Site-directed Mutational Analysis for the Membrane Binding of DnaA Protein

IDENTIFICATION OF AMINO ACIDS INVOLVED IN THE FUNCTIONAL INTERACTION BETWEEN DnaA PROTEIN AND ACIDIC PHOSPHOLIPIDS*

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DnaA protein, the initiator of chromosomal DNA replication in *Escherichia coli*, interacts with acidic phospholipids, such as cardiolipin, and its activity seems to be regulated by membrane binding in cells. In this study we introduced site-directed mutations at the positions of hydrophobic or basic amino acids which are conserved among various bacteria species and which are located in the putative membrane-binding region of DnaA protein (from Asp357 to Val374). All mutant DnaA proteins showed much the same ATP and ADP binding activity as that of the wild-type protein. The release of ATP bound to the mutant DnaA protein, in which three hydrophobic amino acids were mutated to hydrophilic ones, was stimulated by cardiolipin, as in the case of the wild-type protein. On the other hand, the release of ATP bound to another mutant DnaA protein, in which three basic amino acids were mutated to acidic ones, was not stimulated by cardiolipin. These results suggest not only that the region is a membrane-binding domain of DnaA protein but also that these basic amino acids are important for the binding and the ionic interaction between the basic amino acids and acidic residues of cardiolipin and is involved in the interaction between DnaA protein and cardiolipin.

In order to couple the initiation of DNA replication with cell division, the initiator protein should be inactivated soon after the initiation of DNA replication to prevent overinitiation and it should be activated after an appropriate period for the next replication cycle. The inactivation seems to be mediated by the intrinsic ATPase activity of DnaA protein and its activation factor based on the following evidence: (i) the induction of a mutant DnaA protein with decreased ATPase activity led to overinitiation of DNA replication in cells, resulting in a dominant lethal phenotype (10), and (ii) the stimulation factor for the ATPase was found and shown to be identical to the inactivation factor for DnaA protein (11), which was previously suggested to play an important role in the inactivation of DnaA protein (12, 13). As for the activation of DnaA protein for initiation of DNA replication, membrane phospholipids were reported to activate the inactive ADP-binding form of DnaA protein.

Acidic phospholipids, in particular cardiolipin (CL),1 decrease the affinity of DnaA protein for adenine nucleotides (14–17) and activate the DnaA protein from the ADP-binding form to the ATP-binding form in the presence of high concentrations of ATP by stimulating the exchange of ADP with ATP (14). We previously reported that artificial membranes consisting of a mixture of anionic and cationic phospholipids which mimic biological membranes can decrease the affinity of DnaA protein for adenine nucleotides under conditions in which acidic phospholipids form cluster structures (17). Garner and Crooke (18) suggested that the region around Lys372 of DnaA protein is responsible for membrane binding based on their biochemical analysis using degraded products of DnaA protein. A potential amphipathic helix (from Asp357 to Val374 of DnaA protein) located in this region may be a membrane-binding domain (1, 18). A recent study using a cross-linking technique with a photoactivatable phospholipid analog provided further evidence that the amphipathic helix is a membrane-binding domain (19).

In order to better understand how DnaA protein interacts with phospholipids and how it is involved in the regulation of DNA replication, we attempted to identify the amino acids of DnaA protein that are involved in membrane binding. To do this, we examined the effects of site-directed mutations on the membrane binding to DnaA protein.

EXPERIMENTAL PROCEDURES

Materials—A crude extract for oriC complementation assay was prepared from the WM433 strain of *E. coli* as described previously (20, 21). CL was purchased from Sigma. [a-32P]ATP (10 mCi/mmol) and

1 The abbreviations used are: CL, cardiolipin; Tricine, N-[2-hydroxyethyl]morpholinosuccinate; EDTA, ethylenediaminetetraacetic acid; TCA, trichloroacetic acid.

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**RESULTS**

**Site-directed Mutation for the Functional Interaction between Acidic Phospholipids and DnaA Protein—** Since, in general, protein-lipid interactions are mediated by hydrophobic interactions between the hydrophobic moiety of lipids and hydrophobic amino acids in proteins, the amino acids most likely to be involved in the interaction of DnaA protein with phospholipids are hydrophobic ones. The potential amphipathic helix (from Asp\(^{357}\) to Val\(^{374}\) of DnaA protein) contains many hydrophobic amino acids (Fig. 1). A comparison of the amino acid sequences of DnaA protein from various bacteria species revealed that Leu\(^{363}\), Leu\(^{366}\), and Leu\(^{367}\) of *E. coli* DnaA protein are well conserved as hydrophobic amino acids (1). Thus, these three leucines are considered to be important for the interaction between DnaA protein and phospholipids.

It has been reported that anionic residues in phospholipids are indispensable for their interaction with DnaA protein (14–17). Thus, besides the hydrophobic interaction, there is also a possibility that ionic interaction between anionic residues of phospholipids with basic amino acids in DnaA protein are involved in the functional interaction of the protein with phospholipids. As shown in Fig. 1, the amphipathic domain contains three basic amino acids (Arg\(^{360}\), Arg\(^{364}\), and Lys\(^{372}\)), which are well conserved among DnaA protein from various bacteria species (1). Thus, these three basic amino acids may also be involved in the interaction between DnaA protein and phospholipids.

We introduced site-directed mutations into the *dnaA* gene to convert Leu\(^{363}\), Leu\(^{366}\), and Leu\(^{367}\) to Ser (a hydrophilic amino acid) or Arg\(^{360}\), Arg\(^{364}\), and Lys\(^{372}\) to Glu (an acidic amino acid) to construct the mutant *dnaA* genes, *dnaA*\(^{430}\), or *dnaA*\(^{431}\), respectively. Considering the possibility that both groups of amino acids are required for the interaction of DnaA protein with phospholipids, a mutant *dnaA* gene (*dnaA*\(^{432}\)) which has mutations of both Leu\(^{363}\), Leu\(^{366}\), and Leu\(^{367}\) to Ser and Arg\(^{360}\), Arg\(^{364}\), and Lys\(^{372}\) to Glu was also prepared. The coding region of the *dnaA*\(^{430}\), *dnaA*\(^{431}\), or *dnaA*\(^{432}\) gene was conjugated with the promoter of the arabinosine operon to construct a plasmid for overproduction of the mutated DnaA protein (*DnaA*\(^{430}\), *DnaA*\(^{431}\), or *DnaA*\(^{432}\)). To avoid contamination of the wild-type DnaA protein in the fraction of the mutant DnaA protein, the KA450 strain (ΔoriC1701:Tn10, mkiA199[A(m)], dna17[A(m)], tyrE8928[A(m)], tyrA[A(m)], tyrE[A(m)], ilvE[A(m)], and thrA[A(m)]) in which the *dnaA* gene on chromosomal DNA was deleted (12) was used as host cells for overproduction. The viability is not dependent on the function of DnaA protein in KA450. Addition of 1% arabinosine caused overexpression of each mutant DnaA protein (data not shown). Purification of each mutant DnaA protein was done as described under “Experimental Procedures.” At the final step of purification (gel-

![Fig. 1. Amino acid sequence of the potential amphipathic helix (from Asp\(^{357}\) to Val\(^{374}\) of DnaA protein) and strategy for site-directed mutations.](image-url)

10% trichloroacetic acid. Samples were passed through Whatman GF/C glass-fiber filters. The amount of radioactivity on the filters was measured in a liquid scintillation counter, and the amount of DNA synthesized (picomoles of nucleotides) was calculated (20, 21).
filtration column chromatography) both the monomer and aggregated forms of DnaA430 and DnaA432 proteins were recovered (data not shown), as was the case of wild-type DnaA protein (Fig. 2) (23). However, only the monomer form of the DnaA431 protein was recovered (Fig. 2). The recovery of DnaA431 protein after column chromatography was not significantly different from the recoveries of the other types of DnaA protein. The aggregated form of DnaA protein has been suggested to be a complex of DnaA protein with phospholipids, based on the finding that the aggregated form of DnaA protein is activated to the level of the monomer form by phospholipase (28). Thus, the result in Fig. 2 suggests that DnaA431 protein does not have an affinity for phospholipids.

Characterization of ATP and ADP Binding Activity of the Mutant DnaA Proteins—The functional interaction of DnaA protein with phospholipids is monitored by phospholipid-dependent stimulation of the release of ATP (or ADP) from DnaA protein (23). The activation of the ADP-binding form of DnaA protein by phospholipids is mediated by this function of phospholipids on DnaA protein (23). Thus, the mutant DnaA proteins were required to maintain their ATP binding activity in order to examine their functional interaction with phospholipids. The ATP binding activities of these mutant proteins were examined by a filter-binding assay (6). As shown in Fig. 3A, each mutant DnaA protein (DnaA430, DnaA431, or DnaA432) showed nearly the same ATP binding activity as the wild-type protein. The Kd values of DnaA430, DnaA431, DnaA432 and the wild-type protein for ATP were determined to be 28, 19, 28, and 30 nM, respectively. The numbers of ATP-binding sites per DnaA430, DnaA431, DnaA432, and wild-type proteins were calculated to be 0.40, 0.46, 0.39, and 0.42, respectively. The Kd value and the number of ATP-binding sites of wild-type protein were nearly the same as reported previously (5). We also examined the binding of ADP by the mutant DnaA proteins in the same manner. As shown in Fig. 3B, each of the mutant DnaA proteins maintained its ADP binding activity. These results enabled us to examine their functional interaction with phospholipids. The results also suggest that these amino acids in the amphipathic helix domain are not necessary for the adenosine-nucleotide binding activity of DnaA protein.

Functional Interaction of the Mutant DnaA Proteins with CL—To examine the functional interaction of DnaA430, DnaA431, and DnaA432 proteins with CL, we investigated the effect of CL on the release of ATP from DnaA-ATP complex. DNA protein bound to [α-32P]ATP was incubated with CL and the remaining ATP was determined by the filter-binding assay. As shown in Fig. 4, the rates of release of ATP from each of the mutant proteins in the absence of CL were nearly the same as that of the wild type protein, suggesting that the Kd values for ATP of DnaA protein were not significantly affected by these mutations (see above). On the other hand, the release of ATP in the presence of CL was greatly affected by the mutations. CL did not stimulate the release of ATP from DnaA431 protein, whereas it stimulated the release from DnaA430 to nearly the same extent that it stimulated the release from the wild-type protein (Fig. 4). CL slightly stimulated the release of ATP from DnaA432, which has mutations of both DnaA430 and DnaA431 (Fig. 4). These results suggest that the basic amino acids (Arg360, Arg364, and Lys372) but not the hydrophobic amino acids (Leu363, Leu366, and Leu367) in the amphipathic helix domain of DnaA protein are involved in the functional interac-
tion between DnaA protein and CL, and that the interaction is mediated by an ionic interaction between the anionic residues of phospholipids and the basic amino acids of DnaA protein. These results also support the hypothesis that the amphipathic helix domain (from Asp357 to Val374 of DnaA protein) is the membrane-binding domain of DnaA protein (1, 18, 19).

**Replication Activity of the Mutant DnaA Proteins in Vitro**

We measured the activities of DnaA430, DnaA431, and DnaA432 proteins for initiation of DNA replication in an oriC complementation assay (20, 21). As shown in Fig. 5A, each of the mutants showed activity for DNA replication, although the activities were less than the activity of the wild-type protein. The specific activities of DnaA430 and DnaA431 were about one-third and one-fifth, respectively, the activity of the wild-type protein, whereas the activity of DnaA432 was less than one-tenth that of the wild-type protein. DnaA46, and DnaA5 required longer incubation periods for expression of

![Graph showing replication activity](image)

**Fig. 5. Replication activity of the mutant DnaA protein in a crude extract.** DnaA430, DnaA431, DnaA432, or the wild-type DnaA protein was incubated with 1 μM ATP for 15 min at 0 °C (A). DnaA430, DnaA431, or the wild-type DnaA protein (100 ng) was incubated at first with the indicated concentrations of CL for 15 min at 0 °C (B). DNA replication in a crude extract was done for 20 min as described under “Experimental Procedures.” The relative DNA replication activity to that without CL was shown (the 100% values for DnaA430, DnaA431, and the wild-type protein were 152, 185, and 403 pmol, respectively) (B).
their replication activity; a time lag for the DNA replication reaction has been previously reported for these mutant DnaA proteins (7–9). In the case of these mutants, the time course of DNA replication was approximately linear as in the case of the wild-type protein (data not shown). Preincubation with each of the mutant proteins with 1 μM ADP inhibited the replication activity (data not shown) as in the case of the wild-type protein (6).

The relatively low specific activities of the mutant DnaA proteins seems not to be due to denaturation as a result of the purification procedures, because the specific activities of the crude extract fractions of the mutant DnaA proteins were also lower than the specific activity of the wild-type protein. The mutated amino acids may affect the higher order structure of DnaA protein, resulting in the low specific activities.

We also examined the effect of CL on the replication activity of the mutant and the wild-type DnaA proteins. It was shown that CL inhibited the replication activity of the nucleotide-free form of DnaA protein through inhibition of the ATP-binding to that CL inhibited the replication activity of the nucleotide-free mutant and the wild-type DnaA proteins. It was shown that the specific activities of the mutant proteins were lower than the specific activity of the wild-type protein. The crude extract fractions of the mutant DnaA proteins were also subjected to the purification procedures, because the specific activities of the wild-type DnaA protein. The concentrations of CL needed to inhibit 50% inhibition of the replication activity of DnaA430, DnaA431, and the wild-type DnaA protein were determined to be 1.9, 6.5, and 1.8 μM, respectively. The result supports the notion that the functional interaction of DnaA431 but not DnaA430 with CL is weakened by the mutations and thus, the basic amino acids are important for the functional interaction.

DISCUSSION

The replication of chromosomal DNA is initiated in coordination with cell division (membrane division). A replication model was proposed to explain this coordination (29). DNA replication in bacterial cells is initiated on membranes, and the activity of the initiator protein for DNA replication is regulated by membrane components (29). Thus, the interaction between DnaA protein and acidic phospholipids has received much attention because it may provide biochemical support for the replication model. Acidic phospholipids, such as CL, enhance the exchange reaction of ADP bound to DnaA protein with ATP, which results in activation of DnaA protein (14–17). Thus, it was proposed that acidic phospholipids activate DnaA protein to initiate the chromosomal DNA replication in cells (14). To test this hypothesis, identification of amino acids involved in the interaction is important. In this study, we showed that DnaA431, which has mutations (Arg360, Arg364, and Lys372) in the amphipathic helix (from Asp357 to Val374 of DnaA protein) did not have a functional interaction with CL. The behavior of the DnaA431 protein in gel filtration chromatography is consistent with the notion that DnaA431 protein cannot form a complex with CL. These results strongly suggest that the amphipathic helix is the membrane-binding domain of DnaA protein as suggested previously (1, 18, 19).

We also showed that DnaA430 protein, which has mutations of hydrophobic amino acids in the amphipathic helix, maintained an activity for the functional interaction with CL. Thus, the basic amino acids (Arg360, Arg364, and Lys372) but not the hydrophobic amino acids (Leu363, Leu366, and Leu367) seem to be involved in the interaction. It is natural to suppose that the positively charged basic residues interact with the negatively charged phosphates of CL through an electrostatic interaction. Fig. 6 depicts a plausible form of the interaction between DnaA protein and CL. The location of each amino residue is putatively defined by considering the general structural parameters for an α-helical wheel. It is noteworthy that all three of the basic residues are placed on one side of the helical wheel and thus, provide a cationic domain. This configuration allows both of the two phosphates of CL to bind all or part of the three basic residues. A multisite electrostatic interaction may perturb the affinity of DnaA protein for adenine nucleotides.

Garner and co-workers (19) recently proposed a two-step interaction of DnaA protein with acidic phospholipids. At first, DnaA protein adsorbs onto the anionic lipid bilayer surface, and then some part of the protein penetrates into the hydrophobic interior of the membrane (19). The results of this study support the hypothesis that the first step of the interaction is mediated by an ionic interaction between the anionic residues of CL with the basic amino acids of DnaA protein. The second step may be achieved by an interaction between the hydrophobic region of the phospholipids with the hydrophobic amino acids of DnaA protein. However, the present study suggests that hydrophobic amino acids in the amphipathic helix are not involved in this interaction. Besides these amino acids (Leu363, Leu366, and Leu367), there are some other hydrophobic amino acids that are conserved among DnaA proteins from various bacteria species (1). Some of these amino acids may be involved in the second step of the interaction.

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REFERENCES

1. Skarstad, K., and Boye, E. (1994) Biochim. Biophys. Acta 1217, 111–130
2. Hirota, Y., Mordoh, J., and Jacob, P. (1970) J. Mol. Biol. 53, 369–387
Membrane Binding to DnaA Protein

3. Fuller, R. S., Funnell, B. E., and Kornberg, A. (1984) Cell 38, 889–900
4. Bramhill, D., and Kornberg, A. (1988) Cell 52, 743–755
5. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987) Cell 50, 259–265
6. Mizushima, T., Sasaki, S., Oishi, H., Kobayashi, M., Katayama, T., Miki, T., Maeda, M., and Sekimizu, K. (1996) J. Biol. Chem. 271, 25178–25183
7. Hupp, T. R., and Kaguni, J. M. (1993) J. Biol. Chem. 268, 13128–13136
8. Hwang, D. S., and Kaguni, J. M. (1988) J. Biol. Chem. 263, 10633–10640
9. Carr, K. M., and Kaguni, J. M. (1996) Mol. Microbiol. 20, 1307–1318
10. Mizushima, T., Sasaki, S., Maeda, M., Katayama, T., Miki, T., and Sekimizu, K. (1996) J. Biol. Chem. 271, 25178–25183
11. Fuller, R. S., Kaguni, J. M., and Kornberg, A. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7370–7374
12. Fuller, R. S., and Kornberg, A. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5817–5821
13. Garner, J., and Crooke, E. (1996) EMBO J. 15, 3477–3485
14. Garner, J., Durrer, P., Kitchen, J., Brunner, J., and Crooke, E. (1998) J. Biol. Chem. 273, 5167–5173
15. Kurokawa, K., Mizushima, T., Kubota, T., Tschiya, T., Katayama, T., and Sekimizu, K. (1998) Biochem. Biophys. Res. Commun. 243, 90–95
16. Katayama, T. (1994) J. Biol. Chem. 269, 22075–22079
17. Katayama, T., and Kornberg, A. (1994) J. Biol. Chem. 269, 12698–12703
18. Sekimizu, K., and Kornberg, A. (1988) J. Biol. Chem. 263, 7131–7135
19. Yung, B. Y., and Kornberg, A. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7202–7205
20. Castuma, C. E., Crooke, E., and Kornberg, A. (1993) J. Biol. Chem. 268, 24665–24668
21. Mizushima, T., Ishikawa, Y., Obana, E., Hase, M., Kubota, T., Katayama, T., Kunitake, T., Watanabe, E., and Sekimizu, K. (1996) J. Biol. Chem. 271, 3633–3638
22. Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488–492
23. Sekimizu, K., Yung, B. Y., and Kornberg, A. (1988) J. Biol. Chem. 263, 7136–7140
24. Mizushima, T., Kurokawa, K., Tsuchiya, T., and Sekimizu, K. (1998) Biochemistry 33, 11512–11516
25. Kubota, T., Katayama, T., Ito, Y., Mizushima, T., and Sekimizu, K. (1997) Biochem. Biophys. Res. Commun. 232, 130–135
26. Hase, M., Ishikawa, Y., Sekimizu, K., Tsuchiya, T., and Mizushima, T. (1998) J. Biochem. (Tokyo) 123, 680–683
27. Chen, P. S., Jr., Tpribata, T. Y., and Warner, H. (1956) Anal. Chem. 18, 1756–1758
28. Hwang, D. S., Crooke, E., and Kornberg, A. (1990) J. Biol. Chem. 265, 19244–19248
29. Jacob, F., Brenner, S., and Cuzin F. (1963) Cold Spring Harbor Symp. Quant. Biol. 28, 329–348