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A Stimulation Factor for Hydrolysis of ATP Bound to DnaA Protein, the Initiator of Chromosomal DNA Replication in *Escherichia coli*[1]

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Hydrolysis of ATP bound to DnaA protein by its intrinsic ATPase activity negatively controls chromosomal DNA replication in *Escherichia coli.* We developed a new *in vitro* assay system for ATP hydrolysis, which makes feasible a search for factors affecting the ATPase activity of DnaA protein. A crude cell extract enhanced the hydrolysis of ATP bound to DnaA protein, in a dose-dependent manner. Gel-filtration analyses revealed a single entity of the stimulation factor for the ATP hydrolysis and an apparent molecular mass of 170 kDa. The stimulation activity for ATP hydrolysis coeluted with the inactivation activity for DnaA protein initiating an *oriC* DNA replication, as determined by anion-exchange and gel-filtration column chromatographies. Activity of the stimulation factor required DNA and ATP. These observations suggested that IdaA protein, a previously described negative factor for DnaA protein, inactivated DnaA protein through stimulation of the hydrolysis of ATP bound to DnaA protein.

Initiation of DNA replication is strictly regulated so as to be coupled with cell division. In *Escherichia coli* (*E. coli*), DnaA protein, the initiator of chromosomal DNA replication (1-5), specifically binds to DnaA boxes in the *oriC* region, a unique site for the initiation of chromosomal DNA replication, and opens the duplex (6, 7). DnaA protein has a high affinity for both ATP and ADP (8). In an *oriC* DNA replication system reconstituted with purified proteins, the ATP-binding form of DnaA protein is active, whereas the ADP-binding form is inactive (8). We recently reported that synthesized organic compounds designed to block the ATP-binding to DnaA protein specifically inhibited the *oriC* DNA replication *in vitro* (9). These results suggest that adenine-nucleotide binding to DnaA protein functions as 'a molecular switch' for control of initiation.

ATP bound to DnaA protein is slowly hydrolyzed to ADP by its intrinsic ATPase activity (8). Recently, we constructed a mutated DnaA protein with a decreased ATPase activity and found that induction of the mutated DnaA protein caused an overinitiation of DNA replication *in vivo,* resulting in a dominant lethal phenotype (10). Thus, the intrinsic ATPase activity of DnaA protein likely plays an important role in negative regulation of the initiation of chromosomal DNA replication *in vivo* (10) and factors which modulate the ATPase activity of DnaA protein may be involved in the control of DNA replication.

Katayama and Crooke described a protein factor (IdaA) in a crude extract, which inactivates the replication activity at *oriC* of DnaA protein (11). Since mutated DnaA protein (DnaAcos protein), which is insensitive to inactivation by IdaA protein *in vitro,* led to overinitiation of DNA replication *in vivo,* IdaA protein may be involved in negative regulation of the initiation of chromosomal DNA replication *in vivo* (11-14). The molecular mechanism governing inactivation of DnaA protein by IdaA protein has remained uncertain. IdaA protein cannot inactivate the activity of DnaA protein in an ABC primosome system in which the ADP-binding form of DnaA protein is active (11) and DnaAcos protein has no affinity for either ATP or ADP (12). Thus, it seems reasonable to assume that IdaA protein inactivates DnaA protein through stimulation of the ATPase activity of DnaA protein (11). We have now identified a stimulation factor for the hydrolysis of ATP bound to DnaA protein, in a crude extract from *E. coli* cells. In light of these observations, we propose that

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inactivation of DnaA protein by IdaA protein is caused by stimulation of hydrolysis of ATP bound to DnaA protein.

EXPERIMENTAL PROCEDURES

Preparation of DnaA protein and a crude extract. Wild type DnaA protein was purified as described (15) but with some modifications (16). Specific activity of the protein was 0.3×10^6 unit/mg. Purity of the fraction exceeded 90%, as determined by SDS-polyacrylamide (10%) gel electrophoresis. A crude extract was prepared from WM433 strain (*dnaA204*), as described (17, 18). Protein concentrations were determined by the Bradford method (19), using bovine serum albumin (Boehringer Mannheim) as a standard.

Measurement of hydrolysis of ATP bound to DnaA protein. We measured the hydrolysis of ATP bound to DnaA protein under much the same condition as that for the *oriC* replication system with a crude extract (17, 18). DnaA protein (1 pmol) was incubated with 1.5 μM [α-^{32}P]ATP (10^5 cpm/pmol) on ice for 15 min in mixtures (1 μl) under the conditions described elsewhere (8) and then incubated the preparation at 30 °C for 20 min in the reaction mixture (25 μl) containing 40 mM HEPES/KOH, pH 7.6, 10 mM magnesium acetate, 2 mM ATP, 0.5 mM each GTP, CTP, and UTP, 0.1 mM each dATP, dTTP, dCTP, and dGTP, 7% (w/v) polyvinyl alcohol (Mr. 30000-70000), 40 mM creatine phosphate, 100 μg/ml creatine kinase, 200 ng DNA (M13E10(*oriC*) (20) or pKE101), and with or without a crude extract from WM433. pKE101 has the minimal *oriC* sequence of pBS*oriC* (from the *Hinc*II site to the *Pst*I site) (7) in the cloning site of M13mp18 (Takara). Samples were incubated with anti-DnaA serum (5 μl) and 50% slurry of protein A-sepharose (Pharmacia) (60 μl) in buffer L (750 μl) (50 mM HEPES/KOH, pH 7.6, 5 mM magnesium acetate, 1 mM EDTA, 30 mM ammonium sulfate, 0.005% Triton X-100, 100 mM NaCl, 0.1 mM ATP, 0.1 mM ADP, and 5 mg/ml lysozyme) at 4 °C for 30 min with rotation. After centrifugation, precipitates were washed with buffer L and buffer M (buffer L without lysozyme). Nucleotides in the precipitates were extracted with 40 μl of 1M HCOOH containing 5 mM each of ATP, ADP, and AMP and analyzed by thin-layer chromatography on PEI-cellulose plates (Merck) developed with 1M HCOOH and 0.5M LiCl, followed by autoradiography. The radioactive spots corresponding to [α-^{32}P]ADP and [α-^{32}P]ATP were quantitated using Bas2000 (Fujix).

Measurement of inactivation activity for DnaA protein. The inactivation activity for the replication activity of DnaA protein was measured as described (11) but with some modifications. DnaA protein (1.2 pmol) was incubated at 30 °C for 20 min in the reaction mixture (10 μl) containing 40 mM HEPES/KOH (pH 7.6), 2 mM ATP, 11 mM magnesium acetate, 7% (w/v) polyvinyl alcohol (Mr. 30000-70000), 40 mM creatine phosphate, 100 μg/ml creatine kinase, 400 ng of M13mp19 RF I DNA, and a fraction containing the stimulation factor. Portions of the mixtures (5 μl) were added to an *oriC* DNA replication system (25 μl) (17, 18) to measure the activity of DnaA protein.

RESULTS

Immunoprecipitation of the ATP–DnaA complex. To search for factors which affect the hydrolysis of ATP bound to DnaA protein, we first examined the effect of a crude extract from *E. coli* cells on the hydrolysis, using the assay system described elsewhere (8); The [α-^{32}P]ATP-DnaA complex was incubated with the extract and passed through a nitrocellulose filter (Millipore HA 0.45 μm). Under the present conditions we detected considerable amounts of radio-labeled adenine-nucleotides on the nitrocellulose filters, even in the control experiment without DnaA protein (data not shown). Thus, one cannot analyze the hydrolysis of [α-^{32}P]ATP bound to DnaA protein using this method, perhaps because of a non-specific binding of [α-^{32}P]ATP to proteins in the extract. Therefore, we developed a method to separate the ATP-DnaA complex from other ATP-binding proteins in the extract. The [α-^{32}P]ATP-DnaA complex was incubated with anti-DnaA serum and protein A-sepharose in buffer L, and precipitated by centrifugation. As shown in Fig. 1A, [α-^{32}P]ATP in the precipitate fraction, was recovered in a dose-dependent manner of anti-DnaA serum. Little [α-^{32}P]ATP was recovered when anti-DnaA serum was replaced with pre-immune serum (Fig. 1A). In the presence of saturated amounts of anti-DnaA serum (5 μl), [α-^{32}P]ATP was recovered in the immuno-precipitated fraction, in a dose-dependent manner of the [α-^{32}P]ATP-DnaA complex (Fig. 1B).

To examine the specificity of the immuno-precipitation, we did competition experiments with the non-labeled ATP-DnaA complex. As shown in Fig. 1C, the recovery of [α-^{32}P]ATP to the immuno-precipitated fraction was inhibited by pre-incubation of the serum with the non-labeled ATP-DnaA complex, whereas BSA even at high concentrations was without effect. Thus, DnaA protein can be specifically immuno-precipitated, under the present conditions.

Next, we asked if both the ATP-DnaA complex and the ADP-DnaA complex would be precipitated with equal efficiency, under the present conditions. A mixture of the [α-^{32}P]ATP-DnaA complex and the [α-^{32}P]-ATP-DnaA complex of various ratios was immuno-precipitated and adenine nucleotides bound to DnaA protein were analyzed by thin-layer chromatography. The ratio of ATP and ADP bound to DnaA protein remained unchanged after immuno-precipitation (data not shown), indicating that both the ATP-DnaA complex and the ADP-DnaA complex were precipitated with equal efficiency.

Finally, we wanted to determine if adenine-nucleotides bound to DnaA protein would be specifically detected, even in the presence of the crude extract. The [α-^{32}P]ATP-DnaA complex incubated with the extract was precipitated by anti-DnaA serum (Fig. 2A). When DnaA protein was pre-incubated with non-labeled ATP prior to labeling with [α-^{32}P]ATP followed by incubation with the extract, we detected only scanty amounts of radio-labeled adenine-nucleotides (less than 2% of control without the pre-incubation) (data not shown). Therefore, under the present conditions, we can determine the forms of adenine-nucleotide bound to DnaA protein specifically, even in the presence of the crude extract. With this new method, one can examine effects of the crude extract on the hydrolysis of ATP bound to DnaA protein.

Stimulation of hydrolysis of ATP bound to DnaA protein by the crude extract. We examined the effect of

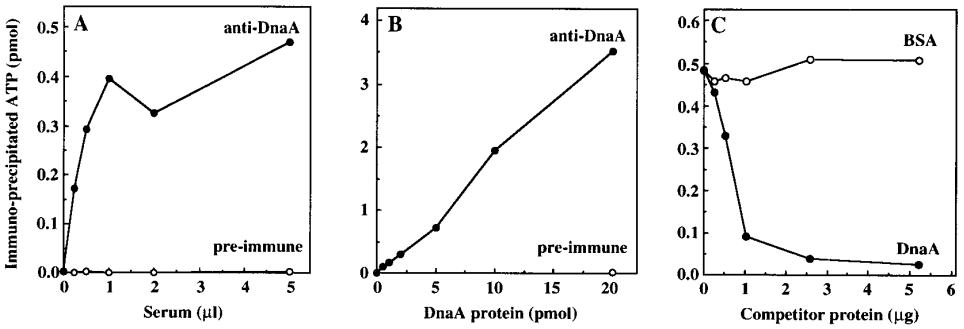

FIG. 1. Immuno-precipitation of the ATP–DnaA complex. *A:* DnaA protein (2 pmol) was incubated with 1.5 μM [α-^{32}P]ATP on ice for 15 min and precipitated with indicated amounts of anti-DnaA serum or pre-immune serum. The precipitated [α-^{32}P]ATP was extracted with 1 M HCOOH and determined in a liquid scintillation counter. *B:* Indicated amounts of DnaA protein were incubated with 1.5 μM [α-^{32}P]ATP on ice for 15 min and precipitated with anti-DnaA serum or pre-immune serum (5 μl). Precipitated [α-^{32}P]ATP was extracted and determined. *C:* Anti-DnaA serum (5 μl) was pre-incubated with indicated amounts of proteins (DnaA protein or BSA) and incubated with the [α-^{32}P]ATP-DnaA complex (2 pmol). Samples were immuno-precipitated and [α-^{32}P]ATP was extracted and determined.

the crude extract from *E. coli* cells on the hydrolysis of ATP bound to DnaA protein. Fig. 2A shows that the extract stimulated ATP hydrolysis; in the presence of the extract, both decrease in the amount of ATP and increase in the amount of ADP proceeded more rapidly than in the absence of the extract. As shown in Fig. 2C, the extract stimulated the hydrolysis of ATP bound to DnaA protein, in a dose-dependent manner. At saturation level of the extract, the hydrolysis of ATP was stimulated about 4-times. These observations suggested that the extract contained a factor which stimulated the intrinsic ATPase activity of DnaA protein. The stimulation activity in the extract was diminished by treatment of the extract at 55 °C for 4 min (data not shown), hence a protein factor is likely to be responsible for the activity. Since creatine kinase and creatine phosphate were present in the reaction mixture, there remained the possibility that we could measure equilibrium between the intrinsic rate of ATP hydrolysis by DnaA protein and the effect of the creatine kinase and creatine phosphate on the hydrolysis. Creatine kinase had no apparent effect on the intrinsic rate of ATP hydrolysis by DnaA protein (data not shown). Therefore, the finding that the crude extract stimulated the rate of hydrolysis was considered to be a direct effect on DnaA protein.

Co-elution of stimulation activity for hydrolysis of ATP bound to DnaA protein with inactivation activity for DnaA protein. Katayama and Crooke reported that a protein factor (IdaA) in a crude extract of *E. coli* cells inactivated the replication activity of DnaA protein (11). Since the ADP-binding form of DnaA protein, the reaction product of the hydrolysis of ATP bound to DnaA protein, is inactive for *oriC* replication (8), we considered the possibility that the factor stimulating the ATP hydrolysis (Fig. 2) is identical to IdaA protein. Thus, we determined if both activities in the crude extract, the stimulation activity of the hydrolysis of ATP bound to DnaA protein and the inactivation activity of the replication activity of DnaA protein, would co-elute on column chromatographies. The extract was applied to a gel-filtration column (Sephacryl S300HR) or an anion-exchange column (DE-52) and both activities in each fraction were measured. As shown in Fig. 3A, a single peak of the activity which stimulated the hydrolysis of ATP bound to DnaA protein was observed for fraction 43, the same fraction number of a peak inactivating the replication activity for DnaA protein, on gel filtration column chromatography. Fig. 3A shows that the crude extract contained a single factor for stimulation of the hydrolysis of ATP bound to DnaA protein, as in the case of IdaA protein (11). Apparent molecular mass of the stimulation factor is 170-kDa, a value much the same as that of IdaA protein (150-kDa) (11). Recovery of the stimulation and of the inactivation activity to pooled fractions (Fr. 41-46) was 83% and 65%, respectively. The pooled fractions had a 2.7-fold and a 2.0-fold increase in specific activity of the stimulation and of inactivation activity, respectively. The difference in recovery and in purification-fold between the two was in error range. The reaction mixture (25 μl) was then replaced, under the same condition, to measure the inactivation activity; GTP, CTP, UTP, dATP, dTTP, dCTP and dGTP were omitted, and 1 μg of M13mp18 DNA was added. One unit of the stimulation activity was defined as two-fold stimulation of the hydrolysis of ATP bound to DnaA protein. On DE-52 column chromatography, one major peak stimulating the ATP hydrolysis was observed for fraction 113, which also corresponded with a peak fraction decreasing the replication activity of DnaA protein (Fig. 3B). Recovery of the stimulation activity and of the inactivation activity in pooled fractions (Fr. 103-138) was 70% and 40%, respectively. The pooled fractions had a 3.9-fold and a 2.2-fold increase in specific activity of the stimulation and of inactivation activity,

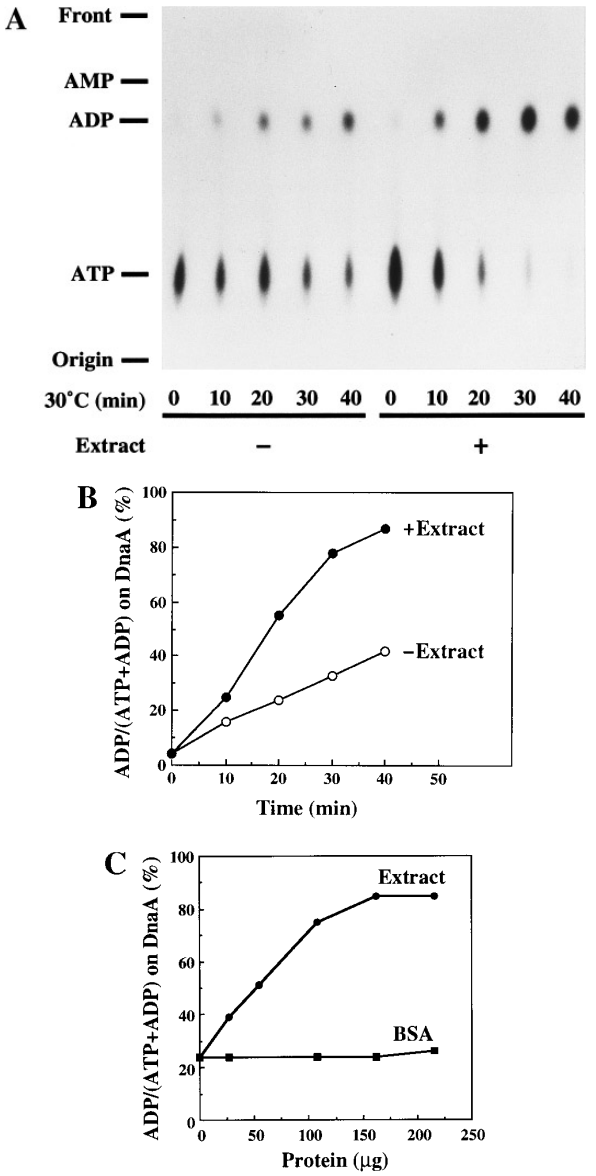

FIG. 2. A crude extract stimulates hydrolysis of ATP bound to DnaA protein. DnaA protein (1 pmol) pre-incubated on ice with 1.5 μM [α-^{32}P]ATP was incubated with 54 μg (*A, B*) or indicated amounts of a crude extract (*C*) in the reaction mixture at 30 °C for the indicated period (*A, B*) or 20 min (*C*). Samples were immunoprecipitated with anti-DnaA serum, and adenine nucleotides were extracted and analyzed by thin-layer chromatography. The plate was autoradiographed (*A*) and scanned using Bas2000 (Fujix), and the ratio of ADP/(ATP and ADP) was determined (*B, C*).

respectively. When pooled fractions of DE-52 column chromatography were applied to Superdex 200 column, the stimulation activity also co-eluted with the inactivating activity and the recoveries of both activities were much the same (data not shown). These observations suggest that IdaA protein inactivates DnaA protein through stimulation of the hydrolysis of ATP bound to DnaA protein.

Requirement of ATP and DNA for stimulation activity. For Figure 2, we examined effects of the extract on the hydrolysis of ATP bound to DnaA protein, under the same conditions as for *in vitro oriC* DNA replication (17, 18). Thus, the stimulation activity was measured in the presence of ATP and DNA. IdaA protein requires ATP and DNA for activity (11). For further examination of the identity of the stimulation factor and IdaA protein, we examined the requirement of these two components for the stimulation. ATP stimulated ATP hydrolysis in the presence of the extract, but it did not affect the hydrolysis in the absence of the extract (Table I). Therefore, a high concentration of ATP seems necessary for activity of the stimulation factor. We also examined the requirement of DNA for the stimulation of

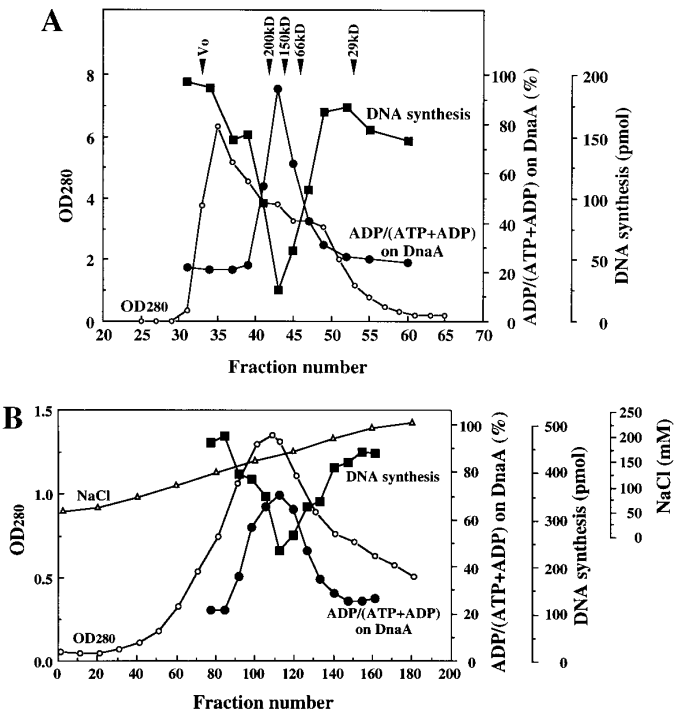

FIG. 3. Stimulation activity co-eluted with inactivation activity for DnaA protein. *A:* The extract (14 mg) was applied to a Sephacryl S300HR column (1 × 27 cm; 21 ml) equilibrated with 25 mM HEPES/KOH (pH 7.6), 0.1 mM EDTA, 2 mM DTT, 20% sucrose, and 50 mM NaCl. Activities were eluted in the same buffer (flow rate 1.5 ml/h). The stimulation activity for the ATP hydrolysis and the inactivation activity of DNA replication in each fraction (5 and 0.8 μl, respectively) were determined as described under "Experimental procedures" except that the inactivation reaction was performed for 30 min. Markers were blue dextran (Vo), β-amylase (200 kDa), alcohol dehydrogenese (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). *B:* The extract (3744 mg) was applied on a DE52 column (5.5 × 12 cm, 285 ml) equilibrated with 25 mM BisTris/HCl (pH 6.0), 0.1 mM EDTA, 2 mM DTT, 15% glycerol, and 50 mM NaCl. The column was developed in the same buffer with a linear gradient of 50 to 250 mM NaCl (2800 ml). The stimulation activity for ATP hydrolysis and the inactivation activity of DNA replication in each fraction (2 and 0.5 μl, respectively) were determined as described under "Experimental procedures."

TABLE I

Requirement of ATP and DNA for Stimulation Activity

Component omitted	ADP/(ATP+ADP) on DnaA	
	+Extract	−Extract
None	%	
	87	24
ATP	33	23
pKE101 DNA	10	17

DnaA protein (1 pmol) pre-incubated on ice with 1.5 μM [α-^{32}P]ATP was incubated with or without 229 μg of the crude extract at 30 °C for 20 min in the reaction mixture as described under "Experimental Procedures". Influence of the omission of ATP (2 mM) or pKE101 DNA (200 ng) was examined. Hydrolysis of ATP bound to DnaA protein was measured as described in the legend of Figure 2.

the hydrolysis of ATP bound to DnaA protein. As shown in Table I, simulation of the ATP hydrolysis in the presence of the extract depended on DNA. DNA did not significantly affect the ATPase activity of DnaA protein in the absence of the extract (Table I). Thus, the stimulation factor for the ATP hydrolysis apparently requires DNA for activity.

It was reported that the intrinsic ATPase activity of DnaA protein is stimulated by DNA (8). The apparent discrepancy can be explained by the different assays used. The present assay system, but not the previous one (8), contained 7% PVA. Depletion of 7% PVA from the present condition resulted in a DNA-dependent stimulation of the intrinsic ATPase activity of DnaA protein (data not shown). On the other hand, by addition of 7% PVA to the previous assay condition (8), we observed DNA-independent ATPase activity of DnaA protein (data not shown). Therefore, independence of ATPase activity of DnaA protein on DNA can be explained by using PVA in the present assay system.

DISCUSSION

We identified a stimulation factor for the hydrolysis of ATP bound to DnaA protein in a crude extract from *E. coli* cells. Characterization of the stimulation factor revealed that stimulation of the ATPase activity of DnaA protein was included in molecular mechanisms for inactivation of DnaA protein by IdaA protein, which was described to inactivate the replication activity of DnaA protein in an *oriC* replication system (11). Since expression of mutant DnaA protein, DnaAcos, which is insensitive to the inactivation by IdaA protein caused the overinitiation of DNA replication *in vivo* (11), IdaA protein is considered to be involved in the negative regulation of initiation of chromosomal DNA replication. We recently reported that induction of artificially mutated DnaA protein which has a decreased ATPase activity led to overinitiation of DNA replication *in vivo*, suggesting that the ATPase activity of DnaA protein also plays an important role in the negative regulation of initiation of chromosomal DNA replication (10). Based on all these observations, we propose that the regulation of the intrinsic ATPase activity of DnaA protein by the stimulation factor may play an important role in negative control of the initiation of chromosomal DNA replication *in vivo*. Further purification of IdaA protein is in progress in our laboratory and the results will be described elsewhere.[3]

Various factors may be involved in the regulation of the activity of DnaA protein through modulation of the ratio of the ATP binding versus the ADP binding forms of DnaA protein. The exchange reaction of ADP bound to DnaA protein with ATP (activation of the ADP-binding form of DnaA protein) is stimulated by acidic phospholipids, such as cardiolipin and phosphatidylglycerol (21-24). Genetic evidence suggests that acidic phospholipids are involved in the activation of DnaA protein *in vivo* (25). Therefore, the activity of DnaA protein seems to be negatively and positively regulated by the stimulation factor for the ATP hydrolysis and acidic phospholipids, respectively. This regulatory mechanism of DnaA protein has a close similarity with that of GTP-binding proteins (G-proteins), which are responsible for the regulation of signal transaction in eukaryotic cells. G-proteins take inactive forms, GDP-binding forms, in the absence of signals (26-28). When a receptor with which a G-protein associate receives a signal, the G-protein takes on the GTP-binding form by the exchange reaction of GDP with GTP (27). GTP bound to G-proteins is hydrolyzed to GDP by intrinsic GTPase activity of the proteins (28). The GTPase activity is stimulated by GTPase activating proteins (GAPs) and the exchange reaction is stimulated by guanine nucleotide release proteins (GNRPs) (26, 28). These factors (GAPs and GNRPs) seem to play an important role in regulation of the activity of G-proteins (26-28). We speculate that roles of the stimulation factor for ATP hydrolysis and acidic phospholipids for DnaA protein correspond to those of GAPs and GNRPs, respectively, for G-proteins.

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