Trigger Factor Interacts with DnaA Protein to Stimulate its Interaction with DnaA Box

Yong Sun Lee†, June Lee, Hak Kyun Kim†, Sukhyun Kang†, Joo Seok Han, Jae Bum Kim and Deog Su Hwang*

Institute of Molecular Biology and Genetics, School of Biological Sciences, Seoul National University, Seoul 151-742, Korea

Key Words:
DnaA protein
Trigger factor
DnaA box
Chaperone

While screening proteins that interact with DnaA protein, the initiator protein for *Escherichia coli* chromosomal DNA replication, we found a 52-kD sized protein which bound to DnaA protein in a salt-dependent manner. This protein was identified as trigger factor, a ribosome-associated peptidyl-prolyl-*cis/trans* isomerase with chaperone activity. Trigger factor was overproduced and purified to near homogeneity, and its effect on the function of DnaA protein was examined. Enhanced binding of DnaA protein to DnaA box with no apparent supershift in the gel-shift experiments suggested that trigger factor, by virtue of its chaperone activity, exerts a change on DnaA protein thus increasing its binding affinity for DnaA box.

DnaA protein is essential for the initiation of *Escherichia coli* chromosomal DNA replication *in vivo* and *in vitro* (Kornberg and Baker, 1992; Messer et al., 1996; Hirata et al., 1970; Fuller et al., 1983; Bramhill et al., 1988). The binding of 20 to 30 molecules of DnaA protein to *oriC* (origin of chromosomal DNA replication) containing 5 DnaA boxes (or 9-mers), which are recognized by DnaA protein, forms an initial complex for the initiation of *in vitro oriC* plasmid DNA replication (Bramhill et al., 1988a; Bramhill et al. 1988b; Crooke et al., 1993).

DnaA protein binds ATP, with a K_D of 0.03 μM, and other nucleotides (Sekimizu et al., 1987). Whereas the ADP or AMP-bound form of DnaA protein is not active in the *oriC* plasmid DNA replication, the ATP-bound form is active. Phospholipid exchanges ADP in the inactive form of DnaA protein with ATP. Also, ATP stabilizes DnaA protein. DnaA protein appears to exist as monomeric and aggregated forms in *E. coli* (Fuller et al., 1983). The inactive and aggregated form of DnaA protein containing phospholipid was converted in an ATP-dependent manner to active monomeric forms by phospholipase or DnaK protein (Hwang, et al., 1990). *In vivo,* DnaA protein predominantly took on the inactive ADP-form, in the presence of the β subunit of DNA polymerase III holoenzyme (Katayama, et al., 1998).

The level of DnaA protein plays an important role in initiation of chromosomal DNA replication, as indicated by overinitiation at *oriC* in response to increased levels of DnaA protein (Meyenburg, et al., 1987; Atlung, et al., 1985; Skarstad, et al., 1989) and transcriptional fluctuation of the *dnaA* gene during the cell cycle (Theisen, et al., 1993). However, other results suggest that the intracellular level of DnaA protein is invariant during cell cycle (Sakakibara, et al., 1982) and cyclic variation of the *dnaA* expression is not necessary for the cell cycle dependent initiation of chromosomal DNA replication (LΦbner-Olesen, et al., 1989). *In vivo* footprinting experiments (Samitt, et al., 1989; Cassler, et al., 1995) indicated that DnaA protein stay bound to the DnaA box R1, R2 and R4 in *oriC* throughout cell cycle, whereas R3 was bound by DnaA protein only at the time of initiation.

Considering above results, it is possible some factor(s) may convert and activate DnaA protein during initiation of chromosomal replication. Alternatively, a factor may load DnaA protein onto DnaA box R3 intiating origin firing. To search for such possible factors, we screened and identified some proteins that interact with DnaA proteins. In this paper, one of the DnaA-interacting proteins, trigger factor, was shown to interact with DnaA protein and increase its binding to DnaA box.

Trigger factor was originally identified by its ability to promote *in vitro* translocation of proOmpA (the precursor of the outer membrane protein A) into membrane vesicles (Crooke, et al., 1987) However, subsequent investigation showed that cells depleted of trigger factor had no proOmpA secretion defect (Guthrie, et al., 1990). Rather, trigger factor depletion or overproduction resulted in filamentation phenotype, caused by a cell division defect.

Currently, trigger factor is known as a ribosome-associated peptidyl-prolyl-*cis/trans* isomerase (PPlase),

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*To whom correspondence should be addressed.
Tel: 82-2-880-7524, Fax: 82-2-874-1206
E-mail: dshwang@plaza.snu.ac.kr

†Supported by BK21 Research Fellowship from Korean Ministry of Education

which assists the folding of nascent polypeptide chains as they emerge from the ribosome (Hesterkamp, et al., 1996; Stoller, et al., 1995). Trigger factor also assists protein refolding and prevents protein aggregation (Huang, et al., 2000; Nishihara, et al., 2000). The importance of trigger factor in protein folding is underscored by the observation that synthetic lethality was caused by the combined deletion of *tig* gene encoding trigger factor and *dnaK* gene encoding Hsp70 homologue chaperone DnaK (Deuerling, et al., 1999; Teter, et al., 1999). In addition, trigger factor cooperates with another chaperone GroEL, as shown by its activity to promote the GroEL-dependent degradation of polypeptides *in vivo* (Kandror, et al., 1995). Unlike most chaperones, which are heat-shock proteins, intracellular levels of trigger factor increased under cold shock and enhanced viability of *E. coli* at low temperatures (Kandror, et al., 1997). These observations and the following presented data suggest that trigger factor, by virtue of its chaperone function, may convert DnaA protein active for binding to DnaA box.

Materials and Methods

Preparation of hexahistidine-tagged DnaA protein

dnaA coding region was PCR-amplified from plasmid pdnaA/dnaN (Burgers, et al., 1981) and subcloned into *Bam*HI/*Hin*dIII restriction sites of pBluescript(+) (Stratagene Corp.) to generate pBSADCHIS, from which a 1404-bp *Nde*I/*Hin*dIII fragment was isolated and inserted to pET21b (Novagen). The resulting plasmid, pETADH1, encodes DnaA protein with a hexahistidine residue at the C-terminus. pETADH1 was transformed into *E. coli* strain BL21 (λDE3) pLysS (Novagen) for the expression of recombinant DnaA protein.

BL21 (λDE3) pLysS containing pETADH1 was grown at 37°C in LB medium with shaking at 200 rpm, to an OD at 595 nm of 0.5. IPTG was added up to 0.5 mM, and the cultures were further grown at 37°C for 2 hr. Cell harvesting and subsequent steps were as previously described (Hwang et al., 1988). Unless indicated, operations were performed at 4°C or less. The thawed lysate was centrifuged for 20 min at 35 krpm in a Beckman Ti45 rotor. The precipitate containing the majority of recombinant DnaA protein was resuspended in buffer A (25 mM HEPES-KOH (pH7.8), 10% glycerol), sonicated and centrifuged at 40 krpm for 20 min in a Beckman 100.3 rotor. The precipitate was solubilized in buffer A containing 6 M guanidine hydrochloride and 50 mM KCl. Insoluble material was removed by centrifugation at 40 krpm for 20 min in a Beckman 100.3 rotor and the supernatant (guanidine hydrochloride-solubilized fraction) was prepared for nickel-charged Chelating Sepharose.

Preparation of fraction II from E. coli

E. coli strain WBT13 (Thöny, et al., 1991) was grown in 300 liter fermentor at 37°C in 200 liters of terrific broth, harvested and lysed as previously described (Hwang et al., 1990) with minor modifications. The lysate was cleared by centrifugation at 35,000 rpm for 30 min in a Beckman Ti45 rotor. The supernatant (fraction I) was precipitated by addition of solid ammonium sulfate (0.37 g per ml of supernatant) with stirring. After an additional 30 min of stirring, precipitate was recovered by centrifugation at 35,000 rpm for 30 min in a Beckman Ti45 rotor, re-suspended with buffer A (fraction II), and dialyzed against buffer A to a conductivity equivalent to buffer A containing 50 mM KCl. The dialyzed fraction II was diluted to 2 mg/ml with the buffer A containing 20 mM imidazole and 50 mM KCl, and loaded on a DnaA-affinity column.

Preparation of DnaA-affinity column

25 ml of Chelating Sepharose Fast Flow (Pharmacia LKB) was charged with nickel according to the manufacturer's instructions and equilibrated with buffer A containing 6M guanidine hydrochloride and 50 mM KCl (equilibration buffer). 100 mg of guanidine hydrochloride-solubilized fraction, containing recombinant DnaA protein, was diluted with 100 ml of the equilibration buffer and loaded to the column. Hexahistidine tagged DnaA protein, immobilized on nickel, was then renatured by a linear gradient from 250 ml of equilibration buffer to the same volume of the buffer containing 0.1 mM ATP instead of guanidine hydrochloride. Then, the column was washed with buffer A containing 20 mM imidazole and 50 mM KCl to remove residual proteins. As a control column, the same volume of Chelating Sepharose Fast Flow was charged with nickel and equilibrated with buffer A containing 20 mM imidazole and 50 mM KCl.

Screening of DnaA-interacting proteins from fraction II through DnaA-affinity column

200 ml of Chelating Sepharose Fast Flow was charged with nickel and equilibrated with buffer A containing 20 mM imidazole and 50 mM KCl. First, 14 g of fraction II in a volume of 7 liters was loaded to this column. The flowthrough was collected and divided in half, one of which was applied to the DnaA-affinity column and the other was to the control column. The proteins retained in each column were washed out with the buffer containing 250 mM, 500 mM and 1M KCl, step by step. Elevation of imidazole concentration from 20 mM to 250 mM allowed hexahistidine tagged DnaA protein to be eluted from the DnaA-affinity column.

Partial purification of 52 kD protein and N-terminal amino acid sequencing

52 kD protein in the 250 mM KCl eluate from the DnaA-affinity column was partially purified through FastQ column

chromatography. 52 kD protein in FastQ fraction was resolved on SDS-polyacrylamide gel electrophoresis, transferred to a PVDF membrane, and submitted to Korea Basic Science Institute Seoul Branch for N-terminal amino acids sequencing. Based on the sequence, the 52 kD protein was identified as trigger factor by BLAST search in *E. coli* database.

Overexpression and purification of trigger factor

E. coli strain W3110 harboring pTIG2 (Guthrie and Wickner, 1990) was grown in 6 liters of Luria-Bertani (LB) media containing 25 μgml^{-1} of ampicillin to an OD at 595 nm of 0.4. L(+)-Arabinose was added to 0.2% to induce the overproduction of trigger factor. After 3 hrs, cells were harvested, resuspended in cell resuspension buffer (20 mM Tris-HCl (pH 7.8), 1 mM EDTA, 2.86 mM β-mercaptoethanol) to an OD at 595 nm of 150 and frozen in liquid nitrogen. Cell lysis and subsequent steps were as described previously (Hwang, et al., 1988), except 0.37 g/ml ammonium sulfate instead of 0.28 g/ml. Trigger factor was purified through Heparin-agarose column and FastS column chromatography.

Gel-shift assay with DnaA box

For the gel-shift assay with DnaA box, we designed a 30-bp oligomer containing the DnaA box R1 and flanking sequences in *oriC* (named as R1 30-mer). The gel-shift assay buffer is 20 mM HEPES-KOH (pH8.0), 60 mM KCl, 1 mM EDTA, 4 mM DTT, 10% glycerol, 0.1% Triton X-100, and 0.5 mg/ml BSA. (Sutton, et al., 1997; Schaper, et al., 1995). The fractions containing trigger factor were first incubated with 10 ng of DnaA protein in 10 μl of gel-shift assay buffer containing 81 μg of BSA and 3.6 mM ATP. After the incubation at 32°C for 12 min, we then added the 8 μl of DNA mixture containing 21.5 fmole of 5'-end labelled R1 30-mer and 1 μg of poly(dI)poly(dC) in gel-shift assay buffer, followed by further 12 min. incubation at 32°C. Then, the reactions were loaded onto 4.5% polyacrylamide gel and subjected to electrophoresis at 100 V for 1 h 15 min in 45 mM Tris-borate (pH 8.3) and 1 mM EDTA.

Results

Protein-affinity chromatography (Sharma, et al., 1997) is a method used to identify and purify proteins associated with a known polypeptide. We used this method to search for proteins which interact with DnaA protein (Fig. 1). Our system includes hexahistidine residue introduced into the C-terminus of DnaA protein and divalent cation such as nickel chelated on Sepharose resin. DnaA protein, immobilized on resin through stable linkage between hexahistidine and nickel, acts as a bait to select putative DnaA-interacting proteins from *E. coli* crude extract.

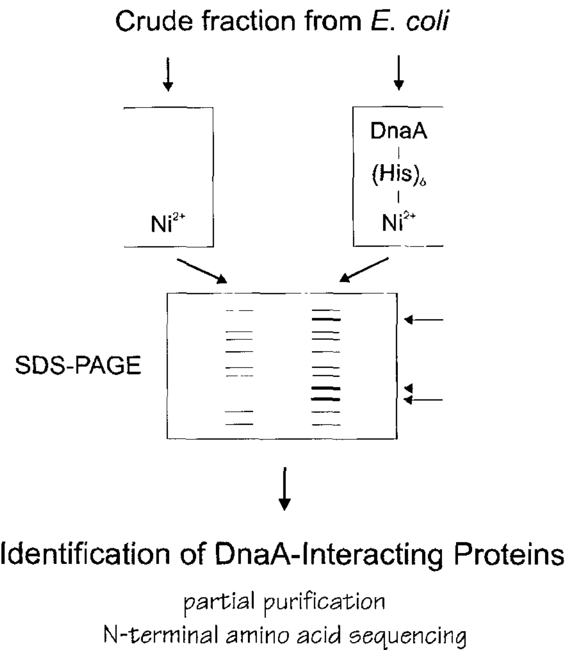

Fig. 1. Experimental scheme of screening putative proteins interacting with DnaA protein.

Crude fraction from *E. coli* was first loaded onto nickel charged Chelating Sepharose without DnaA protein, in order to remove proteins which interact with this column non-specifically. The flowthrough was then loaded onto the DnaA-affinity column under low concentration of salt, 50 mM KCl. Buffer with 250 mM KCl dissociated some of the bound proteins, which were analyzed by SDS-polyacrylamide gel electrophoresis. In each experiment, a parallel experiment was performed with no DnaA protein charged onto the column, as a control (Fig. 1). Some proteins preferentially bound the DnaA-affinity column (lane S in Fig. 2), compared to the nickel column (lane

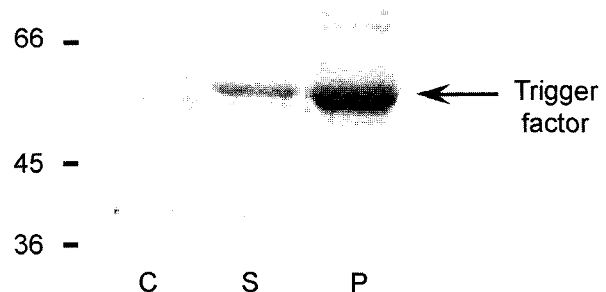

Fig. 2. Trigger factor binds to the immobilzed DnaA protein
Crude fraction from *E. coli* was loaded and eluted from the DnaA-affinity column as described in Materials and Methods. The eluates with buffer containing 250 mM KCl, as well as the partially purified trigger factor from the eluate, were analyzed on a 14% SDS-polyacrylamide gel. The band of trigger factor is indicated by the arrow in the right and molecular weights are marked in the left. S, 250 mM KCl eluate from DnaA-affinity column; C, 250 mM KCl eluate from nickel column; P, partially purified trigger factor from 250 mM KCl eluate from DnaA-affinity column.

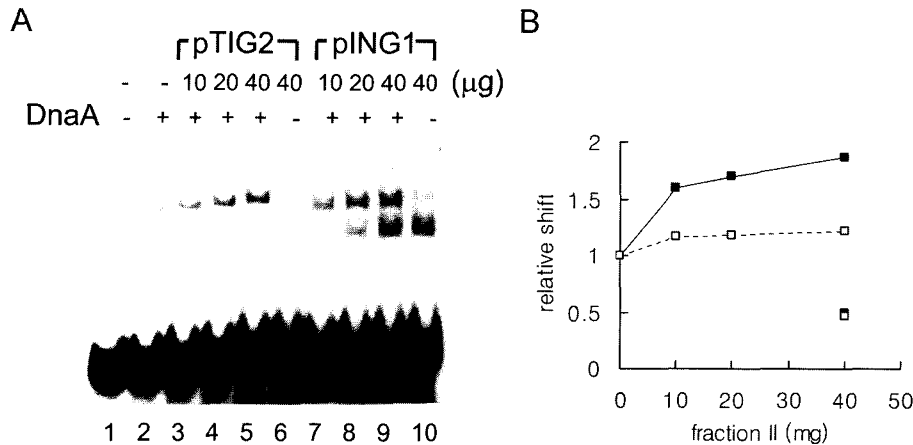

Fig. 3. Stimulation of DnaA protein binding to DnaA box by fraction with enriched trigger factor
Fraction II was prepared from *E. coli* strain W3110 harboring pTIG2 or corresponding vector pING1, after arabinose induction. Gel-shift assay was performed with 10 ng of DnaA protein and indicated amount of each fraction II. DnaA protein and each fraction II were first incubated for 12 min, followed by the addition of the DNA template (see Experimental procedures). After further incubation for 12 min, the reactions were loaded to 4.5% polyacrylamide gel. (A) Gel-shift assay with R1 30-mer was performed as described in Experimental procedures. (B) Radioactivities in the shifted bands were quantitated using FUJIX (BAS1000), normalized to the radioactivity in the shifted band only by 10 ng of DnaA protein, and expressed as "relative shift". These values were plotted against the amounts of fraction II.

C in Fig. 2). Other protein bands, visible in eluates from both columns, could be regarded as proteins interacting non-specifically with Sepharose matrix or nickel.

We focused on the 52 kD sized band from the DnaA-affinity column. The 52 kD protein was partially purified through FastQ column chromatography (lane P in Fig. 2) and subjected to determination of N-terminal amino acid sequence. This enabled us to identify the 52 kD protein in *E. coli* database, by taking advantage of BLAST program. The determined sequence (MQVSVETTQ) perfectly matched the N-terminal amino acid sequence of trigger factor (Guthrie, et al., 1990).

E. coli harboring the plasmid pTIG2 (Guthrie, et al., 1990) overproduces trigger factor upon the addition of arabinose. After arabinose induction, fraction II was prepared from the induced cells and added to the gel-shift assay reaction in order to examine the effect of trigger factor on the DnaA protein-DnaA box interaction (Fig. 3). DnaA protein bound to the 30-mer containing DnaA box R1 and shifted its migration (lane 2 in Fig. 3). As increasing amount of fraction II from the induced cell, which is shown on SDS-PAGE to contain enriched amount of trigger factor (data not shown), was added to the reaction, the binding of DnaA protein to the DnaA box gradually increased (lanes 3-5 in Fig. 3). By contrast, less effect was observed in the parallel control experiment using the origina vector pING1 (lanes 7-9 in Fig. 3). The bands shifted by addition of the vector fraction was caused by contaminating proteins rather than trigger factor. Therefore, trigger factor was assumed to stimulate the interaction between DnaA protein and DnaA box.

Next, we purified this stimulatory activity, presumably by trigger factor. Heparin-agarose and FastS column resulted in about 17% recovery with 9-fold purification (Table 1). The FastS fractions were subjected to SDS-polyacrylamide gel electrophoresis as well as gel-shift assays (Fig. 4). Comparison of these two profiles illustrated that the 52 kD protein is responsible for the stimulation. This result is consistent with the interpretation that trigger factor stimulates the binding of DnaA protein to the DnaA box.

Table 1. Purification table of trigger factor
Purification procedure is described in Experimental procedures. The activity of trigger factor was measured by gel-shift assay with 10 ng of DnaA protein. The radioactivity, measured by Phosphorimager, of the shifted band is normalized by the radioactivity by 10 ng of DnaA protein. The unit of trigger factor activity is expressed as net increase of the norma ized value

	Volume	Protein		Activity		Yield	Fold
	ml	mg/ml	mg	units	units/mg		
Fr I	88	30.2	2657.7				
Fr II	12	187.7	2252.3	133723	59.4	100.0	1.0
Heparinagarose	60	3.0	179.7	47676	265.3	35.7	4.5
FastS	20	2.3	45.4	23058	507.9	17.2	8.6

Discussion

A number of genetic and biochemical data suggest that DnaA protein interacts with several proteins including DnaB helicase, DnaK, GroE, RNA polymerase β subunit, τ, γ subunits of DNA polymerase III holoenzyme, thioredoxin and so on (reviewed in ref. Sharstad, et al., 1994). In these proteins, some chaperones affect the activity of DnaA protein. Amplification of *groEL* and *groES* genes, encoding GroEL chaperonin and its regulator GroES respectively, can suppress the *dnaA46* mutation (Jenkins, et al., 1986). Mutant forms of DnaA protein,

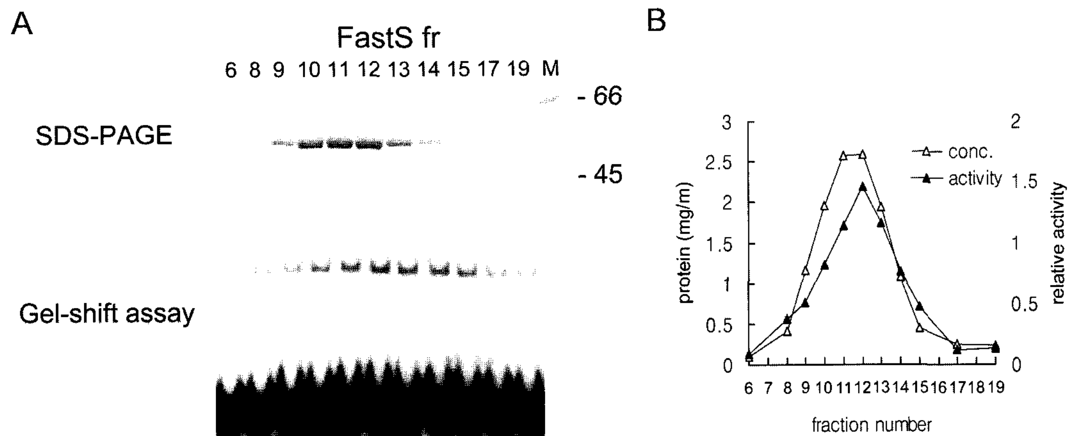

Fig. 4. Purified trigger factor stimulates DnaA protein binding to DnaA box
(A) Upper panel; FastS fractions, 2 μl of each, were subjected to 10% SDS-polyacrylamide gel electrophoresis. Fraction numbers are denoted on the top of each lane and molecular weights in the right. Lower panel; Gel-shift assay was performed with 10 ng of DnaA protein and 0.5 μl of MonoS fractions. The order of incubation was as described in the legend of Fig. 3. (B) The protein concentrations, as well as the activities in panel (A) were plotted against the fraction number.

DnaA5 and Dna46, which are inactive in *in vitro* replication system with purified enzymes, can be activated by DnaK and GrpE heat shock proteins (Hupp, et al., 1993). DnaK protein also can convert the inactive and aggregated form of DnaA protein to an active monomeric form (Hwang, et al. 1990). We showed in this report that trigger factor, a chaperone with PPlase activity, bound to DnaA protein immobilized on Chelating Sepharose matrix. Since DnaA protein was extracted from the insoluble fraction with guanidine hydrochloride prior to immobilization on the column matrix (Materials and Methods), the preparation of DnaA protein used in this system may contain a portion of DnaA protein which is aggregated or denatured and therefore inactive. Actually, the specific activity of this recombinant DnaA protein eluted from the column was about one tenth compared with that of the monomeric DnaA protein purified from soluble fractions (Hwang, et al., 1990), as determined by gel-shift assay with a DNA fragment containing the DnaA box and the flanking *dnaA* promoter region (Fuller, et al., 1981), and *in vitro* complementation assay (Lee and Hwang, 1997) using fraction II from the *dnaA* mutant strain (data not shown). It is possible trigger factor may recognize and bind to partially denatured and unfolded DnaA protein, considering that it associates with nascent polypeptides emerging from the ribosome.

Gel-shift assay results (Fig. 3 and 4) showed that trigger factor stimulated the interaction between DnaA protein and the DnaA box. A lack of supershifting indicates that trigger factor is not present in the protein-DNA complex. Trigger factor, by virtue of its chaperone function, may associate with inactive DnaA protein and convert it, thus activating it for interaction with DnaA box. Although trigger factor was shown to prevent aggregation of proteins (Hwang, et al., 2000; Nishihara, et al., 2000), it is not probable that trigger factor participated in the transition of DnaA protein from aggregated to monomeric form. In these gel-shift assays, we used monomeric DnaA protein isolated by gel-filtration column chromatography. Moreover, a similar extent of stimulation was observed with aggregated DnaA protein (data not shown). Alternatively, this stimulatory effect might be attributed to the PPlase activity of trigger factor. DnaA protein contains 21 proline residues, including three leucine-proline residues which can be preferentially catalyzed by trigger factor (Stoller, et al., 1995), however, this possibility could not be examined.

Overproduction or depletion of trigger factor *in vivo* resulted in a cell division defect (Guthrie, et al., 1990). However, this phenotype is probably not due to abnormal initiation of chromosomal DNA replication caused by the effect of trigger factor on DnaA protein, since filamentation of the trigger factor-overproducing strain could be suppressed by co-overproduction of the essential division gene, FtsZ. Trigger factor may independently affect chromosomal DNA replication and cell division by exerting its effect on DnaA and FtsZ protein, respectively. The *in vivo* significance of our *in vitro* data remains to be investigated.

Acknowledgement

This work was supported by grant from the Korea Research Foundation (KRF-2000-015-DP0320), through the Research Institute of Basic Sciences, Seoul National University.

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[Received January 14, 2003; accepted February 15, 2003]