Protein Associations in DnaA-ATP Hydrolysis Mediated by the Hda-Replicase Clamp Complex

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In Escherichia coli, the activity of ATP-bound DnaA protein in initiating chromosomal replication is negatively controlled in a replication-coordinated manner. The RIDA (regulatory inactivation of DnaA) system promotes DnaA-ATP hydrolysis to produce the inactivated form DnaA-ADP in a manner depending on the Hda protein and the DNA-loaded form of the β-sliding clamp, a subunit of the replicative holoenzyme. A highly functional form of Hda was purified and shown to form a homodimer in solution, and two Hda dimers were found to associate with a single clamp molecule. Purified mutant Hda proteins were used in a staged in vitro RIDA system followed by a pull-down assay to show that Hda-clamp binding is a prerequisite for DnaA-ATP hydrolysis and that binding is mediated by an Hda N-terminal motif. Arg168 in the AAA+ Box VII motif of Hda plays a role in stable homodimer formation and in DnaA-ATP hydrolysis, but not in clamp binding. Furthermore, the DnaA N-terminal domain is required for the functional interaction of DnaA with the Hda-clamp complex. Single cells contain ~50 Hda dimers, consistent with the results of in vitro experiments. These findings and the features of AAA+ proteins, including DnaA, suggest the following model. DnaA-ATP is hydrolyzed at a binding interface between the AAA+ domains of DnaA and Hda; the DnaA N-terminal domain supports this interaction; and the interaction of DnaA-ATP with the Hda-clamp complex occurs in a catalytic mode.

The initiation of chromosomal replication is highly regulated so as to take place once and only once during the cell cycle. In Escherichia coli, this control is ensured by at least three pathways that target the replication origin oriC or the protein DnaA, which binds directly to oriC to initiate replication (1–3). First, newly replicated oriC is temporally inactivated by the binding of the SeqA protein (4, 5). The palindromic sequence occurs in a catalytic mode. Interaction of DnaA-ATP with the Hda-clamp complex supports this interaction; and the AAA+ subunit of the replicase holoenzyme. A highly functional RIDA system promotes DnaA-ATP hydrolysis when DnaA-ATP interacts with the Hda protein in the presence of the DNA-loaded β-sliding clamp, a subunit of the chromosomal replicase. The requirement for the DNA-loaded form of the clamp subunit ensures the timely inactivation of DnaA during the replication cycle. The DnaA-ATP level peaks around the time of replication initiation in vivo and decreases in a RIDA-dependent manner (13). The DnaA R334A mutant, which binds ATP but is inactive for ATP hydrolysis, causes over-initiation of chromosomal replication, a phenotype also conferred by inactivation of the hda gene (16–18).

The minimal oriC region contains five 9-mer DnaA-binding sites, or DnaA boxes. DnaA-ATP molecules complexed with oriC open an AT-rich 13-mer region within this locus (3, 19). DnaB helicase is then loaded onto the unwound DNA, and the single-stranded region is expanded, which allows the single-strand binding protein and DnaG primase (for primer synthesis) to be loaded. The clamp subunit of the DNA polymerase (pol) III holoenzyme, which consists of a β-subunit homodimer that forms a ring, is then loaded onto the primed sites and encircles the duplex strand with the assistance of the clamp-loader γ-complex, another pol III holoenzyme subassembly (20, 21). The DNA-loaded clamp ensures highly processive replication as it slides along duplex DNA and binds to the pol III core, a subassembly with nucleotide-polymerizing activity. After completion of Okazaki fragment synthesis, the pol III core is released from the clamp and binds to a newly loaded clamp at a primed site (22).

pol III core-free clamps remain on replicated DNA and can interact with other proteins such as ligase, MutS, and pol I (23, 24). Recently, we reported that Hda forms a stable complex with the clamp (25). Hda is a 29-kDa protein consisting of a short N-terminal region and an AAA+ domain, which contains conserved motifs seen in other members of the AAA+ protein family (see Fig. 1) (16, 26). AAA+ proteins are ATPases associated with various cellular activities, and AAA+ proteins compose an expanded group that includes these proteins (21, 26, 28). AAA+ family proteins contain unique motifs related to nucleotide binding and hydrolysis in addition to the Walker-type nucleotide-binding motifs.

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1 The abbreviations used are: RIDA, regulatory inactivation of DnaA; pol, DNA polymerase; Ni-NTA, nickel-nitrilotriacetic acid; DAD III–IV, DnaA domains III and IV.
is required both for DnaA-ATP hydrolysis during RIDA and for His-Hda derivatives, we reveal that the N-terminal region of Hda AAA* motifs and shares homology with Hda (28% identity and 48% similarity). The N-terminal region (amino acids 1–26) of Hda includes a putative $\beta$-clamp-binding motif (underlined), and its consensus sequence is shown below (27). The amino acid changes in the mutant Hda proteins used in this study are also indicated (boldface).

We found that at least 40 bp of DNA flanking the clamp is required for RIDA and proposed that the association of DnaA with this DNA supports a functional interaction between DnaA and the Hda-clamp complex (25). In the present study, we analyze the structural and functional relationships between Hda and DnaA to better understand the molecular mechanisms underlying protein-protein interactions relevant to RIDA. First, we purified a highly active form of Hda. We previously studied an Hda derivative conjugated with three tags (Hda*, bearing an N-terminal maltose-binding protein and C-terminal Myc and hexahistidine peptides) because of the difficulty of overexpressing and purifying the native form of Hda (16). The specific activity of Hda* was proposed to be significantly reduced compared with that of native Hda (16). We recently improved the preparation protocol and obtained Hda tagged only with N-terminal hexahistidine (His-Hda). His-Hda is more functional in vitro than Hda*, and we speculate that its activity is comparable with that of native Hda.

Second, using reconstituted in vitro RIDA systems and mutant His-Hda derivatives, we reveal that the N-terminal region of Hda is required both for DnaA-ATP hydrolysis during RIDA and for directing clamp binding. Third, we show that Hda Arg$^{168}$ is required for DnaA-ATP hydrolysis. This residue is located in the AAA* Box VII motif, which is a putative arginine finger proposed to be directly involved in ATP hydrolysis (26, 29). Fourth, we demonstrate that Hda forms a homodimer in solution and that two Hda dimers form a stable complex with a single clamp. Fifth, by immunoblot analysis, we determined the cellular Hda content. Also finally, we found that the DnaA N-terminal domain is required for RIDA. DnaA is subdivided into four domains (Fig. 1): N-terminal domains I and II (amino acids 1–134) specify DnaA-DnaA and DnaA-DnaB interactions; domain III (amino acids 135–373) contains the AAA* motifs; and C-terminal domain IV (amino acids 374–467) contains a helix-turn-helix module with DNA-binding activity (3, 30, 31). Based on these findings, we propose a model that describes a functional mechanism for the hydrolysis of DnaA-ATP that is promoted by its interaction with the DNA-loaded Hda-clamp complex.

**EXPERIMENTAL PROCEDURES**

**Overexpression and Purification of the His-Hda Protein**—The Hda fragment was prepared by PCR using a pACYC184 derivative bearing wild-type hda as a template and primers 5'-CCAGGCTTCGGCATCGCAATAAACCC-3' and 5'-GGCTCAAGGATGCGAAACCTTG-3' and ligated to XhoI-digested pBAD18 (32). To enhance Hda expression, the XbaI-EcoRI region containing the original Shine-Dalgarno sequence was replaced with a primer-amplified fragment, 5'-CTAGAAGGATACATGTGAACTT-3' and ligated to XhoI-digested pBAD18 (32). The resulting plasmid was termed pBAD/Hda. Purification of His-Hda was performed using a Ni-NTA-agarose column (QIAGEN Inc.) pre-equilibrated with buffer A containing 10 mM imidazole. The column was washed with 10 column volumes of buffer A containing 20 mM imidazole and 5 column volumes of buffer A containing 50 mM imidazole. His-Hda was then eluted with buffer A containing 300 mM imidazole. The purity of the His-Hda thus obtained was >90% as judged by SDS-PAGE and Coomassie Brilliant Blue staining.

**Construction and Purification of His-Hda**—His-Hda was constructed by XhoI digestion and self-ligation of a PCR fragment amplifying pBAD/Hda as a template and primer 5'-CTCGAGCTCGGATCC-3' and 5'-GGCTCAAGGATGCGAAACCTTG-3' and ligated to XhoI-digested pBAD18 (32). To enhance Hda expression, the XbaI-EcoRI region containing the original Shine-Dalgarno sequence was replaced with a primer-amplified fragment, 5'-CTAGAAGGATACATGTGAACTT-3' and ligated to XhoI-digested pBAD18 (32). The resulting plasmid was termed pBAD/Hda. Purification of His-Hda was performed using a Ni-NTA-agarose column (QIAGEN Inc.) pre-equilibrated with buffer A containing 10 mM imidazole. The column was washed with 10 column volumes of buffer A containing 20 mM imidazole and 5 column volumes of buffer A containing 50 mM imidazole. His-Hda was then eluted with buffer A containing 300 mM imidazole. The purity of His-Hda thus obtained was >90% as judged by SDS-PAGE and Coomassie Brilliant Blue staining.

**Purification of Native Hda Protein as a Molecular Size Marker**—The Hda fragment was prepared by PCR using a pACYC184 derivative bearing wild-type hda as a template and primers 5'-CCAGGCTTCGGCATCGCAATAAACCC-3' and 5'-GGCTCAAGGATGCGAAACCTTG-3' and ligated to XhoI-digested pBAD18 (32). To enhance Hda expression, the XbaI-EcoRI region containing the original Shine-Dalgarno sequence was replaced with a primer-amplified fragment, 5'-CTAGAAGGATACATGTGAACTT-3' and ligated to XhoI-digested pBAD18 (32). The resulting plasmid was termed pBAD/Hda. Purification of Hda was performed using a Ni-NTA-agarose column (QIAGEN Inc.) pre-equilibrated with buffer A containing 10 mM imidazole. The column was washed with 10 column volumes of buffer A containing 20 mM imidazole and 5 column volumes of buffer A containing 50 mM imidazole. Hda was then eluted with buffer A containing 300 mM imidazole. The purity of the Hda obtained was >90% as judged by SDS-PAGE and Coomassie Brilliant Blue staining.

**Reconstitution of a RIDA System Coupled with Clamp Loading**—The RIDA reaction was performed essentially as described previously (12, 15–17). Briefly, [32P]ATP-bound DnaA (1 pmol) was incubated for 20 min at 30 °C in buffer (25 μl) containing 40 μg of the HEPES-KOH (pH 7.6), 10 mM magnesium acetate, 7% (w/v) polyvinyl alcohol, and 2 mM dithiothreitol). After centrifugation, and the proteins in the fractions were loaded onto a nickel-nitrilotriacetic acid (Ni-NTA)-agarose column (QIAGEN Inc.) and washed with buffer A containing 20 mM imidazole and 50 mM potassium phosphate. The column was then eluted with buffer A containing 300 mM imidazole. The purity of the Hda thus obtained was >90% as judged by SDS-PAGE and Coomassie Brilliant Blue staining.

**Construction and Purification of His-HdaN and Mutant Hda Proteins**—His-HdaN contains amino acids 27–248 of Hda (248 residues). A His-HdaN-overproducing plasmid (pBAD/His-HdaN) was constructed by XhoI digestion and ligation of the fragment containing the His-Hda into the XbaI-digested pBAD18 (32). To enhance Hda expression, the XhoI-HindIII-digested pBAD18 (32). The resulting plasmid was termed pBAD/Hda. Purification of His-Hda was performed using a Ni-NTA-agarose column (QIAGEN Inc.) pre-equilibrated with buffer A containing 10 mM imidazole. The column was washed with 10 column volumes of buffer A containing 20 mM imidazole and 5 column volumes of buffer A containing 50 mM imidazole. His-Hda was then eluted with buffer A containing 300 mM imidazole. The purity of the His-Hda obtained was >90% as judged by SDS-PAGE and Coomassie Brilliant Blue staining.
Interactions in Hda-Clamp Formation and DnaA-ATP Hydrolysis

with buffer C (25). In the second stage, the isolated DNA-loaded clamps (the indicated amounts or 50 fmol of β-subunit dimers loaded onto 0.47 fmol of φX174 circular DNA) were incubated for 20 min at 30 °C in buffer D (25 µl) containing 120 mM potassium glutamate, 0.1% Triton X-100, and 0.1% dimethyl suberimidate (Pierce) and analyzed by SDS-15% PAGE and silver staining. The nucleotides bound to DnaA protein were recovered on a nitrilotriacetic acid filter and analyzed by polyethyleneimine thin-layer chromatography using a Fuji Bioimage BAS2500 analyzer as described previously (12, 25).

**Gel Filtration Analysis**—Gel filtration analyses were performed at 4 °C using a SMART column (bed volume of 2.4 ml) of Superose 12 or Superdex 200 (Amersham Biosciences) equilibrated with buffer D (20 mM Tris-HCl (pH 7.5), 10% glycerol, 150 mM NaCl, 10 mM 2-mercaptoethanol, and 0.1% Triton X-100). The indicated proteins were loaded and eluted at a flow rate of 40 µl/min for Superose 12 or 5 µl/min for Superdex 200. Proteins in collected fractions were analyzed by SDS-PAGE (15 or 12%) and Coomassie Brilliant Blue staining. The nucleotide binding assay and DNA-depending intrinsic ATPase assay were performed as described previously (17, 36), except that cells were incubated at 30 °C for 20 min in buffer A (10.5 µl) before gel filtration. The marker proteins used were apoferritin (443 kDa), β-amylose (200 kDa), γ-globulin (160 kDa), bovine serum albumin (66 kDa), chicken ovalbumin (45 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa).

**Chemical Cross-linking Analysis**—His-Hda protein (0.5 µg) was incubated at 30 °C for 1 h in buffer D (25 µl) containing 2 mM dimethyl suberimidate (Pierce) and analyzed by SDS-15% PAGE and silver staining or immunoblotting. Quantitative Immunoblot Analysis of the Intracellular Hda Content—C600 or MG1655 cells were grown at 37 °C in LB medium and harvested at A600 = 0.5. Cells were immediately suspended in chilled 10% trichloroacetic acid and subjected to immunoblot analysis as described previously (33, 34). An anti-Hda antibody was purified from rabbit anti-Hda* antisera by affinity column chromatography using His-Hda-coupled CNBr-Sepharose 4 (Amersham Biosciences) prepared according to the manufacturer's instructions.

**Ni-NTA Pull-down Assay**—His-Hda (5 pmol as dimer) was incubated at 30 °C for 20 min in 25 µl of buffer E (5 mM Tris-HCl (pH 7.5), 10% glycerol, 1 mM potassium glutamate, 0.01% Brij 99, 8 mM 2-mercaptoethanol, 8 mM magnesium acetate) containing the indicated amounts of the clamp. Reactions were further incubated on ice for 1 h and inverted at 3-min intervals in the presence of Ni-NTA magnetic agarose beads (25 µl of a 2% suspension; QIAGEN Inc.) equilibrated with buffer E containing 20 mM imidazole. The beads and bound materials were collected by magnetic force and resuspended in buffer E (50 µl) containing 50 mM imidazole. This procedure was repeated three times. Proteins were eluted from the beads in buffer E containing 150 mM imidazole and analyzed by SDS-12% PAGE and silver staining.

**Construction and Purification of a Protein Consisting of DnaA Domains III and IV (DAD-III–IV)**—DAD-III–IV consists of amino acids 130–467 of DnaA. A DAD-III–IV-coding fragment was prepared by PCR using a template and primers 5′-GGCGCTACCCGAGTAAACGTCAAACAC-3′ and 5′-CCGAGTTCTTCTTACGGCATGACATGGTCTCGATT-3′ and ligated to NheI-EcoRI-digested pBAD/His-B. The protein was overexpressed and purified by the same method for DnaA protein as described previously (17, 36), except that cells were incubated for 4 h in the presence of the inducer, and 0.28 g/ml ammonium sulfate was used for protein precipitation in cleared cell lysates. The purity of DAD-III–IV was >90% as judged by SDS-PAGE and Coomasie Brilliant Blue staining. The nucleotide binding assay and DNA-dependent intrinsic ATPase assay were performed as described previously (17, 36).

**RESULTS**

**Purification of Hda and Homodimer Formation**—To analyze Hda protein function quantitatively, we constructed an N-terminally hexahistidine-tagged Hda protein (His-Hda). As even slight overexpression of Hda is toxic to wild-type cells, the protein was overexpressed in a host strain bearing dnaAΔoriC mutations and a vector bearing the arabinoose-inducible promoter, which we used for over-initiating DnaA mutants (17). Affinity purification on a Ni-NTA column yielded highly purified His-Hda (Fig. 2A).

In a reconstituted RIDA system (12, 15–17), purified His-Hda efficiently promoted DnaA-ATP hydrolysis (Fig. 2B). We assessed His-Hda activity by comparing it with that of native Hda in a partially purified fraction prepared from a wild-type hda strain. This fraction, previously designated as Idab fraction V, complements Hda activity in a RIDA reaction using DNA-loaded clamps (12, 16). The Hda present in this fraction was quantified by immunoblotting, and its specific activity for DnaA-ATP hydrolysis was assessed. The results indicate that the activity of purified His-Hda was comparable with or even

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higher than that of native Hda in IdaB fraction V (Fig. 2, B and C). When IdaB fraction V was added to a reaction containing His-Hda, no significant inhibition was seen (data not shown). During the course of these experiments, we noticed that Hda activity was considerably reduced by the freeze-thawing procedure in the absence of Triton X-100. The buffer for His-Hda, but not for IdaB fraction V, contained this detergent.

The specific activity of His-Hda was ~15-fold higher than that of Hda* in a reconstituted RIDA system as described above, which is consistent with a previous speculation (16). Intrinsic ATPase activity was not detected for His-Hda.

We next asked whether His-Hda forms an oligomeric structure, as some AAA + proteins are known to form homomultimers (21, 26, 29). Gel filtration analysis showed that Hsd-Hda eluted at a position consistent with a size range of ~50–70 kDa (Fig. 2D), suggesting that it forms a homodimer in its native state since the mass of the His-Hda monomer is calculated to be 32 kDa. A similar elution pattern was seen when His-Hda was incubated and subjected to gel filtration in the presence of 2 mM MgATP. Further analysis using a cross-linking agent showed the formation of a 65-kDa product, confirming that Hda forms a homodimer (Fig. 2E).

**Cellular Concentration of the Hda Dimer**—We determined cellular Hda levels in exponentially growing C600 or MG1655 cells by immunoblot analysis (Fig. 3 and Table I). The specificity of the anti-Hda antibody was confirmed by the absence of a corresponding signal when an hda-deleted strain was used. The amount of Hda was then deduced using purified Hda protein as a quantitative standard (Fig. 3). The results indicate that the cellular level of the Hda dimer is ~50/cell (Table I), a level significantly lower than that of DnaA protein (~500–2000 molecules/cell) (33, 37–39) or of the clamp (~400–5000 molecules/cell) (34, 40).

**Hda-Clamp Complex Formation Is Required for DnaA-ATP Hydrolysis**—Using a pull-down assay with Hda*, we found that the cellular level of the Hda dimer is ~50/cell (Table I), a level significantly lower than that of DnaA protein (~500–2000 molecules/cell) (33, 37–39) or of the clamp (~400–5000 molecules/cell) (34, 40).

**TABLE I**

| Strain | No. of cells/ml of C600 | No. of Hda dimers/cell |
|--------|-------------------------|------------------------|
| C600   | 3.4 × 10^8              | 60 ± 5                 |
| MG1655 | 4.0 × 10^8              | 45 ± 5                 |

The quantitative standard used was the indicated amounts of purified native Hda. To normalize the amounts of background proteins, Hda was included in a whole cell extract prepared from a C600 derivative bearing rnhA17::Tn3 dnaA::KmR Δhda::CmR (A and B) or a MG1655 derivative bearing rnhA17::Tn3 Δhda::KmR (C and D). These strains were grown in the same way as the C600 and MG1655 cells, and the same amounts of these cultures as of the wild-type strains were used for immunoblot analyses. The levels of Hda were determined by densitometric scanning (relative intensity).
FIG. 4. Role for the N-terminal domain of Hda in β-clamp binding and in RIDA. A, to assess β-clamp-binding activity, wild-type His-Hda (WT) or His-HdaΔN (ΔN) (5 pmol as dimer) was incubated for 20 min at 30 °C in buffer (25 μl) containing the indicated amounts of the β-clamp. His-tagged proteins and bound protein were isolated using Ni-NTA magnetic beads, eluted in 150 mM imidazole, and analyzed by SDS-12% PAGE and silver staining. The migration positions of proteins are indicated by arrowheads. —, Hda was not included in the reaction. B and C, the DnaA-ATP hydrolysis activities of His-Hda (●) and His-HdaΔN (○) were assessed in a staged RIDA reaction (see “Experimental Procedures”). In the first stage, the ΔX174 replicative form II-loaded β-clamp was isolated by gel filtration, and the isolated β-clamps were quantified by SDS-12% PAGE and silver staining. In the experiments shown in B, in the second stage, the indicated amounts of Hda were incubated for 20 min at 30 °C in buffer (25 μl) containing [α-32P]ATP-bound DnaA (0.5 pmol) and the DNA-loaded form of the β-clamp (50 fmol as β-dimer). In the experiments shown in C, in the second stage, the indicated amounts of the DNA-loaded form of the β-clamp were incubated in buffer containing [α-32P]ATP-bound DnaA (0.5 pmol) and His-Hda (0.62 pmol as dimer). D, His-HdaΔN (7 μg) was analyzed by Superose 12 PC 3.2/30 gel filtration (column bed volume of 2.4 ml) as described for Fig. 2D. Vo, void volume. E, His-HdaΔN (0.5 μg) was incubated for 1 h at 30 °C in buffer (25 μl) with (+) or without (−) 2 mM dimethyl suberimidate (DMS) and analyzed by SDS-15% PAGE and Coomassie Brilliant Blue staining. The migration positions corresponding to the His-HdaΔN monomer (29 kDa) and homodimer (59 kDa) are indicated.

Roles for the Hda Arginine Finger in DnaA-ATP Hydrolysis—In some AAA+ proteins that adopt a ring-like configuration by multimerization, ATP is located at the interface between two neighboring subunits (29). In some AAA+ proteins that have been well analyzed, an arginine residue in the AAA+ Box VII motif of one subunit reaches an ATP bound to a neighboring subunit and is proposed to participate directly in ATP hydrolysis (41–44). This residue, widely conserved among AAA+ proteins, is called the arginine finger. We asked whether DnaA-ATP hydrolysis is catalyzed in an Hda arginine finger-dependent manner. A comparison of sequences suggested that Hda Arg<sup>168</sup> is an arginine finger (Protein ID 1788842 in Ref. 26) (Fig. 1). This residue is highly conserved in putative Hda orthologs in other bacterial species. When we constructed a homology model of Hda based on the tertiary structure of the AAA+ domain of the Cdc6/Orc1 protein of the archaeabacterium Pyrobaculum aerophilum (17, 45), we found that Hda Arg<sup>168</sup> is exposed on the protein surface.

We constructed and purified Hda R168A and Hda R168M. Both of these mutant proteins were inactive for DnaA-ATP hydrolysis in the staged RIDA reaction (Fig. 7, A and B). Gel filtration analysis suggested that like wild-type Hda, Hda R168M forms a homodimer in solution (Fig. 2D), consistent with the results of chemical cross-linking analysis (data not shown). A pull-down assay revealed that Hda R168M bound to the clamp with an affinity similar to that of wild-type Hda (Fig. 7C). These results indicate that Hda Arg<sup>168</sup> plays a specific role in DnaA-ATP hydrolysis.

Gel filtration analysis showed that Hda R168A eluted with a size greater than that of the Hda dimer, indicating that Hda R168A molecules form homomultimers (Fig. 2D). These results suggest that Hda Arg<sup>168</sup> plays a role in controlling Hda intermolecular interactions. In contrast, the clamp-binding activity of Hda R168A was intact (Fig. 7C).

Role for the DnaA N-terminal Domain in RIDA—We assessed DnaA substructures required for the RIDA reaction. DnaA consists of four functional domains: domains I and II (residues 1–134) mediate protein interactions with DnaB, DnaA itself, and DiaA, a novel factor that coordinates initiation with the cell cycle (46); domain III (residues 135–373) contains the AAA+ motifs that are implicated in ATP binding and hydrolysis; and domain IV (residues 374–467) mediates direct
FIG. 5. Role for the Hda β-clamp-binding motif in RIDA. A, to assess β-clamp-binding activity, wild-type (WT) and mutant (Q21A and L24A) His-Hda proteins (5 pmol each as dimer) were incubated for 20 min at 30 °C in buffer (25 μl) containing the indicated amounts of the β-clamp. Proteins were isolated using Ni-NTA magnetic beads and analyzed as described for Fig. 4A. The migration positions of proteins are indicated by arrows. B and C, the DnaA-ATP hydrolysis activities of His-Hda (○), His-Hda Q21A (●), and His-Hda L24A (□) were analyzed using a staged RIDA reaction as described for Fig. 4 (B and C). In the experiments shown in B, the indicated amounts of the DNA-loaded form of the β-clamp were incubated for 20 min at 30 °C in buffer (25 μl) containing [α-32P]ATP-bound DnaA (0.5 pmol) and wild-type or mutant His-Hda (0.62 pmol as dimer). As the experiments shown in C, the indicated amounts of wild-type or mutant His-Hda were similarly incubated in buffer containing [α-32P]ATP-bound DnaA (0.5 pmol) and the DNA-loaded form of the β-clamp (50 pmol as β-dimer).

In this study, we analyzed the mechanism of RIDA using a highly functional form of Hda, DnaA, and mutant derivatives of both proteins. We found that Hda forms a stable homodimer in solution and that specific N-terminal residues and the AAA + Box VII motifs are required for RIDA activity. The N terminus is required for clamp binding, and Arg168 in AAA + Box VII plays a role in DnaA-ATP hydrolysis and in the maintenance of the homodimer form. Moreover, we revealed a novel role for the DnaA N-terminal region in the functional interaction with the Hda-clamp complex. We also determined the cellular levels of Hda. These data are highly relevant to understanding the mechanism of RIDA.

An activity that promotes DnaA-ATP hydrolysis in a DNA-loaded clamp-dependent manner was previously seen in a partially purified fraction termed IdaB (12). Extracts prepared from an hda-deleted strain do not exhibit RIDA activity, and this defect is complemented by supplying Hda* (16). Also, Hda* can replace IdaB with respect to RIDA activity in a reconstituted system (16). In the present study, we observed, by immunoblot analysis, that the IdaB fraction contains Hda at a level that can function in an in vitro RIDA system (Fig. 2).

A single cell contains ~50 Hda dimers (Fig. 3 and Table I) and 500–2000 DnaA molecules (33, 37–39). In experiments using synchronized cultures of dnaC2 mutant cells, ~80% of the DnaA molecules were shown to be in the ATP form before replication initiation, and as a result of RIDA, this level decreased to ~40% within 20 min after initiation (13). These observations suggest that Hda molecules are recycled to promote DnaA-ATP hydrolysis catalytically. In a reconstituted in vitro RIDA system, a single Hda dimer could promote the isomerization of five DnaA-ATP molecules for 20 min at 30 °C (Fig. 2B). An intermediate containing the Hda-clamp complex plus DnaA is likely very unstable, as such a ternary complex could not be detected with the pull-down assay. These observations are consistent with the above idea.

Our previous immunoblot analysis indicated that ~5000 β-clamps are present in a single cell (34). There might be a system that facilitates the specific loading of Hda onto DNA-loaded clamps, as the number of cellular Hda molecules is much lower. Alternatively, a large proportion of the clamps might be loaded onto chromosomal DNA during replication. This hypothesis is based on the observation that the majority of the clamp molecules are at specific sites in the nucleoid during chromosome replication in vivo, and clamp foci are speculated to be replication fork sites (49).

We have shown that a direct interaction between Hda and the clamp is a prerequisite for RIDA (Figs. 4 and 5). We previously observed a direct interaction between these proteins using Hda* (25). In the present study, we used derivatives of the highly functional His-Hda protein to reveal a requirement for direct binding between the two proteins. Hda L24A showed slight activity for DnaA-ATP hydrolysis when added in excess to the RIDA reaction (Fig. 5C). This can be simply attributed to the reduced affinity of Hda L24A for the clamp (Fig. 5A). No
significant activity for DnaA-ATP hydrolysis was detected with Hda Q21A, although residual affinity for the clamp was also detectable (Fig. 5). A conceivable idea to explain this is that the Hda Q21A-clamp complex takes on an abnormal conformation that inhibits the functional interaction with ATP-bound DnaA.

The clamp-binding motif is conserved among several proteins, including the pol III α-subunit, pol IV, and MutS (27). In a co-crystal consisting of the clamp bound to pol IV little finger domain, this motif can be seen to interact hydrophobically with a specific site in the C-terminal domain of the clamp (50). The Hda-clamp complex could be stably maintained in buffer containing 1 M NaCl (data not shown), consistent with a hydrophobic interaction. The pol III core complex contains the α-subunit, and this subunit binds to the C-terminal hydrophobic region of the clamp via its clamp-binding motif (24). Thus, a reasonable idea is that Hda binds to pol III core-free clamps that remain on the lagging strand during replication. Kinetic and quantitative affinity analyses regarding these processes are under investigation. During the preparation of this manuscript, Kurz et al. (51) reported experiments with mutant Hda* derivatives showing that the Hda N-terminal motif is required for clamp binding.

Inasmuch as the clamp interacts with several factors, including the DNA repair factors pol IV and MutS as mentioned above, Hda might dissociate from the clamp by the competitive binding of those factors after DnaA-ATP hydrolysis. Such competitive usage of the clamp among the clamp-binding proteins has already been proposed (24). Indeed, our experiments indicated that the Hda-clamp complex is used by the γ-complex for clamp loading at an efficiency similar to the case of the Hda-free clamp. During this loading process, the γ-complex dissociates Hda from the clamp.2 These observations are consistent with the idea of competitive usage of the clamp. Further experiments regarding such protein dynamics are in progress.

DnaA and Hda are members of the AAA⁺ protein family (16). Recent progress in x-ray crystallography analysis has revealed the structure of several AAA⁺ proteins such as the N-ethylmaleimide-sensitive factor, p97, RuvB, HslU, and the γ-complex (52–56). A common feature among these is the formation of a multimer consisting of five to six monomers and the presence of an arginine residue on an ATP-interacting interface that is formed by two neighboring subunits (21, 29). Biochemical analysis of RuvB and the pol III γ-complex indicated that this arginine finger motif plays a role in ATP hydrolysis (42, 44). As Hda Arg168 was required for DnaA-ATP hydrolysis in RIDA (Fig. 7), this residue may directly interact with DnaA-ATP (Fig. 9A).

As HdaΔN forms a homodimer, the Hda AAA⁺ domain plays a major role in homodimer formation (Fig. 4). In ring-like complexes containing 5- or 6-mers of the AAA⁺ proteins mentioned above, each protomer interacts in a head-to-tail manner (21, 29). Unlike wild-type Hda and Hda R168M, Hda R168A did not form a stable homodimer, but instead formed higher order multimers (Fig. 2D). These observations can be explained by the idea that Arg168 is exposed on the surface of Hda and that Hda monomers interact in a head-to-tail manner to form a homodimer via the AAA⁺ domains (Fig. 9B). Either a subunit that accepts the arginine finger from another subunit or one that offers it to another subunit can undergo a conformational change that prevents further multimerization. As the replacement of arginine with methionine, but not alanine, did not affect homodimer formation (Fig. 2D), the size of the side chain may be important for this conformational change.

Gel filtration experiments suggested that the Hda-clamp complex contains a single clamp molecule (β-subunit dimer) and two Hda dimers (Fig. 6), although a complex containing only a single Hda dimer bound to a clamp molecule could also be observed in binding reactions containing reduced amounts of Hda (data not shown). As each monomer of the β-dimer has a C-terminal hydrophobic pocket, this binding stoichiometry is reasonable. Hda does not have significant affinity for DNA. Structural analysis of a co-crystal of the clamp and the pol VI little finger domain bearing the clamp-binding motif showed that the two molecules of the little finger domain are symmetrically positioned on the single clamp (50).

The γ-complex consists of single molecules of the 37-kDa δ-subunit and the 39-kDa δ'-subunit and three molecules of the 48-kDa γ-subunit (21). This complex most likely adopts a C-like

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2 H. Kawakami and T. Katayama, unpublished data.
without (by a filter binding assay as described previously (17, 36). The \[\text{ATP}^\text{32P}\] loaded form of the H9252 might be assessed in a staged RIDA reaction as described for Fig. 4 (wild-type DnaA and DAD III–IV (0.5 pmol each) were incubated for the indicated times at 30 °C in buffer (25 μl) with (+) or without (−) dX174 replicative form II DNA (4.3 fmol as circle). The hydrolysis of bound ATP was monitored by thin-layer chromatography as described for the RIDA reaction. D and E, the RIDA sensitivities of wild-type DnaA and DAD III–IV (0.5 pmol each) were assessed in a staged RIDA reaction as described for Fig. 4 (B and C). In the experiments shown in D, the indicated amounts of His-Hda were incubated for 20 min at 30 °C in buffer (25 μl) containing \[\text{ATP}^\text{32P}\] bound DnaA or \[\text{ATP}^\text{32P}\] bound DAD III–IV (0.5 pmol each) in the presence (+) or absence (−) of the DNA-loaded form of the β-clamp (50 fmol as β-dimer). In the experiments shown in E, the indicated amounts of the DNA-loaded form of the β-clamp were incubated in buffer containing \[\text{ATP}^\text{32P}\] bound DnaA or \[\text{ATP}^\text{32P}\] bound DAD III–IV (0.5 pmol each) in the presence (+) or absence (−) of His-Hda (0.62 pmol as dimer).

shape and binds along the clamp ring, as observed for the eukaryotic clamp-loader complex (20). From the viewpoint of molecular size, the idea that one or two DnaA molecules (52 kDa as monomer) interact with an Hda-clamp complex containing one or two Hda dimers (29 kDa as monomer) cannot be excluded. We previously indicated that at least 40 bp of double-stranded DNA loaded with a single clamp is required for Hda-dependent DnaA-ATP hydrolysis and suggested that DnaA interacts with sequences flanking the clamp via its nonspecific DNA-binding activity (25). Taking this prerequisite into consideration, we propose a model that a single DnaA molecule interacts with the Hda-clamp complex during the RIDA reaction (Fig. 9A).

We have revealed that DnaA domains I and II are required for RIDA (Fig. 8). Previously, we found that DnaA R334A, in which an arginine residue in the AAA" domain is replaced, is specifically defective in DnaA-ATP hydrolysis during RIDA (17). DnaA R334A is active for ATP/ADP binding at a level similar to that of wild-type DnaA, and ATP form-dependent initiation activity is also observed for this mutant DnaA. DNA-binding activity is associated with DnaA domain IV, and DnaA-DNA interaction is presumably a prerequisite for the RIDA reaction (25). Thus, we suggest that all DnaA domains (I–II, III, and IV) play unique roles in the RIDA process. DnaA domains I and II function in the intermolecular interaction of proteins (3, 30, 46–48). In this context, DnaA domains I and II are speculated to interact directly with a specific site in the Hda-clamp complex.

Finally, we have presented a model for a RIDA reaction intermediate complex (Fig. 9A). One or two Hda dimers bind to a single DNA-loaded clamp via direct binding between the Hda N-terminal domain and its acceptor hydrophobic pocket on the clamp molecule. A single DnaA-ATP molecule transiently interacts with the Hda-clamp complex. The DnaA N-terminal domain then acts to mediate intermolecular interactions, and DnaA domain IV interacts with DNA flanking the clamp. These interactions support a functional association between Hda and the DnaA AAA" domain, domain III. A plausible hypothesis is that the affinity between the AAA" domains of Hda and DnaA is intrinsically weak and that DnaA domains I–II and IV position the proteins to promote functional AAA" domain interactions. The arginine finger of one monomer of the Hda dimer

![Fig. 9. Model for Hda dimerization and DnaA-ATP hydrolysis in RIDA. A, model for a RIDA reaction intermediate. One or two Hda dimers form a stable complex with the clamp that remains on DNA after completion of an Okazaki fragment. Only one Hda dimer is shown for simplicity. The Hda N terminus (N-terminus) directly binds to the hydrophobic pocket of the clamp. ATP-bound DnaA interacts with this complex in a manner depending on interaction with double-stranded DNA (dsDNA) flanking the β-clamp. The DnaA N terminus (domains I and II) also plays a role in supporting the DnaA-Hda interaction. This DnaA region may interact with the clamp, Hda, or both. For simplicity, the DnaA N terminus is shown as interacting with the clamp. The Hda AAA' domain (AAA+) interacts with the DnaA AAA" domain (domain III), leading to the formation of an ATP hydrolysis catalytic center in which Hda Arg168 (the Box VII motif arginine finger) and DnaA R334A (the Box VIII motif Sensor-2 arginine) participate. B, model for Hda dimerization. Two Hda monomers form a head-to-tail complex. Inter-molecular interactions induce a structural change in one Hda subunit, thereby preventing polymerization. Hda Arg168 plays a role in this interaction.](image-url)
to promote DnaA-ATP hydrolysis in a concerted manner with DnaA Arg334. After DnaA-ATP hydrolysis, the resulting DnaA-ADP is released from the Hda-clamp complex by Brownian movement. This dissociation might be stimulated by a conformational change of DnaA that might be provoked by the energy released from ATP hydrolysis. The hydrolysis of ATP bound to the clamp-loader γ-complex is required for dissociation of the clamp-loader complex from the clamp loaded onto the primed DNA (20, 21).

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