Mutational Analysis of Conserved Hydrophobic Amino Acid Residues in the N-Terminal Region of DnaA Protein

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DnaA is the initiator of chromosomal DNA replication in *E. coli*. We previously reported that conserved hydrophobic amino acid residues in the N-terminal region of DnaA (I26 and L40) are essential for DNA replication *in vivo* and *in vitro* using mutant DnaA proteins (DnaAI26S and DnaAL40S). In this study, we introduced further random mutations to find intragenic suppressors for *dnaAI26S* or *dnaAL40S*. By direct DNA sequence, a mutation which causes substitution of the Ser (Ile, in the wild-type DnaA) with Phe (DnaAI26F or DnaAL40F) was found in all of the suppressors. Site-directed mutational analysis showed that DnaAI26L, and DnaAL40I, but not DnaAI26S or DnaAL40S, were active for *oriC* DNA replication in cells. Furthermore, purified DnaAI26F but not DnaAI26S was active for *oriC* DNA replication in a crude extract. These results strongly suggest that hydrophobic amino acid residues in these positions of DnaA (I26 and L40) are important for the function of this protein as an initiator of DNA replication both *in vivo* and *in vitro*.

Key words DnaA; N-terminal region; DNA replication; hydrophobic amino acid residue

DnaA is the initiator of chromosomal DNA replication in *E. coli*.¹⁾ DnaA has multiple activities, such as *oriC*-specific DNA-binding, sequence-independent DNA-binding, oligomerization (DnaA–DnaA interaction), DnaB-binding, adenine-nucleotide-binding, acidic phospholipid-binding, and ATPase.¹⁾ Therefore, determination of the functional domain (identification of essential amino acid residues) for each activity is important in order to fully understand the function and structure of DnaA. Examining the function of DnaA with mutations in the essential amino acid residue for each activity is an effective way for revealing the role of each activity in DNA replication in cells.²⁾ We identified amino acid residues that are essential for ATP-binding, ATPase activity, sequence-independent DNA-binding, and acidic phospholipid-binding by site-directed mutational analysis, and examined the functions of these mutant DnaA proteins *in vivo* and *in vitro*.^{2–10)} Functional domains for membrane-binding, *oriC*-specific DNA-binding, and DnaA–DnaB interaction have been reported by other groups.^{11–14)}

Previously, we examined the role of the N-terminal region of DnaA in chromosomal DNA replication by use of site-directed mutational analysis. In the N-terminal region of DnaA, we found conserved hydrophobic amino acid residues (I26, L33, and L40) which may form a leucine-zipper structure.¹⁵⁾ We showed that their mutant DnaA proteins (DnaAI26S and DnaAL40S) were active for *oriC*-binding, but not for the duplex opening at *oriC*, suggesting that these hydrophobic amino acid residues are involved in DnaA–DnaA interaction (DnaA oligomerization).¹⁵⁾ Weigel *et al.* supported this notion by directly examining the DnaA–DnaA interaction of N-terminal and C-terminal deleted DnaA.¹⁶⁾ In order to examine the function of these conserved hydrophobic amino acid residues, we here performed intragenic suppressor analysis for the *dnaAI26S* or *dnaAL40S* and found that a mutation which causes substitution of the Ser with Phe (DnaAI26F or DnaAL40F) was found in all of the suppressors. These data showed the importance of hydrophobic amino acid residues in the N-terminal region of DnaA, thus

the importance of the leucine-zipper structure of DnaA for the initiation of DNA replication *in vivo* and *in vitro*.

MATERIALS AND METHODS

Materials [α -³²P]dCTP (6000 Ci/mmol) was from Amersham Pharmacia Biotech. Wild-type DnaA and DnaAI26F were purified as previously described.¹⁷⁾ DnaAI26S was purified as previously described.¹⁵⁾ A crude extract for an *oriC* complementation assay was prepared from the WM433 strain of *E. coli*, as previously described.¹⁸⁾

Bacterial Strains Five strains of *E. coli* from our laboratory stocks were used in this study. They were JM109 (*recA1*, *endA1*, *gyrA96*, *thi*, *hsdR17*, *supE44*, *relA1*, Δ (*lac-proAB*)/F' [*traD36*, *proAB*⁺, *lacIq*, *lacZ*ΔM15]), KS1001 (W3110 *lacZ*), KS1003 (KS1001, *dnaA46*),¹⁹⁾ KA450 (Δ *oriC1071::Tn10*, *rnhA199*(Am), *dnaA17*(Am), *trpE9828*(Am), *tyrA*(Am), *thr*, *ilv*, and *thyA*),²⁰⁾ and WM433 (*dnaA204*, *leu19*, *pro19*, *trp25*, *his47*, *thyA59*, *arg28*, *met55*, *deoB23*, *lac11*, *strA56*, *sull1*, *hsdSK12*).

Site-Directed Mutagenesis and Plasmid Constructions Site-specific mutagenesis was performed as described previously.⁴⁾ Briefly, uracil-containing single-stranded DNA of the M13 phage, containing the coding region of the *dnaA* gene, was hybridized with oligonucleotide primers representing each mutation. The complementary DNA strand was synthesized *in vitro* and the resultant double-stranded DNA was introduced into *E. coli* JM109. Direct DNA sequencing confirmed the mutation, and double-stranded DNA containing the mutation was prepared.

To construct plasmids for the over-expression of DnaA, the *EcoRI*–*HindIII* region of each *dnaA* gene was ligated into pMZ001,⁴⁾ which contains an arabinose promoter.

Plasmids for complementation analysis were constructed by ligating the coding regions of the mutant *dnaA* gene (*Bam*HI–*Hind*III fragment) into pMZ002,⁴⁾ which contains the wild-type promoter of the *dnaA* gene.

Screening for Intragenic Suppressors for *dnaAI26S* or

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***dnaAL40S* by Random Mutagenesis** JM109 with pMZ002 carrying *dnaAI26S* or *dnaAL40S* were treated with 15 μ g/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) for 20 min. After incubation without MNNG, plasmids were isolated and then transformed into a temperature-sensitive *dnaA46* strain. Plasmids were isolated from several temperature-resistant colonies, and DNA sequences of the *dnaA* gene were determined.

***oriC* DNA Replication in a Crude Extract** Replication of minichromosomes in a crude extract (Fraction II) was assayed, as previously described.¹⁸⁾ Template DNA (M13E10) (200 ng, 600 pmol of nucleotides), 200 μ g of Fraction II from WM433 (*dnaA204*), and DnaA were mixed with reaction cocktails¹⁸⁾ and incubated for replication at 30 °C for 20 min. The reaction was terminated by chilling on ice and adding 10% TCA. Samples were then passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filter was measured in a liquid scintillation counter and the amount of DNA synthesized (pmol nucleotides) was calculated, as previously described.¹⁸⁾

RESULTS AND DISCUSSION

DnaAI26S and DnaAL40S were inactive for the *oriC* DNA replication *in vivo* and *in vitro*.¹⁵⁾ Therefore, neither *dnaAI26S* nor *dnaAL40S* could complement the temperature-sensitivity of a *dnaA46* mutant; in other words, *dnaA46* cells with plasmid containing *dnaAI26S* or *dnaAL40S* still showed a temperature-sensitive growth phenotype.¹⁵⁾ In order to obtain intragenic suppressors for the *dnaAI26S* or *dnaAL40S*, we performed two-step experiments. At first, plasmids containing *dnaAI26S* or *dnaAL40S* were randomly mutagenised by MNNG in JM109 cells, then introduced into *dnaA46* to select temperature-resistant colonies. Direct DNA sequence analysis of the *dnaA* gene obtained from colonies showed that all of the suppressors contained further substitution of the Ser (originally I26 or L40 in the wild-type DnaA) with Phe (Table 1).

Results in Table 1 suggest the importance of hydrophobic amino acid residues in these positions (I26 or L40 in the wild-type DnaA). This idea is supported by the fact that these amino acid residues are well conserved among various species of DnaA as hydrophobic amino acid residues (Fig. 1). For further confirmation of this idea, we constructed several mutant *dnaA* genes using site-directed mutation (Fig. 1). To determine whether these mutant *dnaA* genes could function *in vivo*, we conducted plasmid complementation experiments using a *dnaA46* mutant. The coding region of the mutant or the wild-type *dnaA* genes was conjugated with a wild-type *dnaA* promoter on pMZ002.⁴⁾ Each resultant plasmid was introduced into a temperature-sensitive *dnaA46* mutant. As shown in Table 2, the ratio of transformation efficiency of *dnaAI26S* and *dnaAL40S* at 42 °C to that at 30 °C was less than 10^{-5} , whereas that of *dnaAI26F*, *dnaAI26L*, *dnaAL40F*, *dnaAL40I*, and the wild-type *dnaA* was around 1.0. These results confirmed that the I26F mutation is responsible for the temperature-resistant phenotype of clones obtained in the experiments of Table 1. Since leucine (L), isoleucine (I), and phenylalanine (F) are hydrophobic amino acids, and serine (S) is hydrophilic, the results in Table 2 strongly suggest the importance of hydrophobic amino acid residues in these po-

Table 1. Suppressor Mutation in the *dnaA* Gene from *dnaAI26S* or *dnaAL40S* Mutant

Mutant genes	DNA mutation	Amino acid mutation	Number of clones
<i>dnaAI26S</i>	C77T	S26F	30
<i>dnaAL40S</i>	C119T	S40F	34

JM109 cells harboring either *dnaAI26S* or *dnaAL40S* were treated with MNNG. Plasmids were isolated and transformed into a temperature-sensitive *dnaA46* strain. Plasmids were isolated from more than 30 temperature-resistant colonies, and DNA sequences of the *dnaA* gene were determined.

Fig. 1. Amino Acid Sequence and Strategy for Site-Directed Mutations in the N-Terminal Region of DnaA

Amino acid sequences of the N-terminal regions of DnaA (from W25 to W50) are shown. The "*" indicates an amino acid residue that is identical to that found in *E. coli*. These data are from Ref. 1. eco, *E. coli*; sty, *S. typhimurium*; sma, *S. marcescens*; pmi, *P. mirabilis*; bap, *B. aphidicola*; ppu, *P. putida*; sco, *S. coelicolor*; mlu, *M. luteus*; bsu, *B. subtilis*; bbu, *B. burgdorferi*. The conversion of amino acid residues in the mutant DnaA proteins is shown. We introduced indicated amino acid substitutions in the position of I26 or L40 in the wild-type DnaA.

Table 2. Complementation Analysis of Temperature-Sensitivity of a *dnaA46* Mutant with Plasmids Carrying the Mutant *dnaA* Genes

Clones	Colony formation efficiencies (42 °C/30 °C)
pBR322(vector)	1.6×10^{-6}
pMZ002/ <i>dnaA</i> ⁺	1.3
pMZ002/ <i>dnaAI26S</i>	1.1×10^{-5}
pMZ002/ <i>dnaAI26L</i>	1.3
pMZ002/ <i>dnaAI26F</i>	1.0
pMZ002/ <i>dnaAL40S</i>	8.4×10^{-6}
pMZ002/ <i>dnaAL40I</i>	1.2
pMZ002/ <i>dnaAL40F</i>	1.1

KS1003 (*dnaA46*) cells were transformed with pMZ002 with each *dnaA* gene. Cultures were diluted appropriately and spread on LB agar plates containing 100 μ g/ml ampicillin. Plates were incubated at 30 °C or 42 °C, the numbers of colonies were counted, and the ratio of transformation efficiencies (42 °C/30 °C) was determined.

sitions (I26 or L40 in the wild-type DnaA) for the *oriC* DNA replication *in vivo*.

For biochemical analysis, we purified DnaAI26S, DnaAI26F, and wild-type DnaA from over-producing cells and examined their activities for *oriC* DNA replication *in*

Fig. 2. Replication Activities of Mutant DnaA Proteins in a Crude Extract

DNA replication activities of DnaAI26S, DnaAI26F, and wild-type DnaA were measured using the *oriC* DNA replication system of a crude extract. DNA replication was expressed as the amount of DNA synthesized (pmol, nucleotides) at 30 °C in 20 min.

vitro using an *oriC* complementation assay.¹⁸⁾ We also tried to purify DnaAL40F, but due to its poor solubility, we could not obtain purified DnaAL40F. As shown in Fig. 2, DnaAI26F was active for *oriC* DNA replication as much as the wild-type protein in a crude extract. DnaAI26S was inactive for the DNA replication, as described previously.¹⁵⁾ Based on all these observations, we concluded that DnaAI26F is active for the *oriC* DNA replication both *in vivo* and *in vitro*.

Using random mutagenesis and site-directed mutagenesis, we here showed the importance of hydrophobic amino acid residues in the N-terminal region of DnaA (I26 and L40) for the *oriC* DNA replication *in vivo* and *in vitro*. These amino acid residues may form a leucine-zipper structure in which hydrophobic amino acid residues are important. Previously, we showed that these amino acid residues are essential for DnaA–DnaA interaction, which is an essential step for *oriC* DNA replication.¹⁵⁾ Based on all these observations, we pro-

pose that the N-terminal region of DnaA is involved in DnaA–DnaA interaction using a leucine-zipper structure.

REFERENCES

- 1) Skarstad K., Boye E., *Biochim. Biophys. Acta*, **1217**, 111–130 (1994).
- 2) Mizushima T., *J. Biochem. (Tokyo)*, **127**, 1–7 (2000).
- 3) Hase M., Yoshimi T., Ishikawa Y., Ohba A., Guo L., Mima S., Makise M., Yamaguchi Y., Tsuchiya T., Mizushima T., *J. Biol. Chem.*, **273**, 28651–28656 (1998).
- 4) Mizushima T., Nishida S., Kurokawa K., Katayama T., Miki T., Sekimizu K., *EMBO J.*, **16**, 3724–3730 (1997).
- 5) Mizushima T., Takaki T., Kubota T., Tsuchiya T., Miki T., Katayama T., Sekimizu K., *J. Biol. Chem.*, **273**, 20847–20851 (1998).
- 6) Yamaguchi Y., Hase M., Makise M., Mima S., Yoshimi T., Ishikawa Y., Tsuchiya T., Mizushima T., *Biochem. J.*, **340**, 433–438 (1999).
- 7) Makise M., Mima S., Tsuchiya T., Mizushima T., *J. Biol. Chem.*, **275**, 4513–4518 (2000).
- 8) Makise M., Mima S., Tsuchiya T., Mizushima T., *J. Biol. Chem.*, **276**, 7450–7456 (2001).
- 9) Makise M., Mima S., Koterasawa M., Tsuchiya T., Mizushima T., *Biochem. J.*, **362**, 453–458 (2002).
- 10) Makise M., Tsuchiya T., Mizushima T., *J. Biochem.*, **131**, 419–425 (2002).
- 11) Roth A., Messer W., *EMBO J.*, **14**, 2106–2111 (1995).
- 12) Marszalek J., Zhang W., Hupp T. R., Margulies C., Carr K. M., Cherry S., Kaguni J. M., *J. Biol. Chem.*, **271**, 18535–18542 (1996).
- 13) Garner J., Crooke E., *EMBO J.*, **15**, 3477–3485 (1996).
- 14) Sutton M. D., Carr K. M., Vicente M., Kaguni J. M., *J. Biol. Chem.*, **273**, 34255–34262 (1998).
- 15) Mima S., Yamaguchi Y., Kondo T., Tsuchiya T., Mizushima T., *FEMS Microbiol. Lett.*, **176**, 163–167 (1999).
- 16) Weigel C., Schmidt A., Seitz H., Tungler D., Welzeck M., Messer W., *Mol. Microbiol.*, **34**, 53–66 (1999).
- 17) Kubota T., Katayama T., Ito Y., Mizushima T., Sekimizu K., *Biochem. Biophys. Res. Commun.*, **232**, 130–135 (1997).
- 18) Fuller R. S., Kaguni J. M., Kornberg A., *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 7370–7374 (1981).
- 19) Mizushima T., Yokoyama K., Mima S., Tsuchiya T., Sekimizu K., *J. Biol. Chem.*, **272**, 21195–21200 (1997).
- 20) Katayama T., *J. Biol. Chem.*, **269**, 22075–22079 (1994).