The interaction of DiaA and DnaA regulates the replication cycle in *E. coli* by directly promoting ATP–DnaA-specific initiation complexes

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Escherichia coli DiaA is a DnaA-binding protein that is required for the timely initiation of chromosomal replication during the cell cycle. In this study, we determined the crystal structure of DiaA at 1.8 Å resolution. DiaA forms a homotetramer consisting of a symmetrical pair of homodimers. Mutational analysis revealed that the DnaA-binding activity and formation of homotetramers are required for the stimulation of initiation by DiaA. DiaA tetramers can bind multiple DnaA molecules simultaneously. DiaA stimulated the assembly of multiple DnaA molecules on *oriC*, conformational changes in ATP–DnaA-specific initiation complexes, and unwinding of *oriC* duplex DNA. The mutant DiaA proteins are defective in these stimulations. DiaA associated also with ADP–DnaA, and stimulated the assembly of inactive ADP–DnaA–*oriC* complexes. Specific residues in the putative phosphosugar-binding motif of DiaA were required for the stimulation of initiation and formation of ATP–DnaA-specific–*oriC* complexes. Our data indicate that DiaA regulates initiation by a novel mechanism, in which DiaA tetramers most likely bind to multiple DnaA molecules and stimulate the assembly of specific ATP–DnaA–*oriC* complexes. These results suggest an essential role for DiaA in the promotion of replication initiation in a cell cycle coordinated manner.

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In prokaryotes and eukaryotes, the initiation of chromosomal replication is strictly regulated and occurs at a specific time during the cell cycle (Messer 2002; Stillman 2005; Kaguni 2006; Botchan 2007). A crucial aspect of replication initiation is the ordered assembly and conformational change of specific proteins at the replication origin. In *Escherichia coli*, multiple ATP-bound DnaA (ATP–DnaA) molecules form active complexes with the origin of replication (*oriC*) and unwind the duplex DNA within *oriC*, resulting in replication initiation. DnaA monomers specifically bind to sequences within *oriC* called DnaA boxes and interact with other DnaA mol-

ecules. These activities potentiate the formation of DnaA homomultimers on *oriC*. The present study is the first demonstration that DiaA, a DnaA-binding protein (Ishida et al. 2004), forms homotetramers, directly stimulates assembly of multiple ATP–DnaA molecules on *oriC*, and positively regulates a conformational change of the DnaA multimer–*oriC* complex to an initiation-competent state.

A single *E. coli* cell contains ~1000 molecules of the initiator protein DnaA, which exists in either an ATP–DnaA active form or an ADP-bound inactive form (ADP–DnaA) (Messer 2002; Kaguni 2006). The level of ATP–DnaA in the cell temporarily increases during the cell cycle at the same time as the initiation of replication (Kurokawa et al. 1999). The *E. coli oriC* contains several DnaA-binding sites, including several repeats of a 9-mer sequence called the DnaA box. When ATP–DnaA forms a multimeric complex on *oriC*, the DNA duplex is un-

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wound specifically at 13-mer AT-rich repeats within *oriC* (Speck and Messer 2001; McGarry et al. 2004; Kawakami et al. 2005). DnaB helicase is then loaded onto the unwound strands of DNA, and the single-stranded region is extended. DnaG primase and DNA polymerase (Pol) III holoenzyme then assemble on the single-stranded DNA to replicate the complementary strand. After the completion of Okazaki fragments, the β -clamp, which is a component of Pol III holoenzyme, remains on the nascent DNA strand (O'Donnell 2006). Hda forms a complex with the DNA-loaded β -clamp, which promotes hydrolysis of DnaA-ATP to ADP-DnaA (Katayama et al. 1998; Kato and Katayama 2001). This process for inactivating DnaA, termed the regulatory inactivation of DnaA (RIDA), is required for repressing untimely or excessive initiation events.

The timing of replication initiation is tightly coupled with cell cycle progression (Zyskind and Smith 1992). In *E. coli*, when the growth rate of cells is rapid, a single cell contains replication intermediates on multiple chromosomes, and replication initiates on each chromosome at exactly the same time. This level of regulation implies that there are multiple mechanisms in place to ensure that replication initiation is coupled to specific cell cycle events, that ATP-DnaA molecules accumulate to the proper level, and that the formation of ATP-DnaA-*oriC* complexes occurs in a timely manner. These mechanisms of regulation have yet to be fully explored.

In eukaryotes, initiation of DNA replication requires many proteins that directly or indirectly interact with the replication origin (Stillman 2005; Botchan 2007). In *Saccharomyces cerevisiae*, the origin recognition complex (ORC), a nucleoprotein complex that binds to the origin of replication, is preserved during the cell cycle. A pre-replication complex is formed when the MCM helicase complex is loaded onto the ORC-DNA complex, which also contains Cdc6 and Cdt1. The initiation of replication is activated by cyclin-dependent kinase at the G₁/S transition, and several proteins, including Cdc45, the 11-3-2 complex, and the GINS complex are loaded onto the pre-replication complex to form an initiation-competent replication complex in a timely manner.

In *E. coli*, DiaA is required to ensure the timely initiation of chromosomal replication during the cell cycle (Ishida et al. 2004). The *diaA* gene was first identified in a search for intergenic suppressor mutations of the *dnaA* mutant *dnaAcos*, in which there is overinitiation of chromosomal replication, and colony formation at 30°C is inhibited. Replication initiation in the *dnaAcos* mutant is resistant to RIDA at this temperature (Katayama and Crooke 1995). In *diaA* gene-disrupted cells, initiation is retarded, and the synchronous initiation of replication on multiple chromosomes at *oriC* is disrupted (Ishida et al. 2004). In the *diaA* mutant, when two *oriC* copies are present in a single, rapidly growing cell, only one *oriC* initiates, which produces a cell bearing three copies of *oriC*. In wild-type cells, this is an extremely rare event. Mutation of *diaA* also inhibits the stable maintenance of minichromosomes and, in a *dnaA46*-temperature sensitive mutant, causes synthetic lethality

at 37°C. Thus, DiaA is a positive regulator to stimulate the initiation. This function of DiaA is independent of RIDA and SeqA, an *oriC*-binding protein that represses extra initiations (Slater et al. 1995). Purified DiaA directly and specifically binds to DnaA, independent of the nucleotide- or DNA-bound state of DnaA (Ishida et al. 2004). DiaA binds to domains I–II in the N terminus of DnaA. The overall primary sequence of DiaA indicates that it contains a sugar isomerase (SIS) domain (Bateman 1999). This domain is proposed to be a consensus site for binding phosphosugars and is found in several proteins, such as the glucokinase regulator protein.

In the present study, we isolated several plasmid-encoded dysfunctional *diaA* mutants using complementation analysis of the *dnaAcos diaA::Tn5* mutant. In parallel, we determined the crystal structure of DiaA at 1.8 Å resolution. The structure revealed that DiaA forms a homotetramer consisting of a symmetrical pair of homodimers. Analysis of mutant DiaA proteins identified residues specifically required for DnaA binding. We also identified specific sites located in the interdimer interface that were required for homotetramer formation. Another group of mutations was located in the putative phosphosugar-binding region. All the DiaA mutants were inactive in stimulating replication initiation.

We also showed that a single DiaA tetramer can bind multiple DnaA molecules simultaneously. DiaA tetramers stimulated the assembly of multiple DnaA molecules on *oriC*, ATP-DnaA-specific conformational changes of the initiation complex, and unwinding of the duplex DNA at *oriC*. Mutant DiaA proteins were defective in all these activities. We propose that DiaA regulates initiation via a novel mechanism, in which a single DiaA tetramer binds to multiple DnaA molecules to stimulate inter-DnaA interactions, resulting in efficient assembly of multiple ATP-DnaA molecules on *oriC* and stimulation of initiation complex formation. This unique role for DiaA is most likely essential for the promotion of initiation in a cell cycle coordinated manner.

Results

Isolation of dysfunctional *diaA* mutations

To determine the functional mechanism of DiaA-mediated stimulation of replication initiation, we isolated *diaA* mutant alleles using in vitro Mn-PCR mutagenesis and in vivo complementation analysis. The plasmid pTKM601 encodes a histidine (His) fusion of wild-type *diaA* downstream from the arabinose-inducible promoter of pBAD/HisB. The *dnaAcos diaA26::Tn5* double mutant (NA26) grew at 30°C, but colony-forming ability at this temperature was inhibited in the presence of pTKM601 and 0.05% arabinose (Table 1). These results indicated that His-tagged DiaA (hisDiaA) was functional in the stimulation of chromosomal replication. We also introduced several pTKM601 derivatives bearing randomly mutagenized *diaA* alleles into NA26 cells. The mutant *diaA* alleles were unable to complement the *diaA26::Tn5* allele, and the transformants formed colo-

Table 1. *DiaA* complementation test

Strain	Plasmid	Arabinose		Ratio
		0.05%	0%	
		($\times 10^9$) CFU ^a /mL		0.05% / 0%
KH5402-1 (wild type)	pBAD/HisB (Vector)	3.0	3.5	0.86
NA26 (<i>dnaAcos diaA26::Tn5</i>)	pBAD/HisB	3.1	3.1	1.0
	pTKM601 (wild-type <i>diaA</i>)	6.0×10^{-6}	1.6	3.8×10^{-6}
	pMn17 (<i>diaA</i> L23P)	4.4	3.7	1.2
	pMn20 (<i>diaA</i> L45P)	3.2	3.3	0.97
	pMn53 (<i>diaA</i> S52F)	2.9	3.2	0.91
	pMn18 (<i>diaA</i> A53T)	3.1	3.7	0.84
	pMn52 (<i>diaA</i> R71Q)	3.1	3.8	0.82
	pMn31 (<i>diaA</i> L79P)	2.7	3.6	0.75
	pMn57 (<i>diaA</i> N83D)	1.4	2.4	0.58
	pMn30 (<i>diaA</i> K101E)	2.4	2.2	1.1
	pMn51 (<i>diaA</i> E173G)	3.4	2.9	1.2
	pMn6 (<i>diaA</i> L177P)	2.2	2.1	1.0
	pMn14 (<i>diaA</i> C183Y)	3.7	3.1	1.2
	pMn28 (<i>diaA</i> L190P)	4.1	3.7	1.1
	pMn29 (<i>diaA</i> P72S V85A)	3.5	3.1	1.1
	pMn21 (<i>diaA</i> E173G F191S)	4.8	4.4	1.1
	pMn54 (<i>diaA</i> A56V F191L)	3.2	3.0	1.1
	pMn48 (<i>diaA</i> S28P S165P)	3.4	3.3	1.0
	pMn72 (<i>diaA</i> S37T N65D)	2.5	4.0	0.63
	pMn61 (<i>diaA</i> M63K R93H)	3.9	4.3	0.91
	pMn44 (<i>diaA</i> S73R E97G)	3.4	2.8	1.2
	pMn40 (<i>diaA</i> D96G L182P)	4.1	4.0	1.0
	pMn27 (<i>diaA</i> F9S A31T L39P)	3.4	3.0	1.1
	pMn13 (<i>diaA</i> N80Y I116V T178A)	3.2	3.6	0.89
	<i>pdiaA</i> P72A	2.6	2.6	1.0
	<i>pdiaA</i> P72S	2.3	2.5	1.1
	<i>pdiaA</i> L190A	2.6	2.6	1.0
	<i>pdiaA</i> F191L	0.43	0.27	0.63
	<i>pdiaA</i> F191S	0.70	0.68	0.97

Cells of each strain were grown overnight at 30°C, plated on LB plates containing 0.05% or 0% arabinose, and incubated for 19 h at 30°C. Ampicillin and thymine were also included in media.

^aColony forming units.

nies at 30°C in the presence of 0.05% arabinose. We isolated 86 independent transformants and found that 26 of them overproduced a protein that was equivalent in size to full-length hisDiaA expressed in the presence of 0.5% arabinose (data not shown).

The mutant *diaA* alleles were analyzed by nucleotide sequencing (Table 1; Supplemental Table S1), which revealed that there were 22 distinct *diaA* mutants. Based on the sequences of pMn21, pMn29, and pMn54 (Table 1), plasmids encoding DiaA (*pdiaA*) that carried a single amino acid substitution of Pro72 or Phe191 were constructed by site-directed mutagenesis (for Pro72) or by restriction enzyme digestion and replacement (for Phe191). A plasmid encoding *diaA* bearing an alanine substitution of Leu190 (*pdiaA* L190A) was also constructed by site-directed mutagenesis, based on the sequence of pMn28 (*diaA* L190P). These plasmids were reintroduced into NA26 cells, and colony formation was assessed. As seen in Table 1, these mutant alleles were unable to complement the *diaA*-null allele in the growth inhibition of the *dnaAcos* mutant at 30°C and therefore are suggested to be impaired in the stimulation of chro-

mosomal replication. The plasmids were also introduced into NA001 (*dnaAcos*) cells. No *diaA* mutant alleles except for only *diaA* L190P affected the cold sensitivity in colony formation (data not shown). DiaA L190P might inhibit the function of wild-type DiaA.

Classification of *diaA* mutations based on the crystal structure of DiaA

To determine the location of the *diaA* mutations in the DiaA structure, we resolved the crystal structure of DiaA at 1.8 Å resolution (Table 2). The structure revealed a tetrameric DiaA molecule (Fig. 1B). A bundle of three parallel β -sheets that is wrapped by seven α -helices forms a protomer. The two dimers form a symmetrical tetramer via a hydrogen-bond network in the core region formed by the α -helices (Supplemental Fig. S1). Each protomer in the crystal structure is identical. A symmetrical dimer includes disulfide bonds between Cys8 and Cys181 on α -helices of each protomer. A *diaA* mutant allele bearing an amino acid substitution of C181S

Table 2. X-ray data collection, phasing, and refinement statistics

Data collection	
Beam line	BL44B2, SPring-8
Wavelength (Å)	1.000
Resolution (Å) (last shell)	50–1.85 (1.92–1.85)
Unique reflections	29,746
Redundancy	4.0
Completeness (%) (last shell)	99.9 (99.9)
$I/\sigma(I)$ (last shell)	18.5 (8.0)
R_{sym}^a (%) (last shell)	4.2 (17.2)
Refinement statistics	
Resolution (Å)	50–1.85
R_{work} (%)	18.3
R_{free}^b (%)	21.4
Average B -factor (Å ²)	18.1
Minimum B -factor (Å ²)	2.62
Maximum B -factor (Å ²)	61.3
RMSD bond length (Å)	0.006
RMSD bond angles (°)	1.24
RMSD dihedrals (°)	20.7
RMSD impropers (°)	0.702

^a $R_{\text{sym}} = \sum |I_{\text{avg}} - I_i| / \sum I_i$.^b R_{free} is calculated for 10% of randomly selected reflections excluded from refinement.

complemented the *diaA*-null allele of NA26 (*dnaAcos diaA::Tn5*) cells in the cold-sensitive growth (data not shown; Supplemental Table S1), suggesting that the disulfide bonds are not required for the in vivo function (also see below).

The locations of several of the *diaA* mutations in the crystal structure (Fig. 1C) mapped to the protomer surface and were clustered in three structurally distinct regions. The first region, represented by Asn83 and Lys101, was located at the core of the interdimer interface of tetrameric DiaA, which contains a network of hydrogen bonds that creates a “caged structure” (Fig. 1B,C; Supplemental Fig. S1). This structural network appeared to be essential for tetramer formation via the association of a pair of homodimers (see below).

The second region, represented by Arg71, Pro72, Leu190, and Phe191, was located at the outer edge of the DiaA surface (Fig. 1B,C). This region represents a potential interaction site with other proteins, such as DnaA. The third region, represented by Ser52 and Ala53, was located in the putative phosphosugar-binding region (Fig. 1B,C). *Pseudomonas aeruginosa* GmhA has an SIS domain and is highly homologous in sequence to DiaA (Fig. 1A; Brooke and Valvano 1996; Bateman 1999). The crystal structure of GmhA in complex with glycerol-D-mannopyranose-7-phosphate was previously determined (Protein Data Bank ID 1X92) and shows that Ser54 of GmhA, which corresponds to DiaA Ser52, is located on phosphosugar-binding surface (Supplemental Fig. S2).

Regulation of initiation of chromosomal replication is defective in *diaA* mutants

To determine whether the *diaA* mutant alleles described above were functional in the regulation of chromosomal

replication initiation, we performed flow cytometric analysis. Mutant *diaA26::Tn5* cells bearing wild-type or *diaA* mutant alleles on a mini-R low-copy plasmid were grown to exponential phase at 30°C in LB medium in the presence or absence of 0.2% glucose, then allowed to incubate for an additional period of time in the presence of rifampicin and cephalixin to allow run-out replication, and then analyzed by flow cytometry (Fig. 2). We previously revealed by flow cytometry analysis that introduction of *diaA*-carrying pBR322 slightly inhibits the initiation of chromosomal replication but that of *diaA*-carrying mini-R plasmid does not (Ishida et al. 2004). In the present experiments, to avoid overproduction of DiaA, we used a mini-R low-copy vector. The mini-R plasmid used here is a derivative of the R1 plasmid and bears the replication origin, the gene encoding the initiator RepA protein, the copy number-regulating *inc* regions, and the plasmid-partitioning *par* regions (Ishida et al. 2004; Nordström 2006). The copy number of this plasmid is strictly maintained at a level similar to that of the chromosome. After duplication, daughter plasmids are actively and evenly partitioned to two daughter cells.

In cells that expressed wild-type *diaA*, in the presence of glucose, there were two predominant peaks in the flow cytometry histogram, which indicated that four or eight chromosomes are present in these cells (Fig. 2A). In these same cells in the absence of glucose, there were predominantly four chromosomes (Fig. 2B). In these experiments, glucose was added to increase the growth rate of cells. We can observe the effect of DiaA in cells with different growth rates.

In contrast to the profiles of wild-type *diaA*-expressing cells (wild-type cells), in cells expressing mutant *diaA* alleles (mutant cells), the pattern of the peaks in the flow cytometry histogram demonstrated the presence of asynchronous initiation events (Fig. 2A,B). In 0.2% glucose-LB medium, the number of mutant cells carrying eight chromosomes was decreased compared to that of wild-type cells, which indicated that the timing of initiation in mutant cells is delayed, relative to the progression of the cell cycle (Fig. 2A). Inhibition of initiation was also observed in *diaA* mutant cells in LB medium lacking glucose (Fig. 2B). These results indicated that the stimulatory function of DiaA in replication initiation is defective in the mutant cells, and that at least eight amino acid residues—Ser52, Ala53, Arg71, Pro72, Asn83, Lys101, Leu190, and Phe191—are critical for the function of DiaA. The effect of the mutation P72S was limited in inhibition of initiation unlike that of P72A (Fig. 2A,B). Both DiaA P72S and DiaA P72A were clearly defective in the complementation test using the *dnaAcos diaA*-null mutant at 30°C (Table 1). Interaction of DiaA P72S with DnaAcos might be more severely inhibited than that with wild-type DnaA.

Immunoblot analysis showed that the amount of DiaA was similar in cells bearing mutant *diaA* alleles and wild-type cells, with the exception of cells expressing DiaA K101E, in which the level of DiaA was increased approximately twofold compared to wild-type DiaA when cells were grown in LB medium (Fig. 2C,D). These

Figure 1. Structural features of *E. coli* DiaA. (A) Amino acid sequences of *E. coli* (Eco) DiaA, GmhA, and *P. aeruginosa* (Pse) GmhA. Identical residues are highlighted in black, and chemically similar residues are shaded. Arrows indicate the DiaA residues mainly analyzed in this study. (B) Crystal structure of an EcoDiaA tetramer. Each protomer is depicted as a ribbon diagram in different colors. (C) Location of the amino acid residues examined in this study on two EcoDiaA protomers. The two protomers correspond to the left half of the EcoDiaA tetramer depicted in B, left figure, and are rotated 30° along the X-axis. Amino acids belonging to (red) Group I, (purple) Group II, (pink) Group II', and (blue) Group III (see text): Group I, the dimer-dimer interface; Group II, the sites essential for DnaA binding; Group II', the sites near the essential DnaA-binding site; and Group III, the putative phosphosugar-binding region.

results indicated that the inhibition of initiation observed by flow cytometry was not due to decreased amounts of mutant DiaA protein. Cell size was not significantly affected by expression of the mutant *diaA* alleles (data not shown).

Homo-oligomer formation of DiaA and the role of DiaA Asn83 and Lys101

Wild-type and mutant hisDiaA proteins were purified using Ni-NTA affinity column chromatography and then further purified using a gel-filtration column (Fig. 3A). The purity of the final protein fraction was >90% for all DiaA proteins, based on SDS-PAGE analysis and Coomassie Brilliant Blue (CBB) staining (Fig. 3B).

The calculated molecular weight of a DiaA monomer is ~23 kDa. Wild-type hisDiaA eluted from the gel-filtration column at a position corresponding to 50–60 kDa (Fig. 3A), which indicated that DiaA forms homo-oligomers. Similar results were observed for the DiaA mutant proteins, with the exception of DiaA N83D and DiaA K101E. These two proteins eluted at a position corre-

sponding to 40–50 kDa, which is consistent with hisDiaA homodimers (Fig. 3A), which indicated that these two mutant proteins fail to form homo-oligomers.

We further performed glycerol gradient ultracentrifugation experiments using wild-type hisDiaA, DiaA N83D, and DiaA K101E (Fig. 3C). Results were essentially consistent with those of gel filtration experiments. The sedimentation rate of wild-type hisDiaA corresponded to that of 100–60 kDa, which suggests that DiaA can form homotetramers. In contrast, sedimentation rates of DiaA N83D and DiaA K101E corresponded to that of 30–50 kDa.

These results correlated with the crystal structure data, which showed that Asn83 and Lys101 are located at the interface of a pair of homodimers. Together, the data of gel filtration, glycerol gradient ultracentrifugation, and crystal structure indicated that DiaA N83D and K101E form stable homodimers in solution. Wild-type DiaA most likely forms compactly folded homotetramers or equilibrates rapidly between homodimeric and homotetrameric states in the solution used for gel filtration.

Figure 2. Timely initiation of replication is inhibited by *diaA* mutants. NA141 cells (*diaA26::Tn5*) carrying pKP1673 (vector) or pKP1673 derivatives encoding the indicated *diaA* alleles were grown exponentially at 30°C in LB medium containing thymine (50 µg/mL) and chloramphenicol (10 µg/mL) in the (A) presence or (B) absence of 0.2% glucose to an A_{660} of 0.2. Portions of each culture were incubated for an additional 4 h in the presence of rifampicin and cephalixin. DNA content was then quantified using flow cytometry, and the equivalent chromosome number is shown. (C,D) A portion (500 µL) of each culture was analyzed by immunoblot. NA141 cells (*diaA26::Tn5*) were used as the control for background immunoreactivity, and purified DiaA was added to the control whole-cell extract as a quantitative standard (Ishida et al. 2004). Bands that correspond to DiaA are indicated by arrows, and numbers under each panel indicate the amount of DiaA (in nanograms), as determined by the relative densitometric intensities.

In addition, to determine if the disulfide bonds are required for DiaA oligomerization (Fig. 1C), we purified and analyzed DiaA C8S and C181S proteins. Results of gel filtration experiments indicated that DiaA C181S protein formed homo-oligomers like wild-type DiaA (Fig. 3A). DiaA C8S also showed similar results (data not shown). These results coincide with those of the *in vivo* complementation test as mentioned above.

The mutant *DiaA* proteins fail to stimulate minichromosomal replication *in vitro*

Minichromosomal replication *in vitro* is stimulated two- to threefold by DiaA (Ishida et al. 2004). The specific activity of wild-type hisDiaA was similar to that which was previously observed for nontagged DiaA (data not shown; Ishida et al. 2004). We then examined whether mutant DiaA proteins retained the ability to stimulate minichromosomal replication (Fig. 4). Under conditions in which the level of DnaA was limiting, in contrast to wild-type DiaA, none of the *diaA* mutants stimulated minichromosomal replication (Fig. 4A–D). In the presence of increasing amounts of DnaA, wild-type DiaA stimulated replication approximately twofold (Fig. 4E), while the *diaA* mutants had no stimulatory or inhibitory effect (Fig. 4E–G). These results indicated that the mutant *diaA* derivatives were defective in the stimula-

tion of DnaA-dependent initiation of replication *in vitro*, which was consistent with their activity *in vivo* (Table 1; Fig. 2). Since DiaA N83D and DiaA K101E most likely exist as stable homodimers (Fig. 3A), these results are also consistent with the idea that DiaA homodimers are inactive and that the formation of tetrameric DiaA is crucial for the stimulation of replication initiation.

When the representative mutant DiaA proteins (S52F, N83D, and L190A; 2 pmol) were incubated in replication reactions containing 1 pmol of DnaA and various amounts (0.25–2.0 pmol) of wild-type DiaA, the stimulation of replication was not affected (data not shown). Also, mixtures (1:1) of the two different mutant DiaA proteins were inactive in the stimulation of replication *in vitro* (data not shown).

Whereas DiaA P72S was inactive in the initiation stimulation *in vitro* (Fig. 4A), initiation of the *diaA* P72S cells was not inhibited *in vivo* in the presence of the wild-type *dnaA* gene (Fig. 2). DiaA P72S protein might be efficiently stimulated by chaperon proteins *in vivo*, sustaining interaction with wild-type DnaA, but not DnaAcos (Table 1).

DiaA L190 and F191 mutants fail to bind DnaA

It was shown previously using a pull-down assay and a biotin-tagged form of DnaA (bioDnaA) that DiaA binds

Figure 3. Oligomer formation analysis of wild-type and mutant DiaA proteins. (A) Wild-type hisDiaA (WT) and DiaA mutants (S52F, A53T, R71Q, P72A, P72S, N83D, K101E, L190P, L190A, F191S, F191L, and C181S) were subjected to Superdex 75 HR 10/30 gel filtration chromatography (24-mL bed volume), and fractions (5 μ L) were analyzed by 13% SDS-PAGE and CBB staining. Molecular size markers were subjected to the same conditions as above, and their positions are indicated at the top of the gel. (B) Pooled fractions (500 ng of protein) of wild-type hisDiaA or the indicated DiaA mutants were analyzed by 13% SDS-PAGE and CBB staining. (MW) Molecular size markers. (C) Wild-type hisDiaA (WT), N83D, and K101E mutant proteins (20 pmol as monomer) were analyzed using a glycerol gradient (15%–35%) ultracentrifugation method. After centrifugation, 11 fractions (200 μ L) were collected from the bottom of the tube and analyzed by 13% SDS-PAGE and CBB staining. Sedimentation positions of size marker proteins are indicated.

directly to DnaA and that the affinity of DiaA for DnaA was independent of the nucleotide-bound state of DnaA (Ishida et al. 2004). We performed a pull-down assay using bioDnaA and streptavidin-coated beads to determine whether mutant DiaA proteins retained the ability to bind to DnaA (Fig. 5A,B; Supplemental Fig. S3). The results indicated that DiaA L190A, L190P, F191L, and F191S were severely impaired in their ability to associate with DnaA, unlike wild type and the other mutant forms of DiaA. These results indicated that Leu190 and Phe191 of DiaA play a crucial role in its association with DnaA. Taken together, the results demonstrated that direct and specific binding between DiaA and DnaA is crucial for the stimulation of initiation.

The affinity of DiaA P72A and P72S for DnaA was severely decreased compared to wild type, although there was a small level of residual binding when DiaA was present in excess (Fig. 5A,B). This result was consistent with the tertiary structure data, in which Pro72 of DiaA is located close to Leu190 and Phe191 (Fig. 1). The other mutant DiaA proteins exhibited substantial DnaA-binding activity, although the level of binding was ap-

proximately half that of wild-type DiaA, even when DiaA was limiting (Fig. 5B), which indicated that the substitutions have an indirect effect on the interaction of DnaA and DiaA. DiaA N83D and K101E, which fail to form tetramers, were moderately impaired in binding to DnaA, which is consistent with the idea that DiaA tetramer formation is required for optimum binding of DnaA. We do not exclude a possibility that the moderate decrease in affinity for DnaA is an indirect consequence of a conformational change by the mutations. Moderately impaired activity of DiaA S52F and A53T in DnaA binding is consistent with the possibility that the structure of the phosphate-binding region is indirectly related to supporting of DnaA-binding activity. Like the wild-

Figure 4. Analysis of DiaA mutants in DnaA-dependent replication of minichromosome in vitro. A crude replication extract was prepared from TK24 cells (*dnaA204 diaA26::Tn5*) and used to assay replication of the *oriC* plasmid M13KEW101 (200 ng; 600 pmol nucleotide) in the presence of the indicated amounts of DnaA and hisDiaA. (A–D) Reactions were incubated for 20 min at 30°C in the presence of DnaA (0.25 pmol) and the indicated amounts of hisDiaA (WT) and DiaA mutants. (E–G) The indicated amounts of DnaA were incubated as for A–D in the presence of wild-type or the indicated mutants of hisDiaA (4 pmol monomer).

Figure 5. DiaA mutants in DnaA binding. (A) Wild-type hisDiaA (WT) or DiaA mutants (S52F, A53T, R71Q, P72A, P72S, N83D, K101E, L190P, L190A, F191L, and F191S; 20–80 pmol monomer) were incubated for 5 min on ice in buffer (50 μ L) containing bioDnaA (40 pmol). Protein complexes were isolated using streptavidin beads, eluted in buffer containing 1% SDS, and analyzed by 13% SDS-PAGE and CBB staining. (B) The intensities of the bands in A were quantified by densitometry, and the amounts of hisDiaA were determined using a standard curve (data not shown). The symbol key is shown in Figure 4.

type DiaA, these DiaA mutant proteins formed homomultimers (Fig. 3A), which indicated that overall denaturation of the mutant proteins was unlikely.

Interaction of DiaA tetramers with multiple DnaA molecules

To determine if multiple DnaA molecules bind to a single DiaA tetramer, we performed a two-stage pull-down assay. In the first stage of this experiment, we incubated 40 pmol (as monomer) of DiaA molecules for 10 pmol of bioDnaA molecules. The monomer state of DnaA is stable in solution. Excessive amounts of DiaA should avoid that all of the DnaA-binding sites on DiaA tetramers are occupied with bioDnaA molecules only at the first stage. Protein complexes of bioDnaA and DiaA were isolated using streptavidin beads, then at the second stage the isolated complexes were incubated in buffer containing nontagged DnaA, and bound protein complexes were isolated and analyzed by SDS-PAGE. Recovery of nontagged DnaA increased in a manner depending on both DiaA and bioDnaA (Fig. 6A), which indicated that multiple DnaA molecules (bioDnaA and nontagged DnaA molecules) can bind to a single DiaA tetramer simultaneously. We observed similar results using ADP-DnaA (data not shown). The representative DiaA mutant proteins were similarly assessed (Fig. 6A). Recoveries of DiaA N83D and S52F proteins were decreased compared to those of wild-type DiaA. DiaA L190A was inactive in DnaA binding. These results are consistent with those shown in Figure 5, A and B.

Furthermore, we used another pull-down system in which hisDiaA was incubated in the presence of various amounts of DnaA, followed by recovery using Co^{2+} -conjugated beads and determination of the amounts of proteins recovered (Fig. 6B). Results indicated that a single DiaA tetramer bound 1.6–2.3 DnaA molecules if all the DiaA tetramers were complexed with DnaA. If a portion of recovered DiaA tetramers were not associated with DnaA under these conditions, the number of DnaA molecules per DiaA tetramer would be elevated. These results are consistent with those of Figure 6A.

DiaA enhances assembly of DnaA on oriC

Based on the above results, we were interested in whether DiaA enhanced the assembly of DnaA molecules on *oriC*. DiaA binds to DnaA that is bound to the

Figure 6. Binding modes of DiaA and DnaA. (A) Wild-type hisDiaA or DiaA mutants (S52F, N83D, and L190A; 40 pmol as monomer) were incubated for 5 min in the presence (+) or absence (–) of bioDnaA (10 pmol). bioDnaA–hisDiaA complexes were isolated using streptavidin beads, and then incubated for 30 min at 4°C in buffer containing the indicated amounts of nontagged DnaA (ntDnaA). Complexes were isolated and analyzed by 13% SDS-PAGE and CBB staining. The amounts of hisDiaA and ntDnaA were determined using a standard curve (data not shown) and are indicated below the gel image. The number of recovered ntDnaA molecules per DiaA or per DiaA tetramer was deduced (ntDnaA/DiaA and ntDnaA/DiaA₄). For calculation of this number, the slight nonspecific recovery of ntDnaA in the absence of DiaA was subtracted. Arrows indicate the positions of the proteins used in the assay. (ND) Not detected. (B) Wild-type hisDiaA (5 pmol as monomer) was incubated for 15 min on ice in buffer containing indicated amounts of DnaA, followed by isolation using Dynabeads TALON (Invitrogen). Isolated proteins were washed, eluted in 60 mM imidazole, and analyzed by 13% SDS-PAGE and CBB staining. The amounts of hisDiaA and ntDnaA were determined using a standard curve and are indicated below the gel image. The number of recovered DnaA molecules per DiaA tetramer was deduced.

Figure 7. DiaA stimulates assembly of DnaA on *oriC*. (A,C) The indicated amounts of DnaA were incubated for 30 min on ice in the presence or absence of hisDiaA and bio-*oriC*. Proteins bound to bio-*oriC* were isolated using streptavidin beads and analyzed by 13% SDS-PAGE and (A) CBB staining or (C) silver staining. (B,D,E) The intensities of the bands were quantified by densitometry, and the amount of protein in complex with *oriC* was determined using a standard curve. (C) As indicated in A, bio-*oriC* (0.1 pmol) was also included in each reaction. The ratio of the number of DnaA molecules per DiaA monomer in each fraction is shown.

DnaA-box of *oriC* (Ishida et al. 2004). We incubated DnaA and DiaA with biotinylated-*oriC* DNA (bio-*oriC*, 419 bp) on ice, and DnaA-DiaA complexes that associated with *oriC* were isolated using streptavidin beads (Fig. 7). DiaA alone did not directly bind to *oriC*, but DnaA was recovered in an *oriC*-dependent manner, and the amount of DnaA bound to *oriC* increased proportionally with amount of input DnaA (Fig. 7A,B). In the presence of DiaA, the amount of DnaA bound to *oriC* was clearly enhanced in a dose-dependent manner (Fig. 7A-D).

When the amount of input DnaA was fixed at a limiting level, the amount of DnaA bound to *oriC* increased up to threefold in the presence of DiaA (Fig. 7C,E). This result was consistent with our previous results (Fig. 7A-D), and with the stimulatory effect of DiaA on replication initiation (Fig. 4). Of note, this effect was observed when the ratio of DnaA to DiaA monomer was in the range of 2–4, which is similar to the ratio seen in vivo (Ishida et al. 2004). The recovered *oriC*-protein complexes contained DnaA and DiaA at an average ratio of 1.6:1 (Fig. 7C-E). Thus, if a single initiation complex is assumed to contain 20 DnaA molecules, it will contain only three DiaA tetramers. These results demonstrated

that DiaA efficiently stimulates the assembly of DnaA molecules on *oriC*.

DiaA mutants do not stimulate DnaA assembly on *oriC*

We next examined whether three representative DiaA mutants—L190A, N83D, and S52F—stimulated the assembly of DnaA on *oriC*. DiaA L190A was specifically defective in DnaA-binding (Fig. 5). DiaA N83D retained considerable affinity for DnaA, but was defective in homotetramer formation (Fig. 3A). DiaA S52F retained both the modest DnaA-binding activity and the ability to form homotetramers, but was inactive in stimulating replication initiation in vivo and in vitro. As seen in Figure 8, A and B, all the mutant proteins tested were severely impaired in their ability to stimulate the formation of DnaA-*oriC* complexes. The slight stimulatory activity of DiaA N83D and S52F could be due to DnaA-binding activity of the DiaA protomer. These results are consistent with the idea that full stimulatory activity of DiaA depends on both DnaA-binding activity and the

Figure 8. DiaA mutants are defective in stimulating the assembly of DnaA on *oriC*. (A) Stimulation of the assembly of DnaA on *oriC* was assessed as described for Figure 7. The indicated amounts (as monomers) of wild-type hisDiaA (WT) and DiaA mutants (L190A, N83D, and S52F) were incubated for 30 min on ice in buffer containing DnaA (20 pmol) and bio-*oriC* (0.1 pmol). (B) The amounts of DnaA complexed with *oriC* were determined as described for Figure 7. (C) The DnaA-box-binding activity of DnaA was assessed by gel-mobility retardation analysis. Various amounts of DnaA (0, 1.5, 3, 4.5, and 6 pmol) were incubated for 20 min on ice in buffer containing a 15-mer DNA fragment (2.5 pmol) containing DnaA-box R1, in the presence or (None) absence of (WT) wild-type hisDiaA or DiaA mutants (L190A, N83D, and S52F; 20 pmol each). The samples were analyzed at 4°C by 8% PAGE and Gel-star staining. (D) The protein-free DNA (Free DNA) shown in C was quantified, and the relative amounts in each sample were normalized to the level observed in the absence of both DnaA and DiaA [Free DNA (%)]. The symbol key is shown in Figure 4.

ability to form homotetramers. The requirement for Ser52 is consistent with the idea that the putative phosphosugar-binding surface is also an important functional structure. We performed gel-mobility shift experiments to confirm that wild-type DiaA and the three DiaA mutants did not inhibit the affinity of DnaA for the DnaA-box sequence (Fig. 8C,D). Efficiency in decrease of protein-free DnaA-box molecules was not affected by wild-type and mutant DiaA proteins. DiaA did not inhibit or stimulate the initial binding of DnaA to a single DnaA-box. A discrete band corresponding to a DiaA–DnaA–DnaA-box complex was not seen for wild-type DiaA. Most likely wild-type DiaA efficiently promoted oligomerization of the nucleoprotein complexes, resulting in assemblies that have higher molecular mass and cannot migrate into the gel.

DiaA stimulates open complex formation

We next performed a P1 nuclease assay to investigate whether DiaA promoted the formation of the *oriC* open complex (Kawakami et al. 2005, 2006). P1 nuclease specifically degrades single-stranded DNA. When plasmid *oriC* is digested by P1 nuclease, subsequent digestion with the AlwNI restriction enzyme produces a 3.8-kb and a 4.1-kb fragment (Fig. 9). In the presence of limiting amounts of DnaA, DiaA appeared to promote formation of the open *oriC* complex. Open complex formation was not stimulated by DiaA L190A, N83D, and S52F. These results were consistent with earlier results showing that DiaA stimulated the assembly of DnaA on *oriC* (Fig. 8).

Figure 9. The wild-type DiaA, but not DiaA mutants, promotes *oriC* open complex formation. (A) The indicated amounts (as monomers) of wild-type hisDiaA (WT) and DiaA mutants (L190A, N83D, and S52F) were incubated for 3 min at 38°C in buffer containing the *oriC* plasmid M13KEW101 (320 ng) and HU protein (13 ng), in the presence (+) or absence (–) of ATP–DnaA (1.2 pmol), then incubated further in the presence of P1 nuclease. After digestion with AlwNI, the DNA fragments were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. (B) The intensities of the 3.8-kb and 4.1-kb fragments were quantified for each sample by densitometry, and the relative amount of each fragment is presented. The symbol key is shown in Figure 4.

Severe defects of DiaA S52F and N83D proteins in *oriC* unwinding is consistent with those of these proteins in the stimulation of DnaA assembly on *oriC* (Fig. 8A,B). Most likely these mutant proteins were impaired to stimulate the stable assembly of multiple DnaA molecules on *oriC*, and functional initiation complexes were not formed (see below). Stimulation of open complex formation required higher levels of DiaA per molecule of DnaA than the stimulation of minichromosome replication (Fig. 4) and DnaA assembly on *oriC* (Fig. 8). The minichromosome replication reaction contained crude protein extracts, which might include a factor that stimulates the activity of DiaA in promoting a conformational change of assembled DnaA multimers on *oriC*, and open complex formation. ADP–DnaA did not stimulate open complex formation even in the presence of DiaA (data not shown).

DiaA stimulates activated state of initiation complex

To determine whether DiaA promoted the formation of higher-order ATP–DnaA–*oriC* complexes, we performed a DNase I footprinting analysis of *oriC* (Kawakami et al. 2005). The *oriC* region carries high-affinity DnaA-boxes and low-affinity sites that ATP–DnaA specifically binds (Margulies and Kaguni 1996; McGarry et al. 2004; Kawakami et al. 2005). Initiation depends on the interaction of ATP–DnaA with the specific low-affinity sites (McGarry et al. 2004; Kawakami et al. 2005). Our previous study had shown that DnaA specifically binds with high affinity to DnaA-box sequences (termed R1 and R4) at a concentration of 100 nM DnaA, in a manner independent of the nucleotide-bound state of DnaA (Kawakami et al. 2005). At a concentration of 500 nM, DnaA interacts with several additional unique sites (termed I-sites, τ -sites, M, R2, and R3) in an ATP-dependent manner (Kawakami et al. 2005). DiaA promoted the association of ATP–DnaA with each of these specific binding sites, even at a DnaA concentration of 100 nM (Fig. 10). In the absence of DnaA, DiaA alone did not appear to bind to *oriC*.

The DiaA L190A, N83D, and S52F mutants were unable to stimulate formation of ATP–DnaA complexes on *oriC*, although DiaA N83D showed slight residual activity (Fig. 10). In the presence of ADP–DnaA, DiaA stimulated the association of DnaA molecules specifically with a region between DnaA-boxes R2 and R4, but the digestion pattern was different from the ATP–DnaA footprint. These results were consistent with the P1 nuclease assay and indicated that DiaA functions to stimulate the formation of active initiation complexes by promoting the interaction of ATP–DnaA with its specific binding sites.

DiaA-dependent stimulations of initiation in vivo

Disruption of the *diaA* gene exhibits unstable maintenance of minichromosomes and synthetic lethality with specific *dnaA* mutant alleles at 37°C (Ishida et al. 2004).

Figure 10. DiaA promotes ATP-DnaA-specific *oriC* complex formation. The indicated amounts (as monomers) of wild-type hisDiaA (WT) and DiaA mutants (L190A, N83D, and S52F) were incubated for 10 min at 30°C in buffer containing a ³²P-end-labeled *oriC* fragment (419 bp) and 3 mM ATP, in the presence or absence of DnaA, then incubated for 4 min at the same temperature in the presence of DNase I. When ADP-DnaA was assessed (right panel), the reaction buffer included 3 mM ADP instead of ATP. The resultant DNA fragments were analyzed by 5% sequencing gel electrophoresis. The positions of DnaA-binding sequences (boxes R1–R4 and M; sites I1–I3 and τ 1– τ 2) and the 13-mer AT-rich repeats (L, M, and R) are indicated.

To further analyze the *in vivo* role for DiaA in stimulation of initiation complex formation, we assessed the sensitivity of a *diaA*-null strain (NA141) to elevated levels of ATP-DnaA. DnaA R334A has an alanine substitution for the crucial arginine residue within the AAA+ sensor II motif. Our previous study reveals that ATP-DnaA R334A mutant protein is defective in hydrolysis of the bound ATP, whereas this protein is associated with intact activities in ATP/ADP binding and initiation at *oriC* (Nishida et al. 2002). The ATP-form DnaA R334A molecules form active initiation complexes like the ATP-form wild-type DnaA molecules. As ATP bound to DnaA R334A is not hydrolyzed by RIDA, the level of the ATP-form DnaA molecules is increased in cells bearing

this mutant DnaA, resulting in severe overinitiation at *oriC* and inhibition of colony formation of cells (Nishida et al. 2002). Plasmids pSN306 and pSN305 are derivatives of pBR322 bearing a wild-type *dnaA*- or *dnaA* R334A-coding region, respectively, that is located downstream from the *lac* promoter. IPTG (isopropyl- β -D-thiogalactopyranoside) induces moderate increase of DnaA molecules in the cells. In the presence of IPTG, growth of the wild-type *diaA* cells was severely inhibited in a DnaA R334A-dependent manner, whereas that of *diaA*-null cells was not (Fig. 11A). These results are consistent with the idea that DiaA plays an important role in stimulation of initiation complex formation by ATP-DnaA molecules.

Cell cycle parameters of a *diaA* mutant strain (NA141) containing pBR322 or a *dnaA*-carrying pBR322-derivative (pHB10S) were also analyzed using flow cytometry. Results indicated that the number of origins per cell mass was increased in a pHB10S-dependent manner (data not shown), which suggests that overexpression of DnaA can suppress the *diaA* mutation at least in the inhibition

Figure 11. DiaA-dependent activation of DnaA initiation function *in vivo*. (A) Cells of KH5402-1 (wild-type *diaA*) and NA141 (*diaA*::Tn5) carried pHSL99 bearing the *lacI*^q gene and pSN300-derivative plasmid. (*diaA*⁺) KH5402-1 (pHSL99) or (*diaA*[−]) NA141 (pHSL99) cells carrying pSN300 [vector (*none*)], pSN306 [wild-type *dnaA* (WT)], and pSN305 [*dnaA* R334A (R334A)] were grown overnight at 37°C in LB medium containing thymine (50 μ g/mL), ampicillin (50 μ g/mL), and tetracycline (7.5 μ g/mL). Three-microliter serial dilutions of the resultant cells (the A_{660} of 3.0; $\sim 10^9$ cell/mL) were spotted on LB agar plates containing the above chemicals in the presence (+) or absence of IPTG (0.1 mM) and incubated for 20 h at 30°C. (B) Cells of (*diaA*⁺) KA413 (*dnaA46*) and (*diaA*[−]) KA467 (*dnaA46 diaA*::Tn5) bearing pING1, pKA234 (wild-type *dnaA*), or pKK435 (*dnaA* T435M) were grown overnight at 30°C in LB medium containing thymine (50 μ g/mL) and ampicillin (50 μ g/mL). Three-microliter serial dilutions of the resultant cells were spotted on LB agar plates containing the above chemicals and incubated for 24 h at 30°C or for 15 h at 39°C or 42°C.

of initiation. This is consistent with the idea described above. Initiations of multiple origins in a single cell were asynchronous in DnaA-overexpressing cells regardless of the presence or absence of the *diaA* gene.

The *dnaA46* mutant allele produces a mutant DnaA protein bearing substitution mutations within domain III and colony formation of the *dnaA46* cells is temperature (39°C–42°C) sensitive (Erzberger et al. 2002; Ishida et al. 2004). DnaA C-terminal domain IV has a helix–turn–helix DNA-binding motif that is required for DnaA-box binding (Bleasing et al. 2000; Fujikawa et al. 2003). DnaA T435M protein bears a substitution within domain IV and is inactive in initiation due to a specific defect in DnaA-box binding (Sutton and Kaguni 1997; Bleasing et al. 2000; Fujikawa et al. 2003). We introduced plasmids bearing a wild-type *dnaA* or *dnaA* T435M allele (pKA234 or pKK435, respectively) in a *dnaA46* mutant (KA413) and in a *dnaA46 diaA*-null mutant (KA467) at 30°C (Fig. 11B). When KA413 cells bearing pKA234 or pKK435 were incubated at 39°C but not 42°C, colonies were formed at a similar efficiency. This suppression might occur by formation of mixed complexes of DnaA46 and DnaA T435M proteins on *oriC*, resulting in initiation (Kawakami et al. 2005). DnaA46 is intact in the DNA-binding domain, and DnaA T435M is intact in domain III, which carries the DnaA–DnaA interaction activity. Alternatively, the residual activity of DnaA T435M might be somehow elevated at 39°C. This suppression of the *dnaA46* cells bearing pKK435 was dependent on wild-type *diaA* (Fig. 11B). These results are also consistent with idea that DiaA can stimulate DnaA assembly on *oriC*.

Homology analysis of DiaA

We searched for *E. coli* DiaA homologs in a genome database. The DiaA C terminus bears Leu190 and Phe191 residues that are crucial in DnaA binding, whereas *E. coli* GmhA does not conserve these residues at the corresponding position (Figs. 1A and 5A). We used this feature to possibly distinguish between DiaA and GmhA homologs. DiaA homologs were found in various species in the eubacterial domain (Table 3). Especially many species in the γ -Proteobacterial division, to which *E. coli* belongs, conserve DiaA homologs. Of note, a considerable number of species bears a DiaA homolog but does not a GmhA homolog. Only for the β -Proteobacterial division, instead of the Leu–Phe residues, Leu–Leu residues are conserved of the homologs at the corresponding position. We defined these as DiaA homologs, based on similarity of the amino acid sequence and conservation of the putative operon structure. Like the *E. coli diaA* gene, the ORFs of these homologs are located between the ORFs of *yraN* and *yraP* on the genome.

Discussion

DiaA is a DnaA-binding protein that stimulates the initiation of chromosomal replication and is required to

Table 3. Conservation of DiaA homologs among eubacterial species

Phylum	Class	Number of species carrying	
		DiaA homolog and GmhA homolog	DiaA homolog only
Proteobacteria	γ -Proteobacteria	53	41
	β -Proteobacteria	5	27
	δ -Proteobacteria	1	14
	ϵ -Proteobacteria	10	6
Chloroflexi	Chloroflexi	2	2
Spirochaetes	Spirochaetes	3	0
Planctomycetes	Planctomycetacia	0	2
Lentisphaerae		0	1
Firmicutes	Clostridia	0	1
Actinobacteria	Actinobacteria	3	0
Total		77	94

Based on the amino acid sequences, *E. coli* DiaA and GmhA homologs were searched using the BLAST program and the NCBI nonredundant (NR) database. Of homologs that have >40% similarity, proteins that conserve the sequence of Leu–Phe (or Leu–Leu only in β Proteobacteria) corresponding to Leu190 and Phe191 of *E. coli* DiaA are defined as DiaA homologs. Proteins that lack this conservation are defined as GmhA homologs. Genomes of 171 species are found to carry both the DiaA and GmhA homologs or only the DiaA homolog (see also Supplemental Table S2 in detail).

ensure timely initiation during the cell cycle in *E. coli*. In this study, we revealed the crystal structure of DiaA and carried out a structure–function analysis of DiaA to gain a better understanding of the mechanism of stimulation of initiation by DiaA. DiaA forms homotetramers through the symmetrical pairing of stable homodimers. Mutational analysis of DiaA indicated that the tetramer form of the protein is the active form for stimulating the initiation of replication, while the dimerized form is inactive. We defined a specific site in the C terminus of DiaA encompassing Leu190 and Phe191 that is required for binding DnaA. We showed that a single DiaA tetramer can bind multiple molecules of DnaA and that the direct interaction of DnaA and DiaA is crucial for the regulation of initiation by DiaA. Previously, we demonstrated that the DnaA N-terminal domains I–II bind to DiaA (Ishida et al. 2004). Domain III and the C-terminal domain IV of DnaA are associated with ATP/ADP-binding and DNA-binding activities, respectively (Erzberger et al. 2002; Messer 2002; Fujikawa et al. 2003; Kawakami et al. 2005, 2006; Kaguni 2006). DiaA stimulated the formation of DnaA multimers on *oriC*, which stimulated the formation of open complexes. Binding of ATP–DnaA to specific sites within *oriC* was stimulated by DiaA. Thus, we have defined the mechanism by which DiaA stimulates initiation at *oriC*.

The DiaA tetramer can bind multiple molecules of DnaA, and thus can serve as a bridge to bring two DnaA molecules together. Binding of DnaA to the DiaA tetramer may serve as a mechanism to overcome the ef-

fects of random diffusion and facilitate inter-DnaA interactions. This linkage effect would also explain the stimulation of DnaA binding to DNA by DiaA. The linkage effect has been observed for several DNA-binding proteins (Zhou 2001; Stauffer and Chazin 2004). The two domains of RPA70 have modest single-stranded DNA-binding activity alone. When the domains are connected by a flexible linker, the overall affinity for single-stranded DNA is enhanced 1000-fold (Arunkumar et al. 2003). The λ CI repressor has specific domains for DNA binding and dimerization, and dimerization is required for high-affinity binding to DNA (Burz et al. 1994). Similarly, our results suggest that a single DnaA molecule of the DiaA-DnaA complex binds strongly to a DnaA-box sequence, and then this event stimulates binding of a second DnaA molecule in the complex to a flanking DNA site, resulting in the formation of stable DnaA multimers on *oriC*. DiaA had no stimulatory activity for the initial binding of DnaA to a single DnaA-box (Fig. 8C,D), which is also consistent with the above idea.

During the cell cycle, the fluctuation of ATP-DnaA levels is coupled to replication initiation. In the *dnaC2* (Ts) mutant, when the cell cycle is synchronized, the level of ATP-DnaA increases from a basal level and reaches a peak after 10 min, which is the point at which replication initiates (Kurokawa et al. 1999). Thus, there is a limited time span in which ATP-DnaA molecules must assemble on *oriC* to ensure the initiation of replication in a timely and synchronized manner. The results of the present and previous studies indicate that DiaA plays a specific and crucial role in this process, and that when DiaA is absent or inactive, initiation is delayed, resulting in reduced *oriC* copy number and asynchronous initiations. In a cell that contains two copies of *oriC*, replication initiation at only one of the two sites due to improper regulation of preinitiation events results in activation of the RIDA system, and reduction of ATP-DnaA levels. Thus, replication initiation at the second *oriC* is inhibited. Regulation of pre-initiation events to ensure that replication initiation occurs synchronously and at the proper time is profoundly important for proper maintenance of the replication cycle.

In addition, an idea that there might be cross-talk between the *oriC* copies in a single cell to ensure the synchrony of initiation would be possible. Unknown factors and mechanisms might be responsible for cross-talk of the *oriC* copies, which might be affected by the lack of DiaA. We searched for DiaA-binding proteins and found several candidates (data not shown). Functional analysis of these factors is in progress.

Although the DiaA S52F and A53T mutant proteins formed homotetramers and were able to bind to DnaA, they were defective in the stimulation of initiation, open complex formation, and formation of ATP-DnaA-specific *oriC* complexes. These residues are located in the putative phosphosugar-binding region of DiaA, but we cannot exclude the possibility that mutation of these residues disrupts internal interactions of DiaA that affect the overall structure of the protein. Another possibility is that these mutations, indeed, affect the binding

of DiaA to phosphosugars and that this association regulates the activity of DiaA. If phosphosugar binding is essential to activity, mutants that fail to bind phosphosugars would be inactive. Alternatively, a mutation might result in a protein that binds phosphosugars more tightly than wild-type DiaA, which may result in constitutive inactivation. Phosphosugars such as inositol-triphosphate and inositol-hexakisphosphate are known to regulate protein function in sugar metabolic pathways and in signal transduction pathways (Hanson et al. 2004; Kim et al. 2006). The YrbH protein of pathogenic *Yersinia pestis* has an SIS domain and arabinose-5-phosphate isomerase activity, and is required for biofilm formation (Tan and Darby 2006). However, to date, phosphosugars have not been implicated in the regulation of replication initiation. At least, glucose did not affect the affinity of DiaA for DnaA in vitro (data not shown).

We do not exclude a possibility that DiaA function can be regulated during the cell cycle, although at present we do not have concrete evidence to prove the cell cycle-coordinated functional change of DiaA. As DiaA has a phosphosugar-binding motif, one of possible ideas is that a specific phosphosugar binds to DiaA in a cell cycle-dependent manner, which regulates DiaA function such as DnaA binding or tetramer formation.

The *oriC* region contains several DnaA-binding sites of varying affinity (Margulies and Kaguni 1996; Weigel et al. 1997). DnaA R1 and R4 boxes are associated with high affinity for DnaA, independent of the nucleotide-bound state of DnaA. Other DnaA-boxes are associated with lower affinity for DnaA, and some of them specifically interact with ATP-DnaA. ATP-DnaA binding to these weak-affinity sites is crucial for open complex formation (McGarry et al. 2004; Kawakami et al. 2005). We demonstrated that DiaA stimulated the interaction of ATP-DnaA with ATP-DnaA-specific sites of *oriC*, which most likely promotes the formation of open complexes. In contrast, DiaA did not stimulate the binding of ADP-DnaA molecules to ATP-DnaA-specific sites such as I1, I2, τ 1, τ 2, and M sites (Fig. 10). This indicates that the integrity of ATP-DnaA-specific DNA binding is preserved in the presence of DiaA. The formation of ATP-DnaA-specific *oriC* complexes depends on Arg285 of DnaA, which plays a key role in inter-DnaA interactions (Kawakami et al. 2005; Erzberger et al. 2006). One implication of our results is that DiaA reinforces this ATP-DnaA-specific inter-DnaA interaction. This is consistent with the results of the pull-down experiments, the minichromosomal replication assay, and the *oriC* unwinding assay.

In the presence of DiaA and ADP-DnaA molecules, protection from DNase I was seen only for DnaA-boxes R1 and R4 and a region between DnaA-boxes R2 and R4 (Fig. 10). DnaA-boxes R1 and R4 have most potent affinity for DnaA regardless of the nucleotide forms. Unlike DnaA-box R1, box R4 is adjacent to the sites (I3, boxes R3 and R2) that DnaA can weakly bind. The I3 site has a sequence carrying a DnaA-box 9-mer consensus with three mismatches (McGarry et al. 2004; Kawakami et al. 2005). We speculate that DiaA would stimulate interac-

tion of DnaA molecules to DnaA tightly complexed with box R4, resulting in binding of ADP-DnaA to these R4-adjacent weak-affinity sites. Also, DiaA-dependent footprint patterns for this region (R4 to R2) are clearly different between ATP-DnaA and ADP-DnaA. This suggests differences in the mode of inter-DnaA interaction. Most likely this difference can be caused by the presence or absence of interaction between ATP and the DnaA arginine finger Arg285 (Kawakami et al. 2005).

DiaA forms homotetramers, which appear to be the active form of the protein that stimulates replication initiation. The results of gel filtration analysis showed that wild-type DiaA eluted at a position corresponding to a molecular mass of 50–60 kDa, which is smaller than the calculated molecular mass of a DiaA homotetramer. In a dilute solution of DiaA, there may be a state of equilibrium between homotetramers and homodimers. A previously purified preparation of non-tagged DiaA may contain predominantly homodimers (Ishida et al. 2004).

We previously indicated that DiaA binds to an N-terminal domain of DnaA (Ishida et al. 2004). Recently, we revealed the tertiary structure of the DnaA N-terminal domain and reported that the Glu21 residue, which is exposed on the protein surface of the domain, is specifically required for DnaB helicase loading (Abe et al. 2007). The Phe46 residue of this domain is also exposed on the protein surface. We find that DnaA F46A mutant protein is active in ATP binding but is defective in DiaA binding in vitro (data not shown).

Materials and methods

Strains and plasmids

E. coli K12 derivatives KH5402-1 (wild-type), NA001 (*dnaAcos*), NA26 (*dnaAcos diaA26::Tn5*), NA141 (*diaA26::Tn5*), TK24 (*dnaA204 diaA26::Tn5*), and KA413 (*dnaA46*) were previously described (Ishida et al. 2004; Kawakami et al. 2005). KA467 is a KA413 derivative carrying *diaA26::Tn5*. pBAD/HisB was purchased from Invitrogen. The *oriC* plasmid M13KEW101 was previously described (Kawakami et al. 2005). To construct the plasmid encoding hexahistidine-fused DiaA, a *diaA* fragment (0.56 kb) was amplified using pNA159 (Ishida et al. 2004) as the template by PCR, using the primers TAKU15 (5'-CCGGCTAGCATGCAAGAAAGAATTAAAGCT-3') and TAKU4 (5'-CGGAATTCCTCCTTAATCATCCTGGTG-3'). The amplified PCR product was cloned into pBAD/HisB using the *NheI* and *EcoRI* restriction sites to generate pTKM601. The single base substitutions of *pdiaA P72A*, *pdiaA P72S*, *pdiaA L190A*, *pdiaA C8S*, and *pdiaA C181S* were generated using the QuikChange site-directed mutagenesis kit (Stratagene) and pTKM601 as the template (Supplemental Table S1). A 2.4-kb *NdeI* fragment from pMn21 and pMn54, containing *diaA F191S* and *diaA F191L*, respectively, was ligated to a 2.1-kb *NdeI* fragment derived from pTKM601 to generate *pdiaA F191S* and *pdiaA F191L*. All mutations were confirmed by nucleotide sequencing.

A 0.56-kb *HindIII*-*EcoRI* fragment bearing *diaA* with the His-tag deleted was isolated from pTKM601 and inserted into the corresponding sites of the low-copy mini-R (pKP1673) derivative plasmid pNA102, to generate pTKM701. pNA102 encodes non-tagged wild-type *diaA* (Ishida et al. 2004). Derivatives of pNA102 encoding mutant *diaA* alleles were constructed in a similar manner (Table 1; see below).

Plasmids pSN300, pSN305, pSN306, and pHSL99 were previously described (Nishida et al. 2002). Briefly, pSN300 was a pBR322 derivative bearing a *lac* promoter, and pSN305 and pSN306 were pSN300 derivatives bearing a *dnaA R334A* and wild-type *dnaA* coding region, respectively, downstream from the *lac* promoter. pHSL99 is a pACYC184 derivative bearing the *lacI^q* gene. Plasmid pKK435 bearing *dnaA T435M* was constructed using pKA234, the site-directed mutagenesis kit as above, a primer 5'-CGTTTGGTGGCCGTGACCATATGACG GTGCTTCATGCCTGC-3', and its complementary primer. pKA234 was a derivative of pING1 that overproduces wild-type DnaA in an arabinose-inducible manner (Kawakami et al. 2005).

Introduction of mutations and selection of dysfunctional DiaA mutants

The mutagenic PCR method was used as previously described, with minor modifications (Guo et al. 1999). Briefly, *diaA* (0.56-kb fragment) was amplified by PCR using pTKM601 as the template, and the primers TAKU15 and TAKU4, in the presence of 0.05 mM or 0.1 mM manganese. The amplified *diaA*-coding region was inserted in pBAD/HisB using the *NheI* and *EcoRI* restriction sites. To isolate dysfunctional DiaA mutants, the plasmids were introduced into NA26 cells, and transformed cells were obtained by incubation for 20 h at 30°C on LB agar plates containing 0.05% L(+)-arabinose, 100 µg/mL ampicillin, and 25 µg/mL thymine.

Protein purification

NA26 cells carrying pTKM601 were grown at 30°C in 2.4 L of LB medium containing 100 µg/mL ampicillin and 50 µg/mL thymine. When the absorbance at 660 nm (A_{660}) of the culture reached 0.4–0.6, arabinose (0.5%) was added, and cells were incubated for an additional 2 h. The cells were collected by centrifugation, resuspended in buffer E (40 mM HEPES-KOH at pH 7.6, 0.1 mM EDTA, 20% sucrose, and 10 mM β-mercaptoethanol) containing 250 mM KCl, frozen in the presence of 20 mM spermidine-HCl and 0.3 mg/mL lysozyme, and then thawed. The supernatants were collected by centrifugation and applied to a Ni-NTA agarose column (1-mL bed volume; Qiagen). After the column was washed, hisDiaA was eluted in buffer E containing 100 mM imidazole and 250 mM KCl. The eluted fractions were further purified on a Superdex 75 HR 10/30 column equilibrated with buffer C (40 mM HEPES-KOH at pH 7.6, 15% [w/v] glycerol, 1 mM EDTA, and 2 mM dithiothreitol) containing 500 mM KCl, using a flow rate of 3 mL/h. Protein concentrations were determined using a Bradford assay kit (Bio-Rad). DiaA mutant proteins were purified in a similar manner.

Crystallization and structural analysis of DiaA

DNA fragments encoding DiaA or DnaA domains I–II were inserted into pET28a (Novagen) at the *NheI*-*BamHI* sites or the *NheI*-*HindIII* sites, respectively. The resultant plasmids were introduced into *E. coli* BL21 cells to generate N-terminal His₆-tagged fusions of DiaA and DnaA domains I–II. Cells were grown at 37°C to an optical density at 600 nm (A_{600}) of 0.6, and IPTG (1 mM) was added to induce protein expression. After 3 h, the cells were harvested, resuspended in buffer A (25 mM HEPES-KOH at pH 7.6, 200 mM KCl, and 10% glycerol), disrupted by sonication, and centrifuged at 27,000g for 15 min at 4°C. Supernatants containing either His₆-tagged DiaA or DnaA domains I–II were separately loaded onto a HisTrap column (5 mL; Amersham Biosciences), and the column was washed with buffer A containing 20 mM imidazole. Proteins were eluted in a

15-column volume linear gradient of 20–500 mM imidazole in buffer A. The fractions containing his-tagged protein were dialyzed against buffer A, and Thrombin protease (Amersham Biosciences) was added to cleave the His tag. To purify DiaA, fractions were loaded onto a HiTrap Q column (5 mL; Amersham Biosciences) equilibrated with buffer A. The column was washed with 10-column volumes of buffer A, and the protein was eluted with a 10-column volume linear gradient of 0.2–0.6 M KCl in buffer A, and DiaA peak fractions were collected. To purify DnaA domains I–II, fractions were loaded onto a Hilo 26/60 Superdex 75 column (Amersham Biosciences) equilibrated with buffer B (25 mM HEPES-KOH at pH 7.6, 400 mM KCl, and 10% glycerol). The purified DiaA and DnaA domains I–II were mixed at a 2:1 molar ratio, and the mixture was concentrated to 12 mg/mL, with simultaneous exchange of buffer. Crystals of the DiaA–DnaA domains I–II mixture were obtained by the hanging drop method after mixing equal volumes of protein solution and reservoir solution containing 0.1 M sodium citrate (pH 5.4), 25% dioxane, and 20% polyethylene glycol 4000. Crystals were briefly suspended in a cryoprotectant buffer containing 0.1 M Na-cacodylate (pH 6.0), 25% dioxane, 20% polyethylene glycol 4000, and 5% 2-methyl-2,4-pentanediol, and flash-frozen in a stream of N₂ gas (100 K). The data set was collected up to 1.85 Å resolution from beamline BL44B2 at SPring-8 in Harima. The crystals obtained belong to the orthorhombic space group C2, with unit cell constants of $a = 71.36$ Å, $b = 81.21$ Å, and $c = 67.41$ Å. They contained two DiaA molecules per asymmetric unit. The crystal structure was solved by molecular replacement using the MolRep program and the crystal structure of *P. aeruginosa* phosphoheptose isomerase GmhA (PDB ID: 1X92) as a search model. The structure was refined using the CNS program, and the final R_{free} and R_{work} were 21.4% and 18.3%, respectively.

Glycerol gradient ultracentrifugation

The indicated amounts of hisDiaA samples were layered on a 2.2-mL glycerol gradient (15%–30%) in buffer C. A parallel gradient was used for size marker proteins. After centrifugation at 55,000 rpm for 16 h in a Beckman TLS55 rotor at 2°C, fractions (200 μ L) were collected from the bottom of the tube. Proteins used for size markers were β -amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa).

Flow cytometry analysis

Flow cytometry was performed as described previously with minor modifications (Kawakami et al. 2006). Briefly, cells were exponentially grown for ~10 generations at 30°C, followed by incubation in the presence of rifampicin (300 μ g/mL) and cephalixin (10 μ g/mL) for an additional 4 h. Cells were collected in cold 70% ethanol, washed, and resuspended in cold buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM magnesium sulfate, and 2 μ M SYTOX green (Invitrogen), then analyzed using a FACScalibur flow cytometry system (Becton Dickinson). KA413 (*dnaA46*) cells incubated at 42°C and containing one or two chromosomes per cell were used to calibrate for chromosome number.

In vitro minichromosomal DNA replication

The in vitro DnaA-complementation system using minichromosome M13KEW101 and a crude protein extract (TK24 fraction II) was previously described (Ishida et al. 2004).

Pull-down assays for hisDiaA–bioDnaA complex formation and binding of non-tagged DnaA

hisDiaA–bioDnaA complexes were isolated as previously described (Ishida et al. 2004). Briefly, hisDiaA was incubated for 5 min on ice in 50 μ L of buffer F (50 mM HEPES-KOH at pH 7.6, 1 mM EDTA, 2 mM DTT, and 20% sucrose) containing 100 mM KCl, 100 μ g/mL bovine serum albumin, 10 μ M ATP, and 10 mM magnesium acetate in the presence or absence of bioDnaA. Proteins were incubated for an additional 1 h at 4°C in the presence of streptavidin-conjugated magnetic beads (Promega). The beads were collected and washed, and bound material was isolated using magnetic force, then incubated in standard SDS sample buffer (10 μ L) to dissociate bound hisDiaA. Proteins were then incubated in boiling water to dissociate bound bioDnaA. To analyze non-tagged DnaA binding, hisDiaA–bioDnaA complexes bound to streptavidin beads were isolated, washed, and incubated for 30 min at 4°C in the presence of non-tagged DnaA.

Pull-down assay for hisDiaA–DnaA complexes

Wild-type hisDiaA was incubated for 15 min on ice in buffer NP (12.5 μ L) containing 5 mM Tris-HCl (pH 7.5), 10% glycerol, 8 mM 2-mercaptoethanol, 0.01% Brij-58, 8 mM magnesium acetate, 100 mM KCl, and 0.1 mM ATP in the presence of DnaA. Proteins were incubated for an additional 5 min on ice in the presence of Dynabeads TALON (20 μ g, Invitrogen) and 10 mM imidazole in the same buffer (25 μ L) as above. The beads and bound materials were collected and washed in buffer NP (2.5 μ L) containing 30 mM imidazole. Proteins were eluted in buffer NP (8 μ L) containing 60 mM imidazole.

Pull-down assay using bio-oriC

A bio-oriC fragment (419 bp) was amplified by PCR using an oriC plasmid, M13KEW101, a 5'-biotinylated primer (5'-CCC GGGCCGTGGATTCTAC-3'), and an unmodified primer (5'-GAAGATCAACATTCTTGATCACG-3'). DnaA, hisDiaA, and bio-oriC (100 fmol) were incubated for 30 min on ice in buffer F (50 μ L) containing 100 mM KCl, 100 μ g/mL bovine serum albumin, 1 mM ATP, and 10 mM magnesium acetate. The mixture was further incubated for 1 h at 4°C with gentle rotation in the presence of streptavidin-beads (Promega) equilibrated in the same buffer (50 μ L) as above. The beads and bound material were collected, washed in buffer F (50 μ L) containing 100 mM KCl, 1 mM ATP, and 10 mM magnesium acetate (50 μ L), resuspended in 10 μ L of standard SDS sample buffer, and analyzed by 13% SDS-PAGE.

Gel-mobility retardation assay

The gel-mobility assay was performed as previously described (Kawakami et al. 2006; Ozaki et al. 2006). Briefly, DnaA was incubated for 20 min on ice in buffer (10 μ L) containing 2 mM ATP and the 15-mer DnaA R1 box (2.5 pmol) in the presence or absence of hisDiaA (20 pmol). The samples were analyzed by 8% PAGE, followed by Gel-star (Cambrex) staining, then quantified by densitometry. Protein-free DNA was also quantified and is presented as a percentile.

Open complex formation assay using P1 nuclease

The P1 nuclease assay was performed as previously described (Kawakami et al. 2006; Ozaki et al. 2006). Briefly, the indicated amounts of hisDiaA were incubated for 3 min at 38°C in buffer

(50 μ L) containing 5 mM ATP, 16 ng of HU protein, M13KEW101 *oriC* plasmid (320 ng; 61 fmol), and ATP-DnaA (0 or 1.2 pmol), followed by incubation for 200 sec in the presence of P1 nuclease (10 units). DNA was purified, and one-fourth of each sample was digested with AlwNI, then analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The DNA fragments were quantified by densitometry.

DNase I footprinting analysis

DNase I footprinting analysis was performed as described previously (Kawakami et al. 2005). Briefly, the indicated amounts of DnaA and hisDiaA were incubated for 10 min at 30°C in buffer (10 μ L) containing 25 mM HEPES-KOH (pH 7.6), 5 mM calcium acetate, 2.8 mM magnesium acetate, 35 mM ammonium sulfate, 23 mM potassium acetate, 4 mM dithiothreitol, 10% (v/v) glycerol, 0.2% Triton X-100, 0.5 mg/mL bovine serum albumin, 14 μ g/mL poly(dA-dT)-(dA-dT), 14 μ g/mL poly(dI-dC)-(dI-dC), 3 mM ATP, and 32 P-end-labeled *oriC* fragment (5.5 ng), followed by incubation for 4 min at the same temperature in the presence of DNase I (0.63 mU). DNA was then purified and analyzed by 5% sequencing gel electrophoresis and analyzed using a BAS-2500 Bio-imaging analyzer (Fuji Film).

Accession number

Atomic coordinates and structure factor amplitudes of the *E. coli* DiaA have been deposited in the Protein Data Bank under accession code 2YVA.

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The interaction of DiaA and DnaA regulates the replication cycle in *E. coli* by directly promoting ATP-DnaA-specific initiation complexes

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