Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 783–787, January 1995
Biochemistry

In vivo evidence for the involvement of anionic phospholipids in initiation of DNA replication in *Escherichia coli*

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Communicated by Eugene P. Kennedy, Harvard Medical School, Boston, MA, October 31, 1994

ABSTRACT ***In vitro*, anionic phospholipids can reactivate inactivated DnaA protein, which is essential for initiation of DNA replication at the *oriC* site of *Escherichia coli* [Sekimizu, K. & Kornberg, A. (1988) *J. Biol. Chem.* 263, 7131–7135]. Mutations in the *pgsA* gene (encoding phosphatidylglycerophosphate synthase) limit the synthesis of the major anionic phospholipids and lead to arrest of cell growth. We report herein that a mutation in the *rnhA* gene (encoding RNase H) that bypasses the need for the DnaA protein through induction of constitutive stable DNA replication [Kogoma, T. & von Meyenburg, K. (1983) *EMBO J.* 2, 463–468] also suppressed the growth arrest phenotype of a *pgsA* mutant. The maintenance of plasmids dependent on an *oriC* site for replication, and therefore DnaA protein, was also compromised under conditions of limiting anionic phospholipid synthesis. These results provide support for the involvement of anionic phospholipids in normal initiation of DNA replication at *oriC in vivo* by the DnaA protein.**

Substantial evidence indicates that DNA replication in *Escherichia coli* is associated with the cell membrane and may require anionic phospholipids (1–3). From electron microscopy and cell fractionation studies, there appear to be noncovalent interactions among the membrane, the origin of replication, the replication fork, and the termination site of the chromosome (4). DnaA, the protein necessary for initiation of DNA replication at the *oriC* site, is peripherally bound to membranes *in vitro*, copurifies with phospholipids, and specifically binds anionic phospholipids (5, 6). Furthermore, anionic phospholipids facilitate the conversion of the inactive ADP-bound form of the DnaA protein to the active ATP-bound form *in vitro*, resulting in regeneration of the initiation activity of previously inert DnaA protein (2, 3, 7). Only anionic phospholipids containing unsaturated fatty acids are functional in stimulating DnaA function *in vitro* (7), an observation that is also consistent with the observation that initiation of chromosome replication *in vivo* appears to be dependent on a lipid binding site in the membrane containing unsaturated fatty acids (8). However, whether anionic phospholipids are involved in initiating DNA synthesis *in vivo* has not been established.

Initiation of DNA replication requires formation of an RNA primer·DNA hybrid duplex at the initiation site. RNase H, the *rnhA* gene product, restricts DNA replication *in vivo* to the *oriC* site by preferentially degrading these RNA primers at sites other than the DnaA protein-dependent *oriC* initiation site (9). When the *rnhA* gene (10) is inactivated, constitutive stable DNA replication (cSDR) can be initiated at several DnaA protein-independent *oriK* sites by utilizing the undegraded RNA primers (11, 12). Introduction of an *rnhA* mutation into strains lacking the *dnaA* gene or the *oriC* site bypasses the need for these genetic loci for cell viability (13, 14). RecA protein (encoded by the *recA* gene) is required for cSDR at the *oriK* sites (15), but this requirement is suppressed in a *lexA(Def)* mutant (16).

If anionic phospholipids are essential for DnaA protein function *in vivo*, then mutations that suppress the need for DnaA protein-dependent initiation of DNA replication at the *oriC* site may also suppress the growth arrest phenotype of mutants with a limiting level of anionic phospholipids. The phospholipid composition of the *E. coli* cell membrane is 70–80% phosphatidylethanolamine (zwitterionic), 15–20% phosphatidylglycerol (PG, anionic), and 2–5% cardiolipin (CL, anionic) (17). Phosphatidylglycerophosphate synthase (encoded by the *pgsA* gene) catalyzes the committed step in the biosynthesis of PG and CL (18) and is essential for cell growth (19). To systematically study the specific roles of anionic phospholipids in cell functions, a strain bearing a Φ[*lacOP–pgsA*$^+$] gene fusion in a *pgsA*::*kan* null allele background was constructed (20). In this strain, anionic phospholipid content, gene expression, and cell growth rate can be exogenously controlled by the inducer of the *lac* operon isopropyl β-D-thiogalactopyranoside (IPTG). In the absence of IPTG, the basal level of the *pgsA* gene product is insufficient to support cell growth, although cells remain viable, as indicated by resumption of growth upon addition of IPTG. Failure to lose viability indicates that anionic phospholipids are essential for certain cell functions independent of a membrane structural requirement (21).

Based on studies of the suppression of the growth arrest phenotype of the above *pgsA* mutant under conditions of cSDR, we report results that support the involvement of anionic phospholipids in the initiation of DNA replication by the DnaA protein at the *oriC* site *in vivo*.

MATERIALS AND METHODS

Strains, Plasmids, and Cell Growth. Table 1 summarizes the strains and plasmids used in this work and outlines the scheme used to construct strains by P1 transduction (23). To construct strain MRLA12, the *malG*::Tn*10* locus was first introduced into strain MR12 and used to monitor its replacement with the *malG*$^+$ locus along with the closely linked (0.2 min) *lexA(Def)* allele (24, 25). Strain MDL12 was constructed by successive P1 transductions of strain MG1655 (no known mutations) by using strain HDL1001 as donor. Plasmids were introduced into cells by electroporation (Electro Cell Manipulator 600). Cells were grown at 37°C (unless otherwise indicated) in liquid cultures or on agar plates in LB medium supplemented with 100 μM IPTG (where indicated). Antibiotics (26) were present during the growth of plasmid-containing strains unless otherwise indicated and were used only for the initial selection of strains carrying chromosomally located antibiotic-resistance markers.

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Abbreviations: PG, phosphatidylglycerol; CL, cardiolipin; cSDR, constitutive stable DNA replication; IPTG, isopropyl β-D-thiogalactopyranoside.

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Table 1. Strains and plasmids

Strains or plasmid	Relevant characteristic(s)	Source or ref.
Strain		
MG1655	Wild type	G. Weinstock
KA2186	Δ*recA srl*::Tn*10*	G. Weinstock
GE450	*malG*::Tn*10*	G. Weinstock
GE152	*lexA(Def)51*	G. Weinstock
KHG712	*rnhA1*::Tn*3*	T. Horiuchi
HDL1001*	*pgsA30*::*kan* Φ[*lacOP–pgsA*$^+$]*1 lacZ' lacY*::Tn*9 recA srl*::Tn*10*	20
HDL11	*pgsA30*::*kan* Φ[*lacOP–pgsA*$^+$]*1 lacZ' lacY*::Tn*9 lpp2 zdg*::Tn*10*	21
HD30†	*pgsA30*::*kan*	19
HL30†	HD30, *lpp2 zdg*::Tn*10*; P1: HD30 × HDL11	This work
HR30†	HD30, *rnhA1*::Tn*3*; P1: HD30 × KHG712	This work
HLR30†	HL30, *rnhA1*::Tn*3*; P1: HL30 × KHG712	This work
MDL12*	*pgsA30*::*kan* Φ[*lacOP–pgsA*$^+$]*1 lacZ' lacY*::Tn*9*; P1: MG1655 × HDL1001	This work
MR12	MDL12, *rnhA1*::Tn*3*; P1: MDL12 × KHG712	This work
MRA12*	MR12, Δ*recA srl*::Tn*10*; P1: MR12 × KA2186	This work
MRM12	MR12, *malG*::Tn*10*; P1: MR12 × GE450	This work
MRL12	MR12, *lexA(Def)51*; P1: MRM12 × GE152	This work
MRLA12	MRL12, Δ*recA srl*::Tn*10*; P1: MRL12 × KA2186	This work
Plasmid		
pHD102	*pgsA*$^+$ *cam*^{r} *ori*^{ts}	19
pMM5	pBR322, *rnhA*$^+$ *amp*^{r} *tet*^{r}	22
pAC11	*oriC mioC*$^+$ *spec*^{r} *strep*^{r}	J. Zyskind
pAC12	*oriC mioC*$^-$ *spec*^{r} *strep*^{r}	J. Zyskind

Relevant characteristics are a partial list of genetic markers. G. Weinstock is at University of Texas Medical School, Houston; T. Horiuchi is at Kyushu University, Fukuoka, Japan; J. Zyskind is at San Diego State University, San Diego.
*Requires IPTG for viability.
†Requires plasmid pHD102 for viability.

Measurement of Dependence on a Functional *pgsA* Gene. IPTG was washed out of cultures of strain MDL12 and its derivatives after growth overnight in the presence of IPTG (20). Cells were diluted and plated in equal portions on LB agar plates, either with or without IPTG. Colony numbers (10–500 colonies per plate) were scored after incubation of the plates for 24–36 hr, and the percentage of cells forming colonies in the absence of IPTG (IPTG independence index, average of three experiments) was calculated by comparing the colony number in the absence of IPTG to that in the presence of IPTG. A similar approach was used to test the suppression of the temperature-sensitive growth phenotype of strains HR30 and HLR30 (both carrying plasmid pHD102), except with these strains the permissive conditions were growth at 30°C and the restrictive conditions were growth at 42°C. Growth of all the above strains under restrictive conditions for periods longer than 36 hr resulted in formation of additional colonies due to constitutive mutations in the *lac* promoter (20) or temperature-resistant mutants of plasmid pHD102 (19) as reported.

Assay for Plasmid Stability. An overnight culture grown in the presence of IPTG and the appropriate antibiotic [spectinomycin (50 μg/ml) and streptomycin (50 μg/ml) for plasmids pAC11 and pAC12 or tetracycline (10 μg/ml) for plasmid pBR322] was diluted into antibiotic-free LB medium either with or without IPTG. The cultures were kept in exponential growth for 6–14 generations by dilution with fresh medium. At the indicated time points, dilutions of the culture were spread on LB agar plates in the presence of IPTG either with or without the appropriate antibiotic(s) to determine the percentage of cells retaining the corresponding plasmid(s).

Other Procedures. Phospholipid composition of cells uniformly labeled with $^{32}PO_4$ was determined as described (20), except the radiolabel was detected and quantified using a Betascope (Betagene, Waltham, MA). Phosphatidylglycerophosphate synthase activity was determined as described (18).

RESULTS

Suppression of *pgsA* Mutants by Induction of cSDR. Colony formation by strain MDL12 on LB agar plates is IPTG-dependent due to a lack of sufficient anionic phospholipid synthesis to sustain cell growth under repressed conditions for the Φ[*lacOP–pgsA*$^+$] gene (20). Anionic phospholipids in the presence of ATP were demonstrated to reactivate the inactive ADP form of DnaA protein for continuing rounds of initiation of DNA replication at the *oriC* site *in vitro* (2). Since an *rnhA* mutation suppresses the requirement for a functional DnaA protein, such a mutation may also suppress the growth arrest phenotype of the repressed Φ[*lacOP–pgsA*$^+$] mutant thereby linking an anionic phospholipid requirement to DnaA protein function *in vivo*.

With strain MDL12 (*rnhA*$^+$) as control, the IPTG independence index of the strain MR12 (*rnhA*) was determined (Fig. 1); 85% of the MR12 cells were IPTG-independent and only 2% of the MDL12 cells were IPTG-independent. The IPTG independence index of strain MR12 was very similar to the plating efficiency (80%) of *dnaA46*^{ts}-containing cells at the restrictive temperature (42°C) vs. the permissive temperature (30°C) after introduction of an *rnhA* mutation (9). The 2% of MDL12 cells that formed colonies in the absence of IPTG showed a lack of repression of the *lacOP* promoter, which is the most common spontaneous mutation leading to IPTG independence (20). Thus, the growth-arrest phenotype due to a mutation in the ability to maintain a normal level of the major anionic phospholipids can be suppressed by a mutation rendering cells independent of DnaA protein and the *oriC* site, i.e., by utilizing cSDR. Introduction of plasmid pMM5 (*rnhA*$^+$)

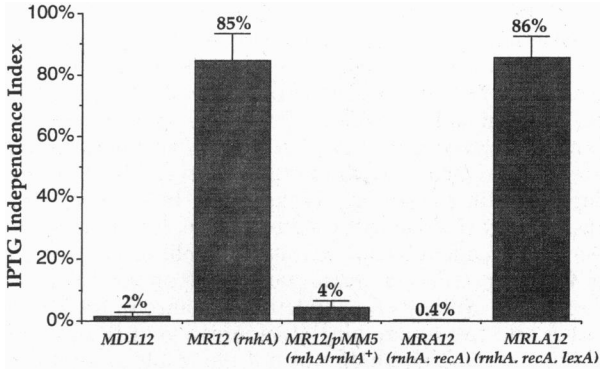

FIG. 1. IPTG dependence of strain MDL12 and its derivatives. All strains were first grown in the presence of IPTG, and the IPTG independence index was determined. Error bars showing the SEM are indicated.

into strain MR12 returned the IPTG independence index to 4% (Fig. 1), thereby verifying that suppression in strain MR12 was directly related to the *rnhA* mutation.

Initiation of DNA replication at the *oriK* sites in a strain carrying an *rnhA* allele is RecA-protein-dependent (15). RecA protein is essential for recombination and the SOS DNA repair system (27, 28) through its interaction with single-stranded DNA. The RecA protein requirement for cSDR may similarly involve the binding and stabilization of single-stranded DNA at the *oriK* sites for assembly of the replication complex in the absence of functional DnaA protein (29). Introduction of the Δ*recA* allele into strain MR12 reduced the IPTG independence index to 0.4% (Fig. 1), further supporting the induction of cSDR as the mechanism for suppression of the *pgsA* mutation.

Besides RecA-protein-dependent cSDR, Torrey and Kogoma (16) have reported two RecA-protein-independent cSDR modes, one of which occurs in cells with the following genotype: *rnhA recA lexA(Def)*. The LexA protein, which is inactivated by the RecA protein in response to DNA damage, normally acts to suppress genes induced in the SOS response of cells to DNA damage; in a *lexA(Def)* strain these genes are constitutively expressed (28). Although the exact mechanism of *recA*$^+$-independent cSDR is not known, it is possible that among the derepressed SOS genes, one gene product is expressed that creates a RecA protein substitute to bypass the requirement for RecA protein in cSDR (16). Strain MRLA12 [*rnhA recA lexA(Def)*] showed an IPTG independence index of 86% (Fig. 1) consistent with a predicted *recA*$^+$-independent cSDR suppression of the requirement for DnaA protein.

Anionic phospholipid metabolism in all the MDL12 derivatives was investigated to assure that induction of cSDR had no effect on phosphatidylglycerophosphate synthase activity levels (Table 2) or phospholipid composition (Table 3) for all the strains. Cells grown in the absence of IPTG for 5 hr showed

Table 2. Phosphatidylglycerosphosphate synthase activities of strains derived from MDL12

Strain	Colony formation (− IPTG)	Specific activity, units/mg	
		+ IPTG	− IPTG
MDL12	−	1.08 ± 0.11	0.25 ± 0.04
MR12	+	1.11 ± 0.13	0.22 ± 0.05
MR12/pMM5	−	1.31 ± 0.03	0.21 ± 0.05
MRA12	−	1.51 ± 0.29	0.21 ± 0.04
MRLA12	+	1.03 ± 0.18	0.18 ± 0.04

Specific activity: 1 unit of enzyme activity is defined as the amount of enzyme that converts 1 nmol of glycerol 3-phosphate into a chloroform-soluble product in 1 min.

Table 3. Phospholipid compositions of strains derived from MDL12

Strain	Colony formation (− IPTG)	Composition, %					
		+ IPTG			− IPTG		
		PE	PG	CL	PE	PG	CL
MDL12	−	69.3	16.6	2.8	84.4	5.8	0.8
MR12	+	69.9	17.7	2.1	85.2	4.5	0.6
MR12/pMM5	−	69.1	19.1	2.5	83.4	5.0	0.7
MRA12	−	70.7	18.7	2.0	85.8	2.7	0.4
MRLA12	+	69.2	18.7	2.5	86.1	3.7	0.6
(Deviation)		(±2.8)	(±1.9)	(±0.2)	(±1.7)	(±0.4)	(±0.1)

PE, phosphatidylethanolamine.

the expected reduction in enzyme activity and anionic phospholipid content.

To test whether the initiation of DNA replication is the only essential cell process dependent on anionic phospholipids, an *rnhA* mutation was introduced into two *pgsA*::*kan* null allele strains lacking the Φ[*lacOP–pgsA*$^+$] allele (19, 21), resulting in strains HR30 and HLR30. The parent strains are dependent for growth on plasmid pHD102 (*pgsA*$^+$), which is itself temperature-sensitive for replication. The *lpp* mutation [normally encodes the major outer membrane lipoprotein (30)] in strain HLR30 is a suppressor of the IPTG dependence of the Φ[*lacOP–pgsA*$^+$] allele but not of a null allele of the *pgsA* gene (21). Experimental results showed that strains HR30 and HLR30 remained temperature-sensitive for growth and, therefore, dependent on a functional *pgsA* gene that cannot be bypassed by induction of cSDR (data not shown). Therefore, there must be essential requirements for anionic phospholipids other than DnaA protein function even in the presence of another known partial suppressor (*lpp*) of the *pgsA* gene (31).

Dependence of *oriC* Plasmid Maintenance on Anionic Phospholipids. As an independent measure of the requirement for anionic phospholipids for DnaA-protein-dependent initiation at the *oriC* site, the stability of plasmids (pAC11 and pAC12) dependent on *oriC* for replication was examined under conditions of limiting anionic phospholipid synthesis. Plasmid pBR322 was used as a control since its replication *in vivo* is not dependent on the DnaA protein (32). Both *oriC* plasmids and pBR322 are partitioned randomly at cell division, and their stability of inheritance is proportional to the copy number in the cell (33). Plasmid pAC11 has a higher copy number than plasmid pAC12 (30 vs. 6 copies; A. Chiaramello and J. W. Zyskind, personal communication) due to the *mioC*$^+$ locus (34, 35) and, therefore, should partition similar to plasmid pBR322 at division.

Neither plasmid pAC11 nor pAC12 yielded significant numbers of transformants (2 ± 2 or fewer transformants per 10 ng of plasmid DNA) of strain MR12 (IPTG independent) when cells were plated in the absence of IPTG. Both plasmids resulted in similarly high numbers of transformants (170 ± 20 transformants per 10 ng of plasmid DNA) when cells were plated in the presence of IPTG. In contrast, the transformation efficiency of strain MR12 by plasmid pBR322 was similar under both plating conditions (plus IPTG, 88 ± 16 transformants per 10 pg of plasmid DNA; minus IPTG, 75 ± 15 transformants per 10 pg of plasmid DNA).

The stability of plasmids pAC11 and pAC12 vs. plasmid pBR322 was further examined in strains MDL12 and MR12 grown in liquid medium in the presence and absence of IPTG. Strain MDL12 will continue to grow slowly in liquid medium even after it reaches its minimum anionic phospholipid content, which requires about seven generations of growth in the absence of IPTG (20). Due to the *rnhA* mutation the growth of strain MR12 is IPTG-independent in liquid medium. Without drug selection, plasmid pBR322 is less stable in *rnhA* mutants than in wild-type cells, but this only becomes apparent

after growth for many more generations than employed here (9). Although the percentage of cells retaining the *oriC* plasmids in the absence of drug selection decreased as a function of generation number even in the presence of IPTG, cells of both strains MDL12 and MR12 growing without IPTG lost the *oriC* plasmids at a significantly faster rate (Fig. 2) while plasmid pBR322 was stable under all growth conditions. Therefore, there is a clear dependence of *oriC* plasmids for stable maintenance on either the level or rate of synthesis of anionic phospholipids.

DISCUSSION

The dependency of cell integrity on membrane phospholipids and the requirement for specific phospholipids for many cell processes are two separate but related functions of phospholipids. Due to the difficulty in systematically altering membrane phospholipid composition without causing cell death, the majority of evidence for specific roles for phospholipids in most cellular processes has come from results obtained by *in vitro* studies with little *in vivo* data available to support these *in vitro* results. In *E. coli*, a mutant in which the PG-plus-CL content of cells can be systematically regulated as a function of IPTG (20) has been used to establish the *in vivo* requirement for anionic phospholipids in the SecA-protein-dependent translocation of proteins across the inner membrane (36, 37). SecA protein has latent ATPase activity that is activated *in vitro* upon association of this cytoplasmic protein with anionic phospholipid-containing membranes (36, 37). The DnaA protein is also a cytoplasmic ATPase that is dramatically altered in its interaction with ATP by association with anionic phospholipids *in vitro* (2, 3). Based on these similarities, the above mutation in anionic phospholipid synthesis (strain MDL12) was utilized to obtain *in vivo* evidence to support the *in vitro* evidence for the dependence of DnaA protein function on anionic phospholipids.

Since a mutation in the *rnhA* gene allows initiation of DNA replication independent of the DnaA protein and the *oriC* site (14), a strain carrying an *rnhA* allele should bypass the need for all cellular components specifically required for initiation at the *oriC* site. Our results clearly demonstrate that an *rnhA* mutation can suppress the growth arrest phenotype of mutants defective in anionic phospholipid synthesis and thus strongly

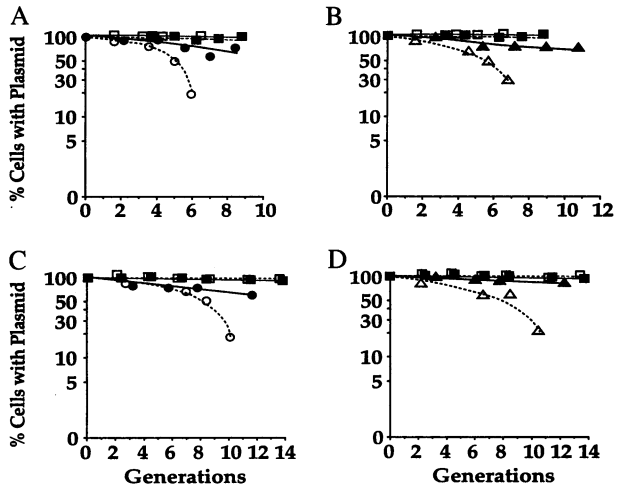

FIG. 2. Stability of *oriC* plasmids in the strains lacking anionic phospholipids. Strains MDL12 (*A* and *B*) and MR12 (*C* and *D*) carrying plasmid pBR322 (■, plus IPTG; □, minus IPTG), pAC11 (●, plus IPTG; ○, minus IPTG), or pAC12 (▲, plus IPTG; △, minus IPTG) were grown in LB medium without antibiotics. The percentage of cells still carrying a plasmid was determined after the indicated number of generations.

support the hypothesis that anionic phospholipids are required for the initiation of DNA replication at the *oriC* site in an *rnhA*$^+$ strain. The suppression by the *rnhA* mutation had the properties expected from the cSDR-dependent bypass of the DnaA protein and the *oriC* site. The suppression was reversed by a plasmid carrying the *rnhA*$^+$ gene, was dependent on RecA protein in a *lexA*$^+$ background, and was RecA-protein-independent in a *lexA(Def)* background. In an independent measure of *in vivo* DnaA protein function, it was shown that plasmids dependent on an *oriC* site for replication, and therefore DnaA protein, were highly dependent on normal anionic phospholipid synthesis for stable maintenance in the absence of other selective forces. Finally, suppression of IPTG dependence by an *rnhA* mutation was not the result of a return to normal anionic phospholipid metabolism.

The model in Fig. 3 summarizes the *in vivo* and *in vitro* data on the involvement of anionic phospholipids in DnaA protein function and the proposed mechanism of suppression of the *pgsA* mutation by an *rnhA* mutation. Based on this model, it should be possible to isolate mutants in the DnaA protein itself that also suppress the IPTG requirement for strain MDL12. *In vitro*, such mutated DnaA proteins should either be independent of anionic phospholipids for activation or be altered in their interaction properties with anionic phospholipids. Initial mutant screenings have generated a collection of mutations in a plasmid borne copy of the *dnaA* gene that appears to suppress the IPTG requirement for strain MDL12 (data not shown).

The inability to suppress a null allele of the *pgsA* gene via induction of cSDR is similar to the results with a mutation in the *lpp* gene that can only suppress *pgsA* alleles capable of some residual level of anionic phospholipid synthesis (21, 31). The limiting level of anionic phospholipid at the point of cell growth arrest in a *pgsA* null strain (19) is very similar to the basal level of anionic phospholipid in the regulated strains grown in the absence of IPTG, which does not result in cell death but simply a reduction in the growth rate of the cell (20). Therefore, there appear to be multiple essential requirements for anionic phospholipids with different minimum thresholds for each essential function.

The effect of reduced anionic phospholipid on protein translocation is not absolute but is reflected in a greatly reduced rate of translocation in proportion to the remaining anionic phospholipids (38, 39). Similarly, the direct relationship between the rate of synthesis of anionic phospholipids (i.e., the level of the *pgsA* gene product) and the growth rate for IPTG-dependent cells (20) might be a result of the reduced rate of initiation by the DnaA protein, which would affect the

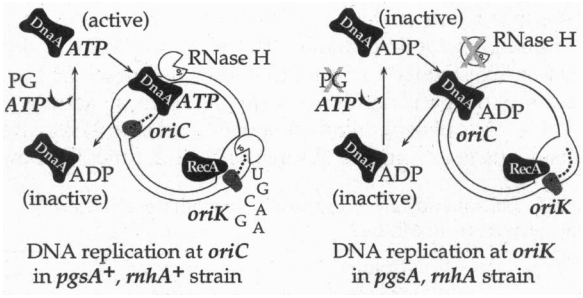

FIG. 3. Model for the initiation of DNA replication in *E. coli*. Under normal conditions initiation of DNA replication is restricted to the *oriC* site and dependent on the DnaA protein due to the digestion of RNA primers at the *oriK* sites by RNase H. Anionic phospholipids (synthesized by the *pgsA* gene product) facilitate the generation of the active ATP form of the DnaA protein. The proposed requirement for anionic phospholipids in maintaining functional DnaA protein can be suppressed through induction of cSDR in an *rnhA* mutant by permitting initiation at the *oriK* sites where RecA protein may substitute for DnaA protein.

growth rate of the cell (40). Bypassing the need for DnaA protein may remove the growth rate dependence of the cell on DnaA protein function and prevent cell growth arrest even at a reduced level of anionic phospholipid. The mechanism of suppression of the growth arrest phenotype of some *pgsA* mutants by the *lpp* mutation might be through increasing the rate at which bulk anionic phospholipid is made available for membrane formation and to sustain the growth rate of the cell dependent on DnaA-protein-directed initiation. Maturation of the *lpp* gene product consumes 7×10^5 molecules of PG per generation in a posttranslation modification event (41). At the relatively deficient level of 5% PG, there are about 10^6 PG molecules distributed mainly between the two leaflets of the inner membrane of *E. coli* (42). Therefore, any process, such as initiation of DNA replication, dependent on the maintenance of a threshold level of free anionic phospholipid on the inner surface of the membrane might be affected by the diversion of newly synthesized PG to this posttranslational event. Any further decline in anionic phospholipids, such as in a *pgsA* null strain even when carrying the *lpp* and *rnhA* suppressor mutations, might begin to compromise a function with a lower threshold requirement for anionic phospholipids and, therefore, still result in arrest of cell growth.

Definitive conclusions about the role of phospholipids in cellular processes cannot be made from either *in vitro* or *in vivo* studies alone but can be made by combining supporting data from both types of studies. It is now clear that protein translocation across the inner membrane in *E. coli* is dependent on anionic phospholipids. The results reported here strongly support a similar involvement of anionic phospholipids in initiation of DNA replication. Other examples exist of the association of cytoplasmic proteins (43–45) with phospholipids, including proteins that also interact with nucleic acids (46, 47), resulting in biologically significant changes. Therefore, a specific requirement for phospholipids in the organization of proteins at the membrane surface in functional complexes appears to be widespread.

We thank Dr. Judith Zyskind for providing us with plasmids pAC11 and pAC12 before their properties were reported. We also thank Dr. Elliott Crooke for helpful discussions during these studies. This work was supported by National Institutes of Health Grant GM-20487.

1. Firshein, W. (1989) *Annu. Rev. Microbiol.* **43,** 89–120.
2. Sekimizu, K. & Kornberg, A. (1988) *J. Biol. Chem.* **263,** 7131–7135.
3. Crooke, E., Castuma, C. E. & Kornberg, A. (1992) *J. Biol. Chem.* **267,** 16779–16782.
4. Ryter, A. (1968) *Bacteriol. Rev.* **32,** 39–52.
5. Sekimizu, K., Yung, B. Y. & Kornberg, A. (1988) *J. Biol. Chem.* **263,** 7136–7140.
6. Hwang, D. S., Crooke, E. & Kornberg, A. (1990) *J. Biol. Chem.* **265,** 19244–19248.
7. Yung, B. Y. & Kornberg, A. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 7202–7205.
8. Fralick, J. A. & Lark, K. G. (1973) *J. Mol. Biol.* **80,** 459–475.
9. Horiuchi, T., Maki, H. & Sekiguchi, M. (1984) *Mol. Gen. Genet.* **195,** 17–22.
10. Kanaya, S. & Crouch, R. (1983) *J. Biol. Chem.* **258,** 1276–1281.
11. Massy, B., Fayet, O. & Kogoma, T. (1984) *J. Mol. Biol.* **178,** 227–236.
12. Torrey, T., Atlung, T. & Kogoma, T. (1984) *Mol. Gen. Genet.* **196,** 350–355.
13. Frey, J., Chandler, M. & Caro, L. (1981) *Mol. Gen. Genet.* **182,** 364–366.
14. Kogoma, T. & von Meyenburg, K. (1983) *EMBO J.* **2,** 463–468.
15. Kogoma, T., Skarstad, K., Boye, E., von Meyenburg, K. & Steen, H. B. (1985) *J. Bacteriol.* **163,** 439–444.
16. Torrey, T. & Kogoma, T. (1987) *Mol. Gen. Genet.* **208,** 420–427.
17. Raetz, C. R. & Dowhan, W. (1990) *J. Biol. Chem.* **265,** 1235–1238.
18. Gopalakrishnan, A. S., Chen, Y., Temkin, M. & Dowhan, W. (1986) *J. Biol. Chem.* **261,** 1329–1338.
19. Heacock, P. N. & Dowhan, W. (1987) *J. Biol. Chem.* **262,** 13044–13049.
20. Heacock, P. N. & Dowhan, W. (1989) *J. Biol. Chem.* **264,** 14972–14977.
21. Dowhan, W. (1992) *NATO ASI Ser. H* **63,** 11–32.
22. Horiuchi, T., Maki, H., Maruyama, M. & Sekiguchi, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 3770–3774.
23. Silhavy, T., Berman, M. & Enquist, L. (1984) *Experiments with Gene Fusion* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 107–112.
24. Bachmann, B. & Low, K. B. (1980) *Microbiol. Rev.* **44,** 1–56.
25. Miki, T., Ebina, Y., Kishi, F. & Nakazawa, A. (1981) *Nucleic Acids Res.* **9,** 529–543.
26. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab. Press, Plainview, NY), pp. 440–444.
27. Witkin, E. (1991) *Biochimie* **73,** 133–141.
28. Cox, M. & Lehman, I. (1987) *Annu. Rev. Biochem.* **56,** 229–262.
29. Skarstad, K. & Boye, E. (1988) *J. Bacteriol.* **170,** 2549–2554.
30. Wu, H. C., Tokunaga, M., Tokunaga, H., Hayashi, S. & Giam, C.-Z. (1983) *J. Cell. Biochem.* **22,** 161–171.
31. Asai, Y., Katayose, Y., Hikita, C., Ohta, A. & Shibuya, I. (1989) *J. Bacteriol.* **171,** 6867–6869.
32. Abe, M. (1980) *J. Bacteriol.* **141,** 1024–1030.
33. Kornberg, A. & Baker, T. (1992) *DNA Replication* (Freeman, New York), pp. 647, 759.
34. Stuitje, A. R. & Meijer, M. (1983) *Nucleic Acids Res.* **11,** 5775–5791.
35. Løbner-Olesen, A., Atlung, T. & Rasmussen, K. (1987) *J. Bacteriol.* **169,** 2835–2842.
36. Lill, R., Dowhan, W. & Wickner, W. (1990) *Cell* **60,** 271–280.
37. Hendrick, J. P. & Wickner, W. (1991) *J. Biol. Chem.* **266,** 24596–24600.
38. de Vrije, T., de Swart, R. L., Dowhan, W., Tommassen, J. & de Kruijff, B. (1988) *Nature (London)* **334,** 173–175.
39. Kusters, R., Dowhan, W. & de, K. B. (1991) *J. Biol. Chem.* **266,** 8659–8662.
40. Helmstetter, C. (1987) in *Escherichia coli and Salmonella Typhimurium: Cellular and Molecular Biology*, eds. Ingraham, J., Low, K. B., Magasanik, B., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), Vol. 2, pp. 1594–1605.
41. Sankaran, K. & Wu, H. C. (1994) *J. Biol. Chem.* **269,** 19701–19706.
42. Neidhardt, F. C. (1987) in *Escherichia coli and Salmonella Typhimurium: Cellular and Molecular Biology*, eds. Ingraham, J., Low, K. B., Magasanik, B., Schaechter, M. & Umbarger, H. E. (Am. Soc. Microbiol., Washington, DC), Vol. 1, p. 4.
43. Rando, R. R. (1988) *FASEB J.* **2,** 2348–2355.
44. Bell, R. M. & Burns, D. J. (1991) *J. Biol. Chem.* **266,** 4661–4664.
45. Yuli, W., MacDonald, J. I. S. & Kent, C. (1993) *J. Biol. Chem.* **268,** 5512–5518.
46. Yoshida, S., Tamiya, K. K. & Kojima, K. (1989) *Biochim. Biophys. Acta* **1007,** 61–66.
47. Parks, W. A., Couper, C. L. & Low, R. L. (1990) *J. Biol. Chem.* **265,** 3436–3439.