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Erratum

This paper was originally published in *The EMBO Journal*, Volume 15, pages 2313–2321 but, due to a publisher's error, ADP–DnaA and ATP–DnaA were wrongly printed as ADP–DnaK and ATP–DnaK, respectively, throughout the paper. The correct version is printed in full below.

Membrane regulation of the chromosomal replication activity of *E.coli* DnaA requires a discrete site on the protein

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The capacity of DnaA protein to initiate DNA synthesis at the chromosomal origin is influenced profoundly by the tightly bound nucleotides ATP and ADP. Acidic phospholipids can catalyze the conversion of inactive ADP–DnaA protein into the active ATP form. Proteolytic fragments of the nucleotide form of DnaA protein were examined to determine regions of the protein critical for functional interaction with membranes. A 35 kDa chymotryptic and 29 kDa tryptic fragment retained the tightly bound nucleotide. The fragments, whose amino-termini are within three residues of each other, but differ at their carboxyl ends, showed strikingly different behavior when treated with acidic phospholipids. The larger chymotryptic fragment released the bound nucleotide in the presence of acidic, but not neutral phospholipids. In contrast, the smaller tryptic fragment was inert to both forms of phospholipids. Acidic membranes, but not those composed of neutral phospholipids, protect from tryptic digestion a small portion of the segment that constitutes the difference between the 29 and 35 kDa fragments. The resulting 30 kDa tryptic fragment, which possesses this protected region, interacts functionally with acidic membranes to release the bound effector nucleotide. Inasmuch as the anionic ganglioside GM_1, a compound structurally dissimilar to acidic glycerophospholipids, efficiently releases the nucleotide from DnaA protein, an acidic surface associated with a hydrophobic environment is the characteristic of the membrane that appears crucial for regulatory interaction with DnaA protein.

Keywords: DnaA/*E.coli*/membranes/*oriC*/replication

Introduction

DnaA protein binds the unique origin, *oriC*, to initiate a cycle of chromosomal replication in *Escherichia coli* (Skarstad and Boye, 1994). Formation of an initial complex, comprised of origin DNA wrapped around a cluster of ~20 DnaA molecules (Funnel *et al.*, 1986; Crooke *et al.*, 1993) is the first discernible stage of initiation (Sekimizu *et al.*, 1988a). In the presence of ATP and architectural proteins HU or IHF, DnaA protein in the initial complex promotes strand opening of the AT-rich 13mer repeats in *oriC* to form an open complex (Bramhill and Kornberg, 1988; Hwang and Kornberg, 1992). Assembly of replisomes at the two forks gives rise to bidirectional replication (Marians, 1992).

The transition from an initial complex to an open complex *in vitro* is influenced profoundly by the high affinity binding of ATP or ADP to DnaA protein [K_d = 0.03 μM and 0.1 μM, respectively (Sekimizu *et al.*, 1987)]. While both forms are capable of forming initial complexes (Yung and Kornberg, 1989; Crooke *et al.*, 1993), only ATP–DnaA is efficient at catalyzing duplex melting; the ADP form is feeble (Sekimizu *et al.*, 1987; Yung *et al.*, 1990). In a DNA-dependent manner, complexed ATP is hydrolyzed slowly to ADP, which remains tightly bound and renders DnaA protein inactive for initiation. Thus, after initiating a round of replication, DnaA protein must be rejuvenated to the ATP form if it is to participate in subsequent initiations.

Acidic phospholipids in a fluid bilayer promote the release of bound ADP and ATP from DnaA protein *in vitro* (Sekimizu and Kornberg, 1988; Yung and Kornberg, 1988; Crooke *et al.*, 1992; Castuma *et al.*, 1993). No distinctions have been observed between the phospholipid-mediated release of bound ADP versus ATP. However, the cellular concentrations of the nucleotides (~250 μM and 3 mM, respectively) are such that, if rebinding of free nucleotide were to occur, formation of ATP–DnaA protein is highly favored. In support, when ADP–DnaA protein is stabilized by *oriC*, acidic phospholipids can facilitate an exchange of ATP for ADP, and thus restore initiation activity to DnaA protein *in vitro* (Sekimizu and Kornberg, 1988; Crooke *et al.*, 1992). The molecular nature of the concerted interaction between ADP–DnaA protein, *oriC* and acidic phospholipids remains unclear.

Normal cellular levels of acidic phospholipids apparently are required to sustain chromosomal replication from *oriC in vivo* (Xia and Dowhan, 1995). A strain of *E.coli* was constructed so that the chromosomal phosphatidylglycerophosphate synthase gene (*pgsA*) is under inducible control (Heacock and Dowhan, 1989). This synthase catalyzes the committed step in the synthesis of the acidic phospholipids. Growth of this strain is absolutely dependent on the presence of the inducer IPTG. Removal

of IPTG from a fully induced culture causes growth arrest with a concomitant 10-fold reduction in the combined levels of acidic phospholipids (Heacock and Dowhan, 1989). These cells remain viable; re-addition of IPTG, even after several hours, results in acidic phospholipid synthesis and consequent cell growth. To determine if the coincident growth arrest seen with reduced levels of acidic phospholipids is due to impaired activation of DnaA protein, conditions that bypass the requirement for DnaA protein in initiating replication were employed. 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 in cells deficient for acidic phospholipids if the primary site of growth limitation is the inability to activate DnaA protein through phospholipid rejuvenation. In agreement, a *rnhA*$^-$, *recA*$^+$ derivative of this strain continues to grow (instead of arresting growth) in the absence of IPTG due to constitutive DNA replication, and indicates the dependency of normal initiations on acidic phospholipids (Xia and Dowhan, 1995).

DnaA protein has been divided into four domains (Figure 3; Fujita *et al.*, 1992). However, this definition is based on sequence conservation of the protein among various species of Enterobacteriaceae. Recently, the localization of a functional site of DnaA protein, that responsible for binding *oriC* DNA, has been described (Roth and Messer, 1995). The 94 carboxy amino acids of DnaA protein appear to be necessary and sufficient to bind origin DNA. Sequence analysis and examination of isolated mutant forms of DnaA protein suggest that a segment which includes Ala184 is involved in the high affinity binding of ADP and ATP (Hwang and Kaguni, 1988a,b; Hansen *et al.*, 1992; Skarstad and Boye, 1994), although this has not been demonstrated directly. Thus, other than the domain for DNA binding, little is known about the location of sites on DnaA protein responsible for critical initiation functions.

Here we describe studies which identify a domain of DnaA protein necessary for membrane reactivation of the protein's initiation activity. A discrete region of DnaA protein appears essential for the acidic phospholipid-mediated exchange of the regulatory molecules ATP and ADP. Features of the membranes necessary for interaction with DnaA protein are also presented.

Results

Bound ATP stabilizes proteolytic fragments during digestion of DnaA protein

Proteolysis conditions were sought such that full-length DnaA protein would be consumed completely, commensurate with the generation of stable intermediate size fragments containing an active, high affinity ATP binding site. Nevertheless, limited tryptic and chymotryptic digestions of nucleotide-free DnaA protein yielded only unstable, transient fragments (data not shown). Treatment of the ATP form of DnaA protein with each protease, however, produced a prominent fragment (Figure 1).

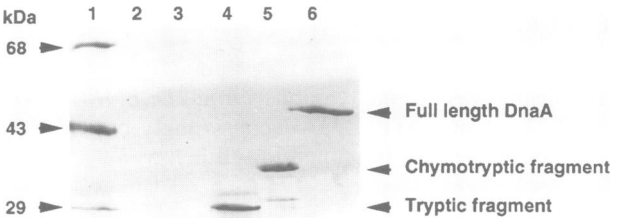

Fig. 1. Proteolysis of ATP–DnaA protein produces stable fragments. ATP–DnaA protein (28 µl buffer A) was subjected to limited proteolysis (30 min, 16°C) by chymotrypsin (10 µg DnaA, 6 mU) or trypsin (20 µg DnaA, 0.75 U). Proteolysis was quenched by addition of aprotinin (10-fold weight excess over protease) and fragments were separated by SDS–PAGE (15%) and stained with Coomassie brilliant blue. Lane 1, molecular weight markers; lane 2, 6.5 µg (0.75 U) trypsin; lane 3, 0.8 µg (6 mU) chymotrypsin; lane 4, tryptic fragment; lane 5, chymotryptic fragment; lane 6, 3 µg DnaA protein.

Digestion with trypsin generated a polypeptide with an apparent mol. wt of 29 kDa (lane 4), while a larger fragment (apparent mol. wt of 35 kDa) was formed during proteolysis with chymotrypsin (lane 5). Residual full-length DnaA protein cannot be detected in either digestion mixture (lanes 4 and 5).

The predominant proteolytic fragments retain the tightly bound ATP

The ATP bound to DnaA protein remained associated with proteolytic fragments in the tryptic and chymotryptic digestions as determined by filter retention assays. The nucleotide binding characteristics of the proteolytic fragments are similar to those for full-length DnaA protein. These include the ATP remaining bound for comparable periods of time over a variety of temperatures (0–38°C), as well as through several cycles of freeze–thawing, even when challenged with excess ATP in solution (data not shown).

The major chymotryptic and tryptic fragments accumulate only when the starting DnaA protein is in its ATP form. Therefore, those fragments were considered likely candidates for the retention of bound ATP. The molar ratios between protein-bound ATP and the isolated 35 kDa chymotryptic and 29 kDa tryptic fragments were measured (Table I). Both the predominant tryptic and chymotryptic fragments have stoichiometries of ~0.5 with protein-bound ATP, values analogous to that seen for full-length DnaA protein (Table I; Sekimizu *et al.*, 1987). No other single proteolytic fragment is present in quantities high enough to account for the levels of protein-bound ATP, although a combination of minor species could give comparable stoichiometries.

To determine independently which fragments retained bound ATP, products of the digestion mixtures were resolved by anion-exchange chromatography. For the chymotrypsin digestion, two peaks of protein-bound ATP were observed; one in the flow-through fractions, another eluting at ~200 mM KCl (Figure 2A). The proteolytic fragment with an apparent mass of 35 kDa was detected in both peaks, proportional to the amount of protein-bound ATP, and was not observed in fractions lacking protein-bound ATP. Similar co-purification of the 29 kDa fragment and protein-bound ATP was observed for the tryptic digestion (data not shown).

Table I. Stoichiometries of ATP bound by the tryptic and chymotryptic fragments and full-length DnaA protein

DnaA	Fragment (pmol)	ATP (pmol)	ATP/fragment
Full-length	4.65	2.76	0.59 ± 0.025
Chymotryptic	3.09	1.67	0.54 ± 0.024
Tryptic	3.40	1.84	0.54 ± 0.010

Radiolabeled ATP–DnaA protein (9 μg; 45 μl buffer A) was digested (30 min, 16°C) with trypsin (1.1 U) or chymotrypsin (5.4 mU). Digestions were stopped by addition of a 10-fold weight excess of aprotinin. A portion of the reactions (1 μl, in duplicate) was spotted on a nitrocellulose filter to measure protein-bound ATP (see Materials and methods) and compared with the ATP binding of full-length DnaA protein. Peptides in samples of the reactions (38 μl chymotryptic, 40 μl tryptic, 10 μl full-length DnaA) were resolved by SDS–PAGE (15%) and transferred to PVDF membranes (see Materials and methods). Bands (visualized by staining with Coomassie brilliant blue) which corresponded to full-length DnaA and the 35 and 29 kDa frament were excised from the membrane and analyzed for amino acid content. Values are normalized for 1 μg of full-length DnaA protein, based on Bradford assay.

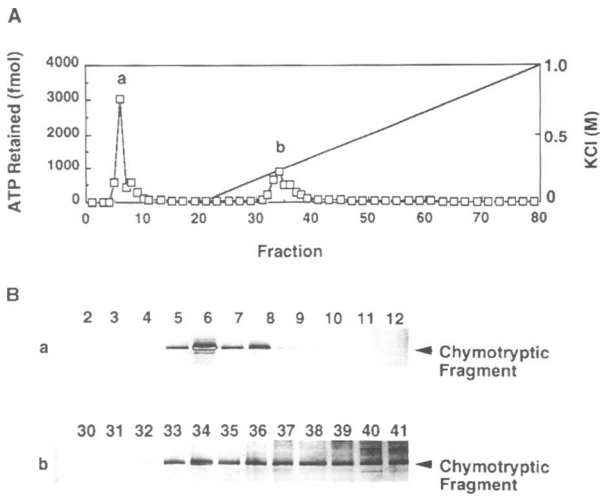

Fig. 2. The prominent chymotryptic fragment co-purifies with protein-bound ATP. [^{32}P]ATP–DnaA protein [60 μg, 200 μl buffer Q (buffer A, pH 8.4)] was digested (30 min, 16°C) with chymotrypsin (36 mU). Proteolysis was quenched by addition of aprotinin (10-fold weight excess). The digestion reaction was loaded (0.05 ml/min) onto a 1 ml Mono-Q column equilibrated in buffer Q (4°C). The column was washed with 3 ml of buffer Q and bound proteins were eluted with a 0–1 M KCl gradient (15 ml; 0.2 ml/min; 0.25 ml/fraction). (**A**) Samples (25 μl) of column fractions were assayed by filter retention for protein-bound ATP. Fractions 1–20, which include peak 'a', are flow-through fractions. Peak 'b' is included in the elution gradient fractions, 21–80. (**B**) Peptides in samples (100 μl) from peaks 'a' and 'b' were resolved by SDS–PAGE and visualized by silver stain. Arrows indicate migration of the prominent chymotryptic DnaA fragment (Figure 1).

A third means to identify fragments having bound nucleotide was provided by cross-linking of a photoactivatible radiolabeled ATP analog to full-length DnaA protein prior to proteolysis. Cross-linking reactions were quenched, the protein was subjected to partial proteolytic digestion and the resulting polypeptides separated by SDS–PAGE. Autoradiography revealed only bands with migrations indistinguishable from those for the predominant tryptic and chymotryptic fragments (data not shown).

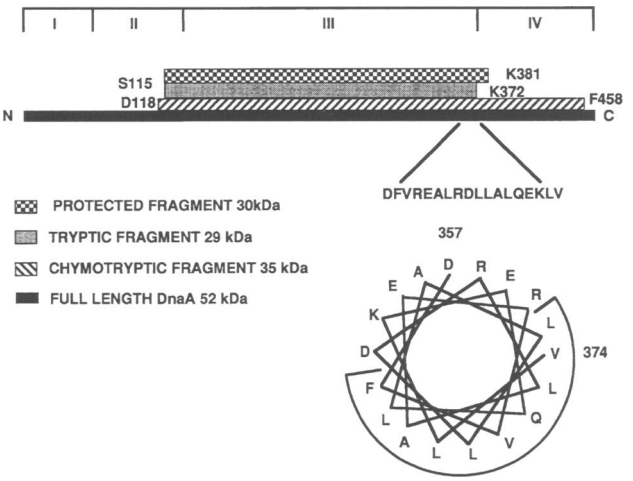

Fig. 3. Identification of the tryptic, chymotryptic and the phosphatidylglycerol (PG)-protected tryptic fragments. ATP–DnaA (6.75 μg, 67.5 μl buffer A) was treated (30 min, 16°C) with trypsin (0.95 U) or chymotrypsin (4.3 mU). Digestions were inhibited by addition of aprotinin (10-fold weight excess). Fragments were separated by SDS–PAGE (15%) and transferred to PVDF membrane (see Materials and methods). Following Coomassie staining, the fragment bands were excised and subjected to Edman degradation (see Materials and methods). The PG-protected tryptic fragment was prepared by incubating ATP–DnaA protein (0.5 mg, 0.75 ml buffer A) with PG vesicles (5 μmol, 10 min, 16°C) before digesting with trypsin (24.2 U, 30 min 16°C). Following inhibition of the protease by addition of aprotinin (100 μg), polypeptides were precipitated by the addition of $CHCl_3$:MeOH (1:3, 114 ml) and collected by centrifugation (27 000 *g*, 60 min, 4°C). Precipitated fragments were dissolved in 1% SDS, 0.1% TFA (500 μl) and purified by reverse phase HPLC (see Materials and methods). The isolated fragment was subjected to Edman degradation to identify the N-terminal sequence. To determine the molecular weight of the tryptic and chymotryptic fragments by mass spectroscopy, the fragments were generated (30 μg DnaA protein, 1 U trypsin, 100 μl buffer A; 30 μg DnaA protein, 37.5 mU chymotrypsin, 100 μl buffer A) and then purified by reverse phase HPLC (see Materials and methods). The isolated fragments were analyzed using a Kratos Kompact MALDI 3. A helical wheel projection of a potential amphipathic helix (G.von Heijne, personal communication; Skarstad and Boye, 1994) involving residues 357–374, and the four consensus sequence domains of DnaA protein (Fujita *et al.*, 1992) are shown.

The tryptic and chymotryptic fragments differ at their carboxy-termini

The chymotryptic and tryptic fragments responsible for bound nucleotide were analyzed for amino-terminal sequence and mass in order to map their locations within DnaA protein. The N-termini of the fragments are in close proximity, at residues Ser115 (tryptic) and Asp118 (chymotryptic); thus, both are missing the N-terminal 25% of full-length DnaA protein. Mass spectroscopy data suggest the tryptic fragment has a mass of 29 220 Da; this value is in agreement with the predicted mass of a fragment (29 111 Da) with a carboxy-terminus at Lys372 of DnaA protein (Figure 3). For comparison, cleavage at the nearest tryptic sites flanking Lys372 would generate fragments of 28 074 Da (Arg364) or 30 136 Da (Lys381).

The mass of the chymotryptic fragment, as estimated by SDS–PAGE, is 35 kDa. A mass of 35 043 Da is predicted for a fragment between Asp118 and Phe458. Mass spectroscopy data also suggest the location of the carboxy-terminus as Phe458; under the non-reducing conditions used to prepare the sample for mass spectroscopy (see Materials and methods), the formation of a

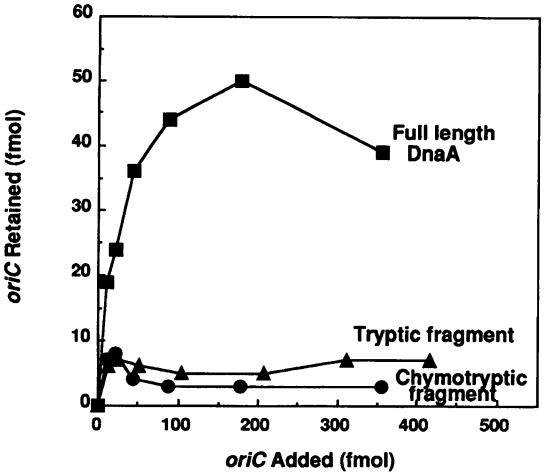

Fig. 4. The chymotryptic and the tryptic fragments of ATP–DnaA are unable to bind *oriC*. [^{3}H]pBS*oriC* was added to mixtures (16 µl) that contained ATP–DnaA protein (2.5 µg) or ATP–DnaA protein (2.5 µg) digested with trypsin (0.29 U, 16°C, 30 min), or chymotrypsin (2 mU, 16°C, 30 min). Samples were incubated with labeled DNA (10 min, 25°C) and then filtered through nitrocellulose. Filters were washed, and retained DNA was measured by liquid scintillation counting.

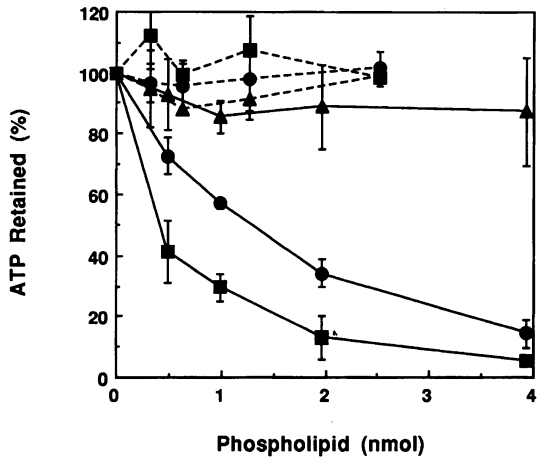

Fig. 5. The chymotryptic fragment retains specificity for acidic phospholipids, the tryptic does not. [^{32}P]ATP–DnaA protein (0.2 µg; 16 µl buffer A) was subjected to limited proteolysis (16°C, 30 min) by chymotrypsin (0.12 mU, ●) or trypsin (24 mU, ▲) to produce the stable fragments. After inhibiting proteolysis with aprotinin (10-fold weight excess), the fragments and undigested [^{32}P]ATP–DnaA protein (■) were treated (10 min, 38°C) with neutral (- - - -) or acidic (——) phospholipid small unilamellar vesicles (see Materials and methods). Samples were filtered through nitrocellulose filters and retained ATP was quantitated by liquid scintillation counting. Values of 100% correspond to the ATP retained by the full-length DnaA protein or the fragments incubated at 38°C in the absence of phospholipid.

disulfide bond between the Asp118–Phe458 fragment (35 043 Da) and the fragment corresponding to Met1–Tyr41 (4778 Da) would give rise to a peptide with a predicted mass of 39 821 Da, a value in agreement with the mass spectroscopy value of 39 835 Da.

The proteolytic fragments fail to bind oriC and initiate replication

ATP–DnaA protein was digested with chymotrypsin and trypsin to produce the 35 and 29 kDa fragments, respectively. The proteolysis reactions were terminated by the addition of a protease inhibitor, and the replicative activities of the fragments in the proteolysis mixtures were assessed. Both the 35 kDa chymotryptic and 29 kDa tryptic fragments are unable to initiate *in vitro* replication of *oriC* plasmids or single-stranded DNA that contains a DnaA binding hairpin (ABC primosome system, Masai *et al.*, 1990). In contrast, mock-digested DnaA protein was virtually identical to untreated DnaA protein, an indication that neither the proteases nor the inhibitor had a deleterious effect on the replication reactions (data not shown). Furthermore, neither fragment was capable of binding *oriC*, in contrast to full-length DnaA protein (Figure 4). Inasmuch as the 94 carboxy amino acids of DnaA protein (domain IV) have been shown to be necessary and sufficient for specific interaction with 9mer DnaA boxes (Roth and Messer, 1995), it is of interest that the chymotryptic fragment fails to bind *oriC* plasmids in filter retention assays. This further indicates that the nine amino acids at the carboxy-terminus of DnaA protein may have been removed from the chymotryptic fragment.

The chymotryptic fragment responds specifically to acidic phospholipids

Incubation of ADP– or ATP–DnaA protein with acidic phospholipids in a fluid bilayer releases the tightly bound nucleotides (Sekimizu and Kornberg, 1988; Yung and Kornberg, 1988; Crooke *et al.*, 1992; Castuma *et al.*, 1993). The 35 kDa chymotryptic and 29 kDa tryptic fragments with bound radiolabeled ATP were examined for their response to treatment with acidic versus neutral phospholipids. The chymotryptic fragment, like full-length DnaA protein, released bound nucleotide when mixed with phosphatidylglycerol, but not with phosphatidylcholine (Figure 5). In contrast, ~ 85% or more of the ATP remained tightly bound to the tryptic fragment when exposed to vesicles composed of either phospholipid (Figure 5).

Acidic phospholipids protect a domain of DnaA protein from tryptic cleavage

ATP–DnaA protein was mixed with phospholipid vesicles before exposure to trypsin to determine if membranes are able to protect portions of the protein from proteolysis. The mixtures were incubated at 16°C to minimize release of bound nucleotide. This temperature, while above the phase transition temperature for the phospholipids employed, does not impart the level of fluidity necessary for nucleotide release (Castuma *et al.*, 1993). Exposure of ATP–DnaA protein to phosphatidylcholine prior to digestion has no effect on the generation of the 29 kDa tryptic fragment (Figure 6, lane 3); the products generated are indistinguishable from those produced in the absence of phospholipids [under the more dilute conditions of this experiment, a doublet is observed (Figure 6, lane 2)]. In sharp contrast, incubation of ATP–DnaA protein with phosphatidylglycerol clearly alters its digestion by trypsin. A fragment with an apparent M_r of 30 kDa becomes the prominent proteolytic product (Figure 6, lane 4), and the 29 kDa fragment generated in reactions lacking acidic phospholipids is not detectable. Peptide sequencing reveals that the 29 and 30 kDa fragments share a common amino-terminus (Ser115 of full-length DnaA protein). Thus, interaction with acidic phospholipids appears to protect a site within DnaA protein (Lys372) normally cleaved by

trypsin. SDS–PAGE data suggest that the carboxy-terminus of the larger tryptic fragment (Figure 6, lane 4) corresponds to residue Lys381 (i.e. fragment mass of 30 136 Da), the next cleavage site following Lys372 of DnaA protein (Figure 3). The next closest trypsin cleavage site after Lys381 is Lys388, which would produce a fragment of 30 991 Da.

The tryptic fragment containing an additional 1 kDa of sequence at the carboxy-terminus responds to acidic phospholipids

Acidic phospholipids are able to protect a region of DnaA protein from proteolytic digestion (Figure 6). This region is absent in the 29 kDa fragment, which is inert to acidic phospholipids, but is present in the 35 kDa fragment, which releases bound nucleotide upon treatment with acidic phospholipids (Figure 3). Thus, the importance of this portion of DnaA protein for functional interaction with membranes was examined.

The region of interest constitutes the difference between the 29 and 30 kDa tryptic fragments (Figure 3). Digestion of ATP–DnaA protein with trypsin produces the 29 kDa fragment which retains bound nucleotide in the presence of acidic phospholipids, even at the elevated temperature of 38°C (Table II, lines A and B; see also Figure 5). Generation of the 30 kDa fragment includes first incubating ATP–DnaA protein with acidic phospholipids at 16°C before addition of trypsin. At this temperature, more than half (56%) of the nucleotide remains bound to full-length protein, as opposed to nearly complete nucleotide release (5% retained) with incubation at 38°C.

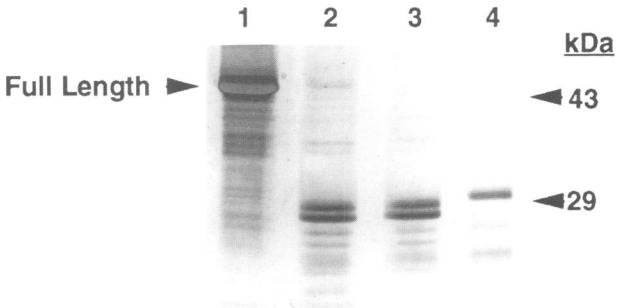

Fig. 6. Acidic phospholipids protect a region of ATP–DnaA from tryptic cleavage. ATP–DnaA protein (3 μg; lane 1) was incubated (256 μl buffer A, 10 min, 16°C) with phosphatidylcholine vesicles (37 nmol; lane 3), phosphatidylglycerol vesicles (38 nmol; lane 4) or buffer (lane 2) before digestion with trypsin (0.36 U, 30 min, 16°C). Digestion was stopped by the addition of aprotinin (7.2 μg). Polypeptides were precipitated by the addition of $CHCl_3$:MeOH (1:3, 800 μl) and collected by centrifugation (14 000 *g*, 20 min, 4°C). Precipitated fragments were dissolved in SDS, separated by SDS–PAGE (15%) and visualized by silver stain.

The subsequent digestion of membrane-associated ATP–DnaA protein by trypsin produces the 30 kDa fragment (Figure 6), which retains its bound nucleotide in the presence of acidic phospholipids at 16°C (Table II, line C). However, when the mixture of the 30 kDa fragment and phosphatidylglycerol is incubated at 38°C, a considerable portion of the bound nucleotide is released (Table II, line D). In contrast, the 29 kDa fragment retains bound ATP in the presence of acidic membranes regardless of the temperature (Table II, lines A and B).

DnaA protein releases bound nucleotide when exposed to acidic gangliosides

A detailed understanding of the membrane binding site of DnaA protein requires an understanding of the pertinent chemical and structural features of the corresponding membranes. The characteristics of the lipids necessary for functional interaction with DnaA protein are understood only to the extent that phospholipid species which possess anionic polar head groups (Sekimizu and Kornberg, 1988; Castuma *et al.*, 1993) and reside in a fluid bilayer (Yung and Kornberg, 1988; Crooke *et al.*, 1992; Castuma *et al.*, 1993) are capable of releasing bound nucleotide from DnaA protein. To define relevant features of the lipids better, ATP–DnaA protein was treated with mixed gangliosides. DnaA protein responds to them in a manner comparable with that when it is incubated with phosphatidylglycerol (Figure 7A). Inasmuch as mixed gangliosides are active at releasing bound nucleotide, the action of defined ganglioside species was examined. While the acidic ganglioside GM_1 is potent at dissociating nucleotide from DnaA protein, a derivative which differs from GM_1 only in that it lacks the sialic acid moiety, is inert (Figure 7B).

The 35 kDa chymotryptic and 29 kDa tryptic fragments of DnaA protein react to gangliosides with the same specificity as that seen for phospholipids. The 35 kDa fragment releases bound nucleotide when treated with acidic GM_1, but not with neutral asialo-GM_1. Neither the acidic nor neutral gangliosides are able to release ATP from the 29 kDa tryptic fragment (Figure 8).

Table II. The 30 kDa fragment responds to acidic lipid treatment

	1st treatment (16°C)	2nd treatment (16°C)	Incubation (10 min)	ATP bound (fmol)	ATP retained (%)
A	trypsin, 30 min	PG, 10 min	16°C	312	100
B	trypsin, 30 min	PG, 10 min	38°C	378	121
C	PG, 10 min	trypsin, 30 min	16°C	237	76
D	PG, 10 min	trypsin, 30 min	38°C	46	0
E	no DnaA protein			51	–

Reactions A and B: ATP–DnaA protein (4 μg, 312 μl buffer A) was digested with trypsin (0.48 U, 16°C) and then treated with phosphatidylglycerol (48 nmol, 16°C). Reactions C and D: ATP–DnaA protein (4 μg, 312 μl buffer A) was treated with phosphatidylglycerol (48 nmol, 16°C) followed by digestion with trypsin (0.48 U, 16°C). Each mixture was then incubated for 10 min (16°C, reactions A and C; 38°C, reactions B and D). Samples (1/10 reaction volume, in duplicate) were assayed by filter retention (see Materials and methods) to measure the amount of protein-bound ATP remaining. The remainder of the reactions were resolved by SDS–PAGE (15%) to confirm the presence of the 29 and 30 kDa fragments and the absence of full-length DnaA protein.

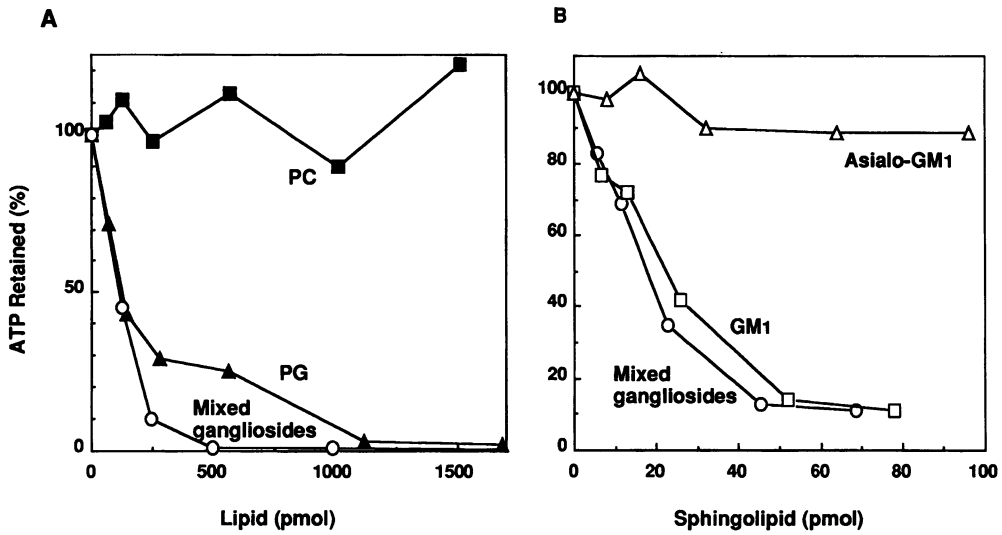

Fig. 7. Acidic sphingolipids promote release of ATP from DnaA protein. (**A**) [^{32}P]ATP–DnaA protein (0.2 μg, 16 μl buffer A) was treated with phosphatidylglycerol, phosphatidylcholine or mixed bovine brain gangliosides (10 min, 38°C). Protein was collected on nitrocellulose filters, washed with buffer G and retained ATP was measured by liquid scintillation counting. (**B**) ATP–DnaA protein (0.2 μg, 16 μl buffer A) was treated with mixed bovine brain gangliosides, GM_1, or asialo GM_1, (10 min, 38°C) and retained ATP was measured by filter retention. Values of retained ATP are a percentage of untreated DnaA protein incubated at 38°C.

Discussion

The activity of DnaA protein probably plays a central role in the timing of primary initiations within the *E.coli* cell cycle (Skarstad and Boye, 1994; Crooke, 1995). Accumulating evidence suggests that this activity may be modulated by a controlled, reversible conversion between active ATP–DnaA protein and the inactive ADP form. A soluble factor that inactivates DnaA protein specifically for initiation *in vitro* at *oriC* is thought to enhance the intrinsic ATPase activity of DnaA protein and, thus, stimulate the shift from ATP–DnaA protein to ADP–DnaA protein (Katayama and Crooke, 1995). A similar negative effect on the initiation activity of DnaA protein has been proposed for SeqA protein (von Freiesleben *et al.*, 1994).

Conversely, acidic phospholipids in a fluid bilayer are capable of reactivating ADP–DnaA protein complexed with the chromosomal origin by promoting the release of the tightly bound ADP (Sekimizu and Kornberg, 1988; Yung and Kornberg, 1988; Crooke *et al.*, 1992; Castuma *et al.*, 1993). The components necessary for such a rejuvenation of inactive DnaA protein appear to be localized at the cellular membrane. Cell fractionation studies suggest that approximately half of the cellular content of DnaA is associated with membranes (Sekimizu *et al.*, 1988a; Hwang *et al.*, 1990). Immuno-gold labeling of thin sections with anti-DnaA protein antibody clearly localizes a vast majority of the cells' DnaA protein in close proximity with their plasma membrane (E.Crooke, unpublished data). Numerous investigations have also demonstrated that *oriC* DNA is found in specific fractions of membrane preparations (Nicolaidis and Holland, 1978; Hendrickson *et al.*, 1982; Kusano *et al.*, 1984; Ogden *et al.*, 1988; Jacq *et al.*, 1989; Landoulsi *et al.*, 1990; Chakraborti *et al.*, 1992; Slater *et al.*, 1995).

Here we have attempted to gain insight into the features of DnaA protein and membranes necessary for the exchange of the tightly bound allosteric effectors ADP and ATP. An appreciation that lipid headgroups can regulate the functions of not only integral membrane proteins, but also those of certain extrinsic proteins, is emerging (Newton, 1993). Extrinsic enzymes regulated by acidic phospholipids include SecA protein (Lill *et al.*, 1990; Hendrick and Wickner, 1991), calcineurin (Politino and King, 1987), phosphatidylinositol-4-phosphate kinase (Moritz *et al.*, 1992), protein kinase C (Nishizuka, 1992; Newton, 1993), sphingosine kinase (Olivera *et al.*, 1996) and glycerol-3-phosphate acyltransferase (Scheideler and Bell, 1989). While the activation of protein kinase C is very specific for phosphatidylserine (Newton and Koshland, 1990; Orr and Newton, 1992a,b; Quest and Bell, 1994), the other enzymes respond to multiple species of acidic phospholipids. An understanding of the nature

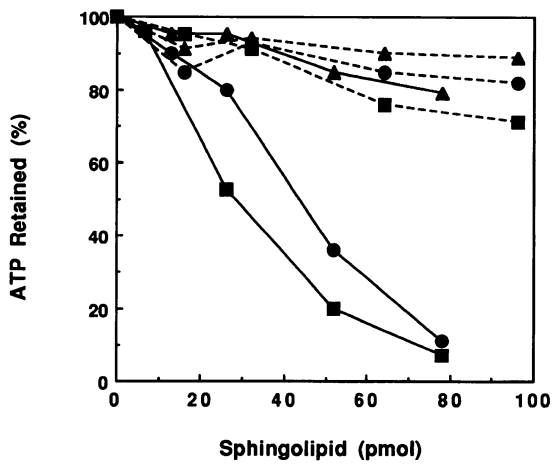

Fig. 8. The proteolytic fragments of ATP–DnaA protein respond specifically to acidic sphingolipids. Full-length [^{32}P]ATP–DnaA protein (■), the chymotryptic fragment (●) and the tryptic fragment (▲) (0.2 μg, 16 μl buffer A) were treated (10 min, 38°C) with gangliosides asialo-GM_1 (– – – –) or GM_1 (——). Bound ATP levels were measured by filter retention. Values are shown as a percentage of ATP retained by the full-length DnaA protein or the fragments incubated at 38°C without lipid.

of the interaction of these proteins with acidic membranes is limiting.

Proteolytic fragments of DnaA protein which retain tightly bound nucleotide were examined for their ability to respond functionally to acidic phospholipids in a manner similar to that for the full-length protein. Two fragments whose amino-termini are within three residues of each other, but differ by their carboxyl ends, show strikingly different behavior when treated with acidic phospholipids (Figure 5). The larger, chymotryptic fragment releases bound nucleotide in the presence of acidic, but not neutral, phospholipids. In contrast, the smaller tryptic fragment is inert to both forms of phospholipids. The portion of DnaA protein which is different between the two proteolytic fragments includes a putative amphipathic helix (Figure 3). Lys372, the likely carboxy-terminus of the tryptic fragment, lies within this sequence and, thus, cleavage of DnaA at this site would most likely disrupt this potential membrane surface-seeking structure.

This region of DnaA protein is protected from digestion by trypsin if the protein is first allowed to associate with vesicles composed of phosphatidylglycerol; no protection is seen with neutral vesicles of phosphatidylcholine. The small, additional protected portion of DnaA protein results in a tryptic fragment (30 kDa) which is responsive to acidic phospholipids (Table II). Thus, a discrete region of ~1–2 kDa is apparently required for DnaA protein to interact functionally with acidic membranes. Cross-linking studies with a radiolabeled photoactivatable phospholipid analog support the hypothesis that this region participates in the association of DnaA protein with membranes. Resolution of fragments generated by chemical cleavage at tryptophans of the DnaA–phospholipid complex with BNPS-skatole [2-(2-nitrophenylsulfenyl)-3-methyl-3′-bromoindolenine] reveal that radiolabel is found solely in a fragment corresponding to residues Gly289–Ser467 of DnaA protein (J.Garner, P.Durrer, J.Brunner and E.Crooke, unpublished data).

In addition to acidic phospholipids, the acidic glycosphingolipid, ganglioside GM_1, is active at releasing bound nucleotide from DnaA protein (Figure 7). The similarity in structure of the headgroup of cardiolipin and the phosphodiester backbone of DNA (Rauch *et al.*, 1984) led to speculation that the DNA binding site of DnaA protein may be responsible for binding of acidic phospholipids (Sekimizu and Kornberg, 1988). However, the lack of specificity for acidic phospholipid species and the structurally dissimilar headgroup of GM_1 argues against this. It is worth noting that while the 35 kDa chymotryptic fragment most probably contains all but the nine carboxyl amino acids of the domain of DnaA protein shown to be necessary and sufficient for *oriC* binding (Roth and Messer, 1995), the fragment was ineffective at retaining supercoiled *oriC* plasmids on nitrocellulose filters (Figure 4). Thus, residues at the extreme carboxyl end of DnaA protein seem critical for origin binding.

ATP–DnaA protein incubated in a mildly acidic buffered solution (pH 6.0) that also contains vesicles composed of phosphatidylcholine retains the tightly bound nucleotide (data not shown). Thus, an overall acidic environment is inadequate for triggering DnaA protein to associate functionally with a membrane bilayer. Instead, an acidic surface with an underlying hydrophobic, fluid phase environment appears to be required for exchange of the regulatory molecules ATP and ADP. DnaA protein may be recruited to membranes through electrostatic interaction with the acidic headgroups, followed by insertion of a portion of the protein into the lipid bilayer, an action which triggers the release of bound nucleotide. A similar mechanism of recruitment and binding has been proposed for other peripheral membrane-associating enzymes (Newton, 1993).

Knowledge of the membrane binding site of DnaA protein will aid in the generation of mutant forms of the protein that lack this function. Examination of these mutant DnaA proteins *in vitro* and *in vivo* will help clarify the importance of the balance of the nucleotide forms of the protein to the cell cycle and the role the cell membrane plays in controlling chromosomal replication.

Materials and methods

Materials

Sources were as follows: HEPES, tricine, CAPS, ATP, *p*-toluene-sulfonyl-L-arginine methyl ester (TAME), benzoyl-L-tyrosine ethyl ester (BTEE), Sigma; [α-^{32}P]ATP, 3000 Ci/mmol, Dupont-NEN; [^{3}H]*S*-adenosyl-methionine, 15 Ci/mmol, Amersham; 1-stearoyl-2-oleolyl-*sn*-glycero-3-[phospho-rac-(1-choline)], 1-stearoyl-2-oleolyl-*sn*-glycero-3-[phospho-rac-(1-glycerol)], Avanti Polar Lipids, Inc.; mixed bovine brain gangliosides, GM_1, asialo-GM_1, Matreya; Mono-Q HR 5/5, Pharmacia; C18 Deltapak (300 Å, 300×3.9 mm), Waters; trypsin (500 U/mg), chymotrypsin (7.5 U/mg), aprotinin, Boehringer Mannheim; type HA nitrocellulose filters, Immobilon P^{SQ} PVDF membrane, Millipore.

Nucleotide binding to DnaA protein

DnaA protein in buffer A [52 mM tricine–KOH, pH 8.25 at 1 M; 2.5 mM magnesium acetate; 0.323 mM EDTA; 8 mM dithiothreitol (DTT); 0.0065% (v/v) Triton X-100; 20% (v/v) glycerol] was mixed with ATP (1 μM; radiolabeled where indicated) and incubated (10 min, 0°C). To quantitate bound nucleotide, samples were filtered through nitrocellulose filters pre-soaked in buffer G [50 mM tricine–KOH, pH 8.25 at 1 M; 0.5 mM magnesium acetate; 0.3 mM EDTA; 5 mM DTT; 0.005% (v/v) Triton X-100; 10 mM ammonium sulfate; 17% (v/v) glycerol]. Filters were washed with 5 ml of buffer G, dried, and retained nucleotide was measured by liquid scintillation counting.

DnaA protein binding of oriC

[^{3}H]pBS*oriC* was prepared by *in vitro* methylation with *Hha*I methylase as described (Yung and Kornberg, 1989). DnaA protein was incubated (10 min, 25°C) with radiolabeled DNA in buffer DB [25 mM HEPES–KOH, pH 7.5 at 1 M; 0.5 mM magnesium acetate; 0.3 mM EDTA; 5 mM DTT; 0.005% (v/v) Triton X-100; 20% (v/v) glycerol]. To quantitate retained DNA, samples were filtered through nitrocellulose filters pre-soaked in buffer DB. Filters were washed with 5 ml of buffer DB, dried, and retained DNA was measured by liquid scintillation counting.

Isolation of proteolytic fragments by reverse phase chromatography

Proteolysis reactions were diluted with 4 volumes of 0.1% trifluoroacetic acid (TFA) and clarified by centrifugation (10 min, 140 000 *g*, 4°C) and loaded onto a Deltapak C18 column (300 Å, 300×3.9 mm; equilibrated in 0.1% TFA, room temperature). A gradient (30 min, 1 ml/min) of 0–70% acetonitrile (in 0.1% TFA), was started immediately following sample injection. Peak fractions were collected, dried *in vacuo* and resuspended in formic acid (88%):ethanol (95%) (7:3; 10 μl). Samples of the resuspended fragments were analyzed by SDS–PAGE and mass spectroscopy (2.5 and 7.5 μl, respectively).

Membrane preparation

Phosphatidylglycerol and phosphatidylcholine in $CHCl_3$ were dried under a stream of nitrogen gas and suspended in water by sonication (15 min, 0°C) with 30% bursts with a microtip probe sonicator (Branson). Sonicated lipids were centrifuged (10 min, 140 000 *g*, 4°C), and the supernatant, which contains small unilamellar vesicles, was collected.

Phospholipids were quantitated by a phospho-polymolybdate colorimetric assay (Ames and Dubin, 1960). Sphingolipids were prepared by suspending the powdered gangliosides in distilled water and storing in liquid nitrogen.

Other methods

Transfer of fragments from SDS–polyacrylamide gel to PVDF membrane was performed as described (Matsudaira, 1987). DnaA protein was purified (Sekimizu *et al.*, 1988b) from BL21(DE3)pLysS/pKA211, a transformed strain in which high level inducible expression of the wild-type *dnaA* gene is under control of a bacteriophage T7 RNA polymerase promoter (T.Katayama, unpublished data). Protein quantitation was performed as described (Bradford, 1976). The proteolytic activity of chymotrypsin was determined by hydrolysis of BTEE as described (Worthington, 1988). Trypsin activity was measured by hydrolysis of TAME as described (Worthington, 1988). Amino acid analysis and N-terminal sequencing was performed by Dr A.Fowler, UCLA Protein Microsequencing Facility, SDS–PAGE and silver staining was as described (Crooke *et al.*, 1988).

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