, Mre11 and Rad50 homologs are arranged in operon-like structures with two genes encoding a nuclease (NurA) and a DNA helicase (HerA). NurA is a 5′–3′ single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) exonuclease and ssDNA endonuclease, while HerA unwinds dsDNA in 5′ to 3′ and 3′ to 5′ directions (Constantinesco et al. 2002, 2004; Manzan et al. 2004). The *nurA*, *rad50*, *mre11*, and *herA* genes are co-transcribed in the crenarchaeon *Sulfolobus acidocaldarius* (Constantinesco et al. 2004). The analysis of transcriptional responses to UV induction revealed that the *nurA*, *rad50*, *mre11*, and *herA* genes were up-regulated 2–3-fold in some strains of *Sulfolobus solfataricus* (Frols et al. 2007) and about 10-fold in others (Rolfsmeier et al. 2010), suggesting that the four proteins are associated and involved in recombinational DNA repair. In fact, NurA was found to interact with HerA physically and functionally in vitro and the two proteins acted cooperatively with Mre11/Rad50 complex and the recombinase RadA to generate recombination products, the joint molecules in the euryarchaeon *Pyrococcus furiosus* (Hopkins and Paull 2008). Our previous study showed that Mre11 was able to stimulate the helicase activity of HerA from *S. tokodaii* through direct interaction (Zhang et al. 2008). Taking together, it is proposed that Mre11/Rad50 is involved in initial processing of DSB. HerA and NurA cooperate to cleave the 5′ strand extensively and efficiently, generating a long 3′ ssDNA overhang (Hopkins and Paull 2008). We have also found that NurA from *S. tokodaii* (StoNurA) physically interacts with SSB, one of the most important DNA metabolism proteins and the nuclease activity of StoNurA is inhibited by SSB (Wei et al. 2008). We proposed that the interaction between NurA and SSB plays a regulatory role in Mre11/Rad50-mediated DSB repair.

Although the structure of a homolog from *Thermotoga maritima* (Tm1739) is available (http://pfam.sanger.ac.uk/structure/1zup and http://pfam.sanger.ac.uk/family?acc=PF09376), no experimental data regarding the functional and structural relationship of NurA have been reported, and the mechanism about the interaction between NurA and SSB is unclear. In the present study, we have analyzed the biochemical properties of site-directed and terminal deletion mutant proteins of StoNurA. The residues D56, E114, D131, and H299 are identified as the essential residues of StoNurA for the nuclease activity. Y291 is found to be involved in the dimer formation. Furthermore, by pull-down and enzyme activity assay, we demonstrate that the C-terminal region of StoNurA is important for the physical and functional interaction with SSB. These results and the information obtained from structure modeling are discussed.

Materials and methods

Plasmids construction

The genes of the wild-type StoNurA and terminal deletion mutants StoNurAΔN (residues 19–331), StoNurAΔNΔC (residues 19–303), StoNurAΔC(residues 1–281), and StoNurAΔC (residues 1–303) were amplified by PCR from *S. tokodaii* genomic DNA using primers listed in Supplementary Table 1. The fragments were digested with *Nde*I and *Sal*I and cloned into the expression vector pET15b. Site-directed mutants D56A, E113A, D131A, Y291A, and H299A were constructed using the overlap PCR method with the common primers as for the wild-type StoNurA and two site-specific primers for each mutant (Supplementary Table 1). After digestion with *Nde*I and *Sal*I, the mutant fragments were inserted back into pET15b. The construction of the expression vectors for StoSSB has been described elsewhere (Wei et al. 2008).

Enzyme expression and purification

All constructs were transformed into host *E. coli* BL21-CodonPlus (DE3)-RIL. The deletion mutants and site-directed mutants were expressed and purified following the procedure for the wild-type StoNurA protein (Wei et al. 2008). The StoSSB protein was purified following the method of SSB from *S. solfataricus* (Wadsworth and White 2001). Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard.

Preparation of the substrates for DNA-binding and nuclease assays

The 34-mer ssDNA (5′-CAAGCTTGCATGCCTGCAG GTCGACTCTAGAGGA-3′) labeled at the 3′ end with FITC and a 34-bp dsDNA were used for the DNA-binding and 5′–3′ exonuclease assays. The dsDNA was prepared by annealing the 3′-FITC-34-mer ssDNA to the 34-mer complementary oligonucleotide (5′-TCCTCTAGAGTCGACC TGCAGGCATGCAAGCTTG-3′). Annealing was performed as follows: 20 pmol of labeled oligonucleotide was mixed with 40 pmol of unlabeled oligonucleotide in 10 μl of annealing buffer (7 mM Tris–HCl, pH 8.0, 50 mM NaCl, and 7 mM $MgCl_2$). The mixture was boiled for 10 min and cooled gradually to room temperature. The mixture was stored at 4°C until use.

Nuclease activity assay

The exonuclease assays of the StoNurA proteins were performed as described previously (Wei et al. 2008).

Various amounts of StoNurA, StoNurAΔN (19–331), StoNurAΔNΔC (19–303), or StoNurAΔC (1–303) were incubated as indicated with StoSSB and 2.0 pmol substrates in the 20 μl reaction mixtures at 65°C. The samples were loaded onto a 15% polyacrylamide gel containing 7 M urea and 1× TBE and electrophoresed for 2 h at 1000 V. After electrophoresis, the products were visualized and quantified using a FluorImager (GE Healthcare).

Cross-linking by formaldehyde

Cross-linking was carried out by incubating 25 μg of wild-type and site-directed mutants of StoNurA in PBS containing 1.5% formaldehyde at room temperature. The reaction was stopped by adding glycine at a final concentration of 0.15 M. The reaction products were separated by 8% SDS-PAGE and were detected by Coomassie Brilliant Blue staining.

Pull-down assay

The untagged StoSSB (500 μg) was pre-incubated with 50% Ni-NTA agarose bead slurry in binding buffer (50 mM Tris–HCl, pH 8.0, 100 mM NaCl, and 10% glycerol) in a volume of 500 μl at 4°C for 1 h, and the beads were recovered by centrifugation at 2000×*g* for 2 min. The supernatant was used for the pull-down assays. Approximately 50 μg of His_6-tagged StoNurA, StoNurAΔN (19–331), StoNurAΔNΔC (19–303), or StoNurAΔC (1–303) immobilized on 50 μl Ni-NTA agarose bead slurry was incubated with 50 μg of untagged StoSSB in 200 μl binding buffer. The beads were washed three times with 400 μl of washing buffer that contained 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 20 mM imidazole, and eluted three times with 100 μl of elution buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, and 300 mM imidazole). The eluate was resolved on 12% SDS-PAGE gels and stained with Coomassie Brilliant Blue.

Results

Construction of vectors and purification of site-directed mutants and deletion mutants of StoNurA

Sequence alignment of representative NurA proteins has revealed conserved regions and three motifs unique to NurA domain proteins (Fig. 1): motif I, DGS; motif II, EXnhhhhDGS; motif III, GYXnH (Constantinesco et al. 2002; Iyer et al. 2004). Although the conserved acidic and basic residues were predicted to participate in catalysis of the NurA domain proteins, no experimental data are available. To determine the functions of these motifs, we performed site-directed mutagenesis on NurA from *S. tokodaii* (StoNurA). Vectors to generate D56A, E113A, D131A, Y291A, and H299A mutants were constructed, and the resultant proteins were expressed in *E. coli*. The proteins were purified using the procedure for the wild-type protein as previously described (Wei et al. 2008), and highly purified proteins were obtained (Fig. S1).

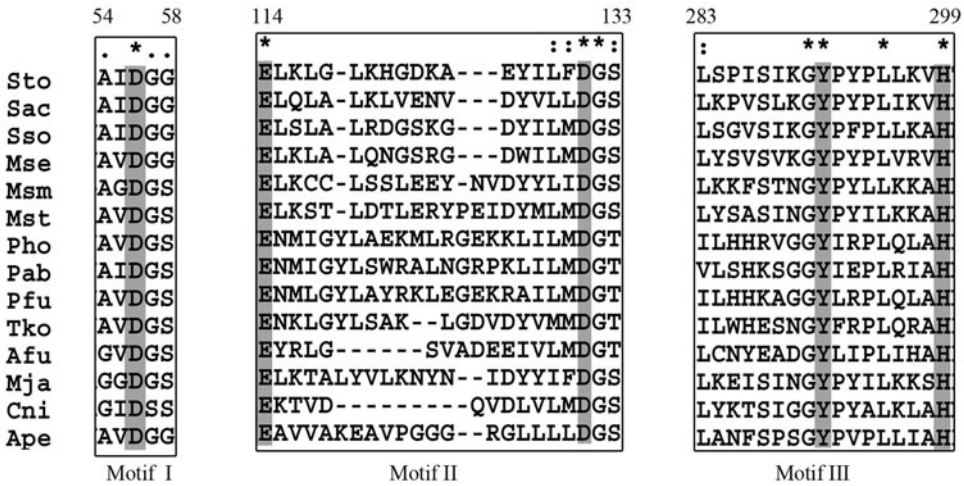

Fig. 1 Three conserved motifs of StoNurA and StoNurA homologs. An alignment was carried out using Clustal W program with manual adjusting and the regions containing the conserved motifs were shown. Numbering of the amino acids is indicated above the sequence. Conserved, conservative, and semi-conservative sites are indicated by *asterisks*, *double* and *single dots*, respectively. Gaps introduced during the alignment are indicated as *dashes*. The selected residues for site-directed mutagenesis with three conserved motifs are in *shadow*. *Sto*, *S. tokodaii*; *Sac*, *S. acidocaldarius*; *Sso*, *S. solfataricus*; *Mse*, *Metallosphaera sedula*; *Msm*, *Methanobrevibacter smithii*; *Mst*, *Methanosphaera stadtmanae*; *Pho*, *Pyrococcus horikoshii*; *Pab*, *P. abyssi*; *Pfu*, *P. furiosus*; *Tko*, *Thermococcus kodakarensis*; *Afu*, *Archaeoglobus fulgidus*; *Mja*, *Methanocaldococcus jannaschii*; *Cni*, *Candidatus nitrosopumilus*; *Ape*, *Aeropyrum pernix*

We also conducted limited proteolysis to identify functional domains of StoNurA, especially the domain interacting with SSB. StoNurA was efficiently degraded into a fragment with an estimated mass of approximately 30 kDa when incubated with trypsin for 30 min or longer (Fig. S2A). The N-terminal cleavage sites of the 30-kDa proteolytic fragment were determined at Gln19 by sequential Edman degradation analysis. Cleavage at the C-terminal end was estimated to be at Lys303 based on trypsin cleavage sites and the size of the stable trypsin-digested fragment. Based on the results from limited proteolysis of StoNurA, three deletion mutants were designed: StoNurAΔN (19–331), StoNurAΔNΔC (19–303), and StoNurAΔC (1–303). Schematic representation of StoNurA and the deletion mutants is shown in Fig. S2B. In a previous report, NurA was predicted to contain at least five conserved α-helices, the last three of which form a triple helical unit (Iyer et al. 2004). To probe the function of the putative triple helical unit of NurA, we also constructed a deletion mutant StoNurAΔC (1–281) that removed the last three α-helices. StoNurAΔN (19–331), StoNurAΔNΔC (19–303), and StoNurAΔC (1–303) were successfully over-expressed in soluble form in *E. coli* (Fig. S2C), but StoNurAΔC (1–281) was expressed solely in insoluble form (data not shown), suggesting that the region containing residues 281–303 is important for proper folding, solubility, or stability of StoNurA.

Nuclease activity of the site-directed mutants of StoNurA

The nuclease activity of the site-directed mutants was examined using ssDNA as a substrate. As shown in Fig. 2, mutants D56A, E114A, D131A, and H299A completely lost the nuclease activity, while Y291A retained only approximately 20% of wild-type StoNurA activity. This indicates that D56, E114, D131, and H299 are critical residues in the catalytic site for nuclease activity of StoNurA, although the possibility that conformation alteration due to mutation leads to the loss of activity cannot be ruled out. The result also suggests that the conserved tyrosine is not essential for catalysis.

Dimer formation by the wild-type and the mutants of StoNurA

It has been shown that NurA from *Pyrococcus furiosus* forms a dimer in solution by gel filtration assay (Hopkins and Paull 2008). We found that StoNurA is also able to form a dimer by gel filtration analysis (data not shown). To confirm and reveal the mechanism of the dimer formation of StoNurA, we performed chemical cross-linking analysis. Wild-type StoNurA and mutant proteins were treated with

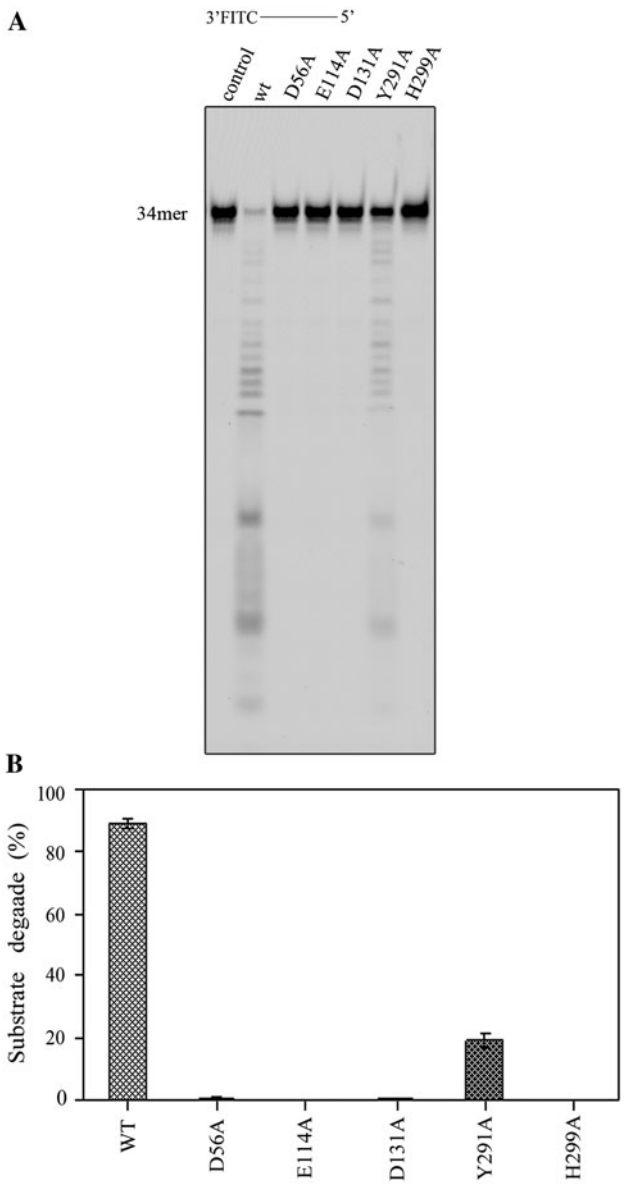

Fig. 2 Comparison of the nuclease activities of wild-type and site-directed mutant StoNurA proteins. The reaction mixtures (20 μl) contained 2 pmol ssDNA and 2.0 pmol of each protein. The products were resolved on 15% polyacrylamide gels containing 7 M urea and visualized using a FluoImager 585. **a** Gel profile of the exonuclease assay samples. **b** Quantification of results in (**a**). Each value was calculated based on the results of three independent reactions

formaldehyde and the samples were subject to SDS-PAGE analysis. Bands corresponding to 76 kDa, in addition to the 38 kDa, were detected with the wild-type, D56A, E114A, D131A, and H299A, mutant proteins (Fig. 3). This result indicates that the proteins have been covalently linked by formaldehyde, thus generating the dimeric 76 kDa band on the SDS-PAGE. Interestingly, the Y291A mutant was only detected at the monomer position of 38 kDa, even though the cross-linking reaction time was extended to 60 min,

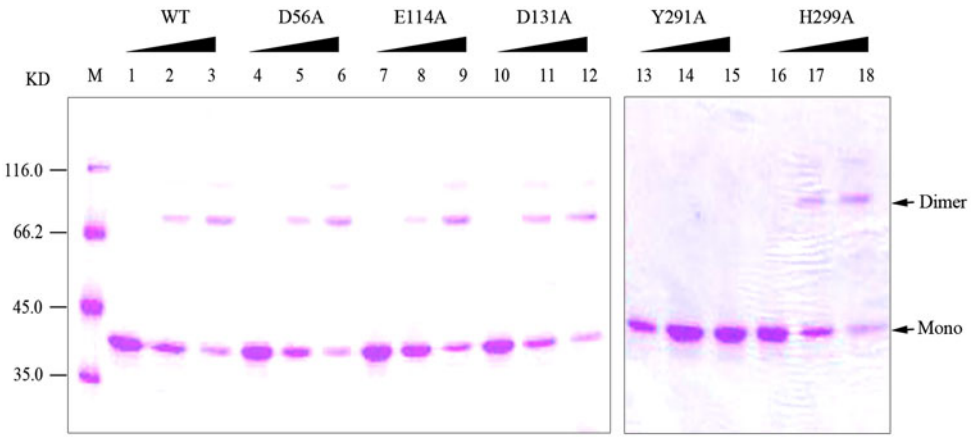

Fig. 3 Chemical cross-linking of StoNurA and the site-directed mutants. Wild-type and site-directed mutants of StoNurA proteins were cross-linked using formaldehyde for 0 min (*lanes 1*, *4*, *7*, *10*, *13*, and *16*), 30 min (*lanes 2*, *5*, *8*, *11*, *14*, and *17*), and 60 min (*lanes 3*, *6*, *9*, *12*, *15*, and *18*). Proteins were separated on 8% SDS-PAGE and stained with Coomassie Brilliant Blue

suggesting that mutation at Y291 has affected the dimer-forming ability of StoNurA. StoNurAΔN (19–331), StoNurAΔNΔC (19–303), or StoNurAΔC (1–303) mutant protein can form dimers in solution as the wild-type StoNurA (data not shown).

StoSSB inhibited the exonuclease activity of StoNurA via the C-terminus of StoNurA

Our previous report demonstrated that StoNurA physically interacts with StoSSB and that StoSSB inhibits the exonuclease and endonuclease activities of StoNurA (Wei et al. 2008). To investigate the mechanism of this inhibition, we analyzed the effect of StoSSB on the exonuclease activity of the StoNurA deletion mutants. As shown in Fig. 4a, the nuclease activity of StoNurAΔNΔC (19–303) and StoNurAΔC (1–303) was not inhibited by StoSSB. In contrast, the activity of StoNurAΔN (19–331) was inhibited by StoSSB, similar to that of the wild-type StoNurA. These results indicate that the C-terminal region of StoNurA is needed for the interaction with StoSSB and suggest that StoSSB inhibits the exonuclease activity of StoNurA via the C-terminus of StoNurA.

To understand if there is a direct interaction between StoSSB and StoNurA deletion mutants, we preformed His-tagged pull-down assay. Untagged StoSSB was pre-incubated with Ni-NTA agarose to eliminate possible non-specific binding. Purified His-tagged wild-type StoNurA and deletion mutants were incubated with the untagged StoSSB and Ni-NTA agarose beads. As shown in Fig. 4b, untagged StoSSB was clearly detected in the eluate with the wild-type StoNurA and StoNurAΔN (19–331). In contrast, StoNurAΔNΔC (19–303) and StoNurAΔC (1–303) were unable to bind to StoSSB. These results indicate that the C-terminal region of StoNurA is necessary for its physical interaction with StoSSB.

Discussion

NurA is implicated to play an important role in DNA metabolism pathways in archaea. The amino acid sequence and the predicted secondary structures of NurA are different from any of the known nuclease in the biological domains (Iyer et al. 2004). The crystal structure of archaeal NurA has not been solved, although the structure of a homolog from *Thermotoga maritima* (Tm1739) is available (http://pfam.sanger.ac.uk/structure/1zup). The present mutation analysis of StoNurA has provided experimental evidences regarding the structure–function relationship of NurA. We have demonstrated that D56, E114, D131, and H299 are the putative catalytic residues for the nuclease activity by site-directed mutagenesis. The nucleases that require divalent cations for catalysis usually have three or four acidic residues within a few conserved motifs (Nishino and Morikawa 2002). The crystallographic study of the homolog from *Thermotoga maritima* (Tm1739) predicts that the residues Asp49, Glu140, Asp157, and Glu280 of Tm1739 are essential for the nuclease activity. Our finding that replacement of the corresponding residues D56, E114, D131, or H299 within the three conserved motifs with alanine (Fig. 1) abolishes the nuclease activity of StoNurA has verified the structural prediction.

We found that the hydrophobic residue Y291 of StoNurA is critical for dimer formation of the enzyme. Mutant Y291A cannot form a stable dimer. The crystal structures of Tm1739 (http://pfam.sanger.ac.uk/structure/1zup) revealed that the dimer formation occurs by inter-subunit interactions, mainly between the N-terminal (beta strand and loop) and C-terminal (helix). We generated a hypothetical structure of StoNurA using the structure of a homolog from *Thermotoga maritima* (Tm1739) as the template by Swiss-PDB-viewer program (data not shown). The secondary structure prediction and manual alignment between the StoNurA and Tm1739 is shown in Fig. 5. StoNurA has

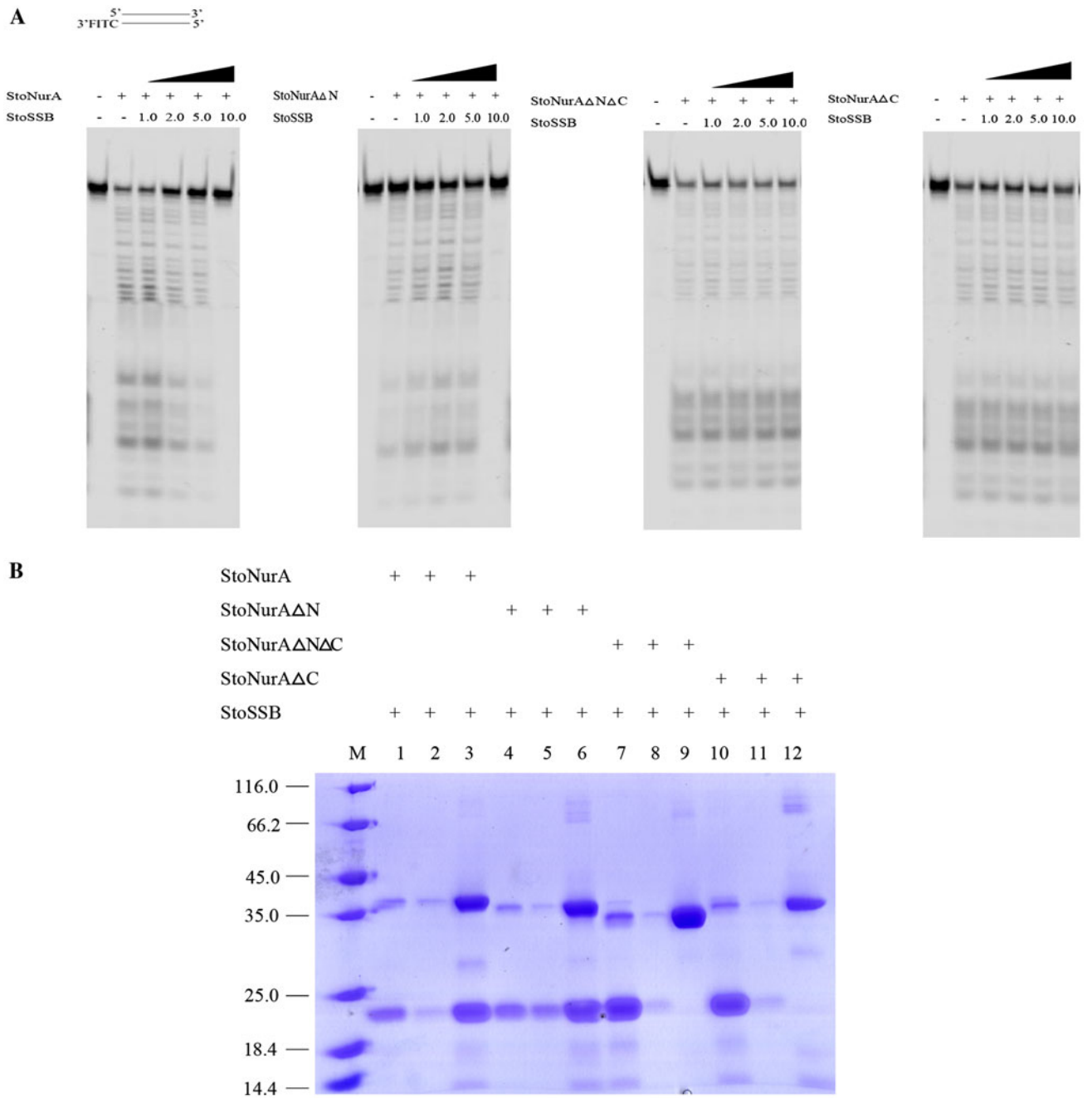

Fig. 4 Physical and functional interactions between recombinant StoSSB and the wild-type StoNurA or its deletion mutants. **a** StoSSB inhibited the exonuclease activities of StoNurA via the C-terminus of StoNurA. Reaction mixtures (20 µl) containing 2 pmol dsDNA, 2 pmol StoNurA or its truncated forms, and increasing amounts (1.0, 2.0, 5.0, and 10.0 pmol) of StoSSB were incubated at 65°C for 30 min. **b** In vitro pull-down assay of untagged recombinant StoSSB and His-tagged wild-type StoNurA and the deletion mutants. *Lanes 1*, *4*, *7*, and *10* represent flow-through when StoNurA and the deletion mutants were immobilized on the beads. *Lanes 2*, *5*, *8*, and *11* represent elution with washing buffer containing 20 mM imidazole. *Lanes 3*, *6*, *9*, and *12* represent elution with buffer containing 300 mM imidazole

at least five α-helices, with the last three (helices 3, 4, 5) at the C-terminal (Fig. 5). The overall predicted structure and topology of StoNurA resembles folding pattern of Tm1739. The hydrophobic residue Y291 is located in the helix-5 of the predicted secondary structure of StoNurA (Fig. 5), and the predicted helix-5 may be involved in the interactions between subunits. The adjacent residue P292 may also be involved in dimer formation of StoNurA. StoNurAΔN (19–331), StoNurAΔNΔC (19–303), or StoNurAΔC (1–303) mutant protein could form dimer in solution as the

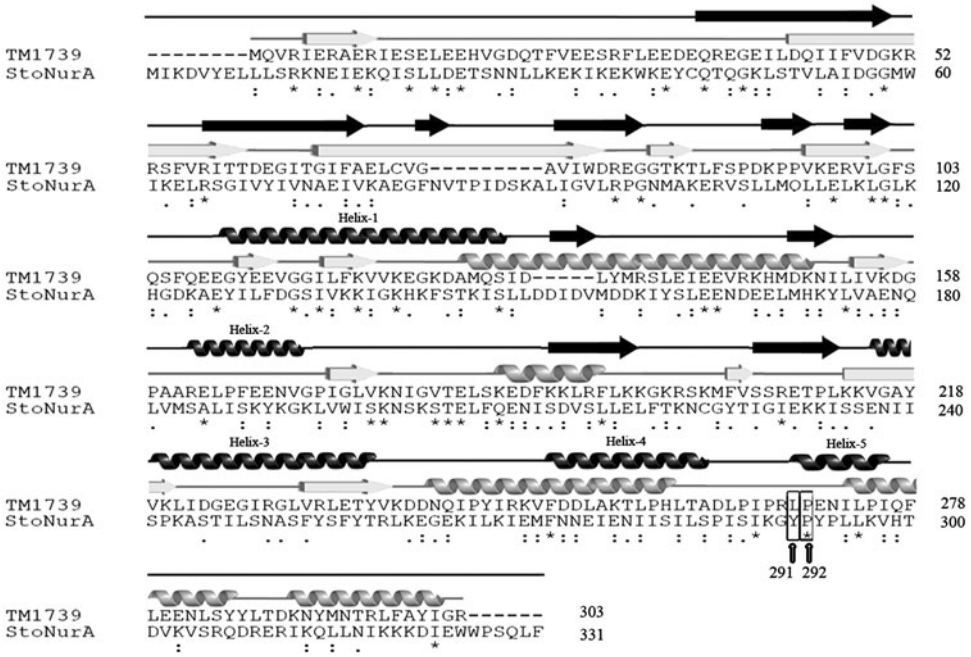

Fig. 5 Sequence alignment and comparison of the secondary structures of the StoNurA and Tm1739 from *Thermotoga maritima*. Residues Y291 and P292 that were suggested to be involved in dimer formation of StoNurA are *boxed*. The predicted α-helices and β-strands of StoNurA are indicated in *solid*, whereas the actual α-helices and β-strands of Tm1739 are indicated in *gray*

wild-type StoNurA (data not shown), indicating that removal of 18 N-terminal (loop) and 28 C-terminal (loop) residues does not impair the dimer-forming ability of StoNurA. The role of the N-terminal residues 1–18 of StoNurA remains to be investigated. By mutation analysis, we also identified residues 19–303 of StoNurA as the core domain for the enzymatic activity of StoNurA (Figs. S3, S4).

We found that the C-terminus of StoNurA is essential for the physical interaction between StoNurA and StoSSB, and for StoSSB-mediated inhibition of StoNurA nuclease activity. It has been demonstrated that helicases and nucleases play central roles in dsDNA break repair (Hopkins and Paull 2008; Mimitou and Symington 2008, 2009). Based on previous results (Wei et al. 2008), we speculated that the exonuclease NurA may function in 5′–3′ nucleolytic degradation to generate ssDNA during archaeal recombinational repair. In the hyperthermophilic euryarchaeon *P. furiosus*, NurA and HerA functionally interact with the Mre11/Rad50 complex in the initial processing of DSB ends during recombinational repair (Hopkins and Paull 2008). The Mre11/Rad50 complex first binds to DSB ends and removes 15–55 nucleotides from the 5′ end, and then the NurA/HerA complex catalyzes 5′-strand resection to generate appropriate 3′-overhangs for subsequent loading of the recombinase RadA. Our findings indicate that during the process of DNA end resection, SSB may function to modulate the cleavage activity of NurA/HerA through direct interaction between NurA and SSB via the C-terminal of StoNurA. The results presented in this study may be informative in elucidating detailed mechanism of Mre11/Rad50-mediated DSB repair not only in archaea but also in eukarya.

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