Inhibitory effects of acidic phospholipids on the binding of origin-recognition complex to origin DNA

Jong-Ryul LEE*, Masaki MAKISE*, Hitomi TAKENAKA*, Naoko TAKAHASHI*, Yoshihiro YAMAGUCHI*, Tomofusa TSUCHIYA* and Tohru MIZUSHIMA*†[1]

*Faculty of Pharmaceutical Sciences, Okayama University, 1-1-1, Tsushima-naka, Okayama 700-8530, Japan, and †PRESTO, Japan Science and Technology Corporation, Okayama 700-8530, Japan

Origin-recognition complex (ORC), a candidate initiator of chromosomal DNA replication in eukaryotes, shares certain biochemical characteristics with DnaA, the initiator of chromosomal DNA replication in prokaryotes. These similarities include origin-specific DNA binding, ATP binding and ATPase activity. DnaA interacts with acidic phospholipids, such as cardiolipin, and its activity is regulated by these phospholipids. In this study, we examined whether *Saccharomyces cerevisiae* ORC also interacts with phospholipids. Among the various phospholipids tested, ORC was found to bind specifically to cardiolipin. This binding was inhibited by excess concentrations of salts but unaffected by ATP, adenosine 5′-[γ-thio]triphosphate or the origin DNA. Cardiolipin weakly inhibited the ATP-binding activity of ORC, whereas it strongly inhibited ORC binding to origin DNA. Acidic phospholipids other than cardiolipin (phosphatidylglycerol and phosphatidylinositol) weakly inhibited ORC binding to origin DNA. Furthermore, total phospholipids extracted from yeast nuclear membranes inhibited ORC binding to origin DNA. We consider that phospholipids may modulate initiation of DNA replication in eukaryotes in a similar manner to that found in prokaryotes.

Key words: ATP binding, cardiolipin, DnaA, DNA replication, origin binding.

INTRODUCTION

Regulation of the activity of initiator proteins is a key step for the control of chromosomal DNA replication in both prokaryotic and eukaryotic cells. DnaA is the initiator of chromosomal DNA replication in *Escherichia coli* [1]. DnaA specifically binds to the origin of chromosomal DNA (*oriC*), forms oligomers to 'open up' the duplex DNA, and recruits DnaB, a replication DNA helicase [1]. The activity of DnaA is regulated by adenine nucleotides bound to the protein. DnaA has a high affinity for both ATP and ADP, but whereas the ATP-binding form is active, the ADP-binding form is inactive during DNA replication [2–4]. ATP bound to DnaA is hydrolysed to ADP by its intrinsic ATPase activity, and this hydrolysis is responsible for inactivating DnaA in order to suppress re-replication [5,6].

The origin-recognition complex (ORC) is a possible initiator of eukaryotic chromosomal DNA replication [7]. ORC was originally identified as a six-protein complex that bound specifically to *Saccharomyces cerevisiae* origins of DNA replication [8] and its homologues have been found in various eukaryotic species, including human cells [9]. Eukaryotic chromosomal DNA replication is initiated by the binding of Cdc6p to ORC followed by recruitment of mini-chromosome maintenance protein (MCM), a replication DNA helicase, by the ORC–Cdc6p complex, which was recently reconstituted *in vitro* [10]. ORC has ATP-binding and ATPase activities [8,11]. ATP binding to Orc1p, one of the two ORC subunits (the other is Orc5p), is essential for ORC binding to origin DNA [11]. The ATPase activity of ORC appears to be involved in disrupting the ORC–Cdc6p complex in order to suppress re-replication [12]. Therefore, adenine nucleotides bound to ORC may regulate the initiation of chromosomal DNA replication in eukaryotic cells, as seen for DnaA.

Acidic phospholipids, in particular cardiolipin (CL), decrease DnaA's affinity for adenine nucleotides and convert the ADP-bound DnaA into the ATP-bound form in the presence of high concentrations of ATP by stimulating the exchange reaction of ADP with ATP [13–16]. It has been suggested that DnaA is regulated by acidic phospholipids *in vivo* [17–19]. CL also inhibits the DNA-binding activity of DnaA [20]. We have previously identified basic amino acid residues of DnaA that are essential for its binding to CL [21–24] and proposed that the ionic interaction between DnaA and CL changes the conformation of DnaA, resulting in decreased affinity of DnaA for adenine nucleotides [24].

As described above, ORC and DnaA share common biochemical characteristics, such as sequence-specific binding to origin DNA, and ATP-binding and ATPase activities. Therefore, we predict that ORC also binds to acidic phospholipids and that acidic phospholipids affect certain biochemical attributes of ORC. Here, this hypothesis is tested using purified *S. cerevisiae* ORC. We found that ORC binds CL and that CL strongly inhibits ORC binding to origin DNA.

MATERIALS AND METHODS

Materials

The ORC from *S. cereviseae* was expressed in Sf9 cells infected with a recombinant baculovirus and purified as described previously [25]. [α-^{32}P]ATP (3000 Ci/mmol) and [γ-^{32}P]ATP (6000 Ci/mmol) were obtained from Amersham Bioscience. T4

Abbreviations used: ORC, origin-recognition complex; MCM, mini-chromosome maintenance protein; CL, cardiolipin; PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

[1] To whom correspondence should be addressed, at the Faculty of Pharmaceutical Sciences (e-mail mizushima@pharm.okayama-u.ac.jp).

polynucleotide kinase was purchased from Takara Co. CL (from bovine heart), phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) from egg yolk, and phosphatidylinositol (PI) from bovine liver were purchased from Sigma.

Origin DNA fragments (*ARS1*; 294 bp) were synthesized by PCR as described previously [10]. DNA fragments were radiolabelled with [γ-^{32}P]ATP mediated by T4 polynucleotide kinase. The specific activity of each probe was 4000–7000 c.p.m./fmol of DNA.

Co-precipitation assay for determining the interaction between phospholipids and ORC

The binding of ORC to phospholipids was examined by applying the assay system for DnaA and phospholipid interactions [26]. Phospholipid liposomes were prepared from dried phospholipids on the bottoms of glass tubes through vigorous vortex mixing in water except for PE, which was suspended in 0.05 % Triton X-100. The amount of phosphorus in the phospholipid fraction was determined using the method of Chen et al. [27].

ORC and phospholipids were incubated in 30 μl of buffer H, containing 50 mM Hepes/KOH (pH 7.5), 0.1 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM magnesium acetate, 0.0002 % Nonidet P-40, 10 % glycerol, 1 mg/ml BSA and 1 mM ATP, at 4 °C for 5 min followed by centrifugation. After washing, precipitates were resuspended in SDS sample buffer, loaded on to 10 % polyacrylamide gels containing SDS, and electrophoresed. The proteins were then immunoblotted with anti-Orc1p monoclonal antibodies (SB16 and SB35) [10].

Filter-binding assays

ATP-binding activity of ORC was determined by a filter-binding assay [2]. ORC (0.15 pmol) was incubated with 0.2 μM [α-^{32}P]ATP at 4 °C for 10 min in 25 μl of buffer H. Samples were passed through nitrocellulose membranes (Millipore HA; 0.45 μm) and washed with ice-cold buffer H. The radioactivity remaining on the filter was measured with a liquid scintillation counter.

To monitor DNA binding to ORC, ORC (0.15 pmol) was incubated with radiolabelled *ARS1* DNA fragments at 30 °C for 10 min in 25 μl of buffer H. Samples were passed through nitrocellulose membranes (Millipore HA; 0.45 μm) and washed with ice-cold buffer H. The radioactivity remaining on the filter was monitored with a liquid scintillation counter.

Gel electrophoretic mobility-shift assay for DNA binding to ORC

A gel electrophoretic mobility-shift assay was performed as described in [10]. ORC was incubated with radiolabelled *ARS1* DNA fragments (90 fmol) for 10 min at 30 °C in 10 μl of buffer T [25 mM Tris/HCl (pH 7.8), 5 mM $MgCl_2$, 70 mM KCl, 2 mg/ml BSA, 5 mM dithiothreitol, 5 % (v/v) glycerol, 1 mM ATP and 1.2 μg/ml poly(dI)/poly(dC) as non-specific competitors]. The reaction sample was loaded on to a 3.5 % polyacrylamide gel containing 0.5 × TBE [0.045 M Tris/borate (pH 8.3) and 1 mM EDTA]. The gel was electrophoresed for 1.5 h at a constant 200 V at 4 °C, dried and autoradiographed.

Preparation of nuclear membranes

Nuclear membranes were prepared from the wild-type *S. cerevisiae* cells as described in [28]. For preparation of liposomes from nuclear membranes, the membranes were extracted with 100 % chloroform and the liposomes were prepared. The amount of phosphorus in the phospholipid fraction was determined using the method of Chen et al. [27], and the amounts of phospholipids were estimated by assuming that the average molecular mass of phospholipids is 700 Da.

RESULTS AND DISCUSSION

Direct interaction between ORC and phospholipids

We applied a co-precipitation assay to examine the direct interaction of ORC with various phospholipids [10]. Major phospholipid components of eukaryotic cell membranes (CL, PE, PI, PC and PG) were used. As shown in Figure 1, Orc1p co-precipitated with CL in a dose-dependent manner. Immunoblotting experiments using antibodies against ORC subunits other than Orc1p showed that all ORC subunits co-precipitated equally with CL, confirming that the whole ORC has some interaction with CL. We did not observe significant co-precipitation of ORC with phospholipids other than CL, even at higher concentrations of these phospholipids (Figure 1).

Next we examined mechanisms for the interaction between ORC and CL. We have reported previously that conserved basic amino acid residues of DnaA are responsible for its interaction with CL, suggesting that the interaction is mediated by ionic interaction between these basic amino acid residues and the acidic moiety of CL [21–24]. In support of this finding, it was reported that this interaction is inhibited by high concentrations of salt [29]. Therefore, we hypothesized that the association between ORC and CL is mediated by an ionic interaction and that this interaction may be inhibited by high concentrations of salt. As shown in Figure 2(A), the co-precipitation of ORC with CL was inhibited by KCl in a dose-dependent manner. Similar results were also obtained with NaCl (results not shown). These results showed that the binding of ORC and CL is mediated by ionic interaction.

Effect of adenine nucleotides and origin DNA on the interaction between ORC and CL

ORC binds to ATP and hydrolyses it to ADP [8,11]. Here, we examined the effect of adenine nucleotides on the interaction of ORC with CL. As shown in Figure 2(B), adenosine 5′-[γ-thio]triphosphate, an analogue of ATP that is difficult to hydrolyse, did not affect the interaction of ORC with CL, suggesting that ATP hydrolysis is not involved in the interaction. Furthermore, ATP was shown to be dispensable for the interaction. Even in the absence of any adenine nucleotides, the efficient interaction between ORC and CL was observed (Figure 2B). These results suggest that CL can interact with any form of ORC (ATP-bound, ADP-bound and nucleotide-free).

We also examined the effect of origin DNA on the interaction of ORC with CL. Since ORC can bind to origin DNA in an ATP-dependent manner [8], we performed experiments in the presence of 1 mM ATP. As shown in Figure 2(C), origin DNA did not affect the interaction of ORC with CL. These results suggest that CL can interact with both DNA-bound and DNA-unbound forms of ORC.

Effect of CL on ATP binding to ORC

As described above, ATP binding to DnaA was inhibited strongly by CL [12–15]. We here tested whether or not CL inhibits ATP binding to ORC, using a filter-binding assay. ORC comprises two subunits (Orc1p and Orc5p) that bind to ATP. ATP binding to Orc1p, but not to Orc5p, requires the presence of origin DNA [11]. Thus we examined the effect of CL on ATP binding to ORC in the presence or absence of origin DNA. In the absence of CL

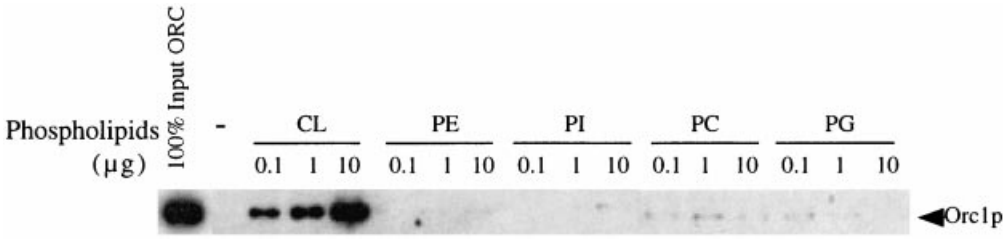

Figure 1 Co-precipitation of ORC with phospholipids

ORC (0.15 pmol) was incubated with the indicated amounts of CL, PE, PI, PC or PG in the presence of 1 mM ATP. After centrifugation, Orc1p in precipitates was visualized by immunoblotting with anti-Orc1p antibodies (SB16 and SB35).

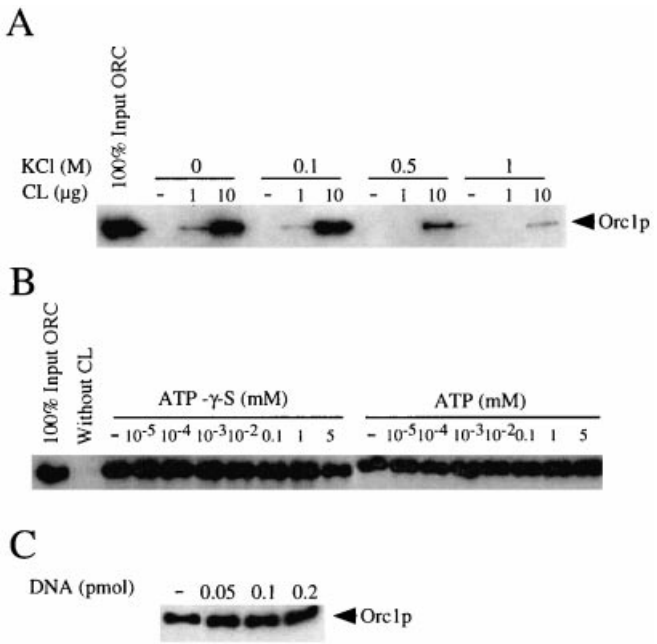

Figure 2 Mechanism of the interaction between ORC and CL

(**A**) ORC (0.15 pmol) was incubated with the indicated amounts of CL in the presence of KCl, as indicated, and 1 mM ATP. (**B**) ORC (0.15 pmol) was incubated with CL in the presence of the indicated concentrations of adenosine 5′-[γ-thio]triphosphate (ATP-γ-S) or ATP. (**C**) ORC (0.15 pmol) was incubated with 10 μg of CL in the presence of the indicated amounts of *ARS1* origin DNA fragments. (**A**–**C**) After centrifugation, Orc1p in precipitates was visualized by immunoblotting with anti-Orc1p antibodies (SB16 and SB35).

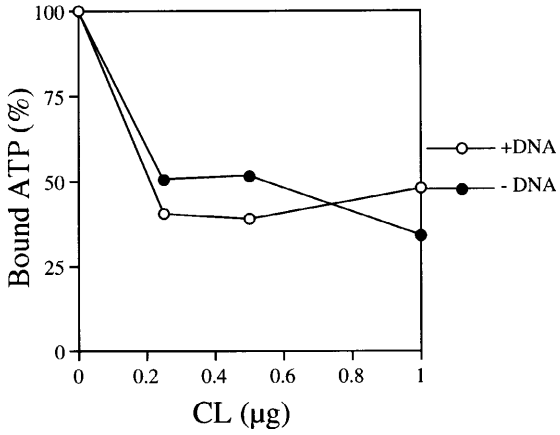

Figure 3 Effect of CL on the ATP-binding capability of ORC

ORC (0.15 pmol) was pre-incubated with the indicated amounts of CL for 10 min at 4 °C and incubated further with or without *ARS1* origin DNA fragments (0.2 pmol) in the presence of 0.2 μM [α-^{32}P]ATP. The amount of bound ATP was determined by a filter-binding assay.

the amount of ATP bound to ORC was higher when origin DNA was supplied in the assay. This may be due to a lack of ATP binding to Orc1p in the absence of origin DNA. As shown in Figure 3, CL partially inhibited ATP binding to ORC in the absence of origin DNA, suggesting that CL partially inhibited the ATP binding to Orc5p. Similar results were obtained in the presence of origin DNA (Figure 3), suggesting that ATP binding to Orc1p was also partially inhibited by CL. Higher amounts of CL (up to 10 μg) did not result in the complete inhibition of ATP binding to ORC in either the presence or absence of origin DNA (results not shown).

Effect of CL on the interaction between ORC and origin DNA

The binding of origin DNA to DnaA is also inhibited by CL [20]. We examined the effect of CL on ORC binding to origin DNA, using a gel electrophoretic mobility-shift assay. As shown in Figure 4(A), CL strongly inhibited ORC binding to the origin DNA. Other acidic phospholipids (PG and PI) also partially inhibited this binding, in a dose-dependent manner (Figure 4A). On the other hand, neutral phospholipids (PC and PE) did not significantly affect ORC binding to origin DNA (Figure 4A). We also examined the effect of various phospholipids on the DNA-binding activity of ORC, using a filter-binding assay. This assay yielded similar results to those observed in the gel electrophoretic mobility-shift analysis (Figure 4B). CL exhibited strong inhibitory effects whereas PG and PI showed weak inhibitory effects on ORC binding to DNA. Taken together, these results suggest that the acidic moiety of phospholipids is important for the inhibitory effect of phospholipids on ORC binding to DNA. However, the results shown in Figure 4 are not consistent with those seen in Figure 1. Neither PG nor PI co-precipitated with ORC (Figure 1), but they were responsible for partial inhibition of the ORC binding to DNA (Figure 4). This may be due to the fact that the interaction between ORC and these phospholipids was so weak that it could not co-precipitate ORC. In other words, an assay of the inhibition of ORC binding to DNA is more sensitive than the co-precipitation assay for the detection of interactions between ORC and phospholipids.

To address the issue of whether or not these results *in vitro* reflect the situation *in vivo*, we examined the localization of ORC

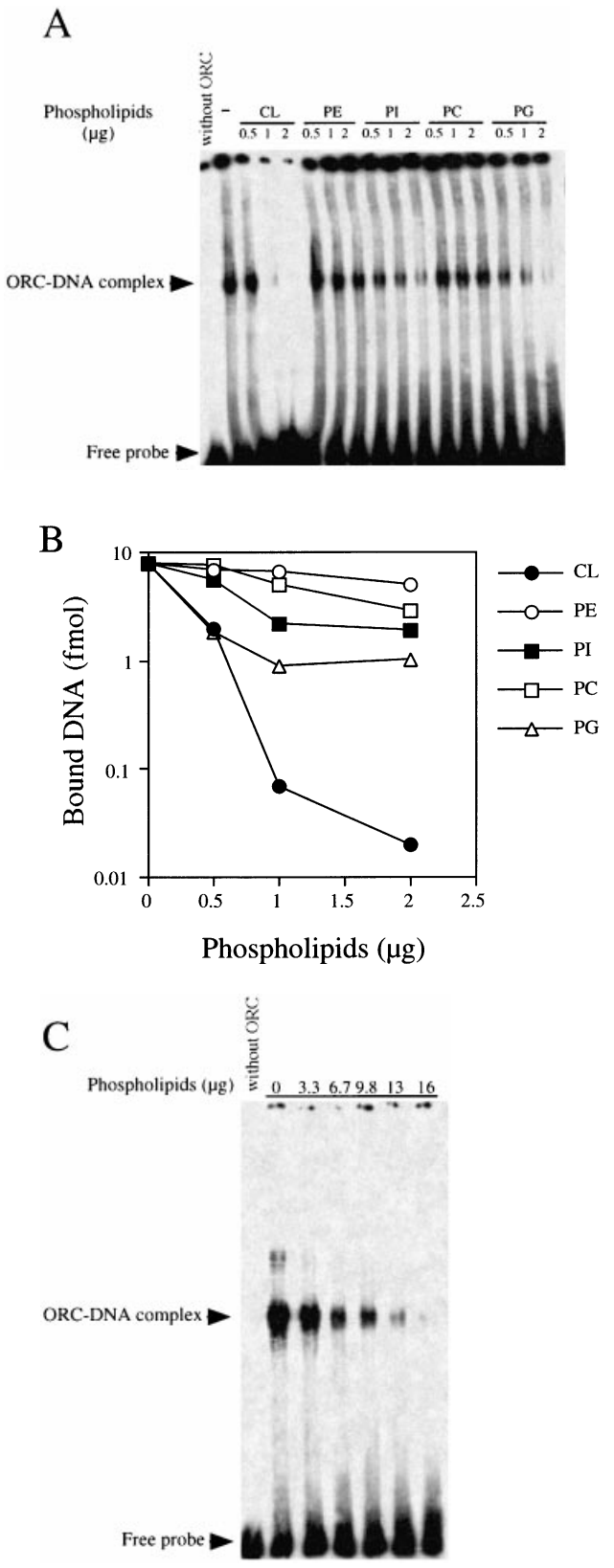

Figure 4 Effect of various phospholipids on the DNA binding of ORC

ORC (0.15 pmol) was pre-incubated with the indicated amounts of CL, PE, PI, PC or PG (**A**, **B**), or total phospholipids of the nuclear membrane (**C**) for 10 min at 4 °C and incubated further with radiolabelled *ARS1* DNA fragments (90 fmol) in the presence of 1 mM ATP and 5 μg/ml poly(dI)/poly(dC). A gel electrophoretic mobility-shift assay was performed and autoradiographed (**A**, **C**). The amount of bound ATP was determined by a filter-binding assay (**B**).

in nuclear membranes. According to a previous study [28], nuclear-membrane fractions were prepared from wild-type yeast cells and the amount of ORC in the fraction determined by immunoblotting. Approx. 10 % of the total ORCs were recovered in the nuclear-membrane fraction. However, we consider that this may have been due to contamination of the nuclear-membrane fraction by the nuclear soluble fraction, since about 10 % of total Mcm2p (one of the subunits of MCM) was also recovered in the nuclear-membrane fraction. Employing the co-precipitation assay, we also examined whether liposomes prepared from total phospholipids of the nuclear membrane could bind to ORC. However, no co-precipitation with ORC was noted in this case (results not shown).

As described above, an assay of inhibition of ORC binding to DNA is more sensitive than the co-precipitation assay for the assessment of interactions between ORC and phospholipids. We examined the effect of total nuclear-membrane phospholipids on ORC binding to DNA. Figure 4(C) shows that total phospholipids inhibited ORC binding to DNA in a dose-dependent manner. We consider, therefore, that the activity of ORC may be modulated by nuclear-membrane phospholipids *in vivo*.

We thank Dr Bruce Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.) for providing antibodies against ORC. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, the Takeda Science Foundation, and the Nagase Science and Technology Foundation. N.T., M.M. and Y.Y. are Research Fellows of the Japan Society for the Promotion of Science (JSPS).

REFERENCES

1 Skarstad, K. and Boye, E. (1994) The initiator protein DnaA: evolution, properties and function. Biochim. Biophys. Acta **1217**, 111–130
2 Sekimizu, K., Bramhill, D. and Kornberg, A. (1987) ATP activates dnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. Cell **50**, 259–265
3 Mizushima, T., Sasaki, S., Ohishi, H., Kobayashi, M., Katayama, T., Miki, T., Maeda, M. and Sekimizu, K. (1996) Molecular design of inhibitors of *in vitro oriC* DNA replication based on the potential to block the ATP binding of DnaA protein. J. Biol. Chem. **271**, 25178–25183
4 Mizushima, T., Takaki, T., Kubota, T., Tsuchiya, T., Miki, T., Katayama, T. and Sekimizu, K. (1998) Site-directed mutational analysis for the ATP binding of DnaA protein. Functions of two conserved amino acids (Lys-178 and Asp-235) located in the ATP-binding domain of DnaA protein *in vitro* and *in vivo*. J. Biol. Chem. **273**, 20847–20851
5 Mizushima, T., Nishida, S., Kurokawa, K., Katayama, T., Miki, T. and Sekimizu, K. (1997) Negative control of DNA replication by hydrolysis of ATP bound to DnaA potein, the initiator of chromosomal DNA replication in *Escherichia coli*. EMBO J. **16**, 3724–3730
6 Katayama, T., Kubota, T., Kurokawa, K., Crooke, E. and Sekimizu, K. (1998) The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. Cell **94**, 61–71
7 Stillman, B. (1996) Cell cycle control of DNA replication. Science **274**, 1659–1664
8 Bell, S. P. and Stillman, B. (1992) ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. Nature (London) **357**, 128–134
9 Dutta, A. and Bell, S. P. (1997) Initiation of DNA replication in eukaryotic cells. Annu. Rev. Cell. Dev. Biol. **13**, 293–332
10 Mizushima, T., Takahashi, N. and Stillman, B. (2000) Cdc6p modulates the structure and DNA binding activity of the origin recognition complex *in vitro*. Genes Dev. **14**, 1631–1641
11 Klemm, R. D., Austin, R. J. and Bell, S. P. (1997) Coordinate binding of ATP and origin DNA regulates the ATPase activity of the origin recognition complex. Cell **88**, 493–502
12 Klemm, R. D. and Bell, S. P. (2001) ATP bound to the origin recognition complex is important for preRC formation. Proc. Natl. Acad. Sci. U.S.A. **98**, 8361–8367
13 Sekimizu, K. and Kornberg, A. (1988) Cardiolipin activation of dnaA protein, the initiation protein of replication in *Escherichia coli*. J. Biol. Chem. **263**, 7131–7135
14 Yung, B. Y. and Kornberg, A. (1988) Membrane attachment activates dnaA protein, the initiation protein of chromosome replication in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **85**, 7202–7205

15 Castuma, C. E., Crooke, E. and Kornberg, A. (1993) Fluid membranes with acidic domains activate DnaA, the initiator protein of replication in *Escherichia coli*. J. Biol. Chem. **268**, 24665–24668
16 Mizushima, T., Ishikawa, Y., Obana, E., Hase, M., Kubota, T., Katayama, T., Kunitake, T., Watanabe, E. and Sekimizu, K. (1996) Influence of cluster formation of acidic phospholipids on decrease in the affinity for ATP of DnaA protein. J. Biol. Chem. **271**, 3633–3638
17 Xia, W. and Dowhan, W. (1995) *In vivo* evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **92**, 783–787
18 Suzuki, E., Kondo, T., Makise, M., Mima, M., Sakamoto, K., Tsuchiya, T. and Mizushima, T. (1998) Alteration in the contents of unsaturated fatty acids in dnaA mutants of *Escherichia coli*. Mol. Microbiol. **28**, 95–102
19 Zheng, W., Li, Z., Skarstad, K. and Crooke, E. (2001) Mutations in DnaA protein suppress the growth arrest of acidic phospholipid-deficient *Escherichia coli* cells. EMBO J. **20**, 1164–1172
20 Crooke, E., Castuma, C. E. and Kornberg, A. (1992) The chromosome origin of *Escherichia coli* stabilizes DnaA protein during rejuvenation by phospholipids. J. Biol. Chem. **267**, 16779–16782
21 Hase, M., Yoshimi, T., Ishikawa, Y., Ohba, A., Guo, L., Mima, S., Makise, M., Yamaguchi, Y., Tsuchiya, T. and Mizushima, T. (1998) 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. J. Biol. Chem. **273**, 28651–28656
22 Yamaguchi, Y., Hase, M., Makise, M., Mima, S., Yoshimi, T., Ishikawa, Y., Tsuchiya, T. and Mizushima, T. (1999) Involvement of Arg-328, Arg-334 and Arg-342 of DnaA protein in the functional interaction with acidic phospholipids. Biochem. J. **340**, 433–438
23 Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2000) Identification of amino acids involved in the functional interaction between DnaA protein and acidic phospholipids. J. Biol. Chem. **275**, 4513–4518
24 Makise, M., Mima, S., Tsuchiya, T. and Mizushima, T. (2001) Molecular mechanism for functional interaction between DnaA protein and acidic phospholipids: identification of important amino acids. J. Biol. Chem. **276**, 7450–7456
25 Bell, S. P., Mitchell, J., Leber, J., Kobayashi, R. and Stillman, B. (1995) The multidomain structure of Orc1p reveals similarity to regulators of DNA replication and transcriptional silencing. Cell **83**, 563–568
26 Hase, M., Ishikawa, Y., Sekimizu, K., Tsuchiya, T. and Mizushima, T. (1998) Effect of glycerol on the affinity of DnaA protein for ATP in the presence of cardiolipin. J. Biochem. (Tokyo) **123**, 680–683
27 Chen, Jr, P. S., Toribata, T. Y. and Warner, H. (1956) Microdetermination of phosphorus. Anal. Chem. **18**, 1756–1758
28 Mann, K. and Mecke, D. (1982) The isolation of *Saccharomyces cerevisiae* nuclear membranes with nuclease and high-salt treatment. Biochim. Biophys. Acta **687**, 57–62
29 Kitchen, J. L., Li, Z. and Crooke, E. (1999) Electrostatic interactions during acidic phospholipid reactivation of DnaA protein, the *Escherichia coli* initiator of chromosomal replication. Biochemistry **38**, 6213–6221

Received 3 September 2001/19 November 2001; accepted 10 December 2001