Molecular Mechanism for Functional Interaction between DnaA Protein and Acidic Phospholipids

IDENTIFICATION OF IMPORTANT AMINO ACIDS

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DnaA protein, the initiator of chromosomal DNA replication in Escherichia coli, seems to be reactivated from the ADP-bound form to its ATP-bound form through stimulation of ADP release by acidic phospholipids such as cardiolipin. We previously reported that two potential amphipathic helices (Lys-327 to Ile-344 and Asp-357 to Val-374) of DnaA protein are involved in the functional interaction between DnaA and cardiolipin. In relation to one of these helices (Asp-357 to Val-374), we demonstrated that basic amino acids in the helix, especially Lys-372, are vital for this interaction. In this study, we have identified an amino acid in the second potential amphipathic helix (Lys-327 to Ile-344), which would also appear to be involved in the interaction. We constructed three mutant dnaA genes with a single mutation (dnaAR328E, dnaAR334E, and dnaAR342E) and examined the function of the mutant proteins. DnaAR328E, but not DnaAR334E and DnaAR342E, was found to be more resistant to inhibition of its ATP binding activity by cardiolipin than the wild-type protein. The stimulation of ADP release from DnaAR328E by cardiolipin was also weaker than that observed with the other mutants and the wild-type protein. These results suggest that Arg-328 of DnaA protein is involved in the functional interaction of this protein with acidic phospholipids. We propose that acidic phospholipids bind to two basic amino acid residues (Arg-328 and Lys-372) of DnaA protein and change the higher order structure of its ATP-binding pocket, which in turn stimulates the release of ADP from the protein.

which causes the duplex DNA to open up at oriC(1). The N- and C-terminal regions of this protein are involved in the oligomerization and in the specific DNA binding, respectively (3–6).

Adenine nucleotides bound to DnaA protein play an important role in the regulation of replication initiation. DnaA protein has a high affinity for both ATP and ADP, but although the ATP-binding form is active, the ADP-binding form is inactive in DNA replication (7, 8). ATP bound to DnaA protein is hydrolyzed to ADP by its intrinsic ATPase activity, and this hydrolysis is involved in inactivating DnaA protein following initiation of DNA replication (9–13). The Lys-178 and Glu-204 amino acids of DnaA protein have been shown to be essential for ATP binding and for ATPase activity, respectively (10, 14). The membrane binding activity of DnaA protein also seems to be involved in the regulation of replication initiation, through modulation of the adenine-nucleotide binding capacity of the protein. Acidic phospholipids, in particular cardiolipin (CL), decrease DnaA protein’s affinity for adenine nucleotides and activate the ADP-bound DnaA protein to the ATP-bound form in the presence of high concentrations of ATP by stimulating the exchange reaction of ADP with ATP (15–18). It has been suggested that DnaA protein is activated by acidic phospholipids to initiate DNA replication in vivo (19–23). However, the precise molecular mechanism by which acidic phospholipids interact with DnaA to decrease the affinity of this protein for adenine nucleotides is still unknown. Identification of essential amino acids involved in the DnaA-membrane interaction is an important first step to identifying this mechanism.

A CL protection assay of DnaA protein from trypsin digestion suggested that a potential amphipathic helix (Asp-357 to Val-374) is involved in the membrane binding activity of DnaA protein (24). We further identified that three basic amino acids in the helix (Arg-360, Arg-364, Lys-372), especially Lys-372, were important for the functional interaction between DnaA protein and CL, suggesting that this interaction is mediated by ionic interaction between these basic amino acids and the acidic moieties of CL (25, 26). A second potential amphipathic helix (Lys-327 to Ile-344), adjacent to the first, has also been demonstrated to be involved in the membrane binding activity of DnaA protein (27). In this paper, we have identified Arg-328 in the second amphipathic helix (Lys-327 to Ile-344) as playing an important role in the functional interaction between the DnaA protein and CL. We propose that a possible molecular mechanism for how acidic phospholipids decrease the affinity of DnaA protein for adenine nucleotides is that the interaction of such phospholipids with two basic amino acids (Arg-328 and

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1 The abbreviations used are: CL, cardiolipin; AAA+, ATPases associated with a variety of cellular activities family of proteins.
Lys-372) of DnaA protein causes a conformational change of this protein’s ATP-binding pocket.

**EXPERIMENTAL PROCEDURES**

**Materials**—A crude extract for an oriC complementation assay was prepared from the WM433 strain of E. coli as previously described (28). CL was purchased from Sigma. [α-32P]ATP (10 mCi/mmol) and [3H]ADP (40 Ci/mmol) were from Amersham Pharmacia Biotech and DuPont, respectively. The mutant DnaA protein and the wild-type DnaA protein were purified, as described previously (29).

**Site-directed Mutagenesis and Plasmid Construction**—Site-specific mutagenesis was performed using the method described by Kunkel (30). Briefly, uracil-containing single-stranded DNA of the M13 phage, containing the coding region of the dnaA gene, was hybridized with oligonucleotide primers representing each mutation (Fig. 1). The complementary DNA strand was synthesized in vitro, and the resultant double-stranded DNA was introduced into E. coli JM109 cells. The mutation was confirmed by direct DNA sequencing, and double-stranded DNA containing the mutation was prepared.

To construct the plasmid for overproduction of each mutant DnaA protein, the EcoRI-HindIII region of the double-stranded DNA was ligated with pMZ001 plasmid (10), which contains the arabinose promoter.

To construct the plasmid for complementation analysis of the mutant dnaA gene, we introduced the coding regions of the mutant dnaA gene (BamHI-HindIII fragments of the double-stranded DNA) into pMZ002, which contains the wild-type promoter of the dnaA gene (10).

**Influence of CL on Release of ATP or ADP from DnaA/ATP or DNA-ADP Complexes**—The stimulation of ATP or ADP release from DnaA-ATP or DNA-ADP complexes by CL was examined as described previously (18). Briefly, CL liposomes were prepared from dried phospholipids on the bottom of glass tubes through vigorous vortex mixing in water. The amount of phosphorus in the phospholipid fraction was determined using the method described by Chen et al. (31). DnaA-ATP or DNA-ADP complexes were formed by incubation of DnaA with 1 μM [α-32P]ATP or [3H]ADP in 40 μl of buffer G (50 mM HEPES-KOH (pH 8.0), 0.5 mM magnesium acetate, 0.3 mM EDTA, 5 mM dithiothreitol, 17% (v/v) glycerol, 10 mM ammonium sulfate, and 0.005% Triton X-100) at 0 °C for 15 min. CL was added, and the mixture was further incubated at 37 °C. Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 μm) and washed with ice-cold buffer G. The radioactivity remaining on the filter was counted with a liquid scintillation counter.

**Filter Binding Assay for ATP or ADP Binding to DnaA Protein**—The ATP or ADP binding activity of DnaA protein was determined by a filter binding assay (7). DnaA protein (2 pmol) was incubated with various concentrations of [α-32P]ATP or [3H]ADP at 0 °C for 15 min in 40 μl of buffer G. Samples were passed through nitrocellulose membranes and counted as described above.

**Inhibition of ATP Binding to DnaA Protein by CL**—Inhibition of ATP binding to DnaA protein by CL was examined as described previously (32). DnaA protein (2 pmol) was preincubated with CL at 0 °C for 5 min and further incubated with 1 μM [α-32P]ATP at 0 °C for 15 min in 40 μl of buffer G. The amount of bound ATP was determined as described above.

**oriC DNA Replication in a Crude Extract**—Replication of minichromosomes in a crude extract (Fraction II) was assayed as described previously (28). Template DNA (13 M13E10) (200 ng, 600 pmol of nucleotides), 200 μg of Fraction II from WM433 (dnaA204), and DnaA protein were passed through ice-cold buffer G. The reaction was terminated by chilling on ice and the addition of 10% trichloroacetic acid. Samples were passed through Whatman GF/C glass fiber filters. The amount of radioactivity on the filters was measured with a liquid scintillation counter, and the amount of synthesized DNA (picomoles of nucleotides) was then calculated.

**RESULTS**

**Strategy for Site-directed Mutagenesis and Purification of Mutant DnaA Proteins**—To reveal DnaA protein’s functional membrane-binding domain, we constructed a series of mutant DnaA proteins using site-directed mutagenesis and examined their functional interaction with CL (25–27). We identified two mutant DnaA proteins, with a triple substitution for Glu of Arg-360, Arg-364, and Lys-372 (DnaA431), and Arg-328, Arg-334, and Arg-342 (DnaA433), which showed a decreased ability to interact with CL (25, 27). These results suggest not only that two potential amphipathic helices (Lys-327 to Ile-344) and the strategy for site-directed mutagenesis.
and the wild-type protein was calculated to be 0.38, 0.28, 0.30, 0.24, and 0.44, respectively. The $K_d$ values and the number of ATP-binding sites for the wild-type protein were nearly the same as reported previously (7).

We also examined the ADP binding activity of these mutant DnaA proteins in the same manner. As shown in Fig. 4, each of the mutant DnaA proteins also demonstrated ADP binding activity. The $K_d$ values of DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein (2 pmol) were incubated with various concentrations of [3H]ADP for 15 min at 0 °C. The amount of bound ADP was determined as described under “Experimental Procedures” and analyzed by the Scatchard plot method.

Because all the mutant DnaA proteins in this study showed both ATP and ADP binding activity, their functional interactions with acidic phospholipids could be examined by monitoring either CL-dependent stimulation of the release of ATP (or ADP) from DnaA protein or CL-dependent inhibition of ATP (or ADP) binding activity of DnaA protein. The observed slight, but significant, effects of these mutations, especially R334E, on the number of ATP- or ADP-binding sites suggests that this helix (Lys-327 to Ile-344) contributes to the adenine nucleotide binding activity of DnaA protein (see “Discussion”).

Inhibition of ATP Binding Activities of the Mutant DnaA Proteins by CL—We previously reported that the ATP binding activity of DnaA433 was found to be less inhibited by CL, suggesting that some of the mutations in DnaA433 affected the functional interaction between DnaA and CL (27). In this study we therefore compared the CL inhibition curve for ATP binding obtained for each single mutation mutant DnaA protein (DnaAR328E, DnaAR334E, and DnaAR342E) with that of DnaA433 or the wild-type protein, to identify which of these amino acids was important for this functional interaction. DnaA protein was preincubated with CL and further incubated with $[^{32}P]ATP$. The amount of bound ATP was determined using a filter binding assay. As shown in Fig. 5, DnaAR328E was found to be more resistant to CL-dependent inhibition of ATP binding than the wild-type protein, as was DnaA433. The inhibition curve for DnaA433 was much the same as reported previously (27). On the other hand, the CL inhibition curve for ATP binding to DnaAR334E or DnaAR342E was much the same as that of the wild-type protein (Fig. 5). These results suggest that Arg-328 is the most important of these three basic amino acids located in the amphipathic helix (Lys-327 to Ile-344) for functional interaction of DnaA protein with CL.

CL-dependent Stimulation of the Release of ADP (or ATP) from the Mutant DnaA Proteins—We also examined the functional interaction between each mutant DnaA protein and CL by monitoring the release of ADP from DnaA protein in the presence or absence of CL. The activation of ADP-bound DnaA protein by CL is mediated by CL stimulation of ADP release from DnaA protein (15). DnaA$[^{32}P]$ADP complex was incubated

FIG. 2. Purification of mutant DnaA proteins. Active Superose 12 chromatography fractions of each mutant DnaA protein were pooled, and 0.2 μg of each protein was subjected to SDS-polyacrylamide gel (10%) electrophoresis and stained with Coomassie Brilliant Blue R-250.

FIG. 3. ATP binding to mutant DnaA protein measured by a filter binding assay. DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein (2 pmol) were incubated with various concentrations of [α-$^{32}$P]ATP for 15 min at 0 °C. The amount of bound ATP was determined as described under “Experimental Procedures” and analyzed by the Scatchard plot method.

FIG. 4. ADP binding to mutant DnaA proteins measured by a filter binding assay. DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein (2 pmol) were incubated with various concentrations of [3H]ADP for 15 min at 0 °C. The amount of bound ADP was determined as described under “Experimental Procedures” and analyzed by the Scatchard plot method.

FIG. 5. CL-dependent inhibition of ATP binding activity of DnaA protein. DnaAR328E, DnaAR334E, and DnaAR342E were preincubated with CL and further incubated with [α-$^{32}$P]ATP. The amount of bound ATP was determined using a filter binding assay. As shown in Fig. 5, DnaAR328E was found to be more resistant to CL-dependent inhibition of ATP binding than the wild-type protein, as was DnaA433. The inhibition curve for DnaA433 was much the same as reported previously (27). On the other hand, the CL inhibition curve for ATP binding to DnaAR334E or DnaAR342E was much the same as that of the wild-type protein (Fig. 5). These results suggest that Arg-328 is the most important of these three basic amino acids located in the amphipathic helix (Lys-327 to Ile-344) for functional interaction of DnaA protein with CL.
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with CL at 37 °C, and the remaining ADP-bound DnaA protein was determined by a filter binding assay. The $k_{app}$ (apparent rate constant) can be calculated from the slope of the resultant graph (Fig. 6A). The $k_{app}$ values for DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein, in the absence of CL, were $2.2 \times 10^{-4}$, $6.0 \times 10^{-4}$, $2.2 \times 10^{-4}$, $1.4 \times 10^{-3}$, and $2.4 \times 10^{-3}$ (s⁻¹), respectively. The $k_{app}$ values for DnaAR328E and DnaAR342E in the absence of CL were nearly the same as that for the wild-type protein, suggesting that both the DnaAR328E- and DnaAR342E-ADP complexes are stable at 37 °C. In contrast, the DnaA433-ADP complex was unstable at 37 °C, even in the absence of CL, as reported previously (27). The DnaA334E-ADP complex was found to be partly unstable, suggesting that Arg-334 is involved in the ADP binding activity of DnaA protein (see “Discussion”). The $k_{app}$ values for DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein, in the presence of CL, were $1.4 \times 10^{-3}$, $2.2 \times 10^{-3}$, $1.8 \times 10^{-3}$, $1.7 \times 10^{-3}$, and $3.4 \times 10^{-3}$ (s⁻¹), respectively.

Compared with the wild-type protein, CL stimulation of ADP release from DnaAR328E and DnaAR342E was found to be less, suggesting that these amino acids contributed to the functional interaction between DnaA protein and CL. We further examined the effect of various concentrations of CL on the release of ADP from DnaAR328E, DnaAR334E, and the wild-type protein (Fig. 6B). DnaAR328E showed the lowest CL stimulation of ADP release (Fig. 6B), again suggesting that Arg-328 is the most important of the three basic amino acids in the amphipathic helix (Lys-327 to Ile-344) for the functional interaction between DnaA protein and CL. The ADP-DnaA433 and ADP-DnaAR334E complexes were unstable in the presence of CL at high temperatures (Fig. 6A).

We also examined the effect of CL on the release of ATP from these DnaA proteins, in the same way (Fig. 7). The results were basically the same as those obtained in relation to ADP release (Fig. 6).

FIG. 5. CL inhibition of ATP binding to the mutant DnaA proteins. DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein (2 pmol) were preincubated with the indicated amounts of CL for 5 min at 0 °C in 40 μl buffer G, and further incubated with 1 μM of [γ-32P]ATP for 15 min in the same buffer at 0 °C. The amount of bound ATP was determined as described under “Experimental Procedures.” Amounts of bound ATP are shown relative to a control (without CL). 100% values of DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein, for ATP binding are 0.32, 0.28, 0.46, 0.32, and 0.63 pmol, respectively.

FIG. 6. The release of ADP from the DnaA-ADP complex in the presence or absence of CL. The dissociation of ADP from DnaAR328E, DnaAR334E, DnaAR342E, and DnaA433 (2 pmol) was compared with that of the wild-type protein (2 pmol) in the presence or absence of CL (0.01 μg/μl), as described under “Experimental Procedures.” $k_{app}$ and $k_{app}$ denote the retained and initial concentrations of ADP-DnaA, respectively. The Co values obtained for DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and for the wild-type protein were 0.62, 0.76, 0.78, 0.54, and 1.12 pmol, respectively. A, the dissociation of ADP from DnaAR328E, DnaAR342E, and from the wild-type protein was examined in the presence of various concentrations of CL. The amounts of ADP remaining on DnaA after 8 min incubation at 37 °C are shown as values relative to a control (before the incubation). B, control values obtained for DnaAR328E, DnaAR342E, and for the wild-type protein, were 0.32, 0.38, and 0.65 pmol, respectively. (Fig. 6A). The $k_{app}$ values for DnaAR328E, DnaAR334E, and DnaAR342E, and the wild-type protein, in the absence of CL, were $3.2 \times 10^{-4}$, $7.3 \times 10^{-4}$, $2.5 \times 10^{-4}$, $1.7 \times 10^{-3}$, and $1.6 \times 10^{-4}$ (s⁻¹), respectively. The $k_{app}$ values for DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein, in the presence of CL, were $7.5 \times 10^{-4}$, $2.8 \times 10^{-3}$, $7.3 \times 10^{-4}$, $2.6 \times 10^{-3}$, and $2.1 \times 10^{-3}$ (s⁻¹), respectively.
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DnaA433, and the wild-type protein’s ability to initiate oriC DNA replication using an oriC complementation assay in a crude extract (28). As shown in Fig. 8, both DnaAR328E and DnaAR342E supported oriC DNA replication in vitro. The specific activities of DnaAR328E and DnaAR342E, and of the wild-type protein, were found to be 0.22, 0.09, and $3.8 \times 10^6$ units/mg of protein, respectively (1 unit of protein promotes the incorporation of 1 pmol of nucleotides/min at 30 °C). DnaAR334E showed no oriC DNA replication activity, and neither did DnaA433 (Fig. 8), as reported previously (27). Thus, the lack of DNA replication activity observed in DnaA433 may be due to the R334E mutation. One possible explanation for this and for the results obtained in relation to DnaAR334E, is the low stability of their complexes with ATP at high temperatures (Fig. 7). They may be unable to form the ATP-bound form long enough to initiate oriC DNA replication. DnaAA184V, DnaA46 and DnaA5 required longer incubation periods for expression of their replication activity; however, a time lag for the DNA replication reaction has been previously reported for these mutant DnaA proteins (33). For these last mutants, the time course of DNA replication was approximately linear, as was the case for the wild-type protein (data not shown).

**FIG. 7.** The release of ATP from the DnaA-ATP complex in the presence and absence of CL. The dissociation of ATP from DnaA, DnaAR328E, DnaAR334E, DnaAR342E, and from DnaA433 (2 pmol) was compared with that of the wild-type protein (2 pmol) in the presence or absence of CL (0.01 μg/ml), as described under “Experimental Procedures.” Ct and Co denote the retained and initial concentrations of ATP-DnaA, respectively. The Co values for DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and for the wild-type protein, were 0.73, 0.83, 0.85, 0.67, and 1.20 pmol, respectively.

**FIG. 8.** Replication activity of the mutant DnaA proteins in a crude extract. DnaAR328E, DnaAR334E, DnaAR342E, DnaA433, and the wild-type protein were incubated with 1 μt of ATP for 15 min at 0 °C. DNA replication in a crude extract was performed for 20 min as described under “Experimental Procedures.”

**TABLE I**

Complementation analysis of temperature sensitivity of a dnaA46 mutant with plasmids carrying the mutant dnaA genes

| Clones | Colony formation efficiency (42 °C/30 °C) |
|--------|----------------------------------------|
| pMZ002 (vector) | $6.9 \times 10^{-6}$ |
| pMZ002/ dnaA46 | $9.8 \times 10^{-1}$ |
| pMZ002/ dnaAR328E | 1.0 |
| pMZ002/ dnaAR334E | $3.7 \times 10^{-7}$ |
| pMZ002/ dnaAR342E | 1.2 |
| pMZ002/ dnaA433 | $2.4 \times 10^{-7}$ |

KS1003 (dnaA46) cells were transformed with pMZ002 containing either the wild-type or each mutant dnaA gene. Cultures were diluted appropriately and spread on LB agar plates containing 50 μg/ml ampicillin. Plates were incubated at 42 °C or 30 °C for 12 and 24 h, respectively, the number of colonies was counted, and the colony formation efficiency was determined. Ratios of colony formation efficiency, at 42 °C to that at 30 °C, are shown.

DISCUSSION

DnaA433, a mutant DnaA protein with three mutations (R328E, R334E, and R342E), shows two important defects. One
such defect is in its functional interaction with acidic phospholipids, such as CL, and the other is a defect in oriC DNA replication. In this study, we constructed three mutant DnaA proteins, each with a single amino acid mutation (DnaAR328E, DnaAR334E, and DnaAR342E), and demonstrated that DnaAR328E shows decreased functional interaction activity with CL and that DnaAR334E has a defect in oriC DNA replication both in vitro and in vivo. We have therefore concluded that the defects described for the DnaA433 mutant in relation to CL interaction and oriC DNA replication are due to the R328E and R334E mutations, respectively.

DnaA protein belongs to the AAA+ (ATPases associated with a variety of cellular activities) family (35). Sequence alignment analysis of various AAA+ family proteins have revealed a number of conserved domains (35). The sensor 2 domain is thought to be important for the adenine nucleotide binding and ATPase activities of AAA+ family proteins (35). DNA polymerase III δ' and γ subunits also belong to the AAA+ family (35). The γ subunit, but not the δ' subunit, has both ATPase and adenine nucleotide binding activities. X-ray structure analysis of the DNA polymerase III δ' subunit has suggested that defects of the δ' subunit in relation to both these activities are partly due to the loss of an arginine amino acid (corresponding to Arg-215 in the sensor 2 domain of the γ subunit), which is conserved among various AAA+ family proteins (36). X-ray structure analysis of the E. coli HslU protein, which also belongs to the AAA+ family, further suggested that the conserved arginine of this protein (Arg-393) can interact with the β and γ phosphates of ATP (37). Interestingly, Arg-334 of DnaA protein corresponds to this arginine (35). From these previous studies, we predicted that DnaAR334E would have a defect in its adenine nucleotide binding activity. In fact, both the ATP-DnaAR334E and ADP-DnaAR334E complexes were unstable at higher temperatures when compared with the wild-type protein (Figs. 6 and 7). We have therefore concluded that the Arg-334 amino acid of DnaA protein is important for the protein’s interaction with adenine nucleotides, as is the case for other AAA+ family proteins.

We previously reported that Lys-372 is the most important of the potential amphiphilic helix (Asp-357 to Val-374) amino acids in the functional interaction between DnaA and CL. In this study, we further found that Arg-328 is the most important amino acid of the second potential amphiphilic helix (Lys-327 to Ile-344) for this interaction. We have therefore concluded that DnaA protein may interact with CL through ionic interactions between the acidic moieties of CL and these two basic amino acids. Interestingly, Arg-334 (see above) is located between these two basic amino acids. Based on these facts, we have considered a possible model for the mechanism by which acidic phospholipids decrease the affinity of DnaA protein for ADP (Fig. 9). In this model, we predict that CL binding to Arg-328 and Lys-372 causes a conformational change of the potential amphiphilic helix (Lys-327 to Ile-344). This conformational change may in turn interfere with Arg-334’s ability to interact with the β phosphate of ADP, resulting in the stimulation of ADP release from the DnaA-ADP complex.

To examine the role played by membrane binding of DnaA protein in oriC DNA replication in cells, it was useful to study a mutant DnaA protein that showed decreased membrane binding activity but normal DNA replication activity. DnaAK372E is such a mutant that has been previously described (26). In this study, we added DnaAR334E to mutant DnaA proteins showing decreased membrane binding activity and normal DNA replication activity. If acidic phospholipids activate DnaA protein in vivo, as discussed above, these mutant DnaA proteins would be inactive in cells. Because acidic phospholipids inhibited in vitro DNA replication under some conditions (15), it is also possible that acidic phospholipids negatively regulate the activity of DnaA protein. In this case, these mutant DnaA proteins defective in acidic phospholipids binding would be hyperactive in DNA replication activity and possibly cause lethality. As was the case for DnaAK372E, the mutant dnaA gene encoding for DnaAR334E was found to be able to normally function for DNA replication in vivo, which would suggest that the regulation of the activity of DnaA protein by acidic phospholipids is not essential for DNA replication in cells. However, at present, the possibility that the remaining membrane interaction activities of these mutant proteins are enough to activate DnaA protein in cells cannot be dismissed. Similarly, the possibility that these mutant DnaA proteins can interact with membranes in cells through the aid of molecular chaperones cannot be overlooked.

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