Identification of functional interactome of a key cell division regulatory protein CedA of E. coli

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Cell division is compromised in DnaAcos mutant Escherichia coli cells that results in filamentous cell morphology. This is countered by over-expression of CedA protein that induces cytokinesis and thus, regular cell morphology is regained; however via an unknown mechanism. To understand the process systematically, exact role of CedA should be deciphered. Protein interactions are crucial for functional organization of a cell and their identification helps in revealing exact function(s) of a protein and its binding partners. Thus, this study was intended to identify CedA binding proteins (CBPs) to gain more clues of CedA function. We isolated CBPs by pull down assay using purified recombinant CedA and identified nine CBPs by mass spectrometric analysis (MALDI-TOF MS and LC–MS/MS), viz. PDHA1, RL2, DNAK, LPP, RPOB, G6PD, GLMS, RL3 and YBC. Based on CBPs identified, we hypothesize that CedA plays a crucial and multifaceted role in cell cycle regulation and specific pathways in which CedA participates may include transcription and energy metabolism. However, further validation through in-vitro and in-vivo experiments is necessary. In conclusion, identification of CBPs may help us in deciphering mechanism of CedA mediated cell division during chromosomal DNA over-replication.

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1. Introduction

Cell division and chromosomal DNA replication in bacteria occur with high accuracy, co-ordination and regulation. In Escherichia coli (E. coli), DnaA protein initiates and regulates the process of chromosomal DNA replication [1,2]. Basically, it binds to the origin of replication (oriC) and initiates formation of the DNA replication initiation complex. Previously, several studies have been performed with DnaA mutants in an attempt to decipher the mechanism(s) of chromosome replication and cell division [3–5]. One such mutant is DnaAcos, a cold sensitive DnaA mutant that carries four amino acid substitutions. It induces excessive initiation from E. coli oriC at 30° C and thus, causes over-replication of chromosomal DNA. Consequently, the process of cell division is compromised in these cells which undergo excessive karyokinesis without cytokinesis and attain a filamentous morphology [1,6,7].

A multi-copy suppressor gene ceda, that encodes a cell division activator protein (CedA), is expressed in dnaAcos mutants and interestingly, its expression controls the cell division. It is an 80 amino acids long DNA-binding protein. Katayama et al. reported that CedA over-expression in DnaAcos mutants started septation, cell division and regular colony formation and thus, inhibited the formation of filamentous morphology at 30° C; however, chromosomal DNA over-replication continued [1]. It is evident from their study that though CedA is important for the initiation of cell division, it does not inhibit dnaAcos to bind at oriC to initiate chromosomal replication. CedA was also identified as one of the components of RNA polymerase complex in E. coli [8]. So, it becomes interesting to decipher how CedA in case of DnaAcos mutants maintains a balance between two entirely integrated events, cell division and chromosomal replication.

Protein–protein interactions are crucial for understanding structural and functional organization of a cell. Their identification helps us in decoding exact physiological function(s) of a specific protein and its binding partners. In addition, interaction network analysis plays a decisive role in exposing underlying mechanism(s) of various related biological processes. Thus, this study was designed to identify functional partners of CedA as a first step to reveal its putative role in cell division associated events. Here, we have cloned, expressed and purified E. coli CedA and then, identified its binding proteins by pull down assay followed by mass spectrometric analysis.

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2. Materials and methods

The experimental set up is shown in Fig. 1.

2.1. Cloning, expression and purification of CedA

The cedA gene (NCBI Ref. Seq. NC_000913.3) was cloned with Ndel and BamH1 restriction sites using the following primer set:

Forward primer: 5'CTTATCATATGGCTTTAGGAAGCC
Reverse primer: 5'ATACTGGATCCTTACTCAGTCACTTCC3'

After PCR amplification and digestion with restriction enzymes, the gene was ligated into pET28a vector using T4 DNA ligase (Thermo Fisher Scientific, United States). CedA clone was then transformed into BL21-Gold (DE3) E. coli expression host. The transformed cells were cultured in LB media (HiMedia, India) containing 50 µg/ml kanamycin and incubated at 37 °C with continuous shaking. Cells were induced with 1 mM IPTG (Sigma-Aldrich, United States) when absorbance at 595 nm was about 0.6. After four hours of induction, cells were harvested by centrifugation and cell lysis was performed by sonication. CedA expression in cell lysate was checked by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and it was further processed for its purification. His6-tagged CedA was isolated by affinity chromatography using Ni-NTA-Agarose beads under native conditions following manufacturer’s recommendations (Qiagen, Germany) and purified CedA was obtained after buffer exchange using PD-10 desalting column (GE Healthcare, Sweden).

2.2. Electrophoresis and mass spectrometry

Purified CedA collected after buffer exchange was run on 15% SDS-PAGE [9]. Its intact mass was determined by UltraflexXTreme MALDI-TOF/TOF Mass Spectrometer (Bruker Daltonik, Germany) using sinapinic acid matrix. Further, its identity was confirmed by MALDI-TOF MS analysis as described earlier [10]. Briefly, single band observed on gel was excised manually and in-gel trypsin digested. The digested peptides were mixed with matrix solution and spotted on MALDI target plate. Generated mass spectra were searched against Swissprot databases for peptide mass fingerprinting using Mascot search engine (Matrix Sciences, UK).

2.3. CedA-binding proteins

2.3.1. Isolation by pull down assay

E. coli CedA-binding proteins (CBPs) were isolated by pull down assay using CedA as a bait. His6-tagged CedA was mixed with Ni-NTA-Agarose beads and incubated at 4 °C for 2 hr to allow it to bind with the beads. These beads were then transferred into a column and washed twice with assay buffer (100 mM sodium phosphate, 50 mM sodium chloride and 10 mM imidazole buffer, pH 8.0). E.coli cell lysate diluted with assay buffer was loaded onto the column, followed by washing with assay buffer containing 50 mM imidazole. Finally, CBPs were eluted with assay buffer containing 250 mM imidazole.

2.3.2. Identification by mass spectrometry

Mass spectrometric identification of CBPs was done by two methods, MALDI-TOF MS and LC–MS/MS. In first set up, CBPs isolated in previous step were separated on 15% SDS-PAGE, protein bands were manually excised, trypsin digested and identified by MALDI-TOF MS analysis. While in second set up, CBPs were identified by LC–MS/MS. Protein bands on gel were excised, cut into small pieces, pooled and trypsin digested. Extraction buffer (1:2 (v/v) 5% formic acid/acetonitrile) was added to digested peptides, vortexed and incubated for 30 min at 37 °C. Extracted peptide samples were desalted using C18 tips (Pierce, Thermo Fisher Scientific, United States) according to manufacturer’s protocol. LC–MS/MS analysis was performed on Easy-nLC II (Thermo Scientific, United States) connected to an ESI mass spectrometer (amaZon SL, Bruker Daltonics). Released peptides were separated by a reversed-phase column (10 cm × 75 µm, 3 µm EASY-Column) using acetonitrile gradient containing 0.1% formic acid in the mobile phase at a flow rate of 0.3 µl/min for 55 min. Peptides generated were analyzed using Compass HyStar 3.2, Data Analysis 4.1 and BioTools 3.2 software. For identification, MS/MS ion search was performed using Mascot search engine (parameters: monoisotopic mass values, mass tolerance ±0.5 Da and maximum missed cleavage = 1).

3. Results

3.1. Purification and identification of CedA

CedA was successfully cloned, expressed and purified as described in methodology section. Its clone was confirmed by nucleotide sequencing. The purification, at each step, was monitored by electrophoresis (Fig. 2). Highly purified CedA was obtained through Ni-NTA-Agarose affinity chromatography and PD-10 buffer exchange, as evident from a single protein band observed on SDS-PAGE gel (Fig. 2D). The intact mass of purified CedA was determined 12.275 kDa by MALDI-TOF (Fig. 3). Further, its identity was confirmed by mass spectrometric analysis. Peptide spectra searched against Swiss-Prot matched to CedA from E.coli with a significant mascot score, 69 (Fig. 4).

3.2. CedA-binding proteins

CBPs isolated by pull down assay using His6-tagged CedA as bait were separated by SDS-PAGE (Fig. 5) and identified by MALDI-TOF MS and LC–MS/MS. CedA and its three functional partners, viz. pyruvate dehydrogenase E1 component (PDHAE1, band 2 on gel, Fig. 5), chaperone protein DnaK (DNAK, band 3) and 50S ribosomal protein L2 (RL2, band 6) were identified by MALDI-TOF MS (Table 1). On the other hand, a total of nine CBPs were identified by LC–MS/MS including the proteins identified by MALDI-TOF MS. Other proteins identified were outer membrane lipoprotein Lpp (LPP), DNA directed RNA polymerase subunit beta (RPOB), glucose-6-phosphate 1-dehydrogenase (G6PD), 50S.
Purified Ni-NTA lysate, Unbound PCR, amino-transferase 4.

Fig. 2. Cloning and purification of CedA. (A) CedA clone confirmation after colony PCR, Lanes- 1: DNA ladder, 2–7: colony PCR products; (B) CedA expression in cell lysate, Lanes- 1: Protein molecular weight markers, 3: supernatant; 5: Pellet; (C) Ni-NTA affinity chromatography, Lanes- 1: Protein molecular weight markers, 2: Unbound fraction, 3: Washing, 4–8: Elution fractions; (D) Purified CedA fractions collected after PD-10 desalting, Lanes- 1: Protein molecular weight markers, 2–4: Purified CedA.

ribosomal protein L3 (RL3), glucoseamine-fructose-6-phosphate amino-transferase (GL3) and an uncharacterized protein ybcj (YBCJ) (Table 1).

4. Discussion

CedA is a ~10 kDa DNA-binding E. coli protein with reported homologues in other Gram-negative enterobacteria including Shigella flexneri and Salmonella typhimurium. The NMR determined CedA C-terminal domain structure is quite similar to that of other known DNA-binding proteins, such as N-terminal DNA-binding domains of λ-integrase and Tn916 integrase proteins and it binds to dsDNA through a β-sheet structure [7]. Previous studies have suggested a putative role of CedA in the regulation of cell division; however, the mechanism via which it regulates the process is still unknown [1,6,7]. It was reported that CedA activated cell division inhibited by chromosomal DNA over-replication in DnaA- cos cells [1]. Lee et al. identified RNA polymerase complex in E. coli using affinity isolation and mass spectrometric analysis and CedA was identified as one of the components of the complex along with several other proteins, such as RpoD, DnaK, NusA, RpoN, DnaJ, RpoS, NusG, etc. [8]. Recently, Abe et al. have identified CedA domains/residues crucial for its binding with DNA and RNA polymerase [6]. These studies provide evidences supporting crucial, but unknown, role of CedA in cell cycle regulation. It is well known that protein interactions play crucial role in cell organization and it is essential to identify specific functional partners of a protein in fully understand its role in a biological process. Thus, we performed this study and identified nine CBPs in E. coli by affinity separation followed by MS analysis, including PDHA1, RL2, DNAK, LPP, RPOB, G6PD, GLMS, RL3 and YBCJ (Table 1).

Binding of CedA with a diverse set of proteins in E. coli suggests that it has a multifaceted role. Manual clustering of CBPs into functional categories reveals that seven of them are associated with energy metabolic pathways (PDHA1, G6PD and GLMS) and transcription (RPOB, RL2, RL3 and YBCJ). Pyruvate dehydrogenase complex converts pyruvate into acetyl-CoA and CO2 and links glycolysis to TCA cycle. This nuclear-encoded multi-enzyme complex consists of multiple copies of three enzyme components (E1, E2 and E3). The E1 component (PDHA1), a hetero-tetramer having two α and two β subunits, plays the key role in the conversion of pyruvate [11,12]. G6PD is an important rate limiting enzyme of pentose phosphate pathway, which is involved in the first step of d-ribulose 5-phosphate synthesis from d-glucose 6-phosphate where it catalyzes the oxidation of glucose 6-phosphate. In addition, it also protects E. coli against the oxidative stress [13,14]. GLMS, also known as glutamine: fructose-6-phosphate aminotransferase, is the first enzyme of hexoseamine biosynthesis pathway. It controls the flux of glucose and converts fructose 6-phosphate into glucosamine 6-phosphate [15,16]. RL2, RL3 and YBCJ are RNA binding proteins. RL2 and RL3 are known to participate in assembly of bacterial ribosomal subunits, while no conclusive information is available for YBCJ function [17–19]. RPOB is DNA dependent RNA polymerase. It is basically one of the five units that form

Fig. 3. Intact mass of purified CedA (~12 kDa) as determined by MALDI-TOF.
RNA polymerase enzyme, which regulates the bacterial transcriptional process [20,21]. CedA interaction with these CBPs hints about its distinctive role(s) in cell cycle regulation as well as in energy metabolism, though unknown to date. DNAK and LPP were other important CBPs identified and we consider that their binding is associated with the septum formation and cell division in E. coli specifically during chromosomal DNA over-replication. DNAK is a multifunctional chaperone of highly conserved HSP70 family which assists in protein folding, disaggregation and remodeling of protein complexes. DNAK interacts with a large number of proteins to assist them and mutations induced in DNAK cause several cellular defects in E. coli [22]. Here, we guess that DNAK binds with CedA and assists in the regulation of cell division during chromosomal DNA over-replication. However, it’s a mere assumption and experimental evidences are required to confirm it. LPP is a protein required for maintaining structural and functional integrity of bacterial cell envelope [23,24]. As mentioned earlier that over-expression of CedA in E. coli ceases the filamentous structure formation in DnaAcos cells, its interaction with LPP is not surprising. It is quite believable that CedA interacts with LPP to regain regular cell morphology during chromosomal DNA over-replication.

A restricted search to experimentally validated interactions over STRING database version 10.5 [25] retrieved only two functional partners of CedA- RNA polymerase subunits rpoC and rpoZ (Fig. 6A). Next, we performed network analysis of CedA and CBPs- including those identified in our study as well as rpoC and rpoZ (Fig. 6B). A weak network was observed as only 6 out of 12 proteins given as input were connected through the constructed network. This was expected as very limited experimental evidences are available for CedA and its interactome. The major nodes were RPOB, rpoC, rpoZ and DNAK and each of them had four connections. Our findings will add new nodes to the network and hence, a strong network can be expected that, in turn, will be crucial for better understanding of CedA associated processes.

### Table 1

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**Fig. 4.** Mass spectra of trypsin digested CedA obtained by MALDI-TOF MS analysis. (Inset) Confirmation of purified CedA by peptide mass fingerprinting followed by database search.
validation through in-vitro and in-vivo experiments. Once validated, these findings would be crucial to decipher exact mechanism of Ceda mediated cell division during chromosomal DNA over-replication.

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