Interplay between CedA, rpoB and double stranded DNA: A step towards understanding CedA mediated cell division in E. coli

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Cell division is compromised in DnaAcos mutant E. coli cells due to chromosome over-replication. In these cells, CedA acts as a regulatory protein and initiates cell division by a hitherto unknown mechanism. CedA, a double stranded DNA binding protein, interacts with various subunits of RNA polymerase complex, including rpoB. To reveal how this concert between CedA, rpoB and DNA brings about cell division in E. coli, we performed biophysical and in silico analysis and obtained mechanistic insights. Interaction between CedA and rpoB was shown by circular dichroism spectrometry and in silico docking experiments. Further, CedA and rpoB were allowed to interact individually to a selected DNA and their binding was monitored by fluorescence spectroscopy. The binding constants of these interactions as determined by BioLayer Interferometry clearly show that rpoB binds to DNA with higher affinity (K_D = 1.0E-12 M) as compared to CedA (K_D = 9.58E-09 M). These findings were supported by docking analysis where 12 intermolecular H-bonds were formed in rpoB-DNA complex as compared to 4 in CedA-DNA complex. Based on our data we propose that in E. coli cells chromosome over-replication signals CedA to recruit rpoB to specific DNA site(s), which initiates transcription of cell division regulatory elements.

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1. Introduction

Cell division activator (CedA), an 80 amino acid long protein located at 38.9 min on the Escherichia coli (E. coli) genome map, is a double stranded-DNA binding protein. Three dimensional structure of CedA was determined by solution NMR and it was found that its C-terminal domain is quite similar to known DNA-binding domains of other proteins [1]. Previous studies have reported that CedA regulates cell division in case of chromosome over-replication. In bacteria, cell division and chromosome replication are highly coordinated and regulated processes that occur with high accuracy [2]. A protein known as DnaA is chief regulator of chromosome replication in E. coli. It binds to the origin of replication (oriC) and initiates formation of the DNA replication initiation complex [3,4]. DnaAcos, a cold sensitive DnaA mutant, induces chromosome over-replication at 30 °C and results in filamentous cell morphology due to compromised cell division [3–5]. Katayama et al. reported that CedA countered this process. They observed that CedA over-expression in DnaAcos mutants started cell division and regular colony formation; however, chromosomal DNA over-replication continued [3]. It is evident from these studies that CedA is crucial for cell division during chromosome over-replication, but mechanism is still unknown.

Proteins participate in various biological processes and perform specific functions through their interaction with other components of native environment [6]. Thus, it becomes crucial to study protein interactions to unfold intermediate steps of a process to reveal a protein’s role as well as mechanism via which it regulates a process. It is well known that DNA binding proteins (DBPs) are key regulatory components in bacterial cell division and the regulatory mechanism is highly dependent on the specificity and affinity of DBPs towards their target sequences [7]. To gain insights of CedA role in the cell division regulation, Abe et al. performed structure based functional analysis and identified its residues important for binding with DNA and RNA polymerase [8]. First, they identified double stranded-DNA (ds-DNA) sequence (5′-CCAGAGTTTTATCC-3′ and 5′-GGATTAAAACTCTTGG-3′) to which CedA preferentially binds and then, searched specific regions of CedA (C-terminal) crucial for binding with DNA and RNA polymerase [8]. In addition, CedA was identified as one of the components of RNA polymerase com-

Abbreviations: BIL: BioLayer Interferometry; CBPs: CedA binding proteins; CD: Circular dichroism; CedA: Cell division activator protein; DBPs: DNA binding proteins; H-bond, Hydrogen bond.

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plex in *E. coli* using affinity isolation and mass spectrometry [9]. In our previous study, we have identified nine CedA-binding proteins (CBPs) in *E. coli* by affinity separation followed by mass spectrometric analysis, viz. pyruvate dehydrogenase E1 component, chaperone protein DnaK, 50S ribosomal protein L2, outer membrane lipoprotein Lpp, DNA-directed RNA polymerase subunit beta (rpoB), glucose-6-phosphate 1-dehydrogenase, 50S ribosomal protein L3, glucosamine-fructose-6-phosphate amino- transferase and an uncharacterized protein yhe) [10]. Based on CBPs identified, we hypothesized that CedA regulates cell division mainly through its participation in energy metabolic pathways and transcription.

In present study, we have performed various biophysical and in *silico* experiments to gain better insights of CedA interaction with rpoB and ds-DNA. First, CedA and rpoB were cloned, expressed and purified using standard molecular biology procedures. Then, their binding was monitored by circular dichroism (CD) and in *silico* docking. Their interaction with ds-DNA was determined by intrinsic fluorescence spectroscopy, biolayer interferometry (BLI) and in *silico* docking. Based on our results, we propose a model to uncover the mechanism through which CedA regulates cell division.

2. Materials and methods

2.1. Cloning, expression and purification of CedA and rpoB

CedA was cloned, expressed and purified as described in our previous study [10]. Same strategy with some modifications was used for cloning and expression of rpoB. Briefly, cedA gene was PCR amplified with *Nde*I and BamH I restriction sites using specific primer set (Supplementary table S1). After amplification and digestion with restriction enzymes, gene was ligated into pET28a vector using T4 DNA ligase (Thermo Fisher Scientific, United States) and transformed into BL21-Gold (DE3) *E. coli* expression host, while rpoB was transformed into BL21 (DE3) pLYS E cells. The transformed cells were cultured at 37 °C and induced with 1 mM IPTG (Sigma-Aldrich, United States). After four hours of induction, cells were harvested. The CedA expressing cells were lysed by sonication in buffer A (100 mM phosphate buffer containing 50 mM NaCl and 1 mM PMSF, pH 8.0) and His-CedA was purified by Ni-NTA-Agarose affinity chromatography followed by buffer exchange using PD-10 desalting column (GE Healthcare, Sweden). The rpoB expressing cells were suspended in buffer B (100 mM phosphate, 10 mM Tris HCl, 8 M urea, pH 8.0) and lysed by sonication. After centrifugation, supernatant was loaded onto Ni-NTA-Agarose column. The column was washed twice with buffer B (pH 6.3) and rpoB was eluted using buffer B of decreasing pH (pH 5.9 and pH 4.5). The eluted fractions were pooled and dialyzed. Purified proteins were analyzed by electrophoresis.

2.2. Confirmation of purified proteins by mass spectrometry

Purified CedA and rpoB were run on 12% SDS-PAGE [11] and their identity was confirmed by MALDI-TOF MS analysis (UltraflexTreme MALDI TOF/TOF Mass Spectrometer, Bruker Daltonik, Germany) as described earlier [12]. Briefly, bands of purified proteins were excised manually from the gel, cut into small pieces and in-gel digested using sequencing grade trypsin (Sigma-Aldrich, United States). The digested peptides mixed with matrix solution were spotted on MALDI target plate. Peptide mass spectra were generated and searched against Swiss-Prot databases using Mascot search engine (Matrix Sciences, UK) and following search parameters- Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Mass values: Monoisotopic, Protein Mass: Unrestricted, Peptide Mass Tolerance: ± 300 ppm, Peptide Charge State: 1+, and Max Missed Cleavages: 1.

2.3. Annealing of oligonucleotides

Two strands of CedA binding DNA (5'-CCAAGAGTTTTAATCC-3' and 5'-GGATTAACCTTGG-3') (Abe et al.) were custom synthesized by IDT. These strands were mixed in annealing buffer (10 mM Tris, pH 7.5, 50 mM NaCl and 1 mM EDTA) and incubated at 95 °C for 5 min. The mixture was cool down gradually to room temperature.

2.4. Circular dichroism spectroscopy

The secondary structural changes in CedA and rpoB due to their interaction were studied by far-UV CD spectrum (250–200 nm). The CD spectra were recorded by a J-815 spectropolarimeter (Jasco Inc. USA) using a quartz cuvette of 1 mm path length and spectral band width 1 nm. Protein samples (CedA and rpoB) were prepared in reaction buffer 1 (100 mM sodium phosphate buffer containing 50 mM NaCl, pH 7.4). The data were acquired separately for CedA, rpoB and their complex. All spectra were average of three measurements.

2.5. Fluorescence spectroscopy

CedA and rpoB interactions with ds-DNA were monitored by intrinsic tryptophan fluorescence spectroscopy. The fluorescence scans were recorded by Cary Eclipse Fluorescence Spectrophotometer (Agilent, USA). The scan parameters were: excitation-295 nm; emission-310–400 nm; cuvette path length- 1 cm; excitation slit width- 5 nm; and emission slit width- 10 nm. A total of 100 μL of 5 μM protein (CedA or rpoB) was prepared in reaction buffer and DNA was titrated into it as continuous injections of 0.5 μL of 50 μM stock.

2.6. Bio-layer interferometry

The kinetics of binding affinity of ds-DNA with CedA, rpoB and CedA-rpoB complex was determined using Octet RED96 instrument (Pall ForteBio, USA) as previously described [13]. Briefly, DNA biotinylation was performed using Biotin 3’ End DNA Labeling Kit (Thermo Scientific, United States). Prior to analysis, streptavidin kinetic grade biosensor (Pall ForteBio) was hydrated in 1X kinetic buffer (Pall ForteBio part no. 18-5032) containing PBS, 0.1% BSA, 0.02% Tween 20 and 0.05% sodium azide. Total reaction volume was 200 μL and assay was performed at 30 °C with constant shaking at 1000 rpm. The assay steps were: equilibration (60s), binding of 10 μg/mL biotinylated-DNA (300s), baseline stabilization (120s), ligand-analyte association (300s) and ligand-analyte dissociation (600s). Analyte (CedA and rpoB) concentrations used were 250 and 750 nM. The kinetic parameters were determined from the acquired data by Octet® Software using global fitting, specifying 2:1 kinetic model.

2.7. In *silico* interaction analysis

CedA (PDB ID: 2BN8) and rpoB (PDB ID: 3LTI) structure coordinate files were retrieved from RCSB protein data bank and processed. Three dimensional structure of ds-DNA was predicted by ‘DNA sequence to structure’ tool [http://www.scfbio-itd.res.in]. CedA and rpoB complex structures were predicted by Cluspro server [14]. For structural insights, interaction between ds-DNA with CedA and rpoB was studied by in *silico* docking experiments performed over the NPDock server for protein–nucleic acid docking [15]. The computational workflow of this program is: global docking using GRAMM → scoring with a statistical potential → clustering of top complex structure models → refinement of the best models. For both of the proteins, the structure coordinates
of top 100 complexes were predicted and refined to attain energy minimized stable states. After refinement, best complex structures were obtained and their structure co-ordinates files in PDB format were downloaded. In addition, the number of simulation steps taken to attain these final complex structures was also determined.

### 3. Results

#### 3.1. Circular dichroism spectroscopy

CedA and rpoB were successfully cloned, expressed and purified as described in Materials and Methods section. Purity of proteins was analysed by 12% SDS-PAGE and to confirm their identity, single bands of CedA and rpoB as observed on gels (Fig. 1) were excised, trypsinized and identified by mass spectrometry. Spectra searched against Swiss-Prot protein database matched to CedA and rpoB of E. coli (Supplementary Fig. S1 and S2, Table 1). The CD spectroscopy results indicate that CedA interacts with rpoB as evident by change in ellipticity. We observed a significant change in ellipticity (mdeg) of CedA and rpoB complex spectra (as a measure of their secondary structures) in comparison to the mathematical sum of their individual CD spectrum (Fig. 2). As determined by system inbuilt Yang’s algorithm, there was slight increase in alpha helix content after complex formation. A significant reduction in beta sheet structures was observed, which contributed to the increase of random structure.

#### 3.2. Fluorescence spectroscopy and bio-layer interferometry

Interaction of CedA with ds-DNA, to which it preferentially binds [8], was studied by fluorescence spectroscopy, BLI and in-silico docking. Further, their interaction was compared with that of same DNA with rpoB. The binding between proteins and DNA was observed by quenching of the fluorescence emission maxima of protein upon binding with DNA compared to that of free protein. As mentioned Materials and methods Section 2.5, 50 µM DNA was titrated as continuous injections of 0.5 µL till the saturation of fluorescence intensity was achieved. A significant decrease in emission maxima was observed in both the proteins (Fig. 3). In case of native CedA, a tryptophan fluorescence intensity maximum was observed at 45 a.u. at a wavelength of 333 nm. Gradually, fluorescence signal quenched after DNA titration and saturated at approximately 7 a.u. (Fig. 3A). Similarly, an intensity maximum of native rpoB was ~100 a.u. at a wavelength of 345 nm and it quenched gradually to

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Table 1: Identification of purified proteins by MALDI-TOF MS.

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<th>S. No.</th>
<th>Protein Match</th>
<th>Mascot Score</th>
<th>Protein Name</th>
<th>Molecular weight (kDa)</th>
<th>Number of peptides matched</th>
<th>Sequence coverage</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>CED,ECOBW</td>
<td>60</td>
<td>Cell division activator CedA</td>
<td>9.37</td>
<td>8</td>
<td>71%</td>
</tr>
<tr>
<td>2</td>
<td>RPOB,ECOBW</td>
<td>568</td>
<td>DNA directed RNA polymerase subunit beta</td>
<td>150.63</td>
<td>82</td>
<td>60%</td>
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Fig. 1. SDS-PAGE profiles of (A) purified CedA, and (B) purified rpoB.
~25 a.u. with addition of DNA (Fig. 3B). BLI results indicate that both CedA and rpoB have two DNA-binding sites as depicted by global fitting into 1:2 kinetic models by Octet software (Fig. 4). As evident by binding constants, the sequence of DNA binding affinity is CedA-rpoB complex > rpoB > CedA. The equilibrium dissociation constants $K_{D2}$ determined for CedA, rpoB and CedA-rpoB complex were $9.58E-09$ M, $<1.0E-12$ M and $<1.0E-12$ M, respectively.

Table 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{D1}$ (M)</th>
<th>$K_{on1}$ (M$^{-1}$ sec$^{-1}$)</th>
<th>$K_{off1}$ (sec$^{-1}$)</th>
<th>$K_{D2}$ (M)</th>
<th>$K_{on2}$ (M$^{-1}$ sec$^{-1}$)</th>
<th>$K_{off2}$ (sec$^{-1}$)</th>
<th>Fitting Model</th>
<th>Docking Score$^*$</th>
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</thead>
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<tr>
<td>CedA</td>
<td>3.13E-07</td>
<td>6.66E+03</td>
<td>2.09E-03</td>
<td>9.58E-09</td>
<td>1.41E+05</td>
<td>1.36E-03</td>
<td>1:2</td>
<td>−4.60</td>
</tr>
<tr>
<td>rpoB</td>
<td>8.61E-06</td>
<td>4.12E+03</td>
<td>3.55E-02</td>
<td>$&lt;1.0E-12$</td>
<td>1.35E+03</td>
<td>$&lt;1.0E-07$</td>
<td>1:2</td>
<td>−5.94</td>
</tr>
<tr>
<td>CedA-rpoB complex</td>
<td>3.10E-06</td>
<td>5.59E+03</td>
<td>1.73E-02</td>
<td>$&lt;1.0E-12$</td>
<td>2.11E+03</td>
<td>$&lt;1.0E-07$</td>
<td>1:2</td>
<td>−7.49</td>
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</table>
Fig. 4. BioLayer Interferometry (BLI) response signal. (A) CedA and ds-DNA, (B) rpoB and ds-DNA, (C) CedA-rpoB complex and ds-DNA.

Fig. 5. Interaction between CedA and rpoB. (A) CedA-rpoB complex predicted by ClusPro server, (B) Intermolecular hydrogen bond, (C) Hydrophobic interactions, (D) Ionic interactions. (Green → rpoB; magenta → CedA).

For more details such as rate of association (K_{on}) and rate of dissociation (K_{off}), please see Table 2.

3.3. In silico docking

Three dimensional structure model of CedA and rpoB complex as predicted by Cluspro server is shown in Fig. 5. In depth analysis of complex structure was performed manually using UCSF Chimera [16] and by Protein Interactions Calculator [17]. It was observed that one main chain intermolecular hydrogen bond (H-bond; formed between Ser21 of CedA and Val353 of rpoB), six hydrophobic and five ionic interactions contributed to the stability of the complex (Supplementary Table S2). The DNA complexes with CedA and rpoB were predicted with NP-Dock server (Figs. 6 and 7) and analysis clearly suggests higher affinity of DNA with rpoB (docking score = −5.94) than CedA (docking score = −4.60). It was observed that rpoB attained a stabilized complex structure with DNA characterized by minimum energy state very quickly (260 steps) compared to CedA (477 steps). Overall, 12 and 4 intermolecular H-bonds were formed in rpoB-DNA and CedA-DNA complexes, respectively. Details of intermolecular H-bonds are given in Supplementary Table S3. Comparative structure analysis of predicted complexes reveals that CedA and rpoB binding site do not overlap with their DNA binding sites, however it was noted that two positively charged residues of CedA (Lys 36 and Arg 80) participate in ionic interaction with rpoB and form intermolecular H-bonds with DNA.

4. Discussion

The cell cycle in bacterial species is highly regulated process that involves accurate coordination of several cellular processes. Cell division is one of them, which starts with the formation of the septum. To ensure equal distribution to the progenies and to main-
tain genome integrity, septum formation essentially needs to take place at the right time and accurate place [7, 18]. The co-ordination of cell division with chromosome replication and segregation is one of the basic biological problems and thus, has been widely explored [19–21]. In E. coli, DnaA protein regulates the process of chromosome replication [22, 23]. More specifically, DnaA participates in the initiation of replication in bacteria, where it binds to specific DNA sequences within oriC and promotes strand opening [24]. DnaAcos, a cold sensitive mutant form of DnaA, lacks the ability to respond the regulatory signals and excessive initiation of chromosome replication occurs in mutant cells at 30°C that leads to formation of filamentous morphology due to inhibition of septum formation [3, 22, 25].

CedA is considered as one of the crucial cell cycle regulatory proteins of E. coli due to three major reports- (i) it is one of the constituent of RNA polymerase complex [9], (ii) it initiates cell division in DnaAcos mutant cells under influence of chromosome over-replication [3], and (iii) it binds preferentially to a specific double stranded DNA [8]. However, a mechanism through which it regulates cell cycle, more specifically cell division under chromosomal over-replication is still unknown. Based on above mentioned facts, this study was intended to reveal possible direction of CedA participation in cell cycle regulation, as an extension to our previous study related to identification of CBPs. As rpoB (one of the nine CBPs identified) primarily catalyzes the transcription of DNA into RNA, it was specifically selected for interaction analysis. The direct interac-
tion between CedA and rpoB was confirmed by BLI (Supplementary Fig. S3). In silico docking analysis over ClusPro server predicted a stable complex formation between CedA and rpoB, however a moderate binding. A single intermolecular H-bond was formed between nitrogen of Ser21 (CedA) and oxygen of Val353 (rpoB), with a distance of 3.42 Å. Amino acid residues Leu (309, 321, 360) and Pro355 of rpoB and Pro (30, 31), Val23, Trp44 and Phe60 of CedA participated in hydrophobic interactions. In addition, complex stability was also favored by ionic interactions between a few residues-Arg202, Asp358, Glu365, Arg368 and Arg378 (rpoB) with Glu58, Lys36, Arg80, Glu58 and Asp38 (CedA), respectively.

As mentioned earlier, Abe et al. identified double stranded DNA sequence to which CedA binds preferentially. We performed binding analysis of this DNA with CedA as well as rpoB and interestingly, we found that rpoB exhibits stronger affinity with this DNA as inferred by biophysical and in-silico interaction analysis. DNA binding with CedA and rpoB was initially confirmed by intrinsic fluorescence spectroscopy followed by evaluation of binding constants by BLI. For both proteins, BLI data fit into 1:2 bivalent analyte models and thus, two sets of rate constants and $K_d$ values were obtained (Table 2). Technically, the first and second sets of values imitate the binding due to the affinity of the interaction and actual binding, respectively. Analysis of second set of values clearly suggests that rpoB has higher binding affinity for DNA immobilized on the biosensor ($K_d$ rpoB = $<1.0E-12$ M; $K_d$ CedA = $9.58E-09$ M). As inferred by rate of association ($K_a$) and rate of dissociation ($K_{off}$), rpoB binds quickly ($K_a = 1.35E+03$ $M^{-1}$ sec$^{-1}$) and dissociates slowly ($K_{off} = 1.0E-07$ sec$^{-1}$) compared to CedA ($K_a = 1.41E+05$ $M^{-1}$ sec$^{-1}$ and $K_{off} = 1.36E-03$ sec$^{-1}$). Though CedA displays comparatively lower binding with DNA, BLI signals of CedA-rpoB complex suggest that it increases binding potential of rpoB with DNA (Fig. 4). These findings were well supported by in-silico docking analysis as superior DNA binding was observed in CedA-rpoB complex (score $= −7.49$) in comparison to rpoB (score $= −5.94$) and CedA (score $= −4.60$).

Based on the variations in binding affinities and rates at which CedA and rpoB bind with DNA, we hypothesize a plausible mechanism to uncover cellular events that re-initiate cell division in E. coli (Fig. 8). We suggest that when cell division in E. coli is compromised due to chromosome over-replication, CedA recruits rpoB to the specific DNA sequence and marks the initiation of transcription of key cell division regulatory protein(s). This, in turn, promotes...
cell division. We believe that chromosome over-replication functions as a signal that triggers CedA to bind with rpoB. Once bound with rpoB, it moves and binds to its target DNA sequence. As rpoB has stronger affinity for DNA, CedA flips sides with rpoB which then binds to DNA. Possibly, a crucial role of two CedA residues (Lys 36 and Arg 80) that stabilize CedA and rpoB complex through ionic interactions can be anticipated here. When in contact, these residues form intermolecular H-bonds with DNA and thus, weaken the ionic interaction between CedA and rpoB. This process may bring some structural changes in close proximity, favorable for rpoB and DNA binding. Several other factors, such as subunits of RNA polymerase and components of transcription factor initiation complex, join in and initiate transcription of specific genes, which we presume translate into cell division regulatory proteins.

5. Conclusions

Here, we have reported biophysical and in silico interaction analysis of CedA protein with RNA polymerase subunit rpoB and specific ds-DNA as an attempt to identify mechanism of CedA mediated cell division in E. coli cells. Based on our findings, we proposed that cell division initiation is controlled by CedA through recruitment of rpoB to specific DNA sequence sites. Though outline of proposed mechanism is placed satisfactorily with preliminary interaction data, further confirmation through advanced experiments at cellular level is desirable.

Conflicts of interest

Authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijbiomac.2017.10.075.

References