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# Acidic Phospholipids with Unsaturated Fatty Acids Inhibit the Binding of Origin Recognition Complex to Origin DNA[1]

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**Origin Recognition Complex (ORC) is a candidate initiator of chromosomal DNA replication in eukaryotes. We recently reported that cardiolipin inhibits the interaction of ORC with origin DNA, as is the case of DnaA, the initiator of chromosomal DNA replication in prokaryotes. We report here that another acidic phospholipid, phosphatidylglycerol (PG), also inhibits the interaction. Synthetic PG with only unsaturated fatty acids inhibits ORC-binding to origin DNA more strongly than PG with only saturated fatty acids. On the other hand, phosphatidylcholine (neutral phospholipid) does not affect the ORC-origin interaction, regardless of the presence of saturated or unsaturated fatty acids. These results suggest that an acidic moiety and unsaturated fatty acids are important factors for the inhibitory effect of phospholipids on ORC binding to origin DNA, as is the case for DnaA. The inhibitory effect of cardiolipin on ORC binding to origin DNA was more apparent at 30°C than at 4°C. Furthermore, chlorpromazine restored the ORC-origin interaction in the presence of cardiolipin. Since the presence of unsaturated fatty acids, low incubation temperatures, and the addition of chlorpromazine all decrease membrane fluidity, these results suggest that membrane fluidity is important for the inhibitory effect of acidic phospholipids on ORC-binding to origin DNA, as is the case for DnaA.**

**Key words: acidic phospholipids, chlorpromazine, DNA replication, DnaA, ORC.**

The mechanism for the initiation of chromosomal DNA replication in eukaryotes is believed to be different from that in prokaryotes. This is mainly because eukaryotes have multiple origins of DNA replication, whereas prokaryotes have a single origin. In addition, the cell-cycle regulation of DNA replication is stricter in eukaryotes than in prokaryotes, so rapidly growing bacterial cells can initiate re-replication before cell division. However, biochemical analyses of eukaryotic and prokaryotic initiators of chromosomal DNA replication have revealed various similarities between them (see below), suggesting that the mechanism of initiation of chromosomal DNA replication in eukaryotes shares some similarities with that in prokaryotes (*1*). The mechanism of DNA replication is well known for prokaryotes but not for eukaryotes. This is primarily due to the lack of an *in vitro* DNA replication system for eukaryotic chromosomal DNA replication. Therefore, the application of information obtained from studies on prokaryotic DNA replication to eukaryotic DNA replication may be of some value (*1*).

DnaA is the initiator of chromosomal DNA replication in *Escherichia coli* (*2*). DnaA binds specifically to the origin of chromosomal DNA replication (*oriC*), forms oligomers to "open-up" the duplex DNA, and recruits DnaB, a DNA helicase (*2*). This opening reaction requires the binding of ATP to DnaA (*3*). The ATP-DnaA complex is active for duplex opening, but the ADP-DnaA complex and nucleotide-free DnaA are inactive (*3–5*). ATP bound to DnaA is hydrolyzed to ADP by its intrinsic ATPase activity, and this hydrolysis is responsible for inactivating DnaA in order to suppress re-replication (*6, 7*).

Origin Recognition Complex (ORC) is a possible initiator of eukaryotic chromosomal DNA replication (*8*). ORC was originally identified as a six-protein complex that binds specifically to *Saccharomyces cerevisiae* origins of DNA replication (*9*), and homologues of ORC have been found in various eukaryotic species, including human cells (*10*). Eukaryotic chromosomal DNA replication is initiated by the binding of Cdc6p to ORC (*8*). The ORC-Cdc6p complex recruits six minichromosome maintenance proteins (MCM) and a DNA helicase (*11, 12*). ORC has ATP-binding and ATPase activities (*9, 13*). The binding of ATP to Orc1p, one of the ORC subunits, is essential for ORC-binding to origin DNA (*13*). The ATPase activity of ORC appears to be involved in disrupting the ORC-Cdc6p complex in order to suppress re-replication (*14, 15*).

Acidic phospholipids, in particular cardiolipin (CL), decrease affinity of DnaA for adenine nucleotides and convert the ADP-bound DnaA to the ATP-bound form in the presence of high concentrations of ATP by stimulating the exchange reaction of ADP with ATP (*16–19*) CL also inhibits the origin-binding activity of DnaA (*20*). We have previ-

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ously identified basic amino acid residues in DnaA that are essential for its binding to CL, and proposed that the ionic interaction between DnaA and CL changes the conformation of DnaA, resulting in a decrease in the affinity of DnaA for ATP or origin DNA (*21–25*). The interaction with acidic phospholipids is not specific for DnaA but is also observed for various DNA-binding proteins, such as histone, DNA topoisomerase I, SV40 T-antigen, and general transcription factors (*26–30*) Furthermore, we recently found that purified *S. cerevisiae* ORC binds to CL through ionic interaction and that CL strongly inhibits ORC binding to origin DNA (*31*). We also found that liposomes prepared from total phospholipids from the yeast nuclear membrane inhibit ORC binding to origin DNA, suggesting that the activity of ORC is modulated by phospholipids in nuclear membranes *in vivo* (*31*).

The functional interaction of DnaA with acidic phospholipids requires membrane fluidity. Phosphatidylglycerol (PG) with only unsaturated fatty acids, which increase membrane fluidity, interacts with DnaA more potently than PG with only saturated fatty acids (*17*). Furthermore, low incubation temperatures and the addition of chlorpromazine (or fluphenazine), both of which are known to decrease membrane fluidity, inhibits the functional interaction of DnaA with acidic phospholipids (*16, 18*). In this study, we examined the effects of changing the membrane fluidity on the inhibitory effect of acidic phospholipids on ORC binding to origin DNA. Synthetic PG with only unsaturated fatty acids was found to inhibit ORC binding to origin DNA more strongly than PG with only saturated fatty acids. The inhibitory effect of cardiolipin on ORC binding to origin DNA was suppressed by low incubation temperatures or the addition of chlorpromazine, suggesting that the inhibitory effect of phospholipids on ORC binding to origin DNA requires membrane fluidity.

## MATERIALS AND METHODS

*Materials*—The ORC from *S. cerevisiae* was expressed in Sf9 cells infected with a recombinant baculovirus and purified as previously described (*32*) [$\alpha$-$^{32}$P]ATP (3,000 Ci/mmol) and [$\gamma$-$^{32}$P]ATP (6,000 Ci/mmol) were obtained from Amersham Pharmacia Biotech. T4 polynucleotide kinase was purchased from TAKARA. CL from bovine heart, phosphatidylcholine (PC) from egg yolk, synthetic PC (18:0), synthetic PC (18:2), PG from egg yolk, synthetic PG (18:0), and synthetic PG (18:2) were purchased from Sigma.

Origin DNA fragments (*ARS1*) (294 bp) were synthesized by PCR as described previously (*11*). DNA fragments were radio-labeled with [$\gamma$-$^{32}$P]ATP mediated by T4 polynucleotide kinase. The specific activity of each probe was about 7,000 cpm/fmol DNA

*Co-Precipitation Assay for the Interaction between Phospholipids and ORC*—Phospholipid liposomes were prepared from phospholipids dried on the bottoms of glass tubes through vigorous vortex mixing in water. The amount of phosphorus in the phospholipid fraction was determined as described previously (*31*).

ORC and phospholipids were incubated in 30 $\mu$l of buffer H, containing 50 mM HEPES-KOH (pH 7.5), 0.1 M KCl, 1 mM EDTA, 1 mM EGTA, 5 mM $Mg(OAc)_2$, 0.0002% NP-40, 10% glycerol, 1 mg/ml BSA, and 1 mM ATP at 30°C for 5 min, and then centrifuged. After washing, the precipitates were re-suspended in SDS-sample buffer, loaded onto 10% polyacrylamide gels containing SDS, and electrophoresed. The proteins were then immuno-blotted with anti-Orc1p monoclonal antibodies (SB16 and SB35) (*11*).

*Filter-Binding Assay for DNA-Binding to ORC*—ORC (0.15 pmol) was incubated with radio-labeled *ARS1* DNA fragments at 30°C for 10 min in 25 $\mu$l of buffer H. Samples were passed through nitrocellulose membranes (Millipore HA, 0 45 $\mu$m) and washed with ice-cold buffer H. The radioactivity remaining on the filter was determined with a liquid scintillation counter.

*Filter-Binding Assay for ATP-Binding to ORC*—ORC (0.15 pmol) was incubated with 0 2 $\mu$M [$\alpha$-$^{32}$P]ATP at 4°C for 10 min in 25 $\mu$l of buffer H. Samples were passed through nitrocellulose membranes (Millipore HA, 0.45 $\mu$m) and washed with ice-cold buffer H. The radioactivity remaining on the filter was measured 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 (*11*). ORC was incubated with radio-labeled *ARS1* DNA fragments for 10 min at 30°C in 10 $\mu$l of buffer T [25 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 70 mM KCl, 2 mg/ml of bovine serum albumin, 5 mM dithiothreitol, 5% (v/v) glycerol, 1 mM ATP, and 5 $\mu$g/ml poly dI/poly dC as non-specific competitors]. The reaction sample was loaded onto 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 200 V at room temperature, dried, and autoradiographed.

## RESULTS AND DISCUSSION

*Effect of Synthetic PG and PC on ORC Binding to Origin DNA*—We recently found that CL (acidic phospholipid) inhibits *S. cerevisiae* ORC-binding to origin DNA (*31*) Other acidic phospholipids, PG (egg yolk), and phosphatidylinositol (bovine liver) weakly inhibit the binding (*31*), whereas neutral phospholipids, PC (egg yolk), and phosphatidylethanolamine (egg yolk) showed no effect on binding (*31*). Here, we have examined this inhibitory effect using synthetic phospholipids.

A gel electrophoretic mobility shift assay showed that PG with only unsaturated fatty acids (18:2) strongly inhibits ORC binding to origin DNA (Fig. 1A) The extent of the inhibition is much the same as that of CL (Fig. 1A). On the other hand, the inhibitory effect of PG with only saturated fatty acids (18:0) is less than that of PG (18:2) or PG (egg yolk) (Fig. 1A). Similar results were obtained when the ORC binding to origin DNA was monitored by a filter-binding assay (Fig. 1B). The only difference was that 20 $\mu$g PG (18.0) completely inhibited ORC binding to origin DNA in the gel electrophoretic mobility shift assay (Fig. 1A) but not in the filter-binding assay (Fig. 1B). DnaA was found to interact preferentially with PG containing only unsaturated fatty acids, compared to PG containing only saturated fatty acids. This phenomenon can be explained by the fact that unsaturated fatty acids increase membrane fluidity, which is required for the interaction between DnaA and acidic phospholipids (*17, 18*). Therefore, we consider that the inhibitory effect of acidic phospholipids on ORC binding to origin DNA also requires membrane fluidity.

We also examined the effect of synthetic PC on ORC

binding to origin DNA. As shown in Fig. 2A, neither PC with only unsaturated fatty acids (18:2), nor PC with only saturated fatty acids (18:0), inhibited ORC binding to origin DNA in the gel electrophoretic mobility shift assay. Similar results were obtained when ORC binding to origin DNA was monitored by the filter-binding assay (Fig 2B). Based on these data (Figs. 1 and 2), and the data in a previous paper (see above) (*31*), we conclude that the presence of both an acidic moiety and unsaturated fatty acids are important for the inhibitory effect of phospholipids on ORC binding to origin DNA.

Since ORC binding to origin DNA requires ATP, acidic phospholipids with unsaturated fatty acids may stimulate ATP release from the ORC-ATP complex, resulting in inhibitory effects on ORC binding to origin DNA. Therefore, we examined the effects of various phospholipids on ATP release from the ORC-ATP complex. As shown in Fig. 3, CL and PG (18:2) stimulated ATP release from the ORC-ATP complex However, PG (18:0), which was less active in inhibiting ORC binding to origin DNA, also stimulated the release to much the same extent as CL and PG (18:2). Therefore, it seems that the inhibitory effect of acidic phospholipids on ORC binding to origin DNA is not caused by the stimulation of ATP release from the ORC-ATP complex by acidic phospholipids

Furthermore, we also examined the effect of various phospholipids on the pre-formed ORC-origin DNA complex. We found that CL and PG (18:2), but not PG (egg yolk) or PG (18:0), slightly stimulated the release of origin DNA from ORC (data not shown).

*Physical Interaction of ORC with Phospholipids*—We used a co-precipitation assay to examine the physical interaction of ORC with various phospholipids. As shown in Fig. 4, only CL co-precipitates Orc1p in a dose-dependent manner. Immuno-blotting experiments using antibodies against ORC subunits other than Orc1p showed that all ORC subunits are equally co-precipitated with CL (data not shown). We did not observe any significant co-precipitation of ORC with phospholipids other than CL, even at higher concentrations (Fig. 4). In particular, PG with only unsaturated fatty acids (18:2) showed no binding activity with ORC,

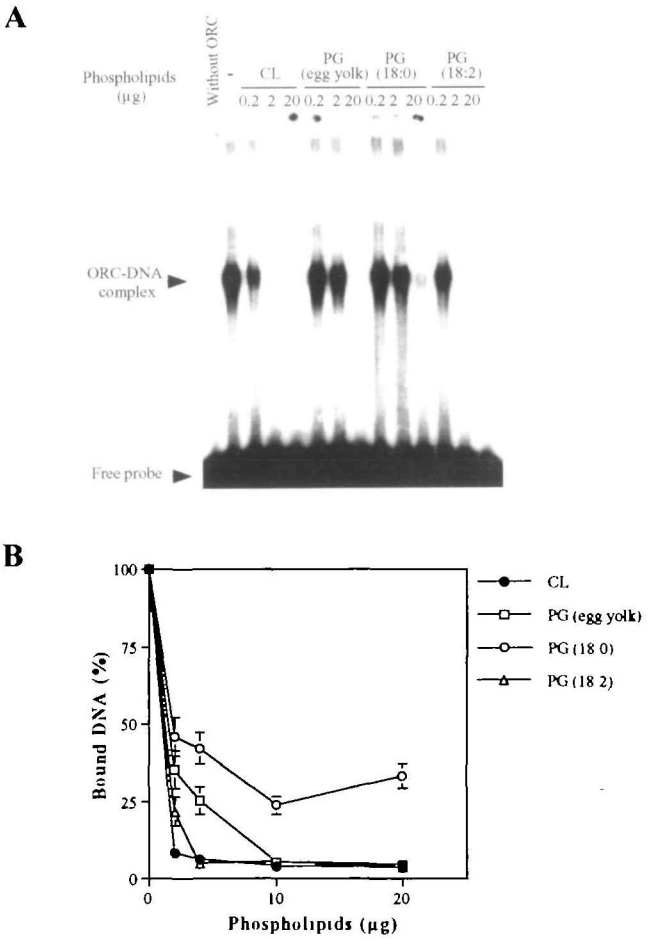


Fig. 1 **Effect of synthetic PG on ORC binding to origin DNA.** ORC (0 15 pmol) was pre-incubated with the indicated amounts of CL, PG (egg yolk), PG (18 0) or PG (18 2) for 10 min at 30°C, and further incubated at 30°C for 10 min with radio-labeled *ARS1* DNA fragments (90 fmol) in the presence (A) or absence (B) of 5 μg/ml poly dI/poly dC (A, B) A gel electrophoretic mobility shift assay was performed and the results subjected to autoradiography (A) The amount of bound DNA was determined by a filter-binding assay Values are mean ± SD, $n$ = 3 (B)

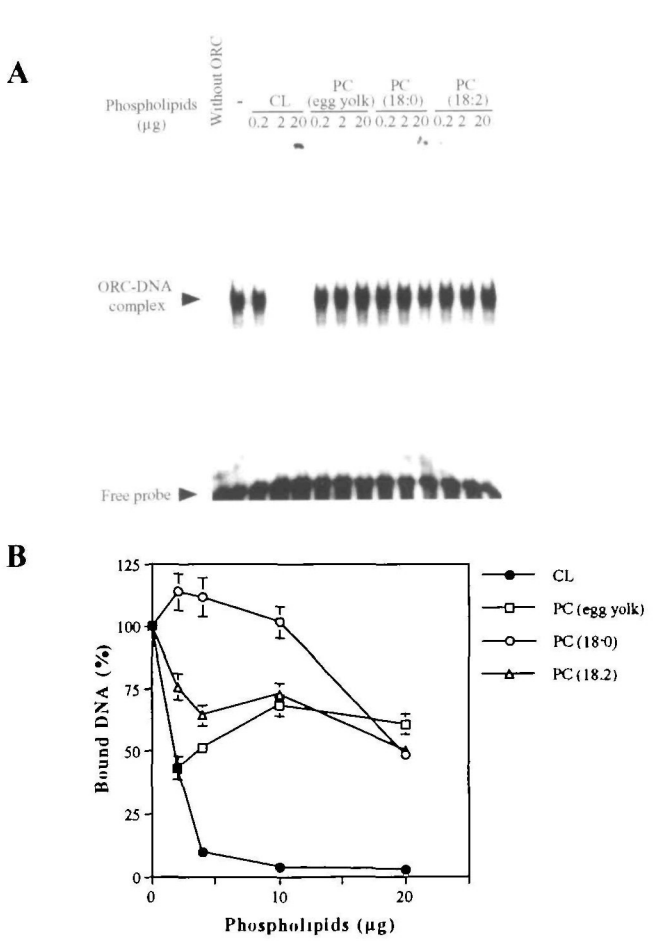


Fig 2 **Effect of synthetic PC on ORC binding to origin DNA.** ORC (0 15 pmol) was pre-incubated with the indicated amounts of CL, PC (egg yolk), PC (18 0), or PC (18 2) for 10 min at 30°C, and further incubated at 30°C for 10 min with radio-labeled *ARS1* DNA fragments (90 fmol) in the presence (A) or absence (B) of 5 μg/ml poly dI/poly dC (A, B) A gel electrophoretic mobility shift assay was performed and the results subjected to autoradiography (A) The amount of bound DNA was determined by a filter-binding assay Values are mean ± SD, $n$ = 3 (B)

although this phospholipid inhibits ORC binding to origin DNA to the same extent as CL (Fig. 1). It seems that the inhibitory effect of acidic phospholipids on ORC-binding to origin DNA is not related to a physical interaction between acidic phospholipids and ORC. The physical interaction between PG (18:2) and ORC may be weaker than that of CL (this weak interaction may be destroyed during centrifugation), but the weak interaction between PG (18.2) and ORC may be enough to inhibit ORC binding to origin DNA.

*Effect of Incubation Temperature and Chlorpromazine on the Inhibitory Effect of CL on ORC Binding to Origin DNA*—As described above, membrane fluidity appears to be important for the inhibitory effect of acidic phospholipids on ORC binding to origin DNA. In order to test this hypothesis, we performed two types of experiment.

Higher incubation temperatures increase membrane fluidity. At physiological temperatures, most membrane lipids are organized in a fluid lamellar liquid-crystalline phase, and as the temperature decreases, the bilayer lipids undergo a transition to a gel phase (*33*). The functional interaction of DnaA with acidic phospholipids (a decrease in the affinity of DnaA for ATP in the presence of acidic phospholipids) requires higher incubation temperatures. This effect has been explained by the requirement for membrane fluidity for the interaction (*16–19*). We then examined the effect of incubation temperature on the inhibitory effect of CL on ORC binding to origin DNA. As shown in Fig. 5, the inhibitory effect of CL on ORC binding to origin DNA was more apparent at 30°C than at 4°C as determined by filter-binding assay. We also observed by gel electrophoretic mobility shift assay that the inhibitory effect of CL on ORC binding to origin DNA is greater when the electrophoresis is performed at 30°C than at 4°C (data not shown).

Some psychotropic drugs and local anesthetics have also been shown to decrease membrane fluidity (*34*). Certain of these drugs, fluphenazine and chlorpromazine, inhibit the functional interaction between DnaA and CL (*16*). Furthermore, mutations in the *dnaA* gene alter the sensitivity of *E.*

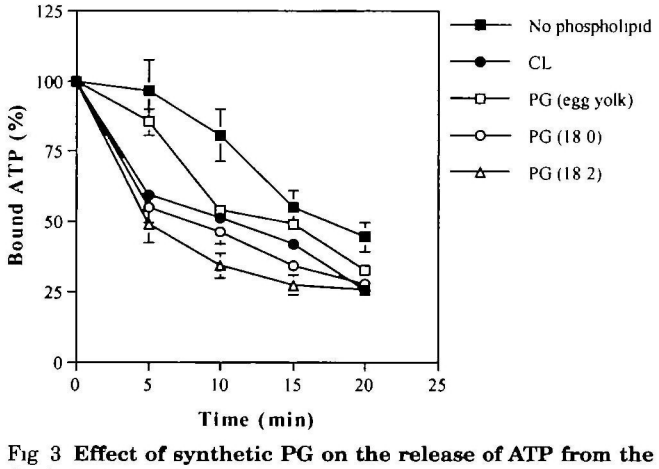


Fig 3 **Effect of synthetic PG on the release of ATP from the ORC-ATP complex.** ORC (0 15 pmol) was pre-incubated with 0 2 mM [α-$^{32}$P]ATP at 4°C for 10 min, and further incubated with or without 10 μg of CL, PG (egg yolk), PG (18 0), or PG (18 2) for the indicated periods at 30°C The amount of bound ATP was determined by a filter-binding assay Values are mean ± SD, $n$ = 3.

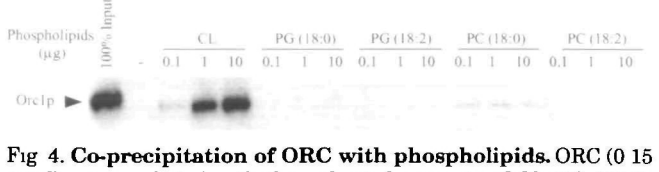


Fig 4. **Co-precipitation of ORC with phospholipids.** ORC (0 15 pmol) was incubated with the indicated amounts of CL, PG (18·0), PG (18.2), PC (18:0), or PC (18 2) at 30°C for 5 min After centrifugation, Orc1p in the precipitates was visualized by immunoblotting with anti-Orc1p antibodies (SB16 and SB35)

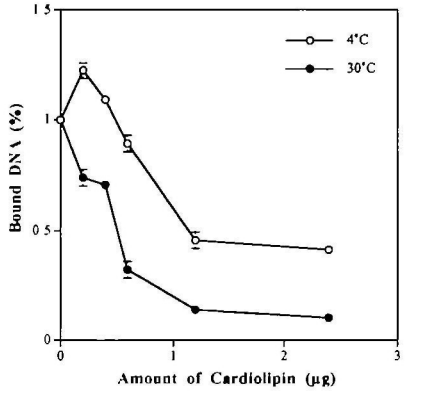


Fig 5 **Effect of incubation temperature on the inhibitory effect of CL on ORC binding to origin DNA.** ORC (0 15 pmol) was pre-incubated with the indicated amounts of CL for 10 min at 4 or 30°C and further incubated with radio-labeled *ARS1* DNA fragments (90 fmol) at 4 or 30°C The amount of bound DNA was determined by a filter-binding assay In the absence of CL, 4 1 or 6 0 fmol of DNA was bound to ORC at 4 or 30°C, respectively Values are mean ± SD, $n$ = 3

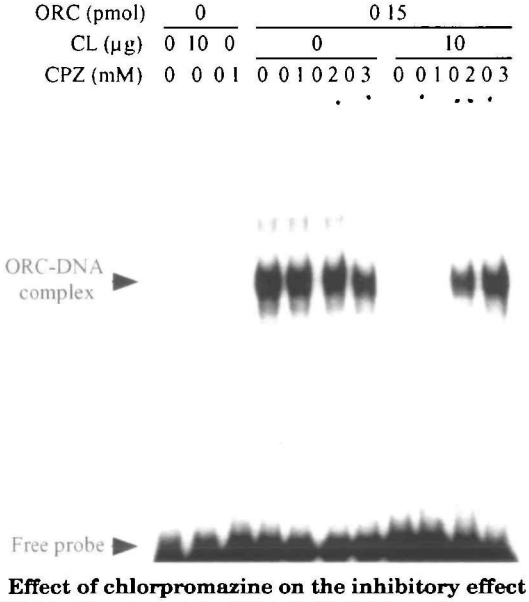


Fig 6 **Effect of chlorpromazine on the inhibitory effect of CL on ORC binding to origin DNA.** ORC (0 15 pmol) was pre-incubated with or without CL for 10 min at 30°C in the presence of the indicated concentrations of chlorpromazine (CPZ) The samples were further incubated with radio-labeled *ARS1* DNA fragments (90 fmol) in the presence of 5 μg/ml poly dI/poly dC at 30°C for 10 min A gel electrophoretic mobility shift assay was performed and the results subjected to autoradiography

*coli* to these drugs (*35, 36*). We here examined the effect of chlorpromazine on the inhibitory effect of CL on ORC binding to origin DNA by a gel electrophoretic mobility shift assay. As shown in Fig. 6, chlorpromazine completely restores the ORC binding to origin DNA that was inhibited by CL. The concentrations of chlorpromazine required to restore ORC binding to origin DNA are much the same as those necessary to produce a decrease in membrane fluidity (*34*). In the absence of CL, chlorpromazine does not affect ORC binding to origin DNA, at least up to a concentration of 0.3 mM (Fig. 6). Since chlorpromazine increases the background in a filter-binding assay for ORC binding to origin DNA, we could not examine the effect of chlorpromazine on ORC binding to origin DNA by means of the filter-binding assay. The results presented in Figs 5 and 6 support our proposal that membrane fluidity is important for the inhibitory effect of acidic phospholipids on ORC-binding to origin DNA.

We previously reported that CL inhibits ORC binding to origin DNA (*31*) However, in eukaryotic cells, CL is predominantly localized in the mitochondrial inner membrane (*37*), and it is not clear whether CL exists in the nuclear membrane. Therefore, the physiological significance of this finding remains unclear. In this study, we show that not only CL, but also another acidic phospholipid (PG), can affect ORC binding to origin DNA if unsaturated fatty acids are present. Since the eukaryotic nuclear membrane has various acidic phospholipids and more than 70% of the fatty acids are unsaturated (*37*), the results presented in this paper suggest that the inhibitory effect of acidic phospholipids on ORC binding to origin DNA *in vitro* is physiologically significant. The remaining question is the role of the inhibitory effect on ORC binding to origin DNA in the regulation of DNA replication in cells. In budding yeast, ORC remains at the origin even soon after the initiation of DNA replication, however, no one knows when the newly replicated origins are occupied by ORC. It would be very interesting if the association of ORC to the newly replicated origins is regulated by membrane acidic phospholipids and membrane fluidity.

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