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A CELL DIVISION INHIBITOR SULA OF *ESCHERICHIA COLI* DIRECTLY INTERACTS WITH FTSZ THROUGH GTP HYDROLYSIS

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Summary *E. coli* SulA is an SOS-inducible protein that inhibits cell division. FtsZ is a protein that plays a central role in bacterial cell division. Using purified SulA protein that was fused to the maltose binding protein, we demonstrate in vitro that SulA interacts with FtsZ to form a stable complex. The reaction requires GTP and Mg ion. GDP and GTPγS cannot substitute for GTP, which suggests that hydrolysis of GTP is required for the reaction. The complex is formed in a molar ratio of approximately one to one of the two proteins. It is likely that the complex formation represents the in vivo mechanism by which SulA inhibits cell division.

The FtsZ protein plays a central role in bacterial cell division and is one of the earliest factors involved in the septum synthesis (1, 2). Immunochemical studies defined the localization of FtsZ during the division cycle. It forms a ring-like structure at the division site prior to invagination of the cell wall (3). The purified FtsZ protein displays binding activities for GTP and GDP, and GTPase activity (4-6). The putative binding domain for GTP contains an amino acid sequence that is highly conserved among eukaryotic tubulins (5). Like tubulins, FtsZ assembles upon incubation with GTP in vitro into filamentous structure (7, 8) that may be comparable to the ring-like structure formed in vivo. However, the role of GTPase activity of FtsZ in septum formation is not clear. GDP can substitute for GTP in the in vitro formation of the filamentous structure (7). Furthermore, FtsZ mutants which do not show detectable GTPase activity are able to support cell division and growth (9).

DNA damage in bacteria results in induction of at least 15 genes named SOS genes, which include those involved in DNA repair (for a review, see reference 10). One of these genes, *sulA*, is a cell-division inhibitor and prevents premature segregation of the damaged DNA into daughter cells during the DNA repair process (11-13). Genetic evidences suggest that the target of SulA protein is FtsZ, because a class of mutations in *ftsZ* (*Rsa*, *sulB*, or *sfiB*) that confer resistance to overproduction of SulA has been isolated (11, 14, 15). Interaction of SulA and FtsZ was further suggested by the observation that the half-life of SulA molecules in maxicells was increased by co-expression of FtsZ (15, 16). In

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a recent study, induction of SulA blocked the formation of ring-like structure of FtsZ (17). On the other hand, it has not been determined whether FtsZ and SulA interact directly with each other, and if they do whether the interaction requires any other factor(s). In this paper, we show a direct interaction between purified FtsZ and SulA in vitro. The reaction requires hydrolysis of GTP.

Materials and Methods

Bacterial strains: *E. coli* strains W3110 (wild type) and GC4276 (carrying *sfiB114* mutation in *ftsZ* gene) were obtained from A. Nishimura of the Stock Center at National Institute of Genetics, Japan. DH5 carrying pMAL-p-SulA(18) and BL21(DE3) carrying pET3Z+ (4) were provided by K. Ishii of Kyushu Institute of Technology and D. RayChaudhuri of Tufts University, respectively. AB1899 (*lon*$^-$) was used for transformation with pMAL-p-SulA, pSulA5, and pACYC184 and its derivatives.

Plasmid constructions: The *ftsZ* genes, wild-type and *sfiB114* mutant, were subcloned between the *Sal*I and *Hind*III sites of pACYC184 (19) from *E. coli* W3110 and GC4276, respectively, by use of polymerase chain reaction (PCR). The two primers used for PCR were 5'-GAAGGTCGAC GCCAACAAGG GGTTAAACAT CACCTG which introduced a *Sal*I site (underlined) starting from the 318th codon of *ftsA* gene and 5'-CAAAGCTTAA TCAGCTTGCT TACGCAGGAA TGCTGG which introduced a *Hind*III site (underlined) 9 bases downstream of the *ftsZ* stop codon. The plasmids thus obtained were named pACYCZ+ (carrying wild-type *ftsZ*) and pACYCZ114 (carrying *sfiB114* mutation), respectively. pSulA5 was constructed by subcloning the wild-type *sulA* gene under the *lacUV5* promoter in pB10a (20) which was provided by H. Nagai of National Institute of Genetics, Japan.

Chemical reagents and enzymes: Restriction enzymes, Taq DNA polymerase and T4 DNA ligase were obtained from Takara Co., Ltd. ^{32}P-labeled guanosine triphosphate was obtained from DuPont.

Wild-type FtsZ protein was prepared from *E. coli* BL21(DE3) carrying pET3Z+, and purified to near homogeneity by the procedure described previously (4). SulA fused to maltose binding protein (MBP-SulA) was prepared from *E. coli* DH5 carrying pMAL-p-SulA plasmid, and purified to near homogeneity through the procedure described by Sonezaki et al (18).

Complex formation between MBP-SulA and FtsZ: 100pmols each of purified MBP-SulA and FtsZ were incubated in a 500ml reaction mixture containing 20mM Tris-HCl (pH7.5), 1mM DTT, 10mM $MgCl_2$, 5mM GTP, and 20mM KCl at 37°C for 30min. The reaction mixture was applied onto an amylose resin column (New England Biolabs) of 50ml bed volume, pre-equilibrated with the reaction buffer. After the pass through fraction was collected, the column was washed with 500ml of the reaction buffer, and the last 50ml from the column was pooled as the washed fraction. Protein(s) bound to the amylose resin was eluted with the reaction buffer containing 10mM maltose. After addition of SDS-loading buffer (21), the samples were boiled for 5min and applied onto 10% SDS-polyacrylamide gel and electrophoresed. The gel was stained with coomassie brilliant blue R250.

GTPase assay: A 50ml reaction mixture contained 5mM (α-^{32}P) GTP (20mCi/mmol), 20mM Tris-HCl (pH7.5), 1mM DTT, 10mM $MgCl_2$, 20mM KCl, and 1mM protein. The reaction was incubated at 37°C, and aliquots were taken out at various times and subjected to thin layer chromatography on polyethyleneimine cellulose (Merck) in 1.5M LiCl. The amount of GTP converted to GDP was determined from radioactivity of each spot.

Results

MBP-SulA fusion protein is active as a cell division inhibitor in vivo: SulA is a cell division inhibitor, and its constitutive synthesis blocks colony formation (15). As shown in Fig. 1, *E. coli* cells harboring pSulA5, which carries the wild-type *sulA* gene under

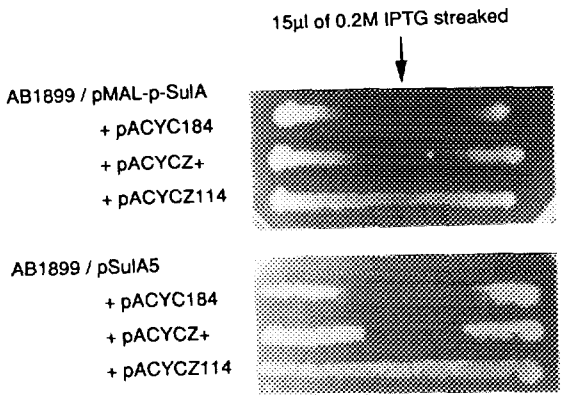


Fig. 1. *Effect of induction of MBP-SulA fusion protein on growth of E. coli cells carrying ftsZ plasmids.* The cells were cross-streaked against 15ml of 0.2M IPTG on LB plates and incubated at 37°C overnight.

lacUV5 promoter, fail to grow in the presence of IPTG. This growth inhibition is depressed by overproduction of mutant FtsZ (SfiB114) which is refractory to the SulA action, but not by that of wild-type FtsZ. pMAL-p-SulA carries a fused gene in which *sulA* is fused to the carboxy terminus of *malE* that codes for maltose binding protein (MBP). As shown in Fig. 1, growth of cells carrying pMAL-p-SulA was also inhibited by IPTG, and the inhibition was depressed by overproduction of the mutant FtsZ (SfiB114), but not by that of the wild-type FtsZ (Fig. 1). Moreover, the wild-type cells carrying pMAL-p-SulA formed filaments upon induction with IPTG, but *sfiB114* mutant cells carrying the same plasmid did not form filaments (data not shown, see also ref. 18). These results strongly suggest that the fused gene product MBP-SulA is as active as the wild-type SulA in inhibiting cell division.

FtsZ directly interacts with MBP-SulA in vitro: The direct interaction of FtsZ and SulA was examined in vitro using purified proteins. Since MBP-SulA binds to amylose resin and is eluted from it with 10mM maltose, it is expected that FtsZ is co-eluted with MBP-SulA if a stable complex is formed between the two proteins prior to application to the resin. The results shown in Fig. 2 demonstrate that FtsZ did form a complex with MBP-SulA during the incubation at 37°C for 30min. If MBP-SulA was omitted, or if MBP-SulA is replaced by MBP, FtsZ was not detected in the eltuate (Fig. 2). These results indicate that a complex was formed by direct interaction of FtsZ and SulA.

The experiment in Fig. 2 was carried out in the presence of 5mM GTP and 10mM Mg ion. To determine if any co-factor(s) are required for the complex formation, effects of Mg and Ca ions, and various nucleotides on the complex formation were tested. The results in Fig. 3A show that the complex formation required both GTP and Mg ion. Ca ion could not substitute for Mg ion. The results in Fig. 3B indicate that various nucleotides, ATP, CTP, UTP, ADP, GDP, and GTPγS all failed to substitute for GTP.

Fig. 4 shows the time course of complex formation during the incubation. After incubation at 37°C for different times, the reaction mixture was applied to amylose resin and the eluted FtsZ was measured. As shown, the complex formation proceeded

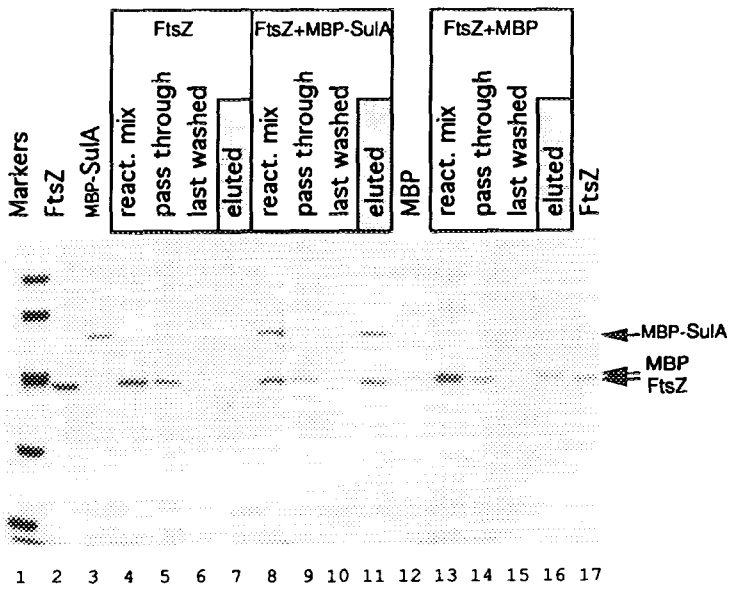


Fig. 2. Complex formation between FtsZ and MBP-SulA as demonstrated by elution with maltose from amylose resin. FtsZ was incubated with (lanes 8-11) or without (lanes 4-7) MBP-SulA, or with 100pmols of MBP (lanes 13-16) at 37°C for 30min in a buffer containing 5mM GTP and 10mM $MgCl_2$, and applied to amylose resin columns as described in Materials and Methods. Samples were collected, electrophoresed on 10% SDS-PAGE, and stained with coomassie blue. Lane 1, molecular weight markers; lanes 2 and 17, purified FtsZ; lane 3, purified MBP-SulA; and lane 12, purified MBP.

progressively with time until the reaction was completed after 20min of incubation. Fig. 5 shows the effect of temperature on the complex formation. After incubation of 20min, the complex formation was nearly complete at 37°C. At 20°C, the complex formation was about 13%, and it was less than 3% at 4°C (Fig. 5). Amount of GTP hydrolysis in each reaction is also shown in Fig. 5, and good correlation between the complex formation and GTP hydrolysis was observed.

In the experiment shown in Fig. 6, the amount of FtsZ that formed the complex was measured as a function of the amount of FtsZ added to the reaction. As seen, amount of FtsZ in the complex was proportional to the amount of FtsZ added, and was saturated when the molar ratio of FtsZ to MBP-SulA reached about one to one (Fig. 6). This suggests that the complex was formed in a molar ratio of approximately one to one of the two proteins.

SulA does not affect GTPase activity of FtsZ: Since the results described above indicated that the complex formation of FtsZ and SulA required GTP hydrolysis, and since FtsZ is known to have GTPase activity in vitro (4-6), we tested whether SulA affects the GTPase activity of FtsZ through the complex formation. The result shown in Fig. 7 indicates that the GTPase activity was not affected by addition of MBP-SulA under the conditions where most of FtsZ formed the complex with MBP-SulA. MBP-SulA itself did not hydrolyze any detectable amount of GTP. Furthermore, specific GTPase activity of the complex that was eluted from amylose resin was identical to that of FtsZ alone (data not

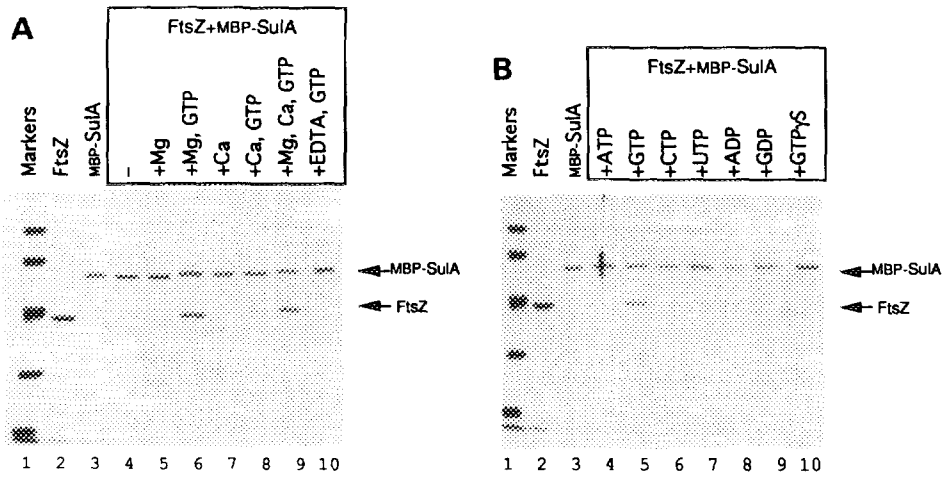
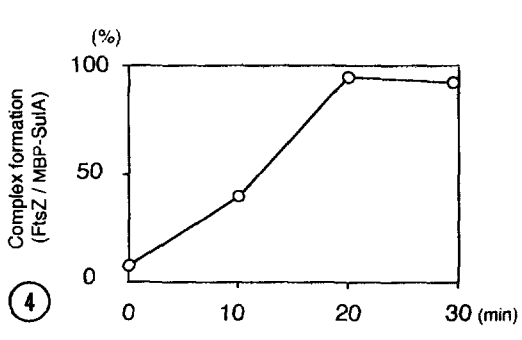


Fig. 3. Requirement for complex formation between FtsZ and MBP-SulA. FtsZ and MBP-SulA were incubated at 37°C for 30min with or without 10mM $MgCl_2$, 10mM $CaCl_2$, or 10mM GTP as indicated (panel A), or with 10mM of indicated nucleotides (panel B). The samples were applied to amylose resin, and the eluates with 10mM maltose were electrophoresed.

shown). These results indicate that the GTPase activity of FtsZ is not affected by formation of the complex with SulA.

Discussion

The experiments described above indicate direct interaction of FtsZ and SulA in vitro. The complex formed appears quite stable, since FtsZ was not eluted off the complex



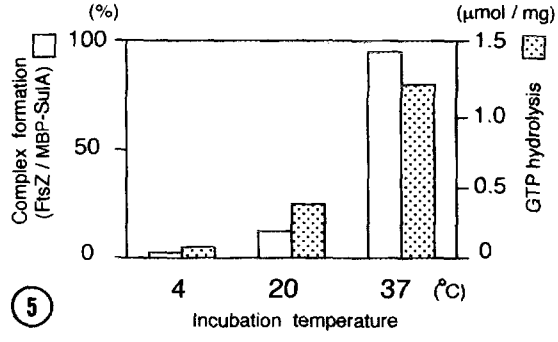
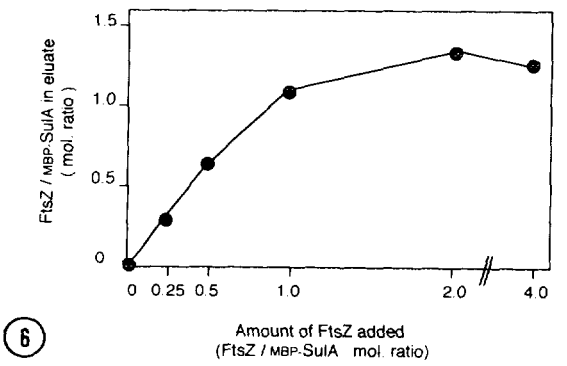


Fig. 4. Time course of the complex formation. FtsZ and MBP-SulA were incubated at 37°C for indicated periods. The amount of complex formed was determined by molar ratio of FtsZ to MBP-SulA in the eluate from amylose resin.

Fig. 5. Effect of temperature on the complex formation and GTP hydrolysis. The complex formation was carried out at the indicated temperature for 20min. After the reaction, the amounts of complex formed and GTP hydrolysis were measured as described in Materials and Methods.



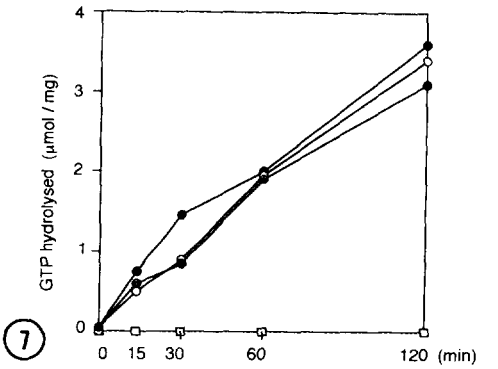


Fig. 6. Stoichiometry of the complex formation. After incubation of indicated amounts of FtsZ with 100pmols of MBP-SulA, the amounts of FtsZ and MBP-SulA in the eluate from amylose resin were measured.

Fig. 7. GTPase activity of FtsZ upon addition of MBP-SulA. GTP hydrolysis was measured in reactions containing 1mM FtsZ(○), 1mM each of FtsZ and MBP-SulA(●), 1mM FtsZ and 2mM MBP-SulA(◉), or 1mM MBP-SulA(□).

that was bound to the amylose resin by washing with a buffer containing 2M KCl (data not shown). The complex formation is not due to covalent interactions because the complex resolves into the components on SDS-PAGE. The complex formation specifically requires GTP. The fact that GTPγS cannot substitute for GTP strongly suggests that the reaction requires hydrolysis of GTP. No other protein factors such as molecular chaperone(s) are required. About 50 molecules of GTP was hydrolyzed in 30min per FtsZ molecule that formed a stable complex with MBP-SulA under the experimental conditions (Fig. 4 and 5). The GTPase activity is due to FtsZ alone, and is not affected by the addition of SulA. Possible roles of GTP hydrolysis in the complex formation include: 1) the complex formation may require the high energy produced by hydrolysis of phosphodiester bond of GTP, 2) a conformational change of FtsZ through the GTP hydrolysis may be needed to accept SulA to form the complex. Recently, Dai et al. reported that all of the six FtsZ mutants tested, which conferred on cells resistance to overproduction of SulA, showed reduced GTPase activity (9). This is consistent with our in vitro result which indicate that the complex formation requires hydrolysis of GTP.

E. coli possesses another division inhibitor, MinCD, that makes the polar sites of the cell inaccessible to the division machinery (22). All of the FtsZ mutants resistant to SulA were reported to be also resistant to MinCD (9). It is conceivable that MinCD may interact with FtsZ in the same way as SulA does. However, no similarities in amino acid sequence have been revealed between SulA and MinCD. In vitro studies similar to the present study using MinCD and FtsZ may shed light on this problem.

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