ATP binding to dnaA protein is essential for its action in initiating the replication of plasmids that bear the unique origin of the Escherichia coli chromosome (oriC). ADP bound to that site renders dnaA protein inactive. Dipalmitoylphosphatidylglycerol (cardiolipin), a diacidic membrane phospholipid, displaces the bound nucleotide, and in the presence of components that reconstitute replication, fully reactivates the inert ADP form of dnaA protein. The monadic phosphatidylglycerol is one-tenth as active as cardiolipin, whereas the neutral phosphatidyethanolamine, the principal E. coli phospholipid, is inactive. Fluphenazine, a tranquilizer drug, blocks cardiolipin activation of dnaA protein, in keeping with the inhibitory action of such agents on phospholipid-dependent enzymes. With the use of this drug to terminate cardiolipin action, dependence of the activation on time, elevated temperature, and high levels of ATP was demonstrated. Cardiolipin binding of nucleotide-free dnaA protein prevents binding of ATP and initiation of oriC replication. Removal of a fatty acid from cardiolipin by phospholipase A reverses this inhibitory effect. The strong and specific interaction of cardiolipin, a cell membrane component, with an essential nucleotide-binding site of dnaA protein, the protein essential for the initiation of chromosome replication, may be an important element in regulating the cell cycle.

A major goal of resolving and reconstituting initiation of chromosome replication at the molecular level is to determine how this crucial event is regulated. One of the key participants in initiation at the origin of the Escherichia coli chromosome (oriC) is dnaA protein (1, 2). Upon examining the properties of the purified protein, we discovered that its action depends on tightly bound ATP which it hydrolyzes to ADP in about an hour, the time interval of a cell cycle (3). Because the ADP renders the protein inert and does not exchange with ATP, this apparent suicide may be programmed to provoke the need for fresh synthesis of dnaA protein to initiate the next chromosome cycle. On the other hand, there may be mechanisms designed to rejuvenate the inactive ADP form by facilitating the replacement of ADP by ATP. To date, no protein has been found to fill this role.

In this report we describe the remarkable property of acidic phospholipids, particularly cardiolipin, to dissociate the adenine nucleotide bound to dnaA protein and in the presence of ATP and the components of the replicative system to restore the inactive ADP-form to full activity. Despite the plausibility of linking chromosome replication to cell membranes (4), chemical evidence for such associations has been lacking (5). In this instance, the strong and specific interaction between a membrane phospholipid and a protein vital for initiation of replication deserves attention for the role it may perform in controlling this process.

EXPERIMENTAL PROCEDURES

Reagents—Sources were as follows: ATP, dNTPs, and HEPES,1 Biochemicals, P-L Biochemicals; GTP, CTP, UTP, and Tricine, Sigma; [γ-32P]ATP (410 Ci/mmol), [3H]ADP (511 mCi/mmol), and [α-32P]dATP (800 Ci/mmol), Amersham Corp; Fluphenazine, E. R. Squibb and Sons.

Enzymes and DNA—Purified DNA replication proteins were prepared as described previously (6). dnaA protein was purified (2) to a specific activity of 1.0 × 109 units/mg and a purity greater than 90% as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Plasmid pCM959 (7), a gift from M. Meijer (University of Amsterdam, The Netherlands), is a minichromosome. It consists solely of E. coli DNA encompassing oriC (base pairs −677 to +3335). Supercoiled plasmid DNA was prepared as described previously (8). Phospholipase A_b (Naja mocambique mocambique, 1490 units/mg) from Sigma was dissolved to 200 units/μl in a buffer containing Tricine-KOH (pH 8.25 at 1 M), 50 mM; magnesium acetate, 13 mM; EDTA, 0.3 mM; glycerol, 20% (v/v); Triton X-100, 0.006%; and dithiothreitol, 8 mM.

Phospholipids—Cardiolipin (bovine heart), phosphatidylglycerol (α-<i>ω</i>-phosphatidyl-δ-glycerol, dipalmitoyl), phosphatidyethanolamine (E. coli), and phosphatidylglycerol (soybean) were from Sigma. Phospholipids (20 mg in methanol-chloroform) were dried under a flow of nitrogen and suspended in 1 ml of distilled water by sonication (Ultrasonics Inc., model W-225R); phosphatidylglycerol and phosphatidyethanolamine was suspended in 0.1% Triton X-100.

Total E. coli phospholipids were prepared by a modification (9) of the Bigh and Dyer (10) method. Frozen HB101 cell paste (1 g) was suspended in 4 ml of 0.12 M sodium acetate, pH 4.8. Chloroform (5 ml) and methanol (11 ml) were added, and phospholipids were extracted for 1 h at room temperature. The solids were removed by centrifugation, and the supernatant was mixed with chloroform (5 ml) and distilled water (5 ml). The lower phase was dried with nitrogen and suspended in 0.8 ml of distilled water by sonication. The amount of phosphorous was determined (11), and the concentration of phospholipids was calculated assuming an average molecular weight of 700.

ATP (ADP)-binding Assay—The standard reaction (19 μl) contained Tricine-KOH (pH 8.25 at 1 M), 50 mM; magnesium acetate, 2.5 mM; EDTA, 0.3 mM; glycerol, 20% (v/v); Triton X-100, 0.007%; dithiothreitol, 7 mM; [γ-32P]ATP (10,000 cpm/pmol) or [3H]ADP (1,100 cpm/pmol), 1 μM; and dnaA protein, 120 ng. After incubation at 0°C for 15 min, the solution was filtered through a nitrocellulose membrane (Millipore HA 0.45 μm, 24-mm diameter) presoaked in wash buffer (50 mM Tricine-KOH, pH 8.25 at 1 M, 0.5 mM magnesium nitrateethanesulfonic acid).

*Fellow of the American Cancer Society, California Division.
†Present address: Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan.

1 The abbreviation used is: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
Cardiolipin Activation of dnaA Protein

RESULTS

Cardiolipin Dissociates ADP or ATP Tightly Bound to dnaA Protein—No E. coli protein has been found that can release or exchange the ADP or ATP tightly bound to dnaA protein (K_D = 0.03 μM). Of special interest is the fact that the ADP form generated by the slow hydrolysis of the ATP bound to dnaA protein is inert in replication (3). Two ways have been found to effect the release of the bound nucleotides. One is by the unphysiological chelation of magnesium (12). The other is through the action of certain phospholipids, particularly diphosphatidylglycerol (cardiolipin) (Fig. 1). Eighty percent of the nucleotide bound to 0.12 μg of dnaA protein (about 2 pmol) was dissociated after 10 min at 38 °C by 0.2 μg of cardiolipin (about 200 pmol). Without cardiolipin the nucleotide-protein complex remains intact (>90%) for at least 60 min.

Among the major phospholipids of E. coli, phosphatidylethanolamine, the principal phospholipid in E. coli membranes, was inert (Fig. 1). Phosphatidylinositol, absent from E. coli, had an activity level near that of phosphatidylglycerol. Hydrolysis products of cardiolipin (diglyceride and glycerophosphate) were inactive (data not shown). Thus, an intact phospholipid is required. Its anionic nature is important, explaining in part why cardiolipin with two phosphate moieties is the most active. Dissociation of bound ATP (or ADP) by cardiolipin required a high temperature (Fig. 2). The

![Fig. 1. Phospholipids dissociate the ADP bound to dnaA protein. The complex with dnaA protein (2.4 pmol) was formed by incubation with 1 μM [α-32P]ATP (6900 cpm/pmol) at 0 °C for 15 min. After incubation with 1 μM DNA polymerase I, 0.35 μg of cardiolipin, and the other replication components, the samples were filtered on membranes. PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; CL, cardiolipin.](image)

![Fig. 2. Effect of temperature on dissociation of ATP from dnaA protein by cardiolipin. A complex with dnaA protein (2.4 pmol) was formed by incubation with 1 μM [α-32P]ATP (6900 cpm/pmol) at 0 °C for 15 min. After exposure to 0.2 μg of cardiolipin at various temperatures, the samples were filtered on membranes. C_a, amount of ATP-dnaA protein remaining; C_i, initial amount (1.15 pmol).](image)

![Fig. 3. Phospholipids restore replication activity to the ADP complex of dnaA protein. A complex with dnaA protein (2.4 pmol) was formed by incubation with 1 μM [α-32P]ATP at 0 °C for 15 min. Replication activity was measured as described under "Experimental Procedures." DNA synthesis with the ATP complex of dnaA protein was 194 pmol. Background synthesis (without dnaA protein) was 14 pmol. A, individual phospholipids. B, total phospholipids extracted from E. coli cultures during stationary or log-phase growth. See Fig. 1 for abbreviations.](image)
activation energy, determined by an Arrhenius plot, was 30 kcal·mol⁻¹. In all measurements of nucleotide dissociation from dnaA protein, phospholipid actions on the ADP and ATP forms were indistinguishable.

Phospholipids Restore Replication Activity to the ADP Complex of dnaA Protein—Release of bound ADP by cardiolipin in the presence of the components needed to reconstitute replication of the oriC plasmid resulted in complete regeneration of the replicative activity of the previously inert dnaA protein (Fig. 3A). The relative activities of the phospholipids and the amounts needed were essentially the same as observed for ADP release from dnaA protein, except that at higher levels, cardiolipin and phosphatidylglycerol became inhibitory.

When the mixture of phospholipids found in *E. coli* was used in place of the pure phospholipids (Fig. 3B), several noteworthy facts emerged. (i) Regenerative activity on ADP·dnaA protein was as great as with cardiolipin alone. (ii) The amount needed was consistent with that anticipated from cardiolipin being present at the usual level of 5–10% of *E. coli* phospholipids. (iii) Phospholipids in the lamellar state were active. (iv) Cardiolipin from *E. coli* was as active as that from a bovine source. (v) No inhibitory action was seen with high levels of the phospholipid mixture as with pure phospholipids. (vi) Despite differences between the metabolic states of phospholipids in logarithmic and stationary growth-phase cells, no distinction was observed in their action on dnaA protein.

**TABLE I**

| Temperature (°C) | DNA synthesis (pmol) |
|-----------------|----------------------|
| 0               | 0                    |
| 16              | 9                    |
| 24              | 12                   |
| 30              | 28                   |
| 38              | 137                  |

**FIG. 4.** Fluphenazine inhibits activation of the ADP·dnaA protein complex by phospholipids. dnaA protein (2.4 pmol) was incubated with 1 μM ATP or ADP at 0 °C for 15 min. Replication was measured as described under "Experimental Procedures." P-Lipid, phospholipids; see Fig. 1 for other abbreviations.

**FIG. 5.** Requirement for a high concentration of ATP for cardiolipin activation of the ADP·dnaA protein complex. The ADP complex of dnaA protein (2.4 pmol) (see Fig. 3) was incubated for 10 min at 38 °C with various concentrations of ATP, 0.35 μg of cardiolipin, and the components of the replication system except dNTPs. The ATP concentration was then adjusted to 5 mM. Fluphenazine (0.15 mM), GTP, CTP, and UTP were added and incubation continued at 38 °C for 10 min. Then (as in Table 1), upon the addition of [32P]dNTPs, DNA synthesis was carried out at 16 °C for 30 min.

**FIG. 6.** Cardiolipin inactivates nucleotide-free dnaA protein for ATP binding. ATP binding of dnaA protein (2.4 pmol) was assayed by membrane binding in the presence of various amounts of cardiolipin. Cardiolipin was added either after a prior incubation of [α-32P]ATP and dnaA protein (see Fig. 2) or mixed with dnaA protein before [α-32P]ATP was added. All reactions were for 10 min.

Whether the regenerative action of cardiolipin on ADP·dnaA protein depended on an elevated temperature as observed for dissociation of ADP and whether ATP influenced the process could not be tested until a means was found (see below) to separate the stage of cardiolipin action from the subsequent initiation stages known to depend on an elevated temperature and high levels of ATP (12, 13).

**Fluphenazine Inhibits Cardiolipin Release of Nucleotides Bound to dnaA Protein**—To separate the cardiolipin action on dnaA protein from subsequent events in which dnaA protein initiates replication, a specific inhibitor of the cardiolipin action was sought. Fluphenazine, a tranquilizer drug, was tried in view of the capacity of this class of agents to inhibit the actions of phospholipid-dependent enzymes (14, 15). Fluphenazine did inhibit the phospholipid activation of ADP·dnaA protein without affecting the replicative activity,
Cardiolipin Activation of dnaA Protein

**FIG. 7.** Cardiolipin inactivates nucleotide-free dnaA protein for DNA replication. dnaA protein (2.4 pmol) was incubated with (ATP-dnaA) or without (dnaA) ATP at 0 °C for 15 min. Cardiolipin was added at 0 °C, followed by the replication reaction (including 5 mM ATP) as described under “Experimental Procedures.”

**FIG. 8.** Time course of phospholipase A2 restoration of ATP-binding activity to dnaA protein inactivated by cardiolipin. dnaA protein (2.4 pmol) was treated with 1 µg of cardiolipin at 0 °C and incubated with phospholipase A2 (100 units) and 1 µM [γ-32P]ATP (66,000 cpm/pmol) at 16 °C. Samples were filtered on membranes.

**FIG. 9.** Phospholipase A2 levels required for restoration of replication activity to dnaA protein inactivated by cardiolipin. dnaA protein (2.4 pmol) was treated with 1 µg of cardiolipin at 0 °C and then incubated with phospholipase A2 and 1 µM ATP at 16 °C for 60 min. Replication activity was assayed as described under “Experimental Procedures.”

protein at high temperatures. Probably binding of ADP (or ATP) to dnaA protein prevents the irreversible inactivation of dnaA protein by changing the conformation of the protein.

**DISCUSSION**

Activation of dnaA protein by cardiolipin (diphosphatidylglycerol) was discovered in our efforts to resuscitate the moribund protein after its attempt at suicide. The dnaA protein with a very tightly bound ATP, essential for its action, hydrolyzes the nucleotide slowly to ADP (3). The ADP product, held just as firmly, renders the protein inert in the initiation of replication. Replacement of the bound ADP by exchange with ATP is very feeble. No counterpart to agents that dissociate GDP from the G proteins (16) was found among the purified proteins involved in replication or those in crude cell extracts. Although the bound nucleotide can be discharged by removing Mg2+ with strong chelation (12), implying a requirement for the metal by the binding site, this mechanism seems unphysiological.

In view of the possible association of dnaA protein and replication events with cell membranes, the influence of phospholipids was tested. Cardiolipin proved to be strikingly effective in the rapid displacement of the bound nucleotide and rejuvenation of the ADP form of the enzyme. Among the E. coli phospholipids, cardiolipin, the least abundant (about 5% of the total), is 10 or more times as effective as the 3-fold more abundant phosphatidylglycerol. Phosphatidylethanolamine, comprising nearly 80% of the phospholipids, is inert. Cardiolipin is equally active whether of bovine origin presented in some micellar form or E. coli origin arranged in a lamellar (vesicular) state among the other E. coli phospholipids. Phosphatidylinositol, not found in E. coli, is about as effective as phosphatidylglycerol, indicating the importance of the acidic head group in this interaction with dnaA protein. Cardiolipin, with two ionized phosphates, is reasonably the most active. Because dnaA protein requires Mg2+ for tight binding of ATP or ADP (12), destabilization of the Mg2+ by cardiolipin is a possible basis for discharge of the nucleoside phosphates.

The isosteric similarity between the cardiolipin head group and the DNA backbone is striking and is likely the basis of

of the ATP form of the protein (Fig. 4). The latter showed the same activity with or without cardiolipin present. The same mixture of cardiolipin and fluphenazine did not inhibit any stage of DNA synthesis (data not shown).

Using fluphenazine to terminate the action of cardiolipin, the need for a temperature near 38 °C for activation of dnaA protein was demonstrable (Table I). ATP at a level about 1000-fold higher than that which saturates the dnaA protein tight-binding site was also needed (Fig. 7). Thus, cardiolipin binding blocks access of ATP to the site essential for dnaA protein action. At 38 °C in the absence of the components of the replication system, cardiolipin, after displacing the ATP, blocks this site and thereby inhibits dnaA protein functions. The ATP binding (Fig. 8) and replication (Fig. 9) activities were restored to the inert cardiolipin-dnaA protein by destroying the cardiolipin through the action of phospholipase A2. Cardiolipin denatures nucleotide-free dnaA protein for DNA replication.
the cross-reactivity between cardiolipin and DNA (17). In
considering where on the dnaA protein cardiolipin might be
located, the DNA-binding site is a possibility that deserves
further study. It is noteworthy, however, that the several
dNA-binding proteins in the replication system are not
detectably affected by cardiolipin in the reaction.

How can we assess the physiological significance of the
influence adenine nucleotides have on dnaA protein function
and the relief of ADP inhibition by cardiolipin? It would
be desirable to know the nucleotide form of the protein during
the cell cycle. Among the thousand dnaA protein molecules
in a cell (18), only a small fraction is engaged at the chromo-
somal origin. The rest are bound to dnaA boxes elsewhere on
the chromosome or to the membrane surface. Thus, a deter-
mination of the form of the protein needs to be coupled with
knowledge of its location. As for attachment of dnaA protein
to cell membranes, particularly cardiolipin, and the influence
of the cell cycle, information about the physiology and ar-
range ment of phospholipids in E. coli membranes is rather
limited.

Turnover of the acidic phospholipids is relatively rapid
compared to that of phosphatidylethanolamine (19, 20), the
breakdown of phosphatidylglycerol appears to be coupled
to a stage in the cell cycle (21), and a requirement for phos-
pholipid synthesis for initiation of replication has been suggested
(22). Yet a mutant deficient in cardiolipin synthesis sustains
apparently normal growth (23). Still, this mutant retains a
low level of cardiolipin, approximately 20,000 molecules, a
number far in excess of that needed to bind the dnaA protein
operating at chromosomal origins, and phosphatidylglycerol
levels in this mutant are elevated. While attempts to delete the
cardi lipin synthetase gene had not succeeded (24), the
recent construction of a null allele of the pgsA gene renders
the cell incapable of synthesizing phosphatidylglycerol or
cardiolipin and is lethal unless rescued by a plasmid-borne
copy of the gene (25). Inasmuch as cells manage a normal
phenotype with very low levels of these acidic phospholipids,
it would seem that these residual amounts are not serving a
structural role in the membrane but rather are supplying
some essential functions such as the orientation of proteins
in key biosynthetic processes (26).

Several arguments can be cited for a physiological mem-
brane base for dnaA protein beyond the interactions with
acidic phospholipids described in this report. In a membrane
fraction which selectively binds the oriC sequence (27), the
dnaA protein, identified by immunoblotting, is highly en-
riched, and the anti-dnaA antibody blocks the binding of oriC
by the membrane fraction. Among a number of cold-sensitive
mutants (often associated with a membrane location), the
dnaA gene locus was repeatedly identified with conditionally
lethal defects in replication. Finally, of the large number of
proteins in a particulate fraction of a cell extract, only one
was found which bound ATP with extraordinary avidity and
this proved to be the dnaA protein (18).

Acknowledgments—We thank K. Kaibuchi (DNAX Research In-
stitute) for helpful discussion. We are grateful to L. Bertsch for
careful reading of the manuscript.

REFERENCES
1. Hirota, Y., Mordoh, J., and Jacob, F. (1970) J. Mol. Biol. 53,
369–387
2. Fuller, R. S., and Kornberg, A. (1983) Proc. Natl. Acad. Sci. U.
S. A. 80, 5817–5821
3. Sekimizu, K., Bramhill, D., and Kornberg, A. (1987) Cell 50,
269–265
4. Jacob, F., Brenner, S., and Cuzin, F. (1963) Cold Spring Harbor
Symp. Quant. Biol. 28, 329–348
5. Kornberg, A. (1982) Supplement to DNA Replication. W. H.
Freeman and Company, San Francisco
6. Kaguni, J. M., and Kornberg, A. (1984) Cell 38, 183–190
7. Meijer, M., Beck, F., Hansen, F. G., Bergman, H. F., Messer, W.,
von Meyenburg, K., and Schaller, H. (1979) Proc. Natl. Acad.
Sci. U. S. A. 76, 580–584
8. Ogawa, T., Baker, T. A., van der Ende, A., and Kornberg, A.
(1985) Proc. Natl. Acad. Sci. U. S. A. 82, 3562–3566
9. Bertsch, L. L., Bonsen, P. P. M., and Kornberg, A. (1969) J.
Bacteriol. 98, 75–81
10. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol.
37, 911–917
11. Chen, P. S., Jr., Torihata, T. Y., and Warner, H. (1964) Anal.
Chem. 28, 1756–1758
12. Sekimizu, K., Bramhill, D., and Kornberg, A. (1968) J. Biol.
Chem. 263, 7124–7130
13. Baker, T. A., Sekimizu, K., Funnell, B. E., and Kornberg, A.
(1986) Cell 45, 53–64
14. Papahadjopoulos, D. (1972) Biochim. Biophys. Acta 265, 169–
166
15. Mori, T., Takai, Y., Minakuchi, R., Yu, B., and Nishizuka, Y.
(1980) J. Biol. Chem. 255, 8378–8380
16. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
17. Rauch, J., Tannenbaum, H., Stoller, B. D., and Schwartz, R. S.
(1984) Eur. J. Immunol. 14, 529–534
18. Sekimizu, K., Yung, B. Y., and Kornberg, A. (1985) Cell 38,
7136–7140
19. Kanfer, J., and Kennedy, E. P. (1963) J. Biol. Chem. 238, 2919–
2922
20. Kanemasa, Y., Akamatsu, Y., and Nojima, S. (1967) Biochim.
Biophys. Acta 144, 382–390
21. Ohkaw, M. (1972) J. Mol. Biol. 68, 249–264
22. Pierucci, O., and Rickert, M. (1985) J. Bacteriol. 162, 374–382
23. Pluschke, G., Hirota, Y., and Overath, P. (1978) J. Bacteriol.
253, 5048–5055
24. Raetz, C. R. H. (1986) Annu. Rev. Genetics 20, 253–295
25. Ohta, A., Waggoner, K., Radominska-Pyrek, A., and Dowhan, W.
(1981) J. Bacteriol. 147, 552–562
26. Miyazaki, C., Kuroda, M., Ohta, A., and Shibuya, I. (1985) Proc.
Natl. Acad. Sci. U. S. A. 82, 7530–7534
27. Kusano, T., Steinmetz, D., Hendrickson, W. G., Murchie, J.,
Benson, A., and Schaechter, M. (1984) J. Bacteriol. 158, 319–
339

L. I. Rothfield, personal communication.