Functional analysis of CedA based on its structure: residues important in binding of DNA and RNA polymerase and in the cell division regulation

Received May 13, 2015; accepted August 17, 2015; published online September 23, 2015

Yoshito Abe^{1,*}, Naoki Fujisaki¹,
Takanori Miyoshi¹, Noriko Watanabe¹,
Tsutomu Katayama² and Tadashi Ueda^{1,†}

¹Department of Protein Structure, Function and Design and

²Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

*Yoshito Abe, Department of Protein Structure, Function and Design, Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81-92-642-6663, Fax: +81-92-642-6667, email: abe@phar.kyushu-u.ac.jp

†Tadashi Ueda, Department of Protein Structure, Function and Design, Graduate School of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka 812-8582, Japan. Tel: +81-92-642-6662, Fax: +81-92-642-6667, email: ueda@phar.kyushu-u.ac.jp

DnaAcos, a mutant of the initiator DnaA, causes over-initiation of chromosome replication in *Escherichia coli*, resulting in inhibition of cell division. CedA was found to be a multi-copy suppressor which represses the *dnaAcos* inhibition of cell division. However, functional mechanism of CedA remains elusive except for previously indicated possibilities in binding to DNA and RNA polymerase. In this study, we searched for the specific sites of CedA in binding of DNA and RNA polymerase and in repression of cell division inhibition. First, DNA sequence to which CedA preferentially binds was determined. Next, the several residues and $\beta 4$ region in CedA C-terminal domain was suggested to specifically interact with the DNA. Moreover, we found that the flexible N-terminal region was required for tight binding to longer DNA as well as interaction with RNA polymerase. Based on these results, several *cedA* mutants were examined in ability for repressing *dnaAcos* cell division inhibition. We found that the N-terminal region was dispensable and that Glu32 in the C-terminal domain was required for the repression. These results suggest that CedA has multiple roles and residues with different functions are positioned in the two regions.

Keywords: CedA/cell division/DNA binding/RNA polymerase.

Abbreviations: HSQC, homonuclear single-quantum coherence; LB, Lysogeny broth; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecylsulphate-polyacrylamide gel electrophoresis; TPCK, L-(tosylamino 2-phenyl) ethyl chloromethyl ketone.

Replication of chromosomal DNA, segregation of the replicated DNA and cell division are known to be repeated in a regular and periodic manner (1, 2). In *Escherichia coli*, DnaA protein is one of the main factors that control the initiation of chromosomal DNA replication (3, 4). Several DnaA mutants are known to cause defects in the regulation of replication initiation and cell division. Cold-sensitive *dnaAcos* mutant cells, which contain a DnaA protein with four amino acid substitutions (Q156L, A184V, H252Y and Y271H), undergo excessive initiations of replication and chromosomal over-replication at 30°C, resulting in the inhibitions of colony formation and cell division (5–7). Similarly, the presence of a multi-copy vector carrying the A184V DnaA mutant, where A184 near the ATP-binding site is substituted for valine, results in a cold-sensitive phenotype (8, 9). To elucidate the mechanism for the inhibition of cell division, Katayama *et al.* (10) isolated multi-copy suppressor genes for the *dnaAcos* mutant. The introduction of a pBR322 derivative bearing one of the suppressor genes, *cedA*, into *dnaAcos* cells did not lead to repression of the over-replication of chromosomes; however, it induced cell division in the host, resulting in the growth of cells with an abnormally elevated chromosomal copy number (10). Therefore, the level of expression of the *cedA* gene appears to be important for inhibiting cell division in *dnaAcos* mutants at 30°C.

CedA is a basic protein with a molecular weight of 10,200 (87 amino acids). To understand the molecular mechanism of cell division reactivation by CedA, the solution structure was determined by nuclear magnetic resonance (NMR) (11). The NMR-determined structure of the C-terminal domain (residues 18–87) of CedA revealed a structure similar to that of DNA-binding proteins. The results of this NMR study also suggested that CedA binds to DNA with a weak affinity (around 100 μ M) via a β -sheet structure. In addition, proteomics research has suggested that CedA interacts with RNA polymerase (RNAP) (12). In this study, we further examined the interaction of CedA with DNA and RNAP. Furthermore, to examine whether cell division reactivation is involved in the interaction between CedA and DNA or RNAP, we introduced several *cedA* mutant genes into *dnaAcos* cells and examined their phenotypes.

Materials and Methods

Protein preparation of *CedA* and mutants

CedA corresponding to amino acid residues 1–87 (full length), 11–87 ($\Delta 10$) or 19–87 ($\Delta 18$) with a His₆-tag was amplified by polymerase chain reaction (PCR). Several mutants were prepared by the mega-primer method. The amplified DNA fragment was ligated into the vector pET-22b(+) (Novagen, Darmstadt, Germany). Each sequence was confirmed by DNA sequencing. The cells were cultured in Lysogeny broth (LB) medium or M9 medium for NMR analysis. The cultured cells were harvested and disrupted by sonication in 50 mM 3-morpholinopropanesulfonic acid buffer (pH 7.0). The supernatants were added to a 1.0 cm i. d. $\times$ 4.0 cm column of Ni²⁺-nitrilotriacetic acid (NTA) agarose (QIAGEN, Hilden, Germany) equilibrated with 50 mM phosphate buffer (pH 7.0) containing 250 mM KCl, and they were eluted in a stepwise manner with 50 mM phosphate buffer (pH 7.0) containing 250 mM KCl and 100 mM imidazole. The protein fractions were collected, and the proteins were precipitated by salting out using 80% ammonium sulphate. The precipitant was re-dissolved in 50 mM phosphate buffer (pH 7.0). The protein solution was purified using a 2.0 cm i. d. $\times$ 30 cm column of TOYOPEARL CM-650 M (TOSOH Co., Tokyo, Japan) equilibrated with 50 mM phosphate buffer (pH 7.0). The protein elution was carried out by buffer exchange from 50 mM phosphate buffer (pH 7.0) to 50 mM acetate buffer (pH 5.0). After the exchange to 50 mM acetate buffer (pH 5.0), the protein solution was eluted with a linear gradient from 0 to 1 M NaCl in 200 ml of 50 mM acetate buffer (pH 5.0). Typical yields of *CedA* were 12 mg protein/l in LB medium and 9 mg/l in M9 medium.

Systematic Evolution of ligand by EXponential enrichment (SELEX)

A synthesized DNA (5'-GGAATTCATATGATTGCACTCAGC(N)₁₂GCATGGTAACCTCGAGGATCCTTG-3') molecule containing a 12-bp random nucleotide sequence was amplified by PCR using the primers 5'-GGAATTCATATGATTGCACTCA-3' and 5'-CAAGGATCCTCGAGGGTTACCA-3'. After purification, the amplified DNA fragment was dissolved in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM MgCl₂, 40 mM KCl and 5% glycerol. A 10- μ l aliquot of the random DNA fragments (30–50 μ M) and various concentrations of *CedA* (0–50 μ M) were incubated on ice for 1 h. The incubated mixture was subjected to 2% agarose gel electrophoresis to separate the free DNA and the *CedA*-bound DNA. The bound DNA was extracted from agarose gel, followed by PCR amplification. After repeating the operation six times, the PCR products were digested with NdeI and XhoI and ligated into the vector pET-22b(+). The ligated vector was then introduced into JM109 cells. After being cultured at 37°C for 10 h, the vectors containing the DNA sequence that preferentially bound to *CedA* were extracted. The nucleotide sequences were confirmed using DNA sequence analysis.

NMR measurements

The NMR spectra were recorded at 25°C on a Varian Unity INOVA 600 spectrometer (Varian Inc., Palo Alto, CA, USA). NMR samples were dissolved in D₂O or 90% H₂O/10% D₂O. The chemical shifts of full-length *CedA* were assigned in reference to those of $\Delta 18$ *CedA*, which have been previously assigned (11). The unassigned chemical shifts in the N-terminal region were assigned by three-dimensional (3D) triple-resonance experiments. The amide proton (NH) signals in the ¹⁵N-¹H homonuclear single-quantum coherence (HSQC) spectra of ¹⁵N-labeled full-length *CedA* are shown in Supplementary Fig. S1.

The DNA titrations were carried out in 20 mM acetate buffer (pH 5.0). The ¹H-¹⁵N HSQC spectra of 0.1 mM ¹⁵N-labeled protein were recorded in the presence of varying concentrations of the purified and annealed Systematic Evolution of ligand by EXponential enrichment (SELEX) selected dsDNA (5'-CCAAGAGTTTTAATCC-3' and 5'-GGATTAAAACTCTTGG-3') or the control dsDNA (5'-CGCCGTTAACGCGCC-3' and 5'-GGCGCGTTAACGGCGG-3') up to 0.2 mM. The concentrations of the proteins were corrected for dilution. The overall volume changes amounted to <10%.

Electrophoresis mobility shift assay

The Electrophoresis Mobility Shift Assays (EMSAs) were performed according to the previous methods (11). Varying amounts of *CedA* or *CedA* mutants were incubated with the indicated DNA for 10 min

at 37°C in 10 μ l buffer [20 mM Tris-HCl, pH 8.0 containing 0.5 mM CaCl₂, 5 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid and 1 mM dithiothreitol (DTT)]. The mixture was analysed using 7.5% polyacrylamide gel electrophoresis (PAGE). DNA was detected by ethidium bromide staining.

Pull-down assay and peptide mass fingerprinting

The *dnaAcos* cell lysates were prepared by sonication in 50 mM phosphate buffer (pH 7.0) and the cell debris was removed by centrifugation (15,000 $\times$ g for 10 min at 4°C). The supernatant was stored at –80°C until use. Three hundred microlitres of a solution of the prepared *CedA*-(His)₆ and the deletion mutants (2 mg/ml) was applied to a Ni²⁺-NTA agarose column pre-equilibrated with three volumes of Tris-buffered saline (TBS) buffer [25 mM Tris-HCl (pH 7.2) containing 150 mM NaCl]. After 800 μ l of *dnaAcos* cell lysate was applied to the column, the column was incubated on ice for 1 h and washed five times with 250 μ l of TBS buffer, and the proteins were eluted in a stepwise manner with increasing concentrations of 200 mM imidazole in TBS buffer. Proteins in the various elution volumes were analysed by sodium dodecyl sulphate (SDS) 7.5% or 15% PAGE, and the gels were subjected to silver staining.

The protein bands were extracted from the gels. After de-staining, the proteins excised from the gels were incubated with acetonitrile for 5 min, and were completely lyophilized. The lyophilized proteins were incubated with reducing solution (25 mM NaHCO₃ solution containing 10 mM DTT) for 1 h at 56°C and then the samples were incubated in the dark with alkylation solution (25 mM NaHCO₃ containing 55 mM iodoacetamide) for 45 min at room temperature. After the samples were washed with a 25 mM NaHCO₃ solution containing 50% acetonitrile, they were again completely lyophilized. The prepared gels were subjected to in-gel digestion with 10 μ g/ml of L-(tosylamino 2-phenyl) ethyl chloromethyl ketone (TPCK)-trypsin for 12 h at 37°C. After digestion, the tryptic peptide solution was recovered and concentrated by lyophilization until the volume of the solution reached 10 μ l. The concentrated sample was desalted using ZipTip C₁₈ (Millipore, Billerica, MA, USA). Matrix-assisted laser desorption ionization time-of-flight mass spectrometric analyses were performed using a Voyager Elite spectrometer (Applied Biosystems, Foster City, CA, USA) with the acceleration voltage set to 20 kV. Data were acquired in the positive-reflect mode of operation. The collected peptides were dissolved in 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA). The peptide solutions (0.5 μ l) were mixed with 0.5 μ l of matrix solution (solution saturated with α -cyano-4-hydroxycinnamic acid containing 0.1% TFA and 50% acetonitrile). To identify the proteins, we used MS-Fit in the Protein Prospector program (<http://prospector.ucsf.edu>). One missed cut and the alkylation of iodoacetamide were allowed.

Cell growth assay

The cell growth assays were performed according to previous methods (10). The deletion mutant $\Delta 18$ *CedA*, which lacks 18 amino acids at the N-terminus, and the single-point mutants were prepared by the quick-change method using pMK11 (10). The prepared vectors were introduced into *dnaAcos* cells by transformation. Cells were grown overnight at 42°C in LB medium containing thymine (25 μ g/ml) and ampicillin (50 μ g/ml). The resultant culture was diluted 100-fold in the same medium and incubated at 42°C until an absorbance of 0.4 at 660 nm was reached. Then, the culture was further diluted 500-fold and cooled to 30°C. After incubation for 8 h, the cell morphology of the mutant cells was checked by microscopy.

Results

Search for a consensus DNA sequence binding *CedA*

The NMR-derived structure of the C-terminal domain (residues 18–87) of *CedA* revealed a structure similar to the DNA-binding proteins (11). *CedA* structural homologs, the GCC-binding box domain of ethylene-responsive transcription factor AtERF1 from *Arabidopsis thaliana*, the N-terminal DNA-binding domain of λ -integrase protein from bacteriophage λ and the N-terminal DNA-binding domain of Tn916 integrase bind double-strand DNA with sequence

Fig. 1 Ceda-binding oligonucleotide sequences from SELEX experiments. Representation of the consensus DNA target-binding motif determined by SELEX using Web Logo (<http://weblogo.threeplussone.com>). The degree of conservation is indicated by the height of the letters and a core sequence 'TTTTXT[T/G]' was found.

specificity via a β -sheet structure (13–15). Based on structural similarity, it is expected that Ceda also preferentially binds to a particular sequence.

Therefore, we carried out a SELEX experiment to find the DNA sequence that Ceda preferentially binds to. DNA molecules containing a 12-bp random nucleotide sequence and full-length Ceda were incubated, and then the tightly bound DNA was separated and extracted by agarose gel and amplified by PCR. After six repeats of selection and PCR amplification, the PCR products were cloned into the vector. The results of 38 DNA sequence analyses in cloned vectors are shown in Supplementary Table SI. The resulting sequences have two prominent features: they are rich in thymine (T: 44%; A: 18%; G: 14%; C: 24%) and frequently have continuous thymine sequences. Taking these features into account, the sequences were aligned, and the consensus pattern of Ceda-binding DNA was found to be TTTTNT[T/G] (Fig. 1).

To evaluate the binding between Ceda and the DNA sequence as determined by SELEX, we performed a DNA titration experiment using NMR. The titrated DNA sequence was 5'-CCAAGAGTTT TAATCC containing the SELEX consensus sequence. The sequence 5'-CCGCCGTTAACGCGCC, which has been previously used for NMR titration (11), was used as the control DNA. We used full-length Ceda instead of $\Delta 18$ Ceda, which lacks 18 residues at the flexible N-terminus. As the concentration of control DNA increased, chemical shift changes in the spectra occurred. The chemical shift changes as a function of the residue number of Ceda at a concentration of 0.2 mM of control DNA are shown in Fig. 2A. Using the control DNA, it was found that the residues causing large chemical shift changes were Arg25, Thr26, Glu27, Glu32, His33 and the $\beta 4$ surface of the Ceda structure (Fig. 2C). From the curve fitting of these chemical shift changes, the value for K_d was estimated to be more than 200 μ M (Supplementary Fig. S2). The signals from Arg25, Thr26, Glu27, Glu32, His33 and the $\beta 4$ surface were broadened as the concentration of SELEX consensus DNA was increased (Fig. 2B). Thus, the intermediate or slow exchange between the free and the bound state occurred, suggesting that the DNA-binding mode changed by selecting the DNA sequence using SELEX.

The N-terminal region is involved in the Ceda–DNA interaction

Previous results investigating DNA binding to Ceda using EMSA have indicated that Ceda tightly binds to 200-bp DNA (11). When we performed EMSA using full-length Ceda and 200-bp DNA by the same method, the DNA–Ceda complex band was detected as indicated previously (Fig. 3A). Conversely, when we performed EMSA using 16-bp DNA containing the SELEX consensus sequence, we did not find a band shift on addition of Ceda (Fig. 3B), suggesting that the short DNA does not stably bind to Ceda and that the binding mode in the EMSA is different from that observed with NMR. Furthermore, we examined the 200-bp DNA binding with $\Delta 18$ Ceda using the EMSA. The DNA band did not shift with the addition of $\Delta 18$ Ceda (Fig. 3C). Similarly, the three-point Ceda mutant, K5A/K9A/K10A, did not bind to 200-bp DNA (Fig. 3C). Therefore, we concluded that the basic residues of the N-terminal region in Ceda contributed to the Ceda–DNA interaction under the conditions of the EMSA. It has been suggested that Ceda tightly binds to DNA through the N-terminal flexible region. Both our and Chen's group (11) performed the NMR experiments under high salt conditions (>150 mM NaCl), because significant precipitation occurs under low salt conditions. High concentrations of salt disrupt the electrostatic interaction between the N-terminal region of Ceda and the DNA; therefore, we could not observe this interaction using NMR. Affinity of the C-terminal region for DNA might be too low to be detected by the EMSA and 16-bp DNA might be too short to interact with both the N-terminal and C-terminal regions (see Discussion).

Ceda binds to RNAP

Proteomics results suggest that Ceda binds to RNAP (12). To confirm the interaction between Ceda and RNAP, we attempted a pull-down assay. His₆-tagged full-length Ceda was preloaded into Ni²⁺-NTA agarose, and the agarose was mixed with the cell lysates from *dnaAcos* mutant cells. Then, the binding proteins were eluted in 250 mM of imidazole solution. The eluted protein solutions were subjected to SDS-PAGE staining by silver (Fig. 4). Three main staining bands appeared in the presence of preloaded Ceda (Fig. 4, lane 4). To identify these proteins, we performed a peptide mass fingerprint analysis after in-gel digestion with TPCK-trypsin. Based on mass spectrometry results (Table I), the RNAP α , β and β' subunits were determined to be possible candidates for these proteins, suggesting that the Ceda protein binds to the RNAP complex. In the absence of preloaded Ceda, no bands derived from RNAP were found (Fig. 4, lane 3).

To examine where in Ceda the interaction with RNAP occurs, we prepared two deletion mutants, $\Delta 10$ and $\Delta 18$ Ceda (Fig. 4). The pull-down assays using deletion mutants and *dnaAcos* cell lysates were performed according to the same method used for full-length Ceda. The results showed that the deletion mutants either did not bind to RNAP, or did so only slightly (Fig. 4, lanes 5 and 6). Therefore, we concluded

Fig. 2 DNA binding to CedA using NMR. (Left) Overlapped two-dimensional ^1H - ^{15}N HSQC spectra of full-length CedA (0.1 mM) in the presence of 0, 0.04, 0.08, 0.12 and 0.2 mM of (A) control DNA (5'-CCGCCGTTAACGCGCC) or (B) SELEX consensus DNA (5'-CCAAGAGTTTTAATCC) at 25°C. (Right) Chemical shift changes between the presence and the absence of 0.2 mM (A) control DNA or (B) SELEX consensus DNA are shown for the backbone amides as a function of the sequence number of full-length CedA. The arrow heads indicate the signals that broadened when the SELEX consensus DNA was added. (C) The schematic secondary structure of CedA showing mapping of the DNA-binding site on the CedA structure (PDB ID, 2BN8). The DNA-binding residues are shown in black. Stick models show the substituted residues from Fig. 5.

that RNAP interacts with CedA via the N-terminal region.

Essential site of CedA for cell division reactivation activity

Small molecules, the nucleotide ppGpp and microsin J25, binding to the β and β' subunits can inhibit the

transcription activity of RNAP and hence, cell division (16–18). These small molecules induce a thermoinducible filamentation phenotype similar to that of dnaAcos cells at 30°C (16). Therefore, we examined whether the interaction of CedA and RNAP is significant for cell division reactivation activity.

Fig. 3 DNA binding to Ceda using an EMSA. (A and C) Ten picomoles of 200-bp or (B) 16-bp DNA were incubated with the indicated molar ratio of (A and B) full-length Ceda or (C) mutant Ceda for 10 min at 37°C in 10 µl buffer (20 mM Tris-HCl, pH 8.0 containing 0.5 mM CaCl₂, 5 mM MgCl₂, 0.1 mM ethylenediaminetetraacetic acid and 1 mM DTT). The mixture was analysed using 7.5% polyacrylamide gel electrophoresis.

In a previous study, Katayama *et al.* (10) evaluated Ceda function by transforming *dnaAcos* cells using a multi-copy vector carrying the *cedA* gene. The resultant transformant *dnaAcos* cells exhibited the phenotype of reactivation of cell division. Therefore, we prepared a multi-copy pBR322 vector carrying $\Delta 18cedA$ that did not possess RNAP- or DNA-binding ability (Figs 3 and 4). This plasmid was then introduced into *dnaAcos* cells. Figure 5 shows

Fig. 4 RNAP binding to Ceda. The RNAP interaction with the wild-type, $\Delta 10$ and $\Delta 18$ Ceda, His₆-tagged at the C-terminus. His₆-tagged Ceda or a blank (no protein) was preloaded into Ni-NTA agarose, and the agarose was mixed with the cell lysates from *dnaAcos* mutant cells. The agarose was mixed with elution buffer containing 250 mM imidazole. The eluted proteins were subjected to SDS-PAGE. Lanes 1 and 2 are markers [lane 1: molecular markers (M), lane 2: RNAP]. Lanes 3–6 are the eluted proteins (lane 3: in the absence of preloaded His₆-tagged Ceda; lanes 4, 5 and 6: in the presence of preloaded wild-type, $\Delta 10$ and $\Delta 18$ Ceda, respectively). The three bands indicated by arrows in lane 4 were determined by the peptide mass fingerprint method to be the following: (i) RNAP β subunit, (ii) RNAP β' subunit and (iii) RNAP α subunit (Table I).

Table I. Ceda-binding proteins

Number of band ^a	Name of protein	Accession no. ^b	Mowse score ^c	Sequence coverage(%) ^c
1	DNA-directed RNA polymerase β' subunit	P00577	668	12.0
2	DNA-directed RNA polymerase β subunit	P00575	8.88	6.0
3	DNA-directed RNA polymerase α subunit	P00574	572	18.0

The proteins were identified by pull-down assay with His₆-tagged Ceda and by matrix-assisted laser desorption ionization time-of-flight mass spectrometry.

^aThe numbers of selected protein bands from SDS-PAGE are presented in Fig. 1.

^bSwiss plot sequence identification number.

^cThe Mowse score and sequence coverage were obtained from the MS-fit search function in the Protein Prospector program.

photographs of *dnaAcos* cells bearing the plasmid at 30°C. *DnaAcos* cells transformed with the vector carrying $\Delta 18cedA$ showed reactivation of cell division similar to that of the vector carrying *cedA* (Fig. 5). These results suggest that the interaction of the N-terminus of Ceda with DNA and RNAP is not essential for the reactivation of cell division in *dnaAcos* cells.

In addition, the other proteins with structural similarity to Ceda have transcription regulation activity (13–15). Therefore, we examined the possibility that the weak interaction of Ceda with DNA via β -sheet region may regulate transcription of cell division regulation proteins, such as FtsZ (19, 20). Based on the

Fig. 5 Cell division reactivation by CedA mutants. Cell morphology of plasmid-bearing CedA mutants. Cells were grown exponentially at 42°C in LB medium and then incubated at 30°C (see Materials and Methods section).

chemical shift data from DNA titration, we prepared the mutants, where Glu27, Glu32, Phe60 and Arg62 were replaced with alanine. The *dnaAcos* cells transformed with vectors carrying each mutant gene showed reactivation, except for the E32A mutant (Fig. 5). E32A mutant cells showed a filamentous phenotype similar to *dnaAcos* cells bearing pBR322. These results suggest that Glu32 is an important residue for cell division reactivation in *dnaAcos* cells. Arg47, which is close to Glu32 on the surface of the 3D structure of CedA, was also replaced with alanine. However, we found cell division reactivation with the R47A mutant as shown in Fig. 5.

Discussion

In this study, we determined the binding site of CedA for DNA or RNAP and examined the binding functions involved in cell division reactivation.

A previous DNA-binding study has suggested that CedA may be categorized as a DNA-binding protein that binds via a β -sheet structure with sequence specificity, based on its structural similarity to other DNA-binding proteins (11). SELEX was used to search for the preferred consensus sequence to CedA. The consensus sequence DNA from SELEX suggests that CedA prefers thymine-rich sequences (Fig. 1). NMR-based DNA-binding experiments indicated that CedA binds to SELEX consensus DNA via a β -sheet structure.

The signals from Arg25, Thr26, Glu27, Glu32, His33 and the β 4 surface were broadened and chemical shift changes in the residues from Gln18 to Val23 were increased rather than when the consensus DNA was used (Fig. 2B). These results suggest that the sequence-specific affinity may increase using DNA produced by SELEX. Possibly, this sequence specificity was caused by the methyl group of thymine and the hydrophobic side chains of Ile19, Ile20, Val23 and possibly Phe60. Therefore, the DNA-binding mode via a β -sheet in CedA is different from that of other proteins, which electrostatically interact with DNA (13–15).

The N-terminus of CedA is necessary for a tight interaction with 200-bp DNA (Fig. 3). The positive charges of the lysine residues (Lys 5, Lys 9 and/or Lys 10) at the N-terminus of CedA may play an important role in binding to the phosphate backbone of DNA. Thus, CedA can interact with DNA using two parts: the β -sheet structure via a sequence-specific, weak hydrophobic interaction and the N-terminal region via a sequence non-specific, tight electrostatic interaction. Our results also suggest that the N-terminus of CedA is important for the interaction with RNAP (Fig. 4). Therefore, the N-terminus amino acid sequence is a flexible but functional region.

In vivo, the function of CedA in *dnaAcos* cells is cell division reactivation. As shown in Fig. 5, Glu27, Glu32, Phe60 and Arg62, which were necessary for weak DNA binding, were not essential for cell division reactivation.

Moreover, the N-terminal region of Ceda, which is a requisite binding site for RNAP or DNA, was not essential for cell division reactivation. Only the E32A mutant showed the thermoinducible filamentation phenotype. Therefore, neither the weak nor the tight bindings of Ceda to DNA and/or RNAP cause the cell division regulation and there must be another mechanism involved in the cell division reactivation activity.

The DnaA mutant molecule in *dnaAcos* cells has a low nucleotide binding affinity, causing impairment of cell division and replication in these cells (7, 8). Recent research has demonstrated that an excess of *datA* sites, which contribute to the inactivation of DnaA molecules, provided on plasmids leads to a delay in cell division (21). Thus, DnaA inactivation plays a key role in cell division. Therefore, Ceda may be involved in the reactivation of DnaA suffering inactivation. In particular, Glu32 in Ceda is an essential residue for cell division reactivation and may interact with the DnaA molecule, directly or indirectly. Future studies of the function of Ceda may provide support for the notion that DNA replication and cell division are closely related at the molecular level.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

We thank Irina Artsimovitch (Department of Microbiology and the RNA Group, Ohio State University) for donating valuable samples, and Dr Yusaku Matsuda (Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Kyushu University) for kind help with the RNA polymerase experiment.

Funding

This work was supported by JSPS KAKENHI grant number 26440031 to Y.A. and T.K. This work was also partly supported by the National Project on Protein Structural and Functional Analyses (NPPSFA).

Conflict of Interest

None declared.

References

1. Zyskind, J.W. and Smith, D.W. (1992) DNA replication, the bacterial cell cycle, and cell growth. *Cell* **69**, 5–8
2. Helmstetter, C. (1996) Timing of synthetic activities in the cell cycle in *Escherichia coli* and *Salmonella typhimurium*. *Cellular and Molecular Biology* (Neidhardt F.C., Curtiss R. III, Ingraham J.L., Lin E.C.C., Low K.B., Magasanik B., Reznikoff W.S., Riley M., Schaechter M., Umberger H.E., eds.) pp. 1627–1639, American Society for Microbiology Press, Washington, DC
3. Bramhill, D. and Kornberg, A. (1988) Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. *Cell* **52**, 743–755
4. Weigel, C., Schmidt, A., Ruckert, B., Lurz, R., and Messer, W. (1997) DnaA protein binding to individual DnaA boxes in the *Escherichia coli* replication origin, *oriC*. *EMBO J.* **16**, 6574–6583
5. Katayama, T. (1994) The mutant DnaAcos protein which overinitiates replication of the *Escherichia coli* chromosome is inert to negative regulation for initiation. *J. Biol. Chem.* **269**, 22075–22079
6. Katayama, T. and Kornberg, A. (1994) Hyperactive initiation of chromosomal replication in vivo and in vitro by a mutant initiator protein, DnaAcos, of *Escherichia coli*. *J. Biol. Chem.* **269**, 12698–12703
7. Katayama, T., Crooke, E., and Sekimizu, K. (1995) Characterization of *Escherichia coli* DnaAcos protein in replication systems reconstituted with highly purified proteins. *Mol. Microbiol.* **18**, 813–820
8. Carr, K.M. and Kaguni, J.M. (1996) The A184V missense mutation of the dnaA5 and dnaA46 alleles confers a defect in ATP binding and thermolability in initiation of *Escherichia coli* DNA replication. *Mol. Microbiol.* **20**, 1307–1318
9. Simmons, L.A. and Kaguni, J.M. (2003) The DnaAcos allele of *Escherichia coli*: hyperactive initiation is caused by substitution of A184V and Y271H, resulting in defective ATP binding and aberrant DNA replication control. *Mol. Microbiol.* **47**, 755–765
10. Katayama, T., Takata, M., and Sekimizu, K. (1997) Ceda is a novel *Escherichia coli* protein that activates the cell division inhibited by chromosomal DNA over-replication. *Mol. Microbiol.* **26**, 687–697
11. Chen, H.A., Simpson, P., Huyton, T., Roper, D., and Matthews, S. (2005) Solution structure and interactions of the *Escherichia coli* cell division activator protein Ceda. *Biochemistry* **44**, 6738–6744
12. Lee, D.J., Busby, S.J., Westblade, L.F., and Chait, B.T. (2008) Affinity isolation and I-DIRT mass spectrometric analysis of the *Escherichia coli* O157:H7 sakai RNA polymerase complex. *J. Bacteriol.* **190**, 1284–1289
13. Allen, M.D., Yamasaki, K., Ohme-Takagi, M., Tateno, M., and Suzuki, M. (1998) A novel mode of DNA recognition by a beta-sheet revealed by the solution structure of the GCC-box binding domain in complex with DNA. *EMBO J.* **17**, 5484–5496
14. Connolly, K.M., Wojciak, J.M., and Clubb, R.T. (1998) Site-specific DNA binding using a variation of the double stranded RNA binding motif. *Nat. Struct. Biol.* **5**, 546–550
15. Wojciak, J.M., Sarkar, D., Landy, A., and Clubb, R.T. (2002) Arm-site binding by lambda-integrase: solution structure and functional characterization of its amino-terminal domain. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3434–3439
16. Vinella, D. and D'Ari, R. (1994) Thermoinducible filamentation in *Escherichia coli* due to an altered RNA polymerase beta subunit is suppressed by high levels of ppGpp. *J. Bacteriol.* **176**, 966–972
17. Magnusson, L.U., Farewell, A., and Nystrom, T. (2005) ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol.* **13**, 236–242
18. Delgado, M.A., Rintoul, M.R., Farias, R.N., and Salomon, R.A. (2001) *Escherichia coli* RNA polymerase is the target of the cyclopeptide antibiotic microcin J25. *J. Bacteriol.* **183**, 4543–4550
19. Bi, E.F. and Lutkenhaus, J. (1991) FtsZ ring structure associated with division in *Escherichia coli*. *Nature* **354**, 161–164
20. Addinall, S.G. and Holland, B. (2002) The tubulin ancestor, FtsZ, draughtsman, designer and driving force for bacterial cytokinesis. *J. Mol. Biol.* **318**, 219–236
21. Morigen, M., Flatten, I., and Skarstad, K. (2014) The *Escherichia coli* *datA* site promotes proper regulation of cell division. *Microbiology* **160**, 703–710