Mutations in DnaA protein suppress the growth arrest of acidic phospholipid-deficient *Escherichia coli* cells

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Cell growth arrests when the concentrations of anionic phospholipids drop below a critical level in *Escherichia coli*, with the insufficient amounts of acidic phospholipids adversely affecting the DnaA-dependent initiation of DNA replication at the chromosomal origin (*oriC*). Mutations have been introduced into the carboxyl region of DnaA, including the portion identified as essential for productive *in vitro* DnaA–acidic phospholipid interactions. Expression of DnaA proteins possessing certain small deletions or substituted amino acids restored growth to cells deficient in acidic phospholipids, whereas expression of wild-type DnaA did not. The mutations include substitutions and deletions in the phospholipid-interacting domain as well as some small deletions in the DNA-binding domain of DnaA. Marker frequency analysis indicated that initiation of replication occurs at or near *oriC* in acidic phospholipid-deficient cells rescued by the expression of DnaA having a point mutation in the membrane-binding domain, DnaA(L366K). Flow cytometry revealed that expression in wild-type cells of plasmid-borne DnaA(L366K) and DnaA(Δ 363–367) reduced the frequency with which replication was initiated and disturbed the synchrony of initiations.

Keywords: DnaA/*Escherichia coli*/flow cytometry/phospholipid/replication

Introduction

DnaA protein initiates *Escherichia coli* DNA replication from the unique chromosomal origin, *oriC*. The activity of DnaA is strongly influenced by its tight binding of the adenine nucleotides ATP and ADP (K_d of ~30 and 100 nM, respectively); *in vitro* ATP–DnaA protein is active for initiating replication, while the ADP form is not (Sekimizu *et al.*, 1987). ATP bound to DnaA protein is slowly hydrolyzed, with the resulting ADP remaining tightly bound, thus producing ADP–DnaA protein. The conversion of ATP–DnaA to the ADP form is enhanced through an action referred to as regulatory inactivation of DnaA (RIDA), carried out by a partially purified factor termed IdaB and the β -subunit sliding clamp component of DNA

polymerase III holoenzyme assembled on DNA (Katayama *et al.*, 1998).

In vitro, the exchange of bound ADP for ATP is slow, with a half-life of ~30 min when ADP–DnaA is incubated with excess ATP (Sekimizu *et al.*, 1987). However, acidic phospholipids in a fluid bilayer can rapidly promote the release of the tightly bound nucleotide (Sekimizu and Kornberg, 1988), and when in the presence of *oriC* DNA, facilitate the reactivation of ADP–DnaA into ATP–DnaA (Sekimizu and Kornberg, 1988; Crooke *et al.*, 1992).

The inner membrane of *E. coli* is composed of zwitterionic phosphatidylethanolamine (~70%), and the anionic phospholipids phosphatidylglycerol (~25%) and cardiolipin (~4%). The committed step in the synthesis of phosphatidylglycerol and cardiolipin is catalyzed by phosphatidylglycerol synthase A, the enzyme encoded by the *pgsA* gene. A strain, HDL1001, has expression of chromosomally encoded *pgsA* dependent on the presence of the inducer isopropyl- β -D-thiogalactopyranoside (IPTG) (Heacock and Dowhan, 1989). When cultured in the absence of IPTG, HDL1001 cells continue to grow for ~10 generations, at which point their content of acidic phospholipids has dropped substantially and they undergo growth arrest. The cells, however, remain viable; even after many hours of quiescence, growth resumes after a brief lag period following the addition of IPTG back to the medium (Heacock and Dowhan, 1989).

Studies with this strain suggested that normal cellular levels of acidic phospholipids are required to sustain chromosomal replication from *oriC in vivo* (Xia and Dowhan, 1995). In the absence of RNase HI, initiations of DNA replication independent of DnaA protein occur (constitutive stable DNA replication) at sites other than *oriC* in a *recA*-dependent manner (Torrey *et al.*, 1984; von Meyenburg *et al.*, 1987). Null alleles of the *dnaA* gene and deletions of *oriC* are suppressed by point mutations and null alleles of *rnhA* (Ogawa *et al.*, 1984). Thus, mutations in *rnhA* should also allow growth of cells deficient for acidic phospholipids if the primary reason for growth limitation is the inability to activate DnaA protein through phospholipid rejuvenation. In agreement, an *rnhA*[–], *recA*⁺ derivative of this strain continues to grow in the absence of IPTG due to constitutive DNA replication, which indicates the dependency of normal initiations on acidic phospholipids (Xia and Dowhan, 1995).

Furthermore, recent studies have provided direct evidence that ATP–DnaA protein is regenerated from ADP–DnaA *in vivo*. *De novo* synthesis of DnaA and its adopting the ATP-form, while necessary, is not always sufficient to support the initiation of replication, and thus, efficient recycling of ADP–DnaA is required (Kurokawa *et al.*, 1999).

Protein–lipid cross-linking studies and analysis of the interaction of DnaA proteolytic fragments with liposomes

reveal that a distinct region of DnaA is responsible for the protein's interaction with acidic phospholipids (Garner and Crooke, 1996; Garner *et al.*, 1998). The region, contained within the approximate residues of amino acids 300–380, is not associated with other known functions of DnaA protein (Roth and Messer, 1995; Sutton and Kaguni, 1997; Sutton *et al.*, 1998; Weigel *et al.*, 1999). Included within this portion of DnaA is a segment predicted as being an amphipathic helix (Skarstad and Boye, 1994), a structure that can act as a membrane surface-seeking domain of peripherally associated membrane proteins.

Here we have introduced mutations into the carboxyl region of DnaA protein, with the aim of altering the lipid-binding properties of DnaA while not affecting the protein's other replicative functions. The mutant DnaA proteins were examined for their ability to suppress the growth arrest of cells lacking normal levels of acidic phospholipids. Members of a subset of the mutant DnaA proteins were able to fulfill this requirement, including some that lack the ability to bind *oriC*. Two mutant forms of DnaA, one with a point mutation and the other with a small deletion in the membrane-binding domain were examined in more detail and their expression was found to perturb greatly the synchronous initiations of replication normally observed.

Results

As a means to assess their altered membrane-binding properties, mutant forms of DnaA protein were examined in a genetic screen for their ability to suppress the arrested growth of cells lacking normal levels of acidic phospholipids. The host strain, HDL1001, has a *pgsA::kan* null background, but harbors an integrated Φ [*lacOP-pgsA*⁺] genetic fusion. The gene *pgsA* codes for phosphatidylglycerol synthase, the enzyme that catalyzes the committed step of acidic phospholipid biosynthesis. HDL1001 cells are able to synthesize anionic phospholipids only when cultured in the presence of the inducer IPTG; without induced expression of *pgsA*, the cellular level of acidic phospholipids reaches a critical threshold after 8–10 generations, and cell growth is arrested (Heacock and Dowhan, 1989).

A series of small, flanking deletions and site-specific and randomly generated mutations causing single amino acid substitutions in the carboxyl region of DnaA protein was constructed. Expression plasmids harboring the mutant forms of *dnaA* under the control of the arabinose promoter were introduced into HDL1001. Transformed cells were propagated in medium containing IPTG and glucose to induce the synthesis of normal cellular levels of acidic phospholipids and repress expression of mutant *dnaA* genes, respectively. The cells were washed to remove IPTG and plated onto three different growth media: LB containing IPTG and arabinose, LB containing glucose and LB containing arabinose.

A distinct boundary exists between deletions that can and can not rescue acidic phospholipid-deficient cells

HDL1001 cells without a plasmid, as well as those transformed with vector alone, were dependent upon IPTG for growth, as expected (Table I). Furthermore, when

Table I. Colony formation of HDL1001 cells transformed with plasmids harboring deletion mutant *dnaA* genes

HDL1001 transformed with plasmids expressing	Number of colonies ^a		
	+ IPTG + glucose	– IPTG	
		+ glucose	+ arabinose
Wild-type DnaA	1000	0	9
DnaAΔ317–322	1000	0	9
DnaAΔ322–329	1000	0	6
DnaAΔ329–333	1000	0	0
DnaAΔ332–337	1000	0	16
DnaAΔ336–340	1000	0	2
DnaAΔ340–345	1000	0	0
DnaAΔ346–356	1000	0	161*
DnaAΔ357–368	1000	0	517
DnaAΔ363–367	1000	0	700
DnaAΔ369–376	1000	0	442
DnaAΔ373–381	1000	0	577
DnaAΔ377–386	1000	0	407
DnaAΔ387–396	1000	0	764
DnaAΔ397–404	1000	0	227*
DnaAΔ405–417	1000	0	9
DnaAΔ418–422	1000	0	407
DnaAΔ432–427	1000	0	262
DnaAΔ428–435	1000	0	362*
DnaAΔ436–445	1000	0	667
DnaAΔ446–455	1000	0	714*
DnaAΔ456–464	1000	0	577
(Vector)	1000	0	0

^aValues are normalized to 1000 colonies for growth in the presence of IPTG and are an average from two or three independent transformations. An asterisk indicates that only very small colonies were obtained.

expression of either the wild-type or mutant plasmid-borne *dnaA* genes was repressed due to the glucose in the medium, IPTG-dependent induction of *pgsA* was still required for growth. Overproduction of wild-type DnaA failed to rescue the growth arrest of acidic phospholipid-deficient cells. Similarly, expression of the mutant forms of DnaA having small deletions from amino acid 317 to 356 failed to restore growth to the acidic phospholipid-deficient cells (Table I). In contrast, the majority of similar size deletions made in the more C-terminal region of DnaA (from residue 357 to 464) effectively suppressed the growth arrest. Thus, there seems to be a distinct boundary that delineates domains of DnaA which when disrupted can and can not rescue cells lacking normal levels of anionic phospholipids.

The colonies that arose with the expression of certain of these deletions were smaller, even with longer incubations. When cells from these small colonies were restreaked onto fresh medium, only small colonies were again observed. These forms of DnaA protein may be less efficient in participating in the initiation of chromosomal replication and may compromise cell growth.

A mutant form of DnaA that lacked the entire putative amphipathic helix, DnaAΔ357–374, failed to rescue HDL1001 cells cultured in the absence of IPTG. However, pulse-chase studies revealed that this form of DnaA protein is rapidly degraded *in vivo* (data not shown).

Table II. Colony formation of HDL1001 cells transformed with plasmids harboring missense mutant *dnaA* genes

HDL1001 transformed with plasmids expressing	Number of colonies ^a		
	+ IPTG + glucose	– IPTG	
		+ glucose	+ arabinose
Hydrophilic face of helix			
DnaA(R360C)	1000	0	0
DnaA(R364E)	1000	0	0
DnaA(R364L)	1000	0	0
DnaA(R364P)	1000	0	0
DnaA(E361K, D365K)	1000	0	0
DnaA(E361Q, E371Q)	1000	0	4
DnaA(E361Q)	1000	0	0
Hydrophobic face of helix			
DnaA(L363R, L366E, L367E, L369K)	1000	0	506
DnaA(L363K, L373R)	1000	0	594
DnaA(L363E)	1000	0	0
DnaA(L363K)	1000	0	0
DnaA(L366E)	1000	0	0
DnaA(L366K)	1000	0	615
DnaA(L367E)	1000	0	0
DnaA(L367K)	1000	0	0
DnaA(L373R)	1000	0	0

^aValues are normalized to 1000 colonies for growth in the presence of IPTG and are an average from two or three independent transformations.

Table III. *In vitro* *oriC*-plasmid-binding activities of purified DnaA proteins

DnaA protein	<i>oriC</i> -plasmid bound/DnaA (molar ratio)
Wild type	0.068
DnaA(L366K)	0.062
DnaA(Δ363–367)	0.042
DnaA(Δ436–445)	$<3.3 \times 10^{-6}$

Certain amino acid substitutions within the membrane-binding domain suppress the growth arrest of cells with limited acidic phospholipids

Missense mutations were introduced into the membrane-binding segment of DnaA (Table II), a region identified by examination of functional proteolytic fragments (Garner and Crooke, 1996) and phospholipid–DnaA protein chemical cross-linking experiments (Garner *et al.*, 1998). The mutations were designed to alter the amphipathic nature of the potential α -helix of residues 357–374; charged residues were placed within the non-polar face of the helix, and charged amino acids on the polar face were replaced with amino acids having an opposite charge or with non-polar residues.

While expressions of most of these mutants had no effect on the arrested growth of acidic phospholipid-deficient cells, three mutant forms of DnaA efficiently facilitated continued growth. Introducing multiple charged residues on the non-polar face [DnaA(L363R, L366E, L367E, L369K) and DnaA(L363R, L373R)] had a strong influence. Interestingly, one form that rescued the acidic phospholipid-deficient cells differed from wild-type DnaA protein by the single amino acid change of leucine 366 to a

lysine. This alteration appears to be highly specific; when leucine 366 was replaced instead with the oppositely charged amino acid glutamate, no suppressing phenotype was observed. Replacing neighboring leucine 367 with lysine also failed to rescue the cells with low levels of acidic phospholipids.

The ability of a mutant form of DnaA to rescue acidic phospholipid-deficient cells is independent of its ability to bind *oriC*

During the initiation of a round of replication, ~15–25 DnaA molecules bind to *oriC* (Fuller *et al.*, 1984; Funnell *et al.*, 1987; Crooke *et al.*, 1993). Since some of the small deletion mutations fall within the DNA-binding domain of DnaA protein, the origin-binding activity of one of them was examined. DnaA(Δ436–445), which efficiently suppresses the growth arrest of acidic phospholipid-deficient cells (Table I) was purified (Li and Crooke, 1999), as was wild-type DnaA, DnaA(L366K) and DnaA(Δ363–367), and their ability to bind *oriC*-plasmids compared (Table III).

Wild-type DnaA, DnaA(L366K) and DnaA(Δ363–367) bound *oriC*-DNA equally well (Table III), yet only expression of DnaA(L366K) and DnaA(Δ363–367) could rescue the acidic phospholipid-deficient cells (Tables I and II). The binding of *oriC*-plasmids by these DnaA proteins was dependent on the plasmids having DnaA-boxes, since they failed to bind the control plasmid, pBluescript. Additionally, DnaA(R364E), which is likely to bind *oriC* as indicated by its ability to support *in vitro* *oriC* replication (Makise *et al.*, 2000), failed to rescue acidic phospholipid-deficient cells (Table II).

Conversely, DnaA(Δ436–445), with a small deletion in its DNA-binding domain, had no detectable origin binding capacity (Table III), yet was efficient at suppressing the growth arrest (Table I). The complete absence of *oriC*-binding activity (at least 20 000-fold less than wild-type DnaA) was not the result of a preparation of totally inactive protein since the purified DnaA(Δ436–445) retained high affinity binding of ATP (10% of wild type).

In summary, neither the capacity to bind *oriC*, nor the lack thereof, seems to be related to the ability of a mutant form of DnaA to restore growth to acidic phospholipid-deficient cells.

HDL1001 cells rescued for growth were still deficient in acidic phospholipids

The acidic phospholipid content of HDL1001 cells expressing DnaA(L366K), but cultured in the absence of IPTG, was only 6% of the total cellular phospholipids (Table IV). This level is similar to that previously described for this strain when expression of its *pgsA* is repressed (Heacock and Dowhan, 1989). The low level of acidic phospholipids observed (Table IV) is in agreement with the cells still showing a dependency on IPTG for growth in the absence of induced expression of DnaA(L366K) (Table II).

Replication initiates at *oriC* in acidic phospholipid-deficient cells in which growth has been restored by the expression of DnaA(L366K)

The growth arrest that occurs with $\Delta pgsA$, $\Phi[lacOP-pgsA^+]$ cells when cultured in the absence of IPTG can be

Table IV. Phospholipid composition of HDL1001 cells with growth restored by the expression of DnaA(L366K)

Strain ^a	Media ^b	Phospholipid species ^c (% of total)			
		PE	PG	CL	PA
MG1655	LB	74.3	17.4	3.1	1.4
HDL1001/ pZL607	LB + IPTG	81.5	11.7	2.6	ND
HDL1001/ pZL607	LB + Ara	87.7	1.2	0.9	4.1

^aPlasmid pZL607 is the pZL606-derived plasmid that expresses DnaA(L366K) under control of the arabinose promoter.

^bLB medium was supplemented with IPTG at 1 mM and arabinose (Ara) at 0.2%, as indicated. Media for transformed strains contained ampicillin (100 µg/ml).

^cPE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; PA, phosphatidic acid.

suppressed by constitutive stable DNA replication, a mode of initiation that occurs with the absence of RNase H at locations other than *oriC* and independent of DnaA (Torrey *et al.*, 1984; von Meyenburg *et al.*, 1987). Therefore, marker frequency measurements were made to see whether initiations proceed normally from *oriC* or occur elsewhere on the chromosome in HDL1001 cells cultured without IPTG and expressing DnaA(L366K). Southern blot analysis of genomic DNA was performed using probes complementary to *dnaA* and *xasA*, which are located 0.9 and ~50 min from *oriC*, respectively. A ratio of 2.9:1 was observed for the genomic content of *dnaA* versus *xasA* in the suppressed mutant strain. The higher frequency of the *oriC*-proximal marker indicates that replication initiates at *oriC*. The ratio was, however, unexpectedly high for a doubling time of 85 min. Analysis of HDL1001 cells grown in the presence of IPTG gave a lower ratio (1.4:1) when grown with a comparable doubling time (60 min). It is not known why the *oriC*-proximal marker frequency is higher than normal in acidic phospholipid-deficient cells rescued for growth by DnaA(L366K). It may indicate that either the C or the D period, or both, is considerably extended.

The marker frequency data indicate that initiation happens in the region of *oriC* in cells rescued by the expression of DnaA(L366K). In agreement, the cells' genotype, *rnhA*⁺ and *recA*⁻, is not supportive of mechanisms, such as inducible stable DNA replication and constitutive stable DNA replication, in which replication is initiated at sites other than *oriC*. Moreover, the restoration of growth of HDL1001 cells is completely dependent upon expression of the mutant form of DnaA; the cells when subsequently cultured in the absence of arabinose do not grow unless IPTG is present in the growth medium (data not shown). Thus, chromosomal replication in acidic phospholipid-deficient cells expressing DnaA(L366K) appears to initiate at *oriC* in a DnaA-dependent manner.

Expression of DnaA(L366K) and DnaA(Δ363–367) disrupts synchronous chromosomal replication

Substitution of the chromosomal *dnaA* allele of a wild-type strain with *dnaA*(L366K) or *dnaA*(Δ363–367) was

attempted. The allelic replacements, which were tried using both rich and poor media and temperatures of 30, 37 and 42°C, were unsuccessful, suggesting that these mutant DnaA proteins can not support all the functions required of DnaA protein *in vivo*. In order to establish how the mutant proteins participate in and influence replication or replication control, the physiology of wild-type cells expressing the mutant proteins was studied by flow cytometry. MC1061 cells harboring pZL606-based plasmids expressing DnaA(L366K), DnaA(Δ363–367), wild-type DnaA or the parental vector pBAD24c were grown overnight in LB with ampicillin, diluted and then grown for four to five generations in the same medium which also contained arabinose to induce expression of the plasmid-encoded proteins. The presence of DnaA(L366K) changed the DNA distribution, shifting it to lower values (Figure 1C, left panel; Table V) and led to an asynchrony of initiation phenotype, as shown by the irregular numbers of chromosomes following run-out of replication in the presence of rifampicin and cephalixin (Figure 1C, right panel). Similar effects were seen after expression of DnaA(Δ363–367) (Figure 1D, right panel). The flow cytometry data show that cells expressing mutant protein have a decreased DNA and origin content (Table V). Thus, expression of these proteins led to an apparent inhibition of the initiation of replication. These results show that the mutant proteins are capable of forming mixed oligomers with the wild-type DnaA protein, although the oligomers do not obey the proper regulation of the initiation event. It is possible that only oligomers containing wild-type DnaA protein function to fire an origin properly, and that origins with complexes made up solely of mutant protein either do not fire at all, or fire in an unregulated fashion. Overexpression of the wild-type DnaA protein led to cell filamentation, over-initiation, lack of replication elongation and eventually impaired cell growth (Figure 1B; Table V), as has been previously observed (Skarstad *et al.*, 1989; Atlung and Hansen, 1993).

Discussion

Results from *in vitro* studies support the argument that interaction with acidic phospholipids plays an important role in regulating the activity of DnaA protein. Yet, evidence that such an association is physiologically relevant and necessary for DnaA to carry out its functions properly is only starting to emerge. It has been demonstrated that normal initiation of chromosomal replication at *oriC* requires the cell membrane to have adequate levels of acidic phospholipids (Xia and Dowhan, 1995). Recently, visualization of the cellular location of DnaA protein via immunofluorescent and immunogold electron microscopy revealed that the bulk of DnaA within a cell is located at the membrane (Newman and Crooke, 2000).

Data presented here suggest that there is a direct link between the lipid composition of the cellular membrane and the ability of DnaA to function *in vivo*. Expression of a mutant form of DnaA protein that contains a single amino acid substitution in its membrane-binding domain restores growth to cells depleted of acidic phospholipids. The capacity of one changed amino acid in the initiator protein to overcome the blocked growth caused by an altered lipid

Fig. 1. DNA versus protein content (FITC) dot plots of exponentially growing cultures (left panels) and DNA histograms of rifampicin- and cephalixin-treated cultures (right panels) of strain MC1061 with vector pBAD24c (A), and strain MC1061 with plasmids expressing wild-type DnaA (B), DnaA(L366K) (C) and DnaA(Δ 363–367) (D) grown for four generations with arabinose (0.2%). Distinct peaks represent accumulation of cells with integral numbers of chromosomes that reflect the numbers of origins at the time of drug action.

content is a persuasive indication that DnaA–membrane interactions are physiologically significant.

The mechanism of how the mutant forms of DnaA described here restore growth to acidic phospholipid-deficient cells remains unknown. Possibilities may involve

nucleotide exchange, including: (i) the mutant forms are able to respond to either neutral phospholipids or the lower content of acidic phospholipids; (ii) the mutant proteins fail to bind nucleotide and therefore don't need acidic phospholipids to rejuvenate ADP–DnaA; (iii) the

Table V. Average cell mass, DNA and origin content as derived from flow cytometry data in Figure 1

MC1061 transformed with plasmids expressing	τ (min)	Cell mass (relative value)	DNA content per cell (chromosome equiv.)	DNA per mass	Origins per cell
(Vector)	30	1	4.7	1	5.7
DnaA	30	1.2	4.9	0.9	>7.0
DnaA(L366K)	30	1.0	3.6	0.8	4.6
DnaA(Δ 363–367)	30	1.1	3.6	0.7	3.7

conversion of mutant ATP–DnaA to the ADP-form is impaired so that nucleotide exchange is not necessary; or (iv) the mutant forms are altered in how they respond to an unknown nucleotide exchange-related factor—they may be less sensitive to a negative acting factor or retain responsiveness to a positive acting factor when levels of acidic phospholipids are low. Some of these possibilities may be less likely since one of the mutant forms of DnaA, DnaA(L366K), has been purified and behaves *in vitro* like wild-type DnaA with respect to retaining affinity for ATP and ADP, the kinetics of hydrolysis of DnaA-bound ATP and specificity toward acidic phospholipids for nucleotide exchange (Z.Li, J.Kitchen and E.Crooke, manuscript in preparation).

The way in which mutant DnaA proteins restore growth to acidic phospholipid-deficient cells may be related to DnaA–membrane interactions independent of nucleotide exchange. One possibility is that the mutant forms may shield the wild-type protein from a negative activity, unrelated to nucleotide binding or hydrolysis, which arises when cells become deficient in acidic phospholipids.

Interestingly, some of the mutations that restore growth to acidic phospholipid-deficient cells are small deletions in the DNA-binding domain of DnaA protein (Table I). The *oriC*-binding activity of one of these mutant DnaA proteins, DnaA(Δ 436–445), was examined; it failed to bind origin DNA (Table III). Moreover, of the proteins that can bind *oriC*, some [DnaA(L366K) and DnaA(Δ 363–367)] are able to rescue acidic phospholipid-deficient cells, while others [i.e. DnaA(L364E)] are not. Combined, these results show that there does not appear to be a link between a mutant protein's ability to suppress the growth arrest and its capacity to bind or not bind *oriC*. Also, while the C-terminus of DnaA is clearly involved in DNA binding, this does not preclude the amino acids responsible for the functions of DNA binding and membrane binding from partially overlapping within the protein's primary sequence.

Because some of the suppressing mutant forms of DnaA can not bind DNA, they must clearly be working in concert with the wild-type DnaA present in HDL1001 cells to support growth. Wild-type protein provides origin-binding function, and the mutant DnaA protein permits some necessary DnaA function to be carried out, which wild-type DnaA is unable to do in the absence of acidic phospholipids. This function may be involved with nucleotide exchange or initiation, or perhaps even some other as yet unappreciated function of DnaA that is independent of the initiation of chromosomal replication (see below).

Failure to obtain a strain in which the chromosomally encoded wild-type *dnaA* gene has been replaced with the

allele for DnaA(L366K) further indicates that at least some of the mutant forms of the protein can not support cell growth on their own (W.Zheng and E.Crooke, unpublished data). In agreement, DnaA(L366K) is feeble at initiating DNA replication *in vitro*, but is excellent at augmenting a limiting quantity of wild-type DnaA (Z.Li, J.Kitchen and E.Crooke, manuscript in preparation). Thus, it appears that in rescuing acidic phospholipid-deficient cells, mixed oligomers of the chromosomally encoded wild-type DnaA and plasmid-encoded mutant DnaA protein complement each other, with each form carrying out an essential function that the other is unable to perform. Interestingly, none of the deletion or missense mutant forms of DnaA protein was lethal when overexpressed in cells having normal levels of acidic phospholipids.

Lack of a necessary replicative function or failure to respond to regulatory signals was also seen for the mutant forms of DnaA protein by flow cytometry analysis. Initiation of replication became asynchronous after overexpression of DnaA(L366K) and DnaA(Δ 363–367) in wild-type cells (Figure 1). Asynchrony probably occurred either because initiation complexes contained insufficient amounts of wild-type DnaA protein to fire at the correct time or to respond correctly to regulatory signals. It also seems that the average initiation frequency was decreased because the cellular DNA and origin content were significantly lower than those found in cells not expressing the mutant proteins (Table V). This, however, did not affect the growth rate.

Overexpression of wild-type DnaA protein resulted in growth inhibition after approximately six generations. There may be several reasons for this. One may be that the DnaA protein negatively regulates the expression of important genes, among these its own. It is thus reasonable that overproduction of DnaA may lead to scarcity of essential factors and subsequent impairment of cell growth. The fact that expression of the mutant DnaA proteins did not lead to growth impairment indicates that they may be less efficient as negatively acting transcription factors.

A form of DnaA carrying a site-directed mutation in the membrane-binding domain was reported to have a slightly decreased rate of nucleotide release when treated *in vitro* with limiting levels of cardiolipin liposomes (Hase *et al.*, 1998; Makise *et al.*, 2000). However, when a sample of this mutant DnaA was kindly given to us, we found that it responded comparably to wild-type DnaA when treated over a concentration range of cardiolipin liposomes or vesicles composed of *E.coli* phospholipids.

Matsumoto and colleagues recently reported the viability of cells lacking detectable levels of phosphatidylglycerol and cardiolipin (i.e. *pgsA::kan* genotype)

(Kikuchi *et al.*, 2000). However, this was only the case when the cells also had the *lpp-2* mutation, and thus, were unable to synthesize the major outer membrane lipoprotein; otherwise, accumulation in the inner membrane of an intermediate in lipoprotein synthesis compromises the integrity of the membrane and is lethal. Although the cells lacked phosphatidylglycerol and cardiolipin, the content of the anionic lipid phosphatidic acid was significantly elevated in these cells. Based on the *in vitro* capability of phosphatidic acid to satisfy the acidic phospholipid requirement for nucleotide exchange on DnaA protein, it was proposed that the phosphatidic acid that accumulates in *pgsA::kan*, *lpp-2* cells is sufficient to support chromosomal replication (Kikuchi *et al.*, 2000). Moreover, these cells, although viable, are not entirely normal in that they cease to grow at temperatures $>37^{\circ}\text{C}$ and are not viable in a minimal medium nor in media with low osmolarities. An examination of the initiations in the cells will be informative on the importance of having, specifically, the phospholipids cardiolipin and phosphatidylglycerol for normal chromosomal replication.

DnaA has been implicated in functions independent of its role in initiation. Overexpression of DnaA protein stabilizes the inheritance of partitioning-defective pSC101 plasmids. However, high level expression of a mutant DnaA protein that has an in-frame deletion which removes a region that includes the membrane-binding domain, or a DnaA protein with an amino acid substitution in the membrane-binding domain, fails to provide this stability (Miller and Cohen, 1999). In light of these findings and of DnaA being localized at the membrane (Newman and Crooke, 2000), potentially as discrete foci, as detected with a functional green fluorescent protein–DnaA fusion (G.Newman and E.Crooke, unpublished data), future studies should focus on whether the association of membranes and DnaA protein is important for spatial as well as temporal control of chromosomal replication and segregation.

Materials and methods

Materials

Unless otherwise stated, reagent grade chemicals were from J.T.Baker, Sigma Chemicals or Boehringer Mannheim Biochemicals. Rifampicin and cephalixin were from Fluka and Eli Lilly, respectively. Growth media components were from Difco Laboratories. Restriction endonucleases were from New England BioLabs. Phospholipid standards were from Avanti Polar Lipids. PROTRAN nitrocellulose membranes were from Schleicher & Schuell. Thin layer PEI cellulose plates were from EM Science; $[\alpha\text{-}^{32}\text{P}]\text{TTP}$ (3000 Ci/mmol) and $^{32}\text{PO}_4$ (8500–9120 Ci/mmol) were from Dupont-NEN.

Bacterial strains and plasmids

Strain HDL1001 is *pgsA30::kan* ϕ [*lacOP*-*pgsA*⁺]*lacZ'* *lacY*::Tn9 *recA* *srl*::Tn10. Strain MC1061 is F⁺*araD139* Δ (*ara-leu*)7696 *galE15* *galK16* Δ (*lac*)X74 *rpsL*(*Str*)*hsdR2*(*r_K*[−] *m_K*⁺)*mcrA* *mcrB1*. Strain MG1655 is λ [−], *rph*-1. The wild-type DnaA expression plasmid pZL606 was constructed by digesting pZL411 (Li and Crooke, 1999) with *Nde*I and *Hind*III and ligating the 1.8 kb fragment that contains the wild-type *dnaA* gene into pBAD24c (Guzman *et al.*, 1995) that had been digested with the same restriction enzymes.

Site-directed mutagenesis of *dnaA*

Site-directed mutagenesis was carried out with the MutaGene mutagenesis kit (Bio-Rad) based on the method of Kunkel *et al.* (1987). Briefly, uracil-containing single-stranded phagemid pZL606 DNA, which contains the coding region of wild-type *dnaA*, was

hybridized with oligonucleotide primers that introduced point mutations or small deletions in the membrane-binding region of DnaA protein. The complementary DNA strand was synthesized *in vitro*, and the resulting double-stranded DNA was introduced into DH5 α cells cultured in the presence of glucose. Double-stranded DNA plasmids that contained the mutated *dnaA* genes were prepared from the transformants and mutations were confirmed by DNA sequencing.

Screening mutant DnaA proteins for the suppression of arrested growth of acidic phospholipid-deficient cells

HDL1001 cells were transformed with the pZL606-based plasmids carrying the mutant *dnaA* genes and plated onto three different ampicillin-containing media: LB + IPTG (1 mM) and glucose (0.2%); LB + arabinose (0.2%); and LB. Plates were incubated (37°C) for 24 h (with IPTG) or 48 h (without IPTG) and colonies were counted. Suppression indices were determined by inoculating liquid LB that contained ampicillin (100 $\mu\text{g/ml}$), IPTG (1 mM) and glucose (0.2%) with a single colony of transformed HDL1001 grown on LB + IPTG + ampicillin. The liquid culture was incubated overnight (37°C). The cells were washed twice with LB and plated onto three different ampicillin-containing media: LB + IPTG (1 mM) and glucose (0.2%); LB + arabinose (0.2%); and LB. Colonies were counted after 24 and 48 h for plates that did and did not, respectively, contain IPTG.

oriC-DNA binding assays

DnaA proteins (2 pmol) were incubated (10 min, 25°C) with $[\text{H}^3]\text{pBSoriC}$ plasmids or $[\text{H}^3]\text{pBluescript}$ plasmids (10–400 fmol). DNA bound by DnaA protein was retained on nitrocellulose filters and measured by liquid scintillation counting (Li and Crooke, 1999).

Analysis of cellular phospholipids

Phospholipids were analyzed as previously described (Heacock and Dowhan, 1989). Cells were cultured for multiple generations in the presence of $^{32}\text{PO}_4$ to uniformly radiolabel cellular phospholipids. Phospholipids were extracted by vigorously suspending a sample (1.6 ml) of the culture in a mixture of chloroform:methanol (1:2; 6 ml), and unlabeled carrier phosphatidic acid (50 μg), cardiolipin (50 μg) and *E.coli* phospholipids (0.1 ml of a stationary culture of the wild-type strain MG1655) were added. The mixture was made monophasic by the addition of a solution of 0.5 M NaCl–0.1 N HCl (2 ml) and mixed thoroughly. The sample was made biphasic by the addition of chloroform (2 ml). The phases were mixed and then separated by centrifugation. The organic phase was saved and the aqueous phase was re-extracted once with additional chloroform (2 ml). The pooled chloroform phase was concentrated by evaporation under a stream of $\text{N}_2(\text{g})$, and the phospholipids were separated by thin layer chromatography on silica gel with solvent systems of chloroform:methanol:acetic acid (65:25:10) and chloroform:methanol:acetic acid:water (25:15:4:2). Phospholipids were visualized after exposure to Haines Reagent and radioactive phospholipids were visualized and quantitated with a Storm 840 Imager (Molecular Dynamics).

Marker frequency analysis

Samples (5 ml) of cells from exponentially growing cultures ($\text{OD}_{600} = 0.5$) were harvested and total cellular DNA was prepared using Wizard Genomic DNA Purification (Promega). DNA (~ 2 μg in 5–15 μl) was digested (3 h) with *Nco*I and *Pst*I, and the fragments were separated by electrophoresis (80 V, 0.8% agarose). The gel was treated (40 min) with 0.5 M NaOH and 1.5 M NaCl, washed twice with water, and soaked (45 min) in 0.5 M Tris–HCl pH 7.5 and 3 M NaCl. DNA was transferred from the gel to a nitrocellulose membrane by capillary action and hybridized with a mixture of radiolabeled probes (1.4 kb) complementary to segments of *dnaA* and *xasA*. The probes were generated by PCR using the following primers: *dnaA*, 5'-CCGGAATTCATGTCACTTTCGCTTTGGCAG-3' and 5'-CCGGATCCTTACGATGACAATGTTCTGAT-3'; for *xasA*, 5'-TGTTTCTGTGCATTCATCAC-3' and 5'-GGCTGTTTATGAATACCCTA-3'. The PCR products were labeled with $[\alpha\text{-}^{32}\text{P}]\text{TTP}$ using hexanucleotide random-primed DNA labeling (Boehringer Mannheim Biochemicals). Band intensity on the blot was quantitated using a Storm 840 Imager (Molecular Dynamics). Each gel included samples of DNA prepared from cells that had been treated (3 h) with rifampicin so that all ongoing rounds of replication were completed. These samples served as an internal standard for normalization of the labeling and hybridization of *dnaA* and *xasA* probes.

Treatment with rifampicin and cephalixin for flow cytometry analysis

Rifampicin (150 µg/ml) (Fluka) and cephalixin (10 µg/ml) (Eli Lilly) were added to exponentially growing cells ($OD_{450} = 0.15$) and incubation continued for three to four doublings to complete ongoing rounds of replication. The cells were fixed and stained as described below.

Rifampicin inhibits transcription, which results in inhibition of initiation of DNA replication, whereas cephalixin inhibits cell division (Skarstad *et al.*, 1986; Boye and Løbner-Olesen, 1991). Thus, after treatment with these drugs cells end up with an integral number of chromosomes (Skarstad *et al.*, 1986) that represents the number of origins present in each cell at the time of drug action. In a culture of cells with synchronous initiation the integral number of chromosomes is 2, 4 or 8. With increasing asynchrony, an increasing number of cells with 3, 5, 6 or 7 chromosomes appear.

Fixation and staining

Exponentially growing cells ($OD_{450} = 0.15$), or cells treated with rifampicin and cephalixin, were washed and suspended in TE buffer and then diluted 10-fold in ethanol (77%) for fixation. The cells were washed in 0.1 M phosphate buffer pH 9.0 and stained overnight (4°C) in 1.5 µg/ml fluorescein isothiocyanate (FITC) in the same buffer (made from a freshly made 3 mg/ml stock solution) (Wold *et al.*, 1994). This dye binds covalently to cellular proteins. The cells were washed twice in 0.02 M phosphate-buffered saline pH 9.0 to remove unbound dye, and were resuspended in the same buffer. The DNA was stained by the addition of an equal volume of Hoechst 33258 (3 µg/ml in the same buffer; final concentration of 1.5 µg/ml).

Staining of the flow cytometry DNA standard

An ethanol-fixed sample of slowly growing wild-type cells (the majority of which contained either one or two chromosomes) was used as an internal standard in all samples. The standard, which was not stained with FITC, was included with every sample during incubation with the Hoechst 33258 stain (see above).

Flow cytometry

Flow cytometry was performed with a FACStar+ (Becton Dickinson) equipped with an Argon ion laser emitting 0.5 W at 488 nm (Spectra Physics) and a Krypton laser emitting 0.5 W in multi-line UV-mode (351 and 357 nm; Spectra Physics). The fluorescence of FITC was filtered through a 510–545 nm interference filter. A 488 nm bandpass filter was employed for collection of the 90° light scatter. A combination of a longpass 400 nm and a shortpass 480 nm interference filter was used for collecting Hoechst 33258 fluorescence. 90° light scatter was used as the threshold parameter for calibration of the DNA content axis (Hoechst 33258 fluorescence). The standard (see above) in each sample was selectively measured for Hoechst 33258 fluorescence by employing a software gate to include only the FITC-negative cells (Torheim *et al.*, 2000).

Analysis of flow cytometry histograms

The average cell mass was determined as average FITC fluorescence per cell. The average DNA content per cell was determined as average Hoechst fluorescence per cell. The average DNA per mass was found by dividing average DNA content by average cell mass.

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