Restoration of Growth to Acidic Phospholipid-deficient Cells by DnaA(L366K) Is Independent of Its Capacity for Nucleotide Binding and Exchange and Requires DnaA*

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In the absence of adequate levels of cellular acidic phospholipids, Escherichia coli remain viable but are arrested for growth. Expression of a DnaA protein that contains a single amino acid substitution in the membrane-binding domain, DnaA(L366K), in concert with expression of wild-type DnaA protein, restores growth. DnaA protein has high affinity for ATP and ADP, and in vitro lipid bilayers that are fluid and contain acidic phospholipids reactivate inert ADP-DnaA by promoting an exchange of ATP for ADP. Here, nucleotide and lipid interactions and replication activity of purified DnaA(L366K) were examined to gain insight into the mechanism of how it restores growth to cells lacking acidic phospholipids. DnaA(L366K) behaved like wild-type DnaA with respect to nucleotide binding affinities and hydrolysis properties, specificity of acidic phospholipids for nucleotide release, and origin binding. Yet, DnaA(L366K) was feeble at initiating replication from oriC unless augmented with a limited quantity of wild-type DnaA, reflecting the in vivo requirement that both wild-type and a mutant form of DnaA must be expressed and act together to restore growth to acidic phospholipid-deficient cells.

The cycle of chromosomal DNA replication in Escherichia coli is largely controlled at the initiation stage. A cluster of multiple DnaA protein molecules binds to DnaA recognition sites in the unique origin (oriC) (1–4). Aided by architectural proteins HU and integration host factor (IHF), DnaA protein promotes strand opening of the AT-rich 13-mer repeats of oriC and mediates the delivery of DnaB helicase from DnaB-DnaC complexes onto the exposed single strands. The complementary strands are subsequently synthesized by replisomes that have been assembled at the two DNA forks (5). The transition of the origin from duplex to melted DNA is influenced by the high affinity DnaA protein has for ATP and ADP; although both forms of DnaA protein are capable of producing very similar nucleoprotein complexes at oriC, only ATP-DnaA is active for promoting strand opening and subsequent replication steps (6).

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Regulatory inactivation of DnaA (RIDA),1 one of the mechanisms to ensure that a round of chromosomal replication happens only once per cell cycle, stimulates the hydrolysis of DnaA-bound ATP, generating ADP-DnaA that is feeble at initiating replication (7–9). RIDA activity is dependent on the β-subunit of DNA polymerase III loaded as a sliding clamp onto template DNA and Hda, a protein important for controlled replication of the E. coli chromosome and plasmid RK2 (10–12).

Acidic phospholipids in a fluid bilayer promote the release of bound nucleotide from DnaA in vitro (13, 14). When ADP-DnaA is stabilized by being bound to oriC, acidic phospholipids facilitate an exchange of ATP for ADP and thus can restore initiation activity to DnaA protein (15). Analysis of functional DnaA proteolytic fragments identified a domain of DnaA protein that is necessary for membrane reactivation of the initiation activity of the protein (16). Cross-linking studies that employed a radiolabeled, photoactivatable phospholipid analog revealed that this essential domain of DnaA protein inserts into the hydrophobic portion of the lipid bilayer during the nucleotide release process (17). Analysis of the amino acid sequence of the DnaA protein suggests that this membrane-interacting segment of DnaA protein forms an amphiphatic helix (16), a structural motif that can act as a membrane surface-seeking domain for peripherally associated membrane proteins. Homology modeling of the E. coli DnaA sequence onto the recently solved crystal structure of a truncated version of Aquifex aeolicus DnaA supports this region of E. coli DnaA being such a structure (18).

In general, DnaA protein is composed of an amino-terminal domain I involved in helicase recruitment (19, 20) and with specific residues needed for oligomerization at oriC (21), a variable domain II, a core domain III that contains the nucleotide binding site, and a carboxyl-terminal DNA binding domain (22, 23). Domain III and the helix-turn-helix motif of domain IV are connected by the membrane-binding long amphiphatic helix mentioned above.

In vivo, a close link between DnaA protein function and the state of the cellular membrane is likely. When the synthesis of acidic phospholipids is impeded via repressed transcription of the gene for phosphatidylglycerophosphate synthase, cells become arrested for growth after a few generations but remain viable (24). The growth arrest can be relieved by expression of certain plasmid-borne mutant forms of DnaA protein, including DnaA(L366K), with initiation of replication occurring at or near oriC (25). In support of the idea that the arrested growth in acidic phospholipid-deficient cells is associated with im-

1 The abbreviations used are: RIDA, regulatory inactivation of DnaA; PIPES, 1,4-piperazinediethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
paired chromosomal replication from oriC, the growth arrest can also be alleviated if the cells are permitted to undergo constitutive stable DNA replication, a process that allows DNA-independent initiation of replication at sites other than oriC (26).

The mechanism of how DnaA(L366K) restores growth to acidic-phospholipid deficient cells has been speculated (25) to be related to its altered nucleotide state or reactivation by membranes. Possibilities include: (i) the ability of ADP-DnaA(L366K) to be rejuvenated by neutral, waxyteric membranes, (ii) DnaA(L366K) never being in its ADP form because it fails to bind ATP in the first place, thus negating the need for acidic membrane-mediated reactivation, (iii) DnaA(L366K) binds ATP but fails to hydrolyze it to produce ADP-DnaA or is inert to RIDA, therefore bypassing the necessity to be reactivated by acidic phospholipids, and (iv) ADP-DnaA(L366K) is active at initiating chromosomal replication. A biochemical study of these possibilities is presented here. The membrane reactivation characteristics of another mutant form of DnaA protein that possesses other amino acid substitutions in the membrane-binding region are also presented.

EXPERIMENTAL PROCEDURES

Materials—Unless otherwise stated, reagent grade chemicals were from J. T. Baker, Sigma Chemicals, Fisher Scientific, or Roche Applied Science. Growth media components were from BD Biosciences. Phospholipids 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-rac-(1-choline), 1-stearoyl-2-oleoyl-sn-glycero-3-phospho-rac-1-glycerol, E. coli phosphatidylethanolamine, and bovine heart cardiolipin were from Avanti Polar Lipids. [1-32P]ATP or [2,8-3H]ADP in 25 mM HEPES (pH 7.6 at 1 M); 10 mM magnesium acetate; 40 mM phosphocreatine; 2.5 mM magnesium acetate, 7 mM dithiothreitol. Nucleotides ADP standards were visualized by UV absorption. Internal ATP concentrations were measured by liquid scintillation counting.

DNA Replication in a Crude Cell-free Extract—In vitro replication of oriC-plasmid M13mpRE85 in a crude enzyme fraction (Fraction II) was performed basically as described (29, 30). Briefly, a crude protein fraction prepared was washed with 5 ml of ice-cold buffer DB, dried, and retained DNA was measured by liquid scintillation counting.

DNA Replication with Defined Components—In vitro replication of pBSoriC, dependent on eight purified replication proteins, was largely performed as described (30). Reactions (25 ml) contained: 30 mM Tricine-KOH (pH 8.25); 7 mM magnesium acetate; 2 mM ATP; 0.5 mM each GTP, CTP, and UTP; 0.1 mM each dATP, dGTP, dCTP, and dTTP; (250 ml of the crude protein extract prepared from WM433; and 200 ng (600 pmol as nucleotide) of M13mpRE85 replicative form I DNA. Histidine-tagged wild-type or mutant DnaA protein was added to the reaction, and the preparation was incubated at 30 °C for 20 min. Nucleotides incorporated into acid-insoluble materials were retained on GF/C filters (Millipore) and measured by liquid scintillation counting.

RESULTS

Nucleotide Binding by DnaA(L366K) and Wild-type DnaA Is Similar—DnaA(L366K) possesses high affinities for ATP and ADP with dissociation constants of 135 and 58 nM, respectively.
retains the intrinsic ATPase activity of wild-type DnaA. Bound ATP is slowly hydrolyzed with half being consumed in \( \sim 45 \) min (Fig. 2), kinetics comparable with what has previously been seen for purified DnaA under similar conditions (6, 27). And as with the wild-type protein, the resulting ADP from ATP hydrolysis remains bound to DnaA(L366K) (Fig. 2). Therefore, the point mutation in DnaA(L366K) does not affect the protein conversion from the active ATP form to inactive ADP-DnaA.

DnaA(L366K) Is Sensitive to RIDA—DnaA(L366K) treated with crude cell extracts showed diminished replication activity (data not shown), indicating that it is sensitive to RIDA. Furthermore, in vivo experiments have shown that deletion of the hda gene (12), an essential component of RIDA (10), failed to alleviate the growth arrest in cells lacking adequate levels of acidic phospholipids (data not shown). Both of these findings support the conclusion that the growth rescuing phenomenon of DnaA(L366K) is not caused by its resistance to Hda-mediated inactivation.

DnaA(L366K) Is Feeble at Initiating Replication from oriC in Vitro—DnaA(L366K), although indistinguishable from the wild-type protein in its nucleotide binding and hydrolysis and requirement for acidic phospholipids to promote nucleotide release, is poor at directing replication from oriC in a system reconstituted with purified proteins (Fig. 3A). Wild-type DnaA produced maximal DNA synthesis when levels of protein that represent DnaA to oriC ratios of \( \sim 10-12 \) DnaA molecules per oriC were used, similar to previous data. However, when DnaA(L366K) was used, even at a level of 18 mutant protein molecules per oriC, only a very low amount of DNA synthesis resulted (Fig. 3A). Moreover, DnaA(L366K) was poor at initiating replication in a crude cell extract that lacked DnaA activity because it was prepared from a dnaA temperature-sensitive strain. Again, maximal synthesis was seen when the reactions contained \( \sim 10 \) wild-type DnaA molecules per oriC. In contrast, DnaA(L366K) was completely inactive despite being present at up to 20 protein molecules per oriC (Fig. 3B).

oriC Binding Activity of DnaA(L366K) Is Comparable with That of DnaA—The inability of DnaA(L366K) to initiate replication is not due to its failure to bind oriC. The wild-type and the mutant forms of DnaA were equally effective at binding to supercoiled oriC plasmids as determined by a filter-retention assay (Fig. 4). In agreement with the in vitro DNA replication results, DNA binding reached a plateau of about 130 fmol of template bound, corresponding to \( \sim 15 \) DnaA or DnaA(L366K) molecules per origin.

**ATP-DnaA(L366K) Augments Limiting Levels of Wild-type DnaA in Initiating Replication from oriC in Vitro**—The mutant form of the protein, although feeble at initiating replication, is able to participate in efficient replication when coupled with minimal levels of wild-type DnaA. Only 53 pmol of DNA synthesis occurred when ATP-DnaA was added at the level of
Chromosome Replication Properties of DnaA(L366K)

2 DnaA molecules per oriC (Fig. 5A). When that limiting level of DnaA was combined with either additional wild-type DnaA or DnaA(L366K) protein, robust DNA synthesis ensued with only slightly more DnaA(L366K) needed to obtain the same level of synthesis possible with wild-type DnaA (Fig. 5A).

Like wild-type DnaA, DnaA(L366K) needs to be in its ATP form to actively initiate replication from oriC. As expected, the limiting amount of 2 ADP-DnaA molecules per oriC completely failed to initiate replication, being indistinguishable from reactions lacking any DnaA protein (Fig. 5B). However, when supplemented with ATP-DnaA(L366K), over 500 pmol of DNA synthesis was obtained with a total of 13 DnaA molecules per oriC (Fig. 5B). On the other hand, supplementing the ADP-DnaA with ADP-DnaA(L366K) resulted in poor DNA synthesis. Thus, the nucleotide requirements for DnaA(L366K) to initiate chromosomal replications are similar to those previously seen for the wild-type protein (6, 15).

DnaA(L366K) Must Act in Conjunction with DnaA in Vivo—Replacement of the chromosomal copy of dnaA with the gene for DnaA(L366K) by homologous recombination (32) occurred efficiently when cells carried a low copy plasmid expressing dnaA under its own promoter. Specifically, following the second cross-over event, 18 of 20 candidates screened had replaced their chromosomal dnaA with the gene encoding the mutant protein. When cells arising from successful allelic replacement were cured of the expression plasmid harboring wild-type dnaA, 10 of 10 candidates examined had reverted to the wild-type gene at the dnaA locus. Thus, cells cannot tolerate dnaA(L366K) as the sole allele for dnaA. In agreement, in the absence of a plasmid-encoded dnaA, allelic replacement completely failed, with 0 of 100 candidates having exchanged wild-type dnaA for the mutant allele following the second cross-over event. Similar results were seen when cells were depleted of their acidic phospholipids prior to selecting for the second crossover event.

Another DnaA with Membrane-binding Domain Mutations Behaves Like DnaA(L366K)—Mizushima and co-workers (33) have also constructed DnaA proteins that contain alterations in the membrane-binding domain. Specifically, basic lysine and arginine residues, singularly or in tandem, were substituted with the acidic amino acid glutamate. The purified mutant proteins retained their capacity to bind adenine nucleotide, and it was reported that DnaA proteins that had the lysine at position 372 substituted with glutamate (K372E) had ~4-fold decreased rates for the release of bound nucleotide when compared with wild-type DnaA (33).

These kinetic measurements were performed with a low molar ratio of cardiolipin to protein. Working with DnaA(R360E, R364E, K372E) (termed DnaA431 in Ref. 33) that we purified, as well as some kindly provided by T. Mizushima, we obtained a similar 3- to 4-fold decreased rate in nucleotide release from DnaA431 compared with wild-type DnaA when examined at the same low cardiolipin to DnaA ratio (Fig. 6A, squares). However, when cardiolipin levels were increased just 2-fold, the difference seen between DnaA431 and wild-type decreased (Fig. 6A, triangles), and with the concentration of cardiolipin increased 4-fold, the rate of release of nucleotide from DnaA431 approached that for wild-type DnaA (Fig. 6A, circles). The similar behavior of DnaA and DnaA431 when exposed to acidic phospholipids is further illustrated by their comparable response to treatment with cardiolipin vesicles over a 10-fold concentration range (Fig. 6B).

We further examined DnaA431 using vesicles composed of lipids other than 100% cardiolipin. When treated with acidic liposomes composed of 80% phosphatidylglycerol and 20% phosphatidylcholine nucleotide release from DnaA431 and wild-type DnaA were indistinguishable, and DnaA431, like wild-type DnaA, was inert to 100% phosphatidylcholine vesicles (Fig. 6C). Moreover, DnaA431 and wild-type DnaA bound equally well to the acidic and not neutral liposomes (100% phosphatidylcholine) as analyzed by isopynic centrifugation as previously described (34) (data not shown). Furthermore, DnaA431 protein responded like wild-type DnaA when treated with vesicles composed of phospholipids extracted from E. coli cells (Fig. 6D). Thus, DnaA431 and wild-type DnaA behave in a largely similar manner when treated with acidic phospholipid-containing membranes other than the specific case of low levels of liposomes composed solely of cardiolipin.

Like DnaA(L366K) (25), DnaA431 restored growth to acidic phospholipid-deficient cells but cannot be the sole source of cellular DnaA (data not shown). The biochemical properties of two different DnaA proteins that possess mutations in the membrane-binding region are similar, and both proteins permit growth in cells lacking acidic phospholipids, perhaps through a common mechanism.
A genetic and mutational analysis provided the most direct evidence that there is an in vivo link between DnaA protein function and the lipid composition of the cellular membrane (25). Specifically, cells that have depleted levels of acidic phospholipids, which normally would be arrested for growth, are able to keep growing when DnaA(L366K) is expressed. That a single amino acid substitution in the initiator of chromosomal replication, DnaA protein, can restore growth to cells that have stopped growing because of an altered membrane lipid composition argues for a physiologically relevant relationship between DnaA and the cell membrane.

Previous biochemical studies suggest anionic lipids participate in the regulation of DnaA protein. Fluid bilayers possessing adequate levels of acidic phospholipids promote the release of ADP from DnaA, and when the protein is bound to DNA an exchange of ATP for ADP can occur, thereby rejuvenating inactive ADP-DnaA into active ATP-DnaA (13–15). Recently, it was observed that basic and neutral phospholipids block acidic phospholipids from promoting nucleotide release from Staphylococcus aureus DnaA (35).

In view of these biochemical findings, purified DnaA(L366K) was examined here to determine whether the molecular mechanism of how the mutant protein supports growth of cells lacking acidic phospholipids is based on the protein being altered in its adenine nucleotide binding and hydrolysis properties or its response to treatment with fluid lipid bilayers. For each of these aspects studied, DnaA(L366K) was largely similar to wild-type DnaA, thereby suggesting that the function of wild-type DnaA adversely affected in acidic phospholipid-deficient cells is something other than nucleotide binding, hydrolysis, and exchange.

Although DnaA(L366K) has origin binding activity, the mutant protein is unable to initiate replication from oriC in vitro (Fig. 3) or serve as the sole source of DnaA activity in vivo (25). Thus, based upon known initiation functions of DnaA protein, DnaA(L366K) acting on its own may not possess the ability to properly oligomerize at oriC and promote strand opening of the AT-rich 13-mers or to successfully recruit the replicative helicase, DnaB, to the opened origin DNA.

Analogously, wild-type DnaA is no longer able to carry out all of its necessary chromosomal replication functions in cells lack-
ing sufficient levels of acidic phospholipids (25). The absence of acidic phospholipids does not appear to cause growth arrest simply by depleting the concentration of available DnaA because high level expression of additional wild-type DnaA failed to restore growth (25). Instead, an essential DnaA function is most likely perturbed in acidic phospholipid-deficient cells, and growth only occurs when DnaA(L366K) provides this missing function. In such a case, the function of wild-type DnaA affected in acidic phospholipid-deficient cells must be distinct from the missing function of DnaA(L366K). Because both proteins have similar nucleotide binding, hydrolysis, and exchange characteristics in vitro as shown here, none of these functions seem to be the DnaA function adversely affected by the absence of acidic phospholipids, as originally speculated (25).

The results from the in vitro studies presented here support the argument that DnaA(L366K) acts in conjunction with wild-type DnaA, with DnaA and DnaA(L366K) forming functional mixed oligomers at oriC. Specifically, DnaA(L366K) on its own is unable to support DNA replication (Fig. 3) as is an insufficient concentration of wild-type DnaA (Fig. 5). Yet, origins with the same origin, acting as a mixed oligomer.

The results presented here suggest that the mechanism by which DnaA(L366K) supports growth in cells with low levels of acidic phospholipids is independent of the nucleotide binding and hydrolysis characteristics of the protein as well as how it responds to acidic versus neutral lipids with respect to nucleotide release. Supporting this argument, another DnaA protein with amino acid substitutions in the membrane-binding domain, DnaA431, also rescues acidic phospholipid-deficient cells in vivo and behaves like DnaA(L366K) when exposed to phospholipids in vitro.

Defining what domains of DnaA(L366K) are essential versus dispensable for growth to occur in acidic phospholipid-deficient cells will likely give insight into the essential function of DnaA that is perturbed in acidic phospholipid-deficient cells and may reveal a novel acidic phospholipid-dependent function of DnaA in the chromosomal replication cycle.