Escherichia coli PriA Protein, Two Modes of DNA Binding and Activation of ATP Hydrolysis

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Escherichia coli PriA protein plays crucial roles in processing of arrested replication forks. PriA serves as a sensor/stabilizer for an arrested replication fork and eventually promotes restart of DNA replication through assembly of a primosome. PriA carries a 3’ terminus binding pocket required for its high affinity binding to a specific arrested fork as well as for its biological functions. We show here that PriA binds to DNA in a manner either dependent on or independent of 3’ terminus recognition. The former mode of binding requires the 3’ terminus binding pocket present at the N-terminal half of the 181-residue DNA binding domain and exhibits specific bipartite interaction on the template DNA. The latter mode is independent of the pocket function, but requires the C-terminal half of the same domain. ATP hydrolysis activity of PriA can be stimulated in vitro by either of the two binding modes. We propose architecture of PriA bound to various arrested replication fork structures and discuss its implication in helicase activation and ATP hydrolysis.

The DNA chain elongation process of chromosome replication is prone to various challenges that may stall or delay the progression of replication forks. These include DNA damages, DNA-binding proteins interfering with the moving replication forks, shortage of nucleotide precursors, and secondary structures of DNA template. Cells need to cope with these situations to successfully complete the replication of the entire chromosomes. The first step of the cellular responses to the replication fork block is the “sensing” of the arrested replication fork (1–3). Genetic analyses in yeasts have implicated a set of proteins in this process (4, 5), but precise biochemical basis for recognition of arrested replication forks has not been elucidated.

Escherichia coli PriA protein was originally identified as a component essential for conversion of the dX174 single-stranded genome to double-stranded replicative form. PriA binds to n′-pas (n′-primosome assembly site; a single-stranded DNA hairpin structure recognized by PriA protein) and assembles a primosome, a complex responsible for primer RNA synthesis and duplex unwinding at a replication fork (6, 7). Genetic analyses of priA mutants have suggested that PriA plays crucial roles in cellular responses to replication fork arrest. PriA null cells exhibit severe sensitivity to DNA damages including UV and mitomycin C (8, 9), and are defective in RecA-dependent stable DNA replication induced by replication fork blocks (10). Indeed, PriA can specifically recognize and bind to D-loop like and arrested fork structures in vitro (11–13) and establishes a primosome on these structures (14–17). Other biochemical activities of PriA protein include ATP hydrolysis that strictly depends on binding to specific DNAs (7, 18) and 3’ to 5’ DNA helicase actions (19, 20).

PriA is composed of two domains, namely the N-terminal DNA binding domain and the C-terminal DEXH-type helicase domain (21–23). The former contains a 3’ terminus binding pocket that specifically recognizes and binds to a 3′-end of DNA. The presence of this 3′-end binding structure facilitates high affinity and stable binding to D-loop or arrested fork structures carrying a 3′-end at the branch point (24).

PriA is a vital protein for cellular responses to replication fork blocks in bacterial cells, and it may serve as a sensor and stabilizer for the arrested replication forks (25–27). Therefore, it would be very important to dissect the architecture of PriA protein bound to the arrested forks and other substrates to elucidate the mechanism by which this protein recognizes blocked replication forks. It is also an intriguing question, which domain of PriA is required for activation of ATP hydrolysis.

To address these issues, we have conducted detailed analyses of protein-DNA complexes and ATPase activation using various DNA substrates and mutant PriA proteins. The results indicate that the PriA binds to DNA in two distinct modes, either dependent on or independent of the 3′ terminus recognition. In the former, PriA makes a stable, bipartite interaction at the fork junction. The N-terminal DNA binding domain contains a module that may recognize the duplex to single-strand DNA junction, in addition to the 3′ terminus binding pocket. DNA binding through either module can stimulate ATP hydrolysis by PriA, and engagement of the both modules leads to the most stable binding and maximum activation of the ATPase activity.

EXPERIMENTAL PROCEDURES

Proteins—Wild-type or the LFY mutant (L12G, F16G, Y18G) form of the full-length PriA protein and PriA-(1–105), PriA-(1–181), and PriA-(361–541) were expressed in E. coli and purified with N-terminal His tag as described previously (18).
Interaction of PriA with DNA and Activation of ATPase

A

B

C

D

E

F

G

Y-fork 49
(P1 nuclease)

Y-fork

A-fork [3', 5']

A-fork [3']

(A-fork [3'] (Exonuclease III))

A-fork [3']

PriA-(1–732)

PriA-(1–193)

CHAPS
(20 mM)

Interaction of PriA with DNA and Activation of ATPase

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B

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Y-fork 49
(P1 nuclease)

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A-fork [3', 5']

A-fork [3']

(A-fork [3'] (Exonuclease III))

A-fork [3']

PriA-(1–732)

PriA-(1–193)

CHAPS
(20 mM)
(1–181), or PriA-(1–193) N-terminal polypeptides were expressed and purified as previously described (22, 24, 28). The expression vectors for the C-terminal polypeptide, PriA-(194–732) and PriA-(109–181), were constructed by insertion of the PCR fragments amplified from plasmid carrying wild-type PriA sequence (22) using a primer set (sense, 5′-gcaatcatatgggtg-agcgttggcagtaa-3′ and antisense, 5′-ttagacttaccct cat gcgtgtaaca-3′) and sense, 5′-gcaatcatatgcgcggcgaagcgc-3′ and antisense, 5′-attaggatccctaggttctacgcg-3′, respectively) at the NdeI-BamHI site of pET15b (Novagen). The tagged polypeptides were purified through nickel-nitritotriacetic acid-agarose resin, and were digested by thrombin for removal of the His6 segment, followed by further purification through ion exchange column chromatography and gel filtration. Protein concentrations were measured by UV absorbance at 280 nm (with a coefficient of 1.07 or 2.21 for PriA-(194–732) or PriA-(109–181), respectively, at 1 mg/ml) or by the Bradford method using bovine serum albumin as standard.

**DNA Substrates**—Forked or extension substrates were constructed by annealing the oligonucleotides as indicated in supplemental Table S1. Substrates designated [32P]-P carries the leading strand whose 3′-end is phosphorylated. The 5′-end of the labeled oligonucleotide was phosphorylated by T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (GE Healthcare) prior to annealing. Each substrate was isolated from the polyacrylamide gel as described previously (22).

**Nuclease Protection Assay**—Reaction was performed in a mixture (25 µl) containing 40 mM Hepes/KOH (pH 7.6), 8 mM magnesium acetate, 0.5 mM CaCl2, 40 mM potassium glutamate, 20 µg/ml bovine serum albumin, 0.8–3 mM substrate, and 4–64 mM PriA full-length protein as indicated in the figures. For other polypeptides, the concentrations are shown in the figures. To prevent aggregation of the polypeptides in binding assays, 20–30 mM CHAPSO3 was added in the reactions, where indicated. After incubation for 20 min at 30 °C, 0.001 unit of DNase I (TAKARA), 0.04–0.9 units of P1 nuclease (Roche), or 50 units of exonuclease III (New England Biolabs) were added to the reaction and incubation was continued for 1 min, followed by addition of an equal volume of 2× stop solution containing 20 mM EDTA, 1% SDS, 200 mM NaCl, and 125 µg/ml yeast tRNA. Digested fragments were extracted by phenol and recovered by ethanol precipitation before being resolved in 80% formamide.

The abbreviations used are: CHAPSO, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; A-fork, arrested fork-like structure; AFM, A-fork [3′] mimic.

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**RESULTS**

**Nuclease Protection Analyses on the Interaction of PriA with DNA—**Complex formation of PriA with arrested fork-like DNA structures (A-fork) can be detected by gel shift assays (11.

After heating at 96 °C for 5 min, each sample was applied to 10 or 12% denaturing polyacrylamide sequencing gels (40 cm) containing 8 m urea. Electrophoresis was conducted in 0.5 × Tris borate EDTA at 40 W constant electric power for 1 h. Digestion patterns were visualized by autoradiography.

**ATPase Assay**—ATP hydrolyzing activity of the wild-type or mutant PriA protein, N- or C-terminal polypeptide was measured by thin layer chromatography as previously described (22). Reactions were performed in a 12.5-µl mixture containing 40 mM Hepes/KOH (pH 7.6), 8 mM magnesium acetate, 40 mM potassium glutamate, 1 mM dithiothreitol, 10% glycerol, and effector DNA. Protein titration was performed as indicated in the figures. Each reaction was started by addition of 0.2 mM ATP with 0.5 µCi of [γ-32P]ATP and incubation was conducted at 30 °C with or without 4 mM effector DNAs. After a 20-min incubation, 1-µl samples were withdrawn and spotted on the polyethyleneimine cellulose thin layer chromatography plate (Merck). Dried spots were developed by 0.5 M LiCl and 1 M formic acid. Each plate was autoradiographed. Free [32P]phosphate was quantified by Fuji Photo Film Multi Gauge (version 3.0) software and values of hydrolyzed ATP were plotted against protein concentrations.

**BIAcore Analyses of DNA Binding**—BIAcore analyses were conducted as described previously (24). The amount of the immobilized oligonucleotide is indicated in the figure as resonance units. Sequence of the oligonucleotide for A-fork [3′] mimic (AFM) is listed in supplemental Table S1. For AFM [3′]-P, the 3′-end of the oligonucleotide was phosphorylated as described above. The dissociation constants were determined by a global fitting method using the BIA evaluation software 3.1.

**Gel Shift Assay**—Reactions were conducted as described previously (22). Gels were dried and autoradiographed. DNA binding activity of PriA-(109–181) was assayed by mixing the polypeptide (at 50 µM) with a substrate DNA (at 10 µM). After incubation for 10 min at room temperature, glycerol was added at a final concentration of 5% and samples were directly applied to 0.7% agarose gel, followed by electrophoresis with 50 V constant voltage for 40 min at 4 °C in 1× Tris borate buffer. Gel was stained with ethidium bromide and photographed under UV illuminator.

**RESULTS**

**Nuclease Protection Analyses on the Interaction of PriA with Arrested Fork Structures**—Revealed Bipartite Interaction of PriA with DNA—Complex formation of PriA with arrested fork-like DNA structures (A-fork) can be detected by gel shift assays (11,
12, 23, 24). The synthetic DNA substrates used in this study are summarized in supplemental Table 1. The preferred substrate of PriA is the A-fork with a nascent leading strand (A-fork [3']) (12, 24). It also binds to A-fork with both leading and lagging strands (A-fork [3', 5']) with high affinity, whereas it binds to A-fork [5'] carrying a nascent lagging strand with much less affinity (24).

We have conducted DNase I protection assays on A-fork [3'] with the full-length protein, PriA-(1–732), and the N-terminal polypeptide, PriA-(1–193) (Fig. 1B). PriA-(1–193) is composed of the N-terminal 193 amino acids of PriA protein carrying its DNA binding domain but lacking the C-terminal helicase domain, and is capable of recognizing arrested forks or D-loop structures on its own (22). It carries a 3' terminus DNA binding pocket and binds to A-fork [3'], albeit with 1 order of magnitude lower affinity than the full-length PriA (24) (supplemental Fig. S1B). This polypeptide was used to determine the contribution of each domain on binding to the fork structures.

With increasing amounts of full-length PriA protein, two areas were found to be protected; one centered on the branch point (−12 to +10) and the other from −22 to −16 within the unreplicated duplex region. A hypersensitive site was detected at +10 in the leading strand arm (Fig. 1B, lanes 1–8). When PriA-(1–193) was used, weak protection was detected near the branch point through the leading strand arm at lower concentrations (80–160 nM) of the polypeptide, and weak protection near the branch point of A-fork [3', 5'] (Fig. 1D). However, the protection within the unreplicated arm was not as localized as that seen on A-fork [3']. The hypersensitive site detected at +10 on A-fork [3'] was not seen on A-fork [3', 5']. Protection extended over the 13-bp segment in the leading strand arm as more PriA was added. Strong protection was also detected on the lagging strand arm of A-fork [3', 5'] (+7 to +1, solid line; Fig. 1E, lanes 9–16). A second, weak protection (+17 to +8; dashed line) was also detected on this arm of A-fork [3', 5']. In contrast, interaction of PriA with the lagging strand arm on A-fork [3'] was limited. P1 nuclease protection assays on A-fork [3'] showed very weak protection of only 7 nucleotides from the junction (+1 to +7, Fig. 1E, lanes 17–24). On this strand of A-fork [3', 5'], rather strong protection in the unreplicated segment was also detected, and the protected segment extended as more PriA was added (up to −17, Fig. 1E, lanes 9–16). On A-fork [5'], feeble protection was detected on the unreplicated arm near the branch point (−1 to −6), which extended over the larger segment on both the lagging strand and unreplicated arms, when more PriA was added (Fig. 1E, lanes 1–8), consistent with the lower affinity of PriA to this substrate in gel shift assays (12, 24). Y-fork structure, to which PriA binds with 1 order of magnitude lower affinity than to A-fork [3'] in the gel shift assay (data not shown), was only very weakly protected from DNase I digestion on both leading and lagging strand templates (Fig. 1F). Weak periodic protection (alternate appearance of protected and non-protected regions) was detected on the duplex arm as well. We also conducted P1 nuclease footprinting assays on Y-fork 49 to probe interactions on the single-stranded arms more precisely. A 49-mer Y-fork was used to reduce the possibility of a secondary structure in the single-stranded DNA segments. Significant protection was not detected on both arms in this assay (Fig. 1G), indicating that interaction between PriA and single-stranded segments, if any, is weak and maybe transient. These results may indicate that PriA interacts with Y-fork at multiple sites in a rather scattered and transient manner. The bindings of PriA to A-fork [5'] and Y-fork do not involve the 3' terminus and these binding modes

### FIGURE 2. Interactions of PriA with 3'- or 5'-extension structures, partial duplexes carrying a single-stranded tail at one side.

DNase I (A and B) or P1 nuclease (C) protection assays were conducted with the PriA-(1–732) as described under “Experimental Procedures.” Protection by PriA on long (105-mer with 50-bp duplex; A) or short (49-mer with 27-bp duplex; B and C) extensions. The structures of substrates used are schematically drawn at the top of the each panel. The regions protected by PriA are indicated by vertical lines along the panels as well as by gray lines along the schematic drawings of the substrates on top of the panels. The positions of the single to duplex transition are indicated by filled arrowheads. The nucleotides are numbered as described in the legend to Fig. 1. Single-stranded (numbered in minus) and double-stranded (numbered in plus) segments correspond to unprotected and replicated arms of arrested forks, respectively. In both A and B, the 5'-ends of longer (lanes 1–16) or shorter (lanes 17–32) strands were 32P-labeled. In C, the 5'-ends of longer strands were 32P-labeled. Lanes 1, 2, and 9, 10, or lanes 17, 18, and 25, 26 in A and B and lanes 1, 2, and 9, 10 in C represent undigested samples without and with PriA, respectively.
appear to be different from that on fork structures carrying a 3'-terminus in that the former does not give strongly localized protection.

We next examined a set of partially duplex DNA, 5'- and 3'-extension substrates. There have been some discrepancies in binding of PriA to these structures. Initially, McGlynn et al. (11) reported that PriA does not bind to either structure, whereas Nurse et al. (12) reported that PriA binds to the 3'-extension with high affinity but not to 5'-extension. They also showed that the binding affinity increases significantly with the increase of the 3'-extension tails. More recently, Chen et al. (23) reported that PriA binds to both structures albeit with affinity lower than that to fork structures. We prepared two sets of 3'- and 5'-extension substrates (49 and 105 nucleotide long). In our hands, they were bound by PriA with roughly the same affinity in gel shift assays (supplemental Fig. S2). We then conducted DNase I footprinting analyses on these substrates. Specific and localized protection was detected in both duplex and single-stranded segments spanning the 3'-end of the hybridizing DNA on the 105-mer 5'-extension (−10 to +10, Fig. 2A, lanes 1–8). This is likely due to specific recognition of the 3'-end at the single to double strand DNA transition, because block of this 3'-terminus by phosphorylation significantly reduced the affinity of PriA (24). Protection on 3'-extensions (3'-extension 105 and 3'-extension 49) was detected over several residues near the transition (+1 to +10, Fig. 2A, lanes 9–16, and −5 to +6, Fig. 2B, lanes 9–16). No or very little protection was observed at the two 3'-ends of the 3'-extension (Fig. 2, A and B, lanes 9–16 and 25–32) and at the 3'-end of the duplex segment of the 5'-extension structure (Fig. 2, A and B, lanes 17–24), suggesting that PriA does not form a stable complex at the 3'-end of a single-stranded or duplex DNA. Similar protection over the single to double strand junction was detected on the 49-mer 5'-extension structure as well (Fig. 2B, lanes 1–8). We also conducted P1 nuclease footprinting on the 49-mer 5'- and 3'-extension structures. On the 5'-extension, we did not detect any significant protection on the single-stranded segment (Fig. 2C, lanes 1–8), whereas weak protection was detected at positions +3, +5 to +8, +13, and +14 in the single-stranded DNA segment of the 3'-extension (Fig. 2C, lanes 9–16), indicating that PriA interacts more broadly with the single-stranded DNA segment on a 3'-extension. These results suggest that PriA binds to a single to double strand DNA junction in two modes, one that involves the 3'-end recognition (more localized) and the other that is independent of this (less localized and scattered).

**Arrested Fork DNA Activates PriA ATPase Activity through Both 3'-Terminus-dependent and -independent Interactions**—PriA is a DNA-dependent ATPase, and ATP hydrolysis by PriA strictly depends on its binding to DNA (7). We previously reported that a D-loop like structure strongly stimulated ATPase activity of PriA (29). Similarly, A-fork [3'] DNA significantly activated PriA-mediated ATP hydrolysis (Fig. 3, A and C), and the phosphorylation of the 3'-end of the arrested leading strand reduced the level of ATP hydrolysis (Fig. 3, B and D). We also used A-fork [3'] carrying phosphorylated 3'-ends at duplex ends in ATPase assays. On this template as well, phosphorylation of the 3'-end present at the junction (A-fork [3'-P]) reduced the level of ATP hydrolysis with the wild-type PriA, albeit to a lesser extent (Fig. 3D). The L12G,F16G,Y18G mutant PriA (LFY), in which the 3'-terminus binding pocket is specifically disrupted by the amino acid substitutions of the critical residues (24), showed an identical level of ATP hydrolysis with A-fork [3'] or A-fork [3'-P] (Fig. 3C), suggesting that 3'-terminus-independent interaction also contributes to the stimulation of ATP hydrolysis with this effector DNA. The C-terminal
helix domain (PriA-(194–732)) exhibits only a basal ATP hydrolysis activity, whereas the N-terminal fragment, PriA-(1–193), is completely defective in ATP hydrolysis (Fig. 3E), suggesting that the N-terminal DNA binding domain suppresses the basal ATP hydrolysis activity of the C-terminal domain in the absence of effector DNA, as discussed previously (23). Basal helicase activity of the C-terminal helicase domain polypeptide was not further stimulated by the presence of DNA (Fig. 3, A and E, Fig. 4, A and C). Thus, ATP hydrolysis by PriA may be conducted through coordinated conformational changes of the N-terminal DNA binding and C-terminal helicase domains.

**PriA ATPase Activation by Single-stranded DNA Depends on Its Length and 3′ Terminus Recognition**—It has been known that single-stranded DNA without any particular secondary structure can activate ATP hydrolysis by PriA in the absence of single-stranded DNA-binding protein (30). Indeed, a 50-mer single-stranded DNA activated ATP hydrolysis activity of PriA (1–105) to a level about one-fifth of that achieved by A-fork [3′] (Fig. 4A). What would be the basis for this activation? We first examined the effect of the length of the single-stranded oligonucleotide on the level of ATP hydrolysis (Fig. 4B). There was a general tendency that the increase of the oligonucleotide length resulted in increased ATP hydrolysis. Very little ATP hydrolysis was observed with a 20-mer oligonucleotide, whereas the 41- or 61-mer oligonucleotide promoted a significant level of ATP hydrolysis (Fig. 4B). We then examined whether the 3′-end of the single-stranded DNA oligonucleotide plays any role in ATPase activation. The 3′-phosphorylated 50-mer oligonucleotide supported only a significantly reduced level of ATP hydrolysis (Fig. 4, C and D). Furthermore, LFY mutant (24) promoted very little ATP hydrolysis compared with the wild-type even in the presence of a non-phosphorylated 50-mer (Fig. 4D). These results indicate that the activation of PriA ATPase activity by non-structured single-stranded DNA requires a length at least more than 40–50 nucleotides long and is highly stimulated by the recognition of its 3′-end. It should be noted, however, that the low ATP hydrolysis exhibited by LFY still depends on added DNA, because the LFY mutant and wild-type PriA showed only the background level ATPase activity in the absence of DNA (supplemental Fig. S3), suggesting that non-structured DNA can interact with PriA without involving 3′ terminus recognition for activation of ATP hydrolysis.

The N-terminal DNA Binding Segment, PriA-(1–193), May Contain an Additional Domain Recognizing a Feature in the Forked Structure—The ability of A-fork [3′] to activate PriA ATPase activity in the absence of 3′ terminus interaction suggests the presence of an additional domain involved in interaction with structured DNAs (Fig. 3, C and D). Because the N-terminal 193- or 181-amino acid polypeptide can bind to 3′-extension structures that do not carry a 3′ terminus at the single to duplex junction (Fig. 5A and data not shown), the predicted DNA recognition domain should be present on this polypeptide. Note that there is no significant functional difference between PriA-(1–193) and PriA-(1–181) polypeptides (supplemental Fig. S1). It is most likely that this domain spans the segment 106 to 181. We then compared the binding properties of the two polypeptides, namely PriA-(1–105) and PriA-(1–181) in BLAcore assays. As reported previously, binding of PriA-(1–105) and PriA-(1–181) to a 17-mer oligonucleotide with a free 3′-end could be detected in the BLAcore assays (K_D, 20 and 4.9 µM, respectively), although the 3′ phosphorylated 17-mer could not be detected under the same condition (Fig. 5B) (24). This suggests that a 3′-end is the only determinant for the observed binding between the 17-mer and these polypeptides and that the 3′ terminus binding pocket is present on the PriA-(1–105) polypeptide (24, 31). To measure binding of these polypeptides to fork-like structures in BLAcore assays, we then developed a novel ligand, “A-fork [3′] mimic” (AFM; Fig. 5C), which self-generates a secondary structure mimicking A-fork [3′]. In contrast to the 17-mer, the PriA-(1–181) bound to the AFM with 1 order of magnitude higher affinity (K_D, 390 nM, Fig. 5, C and D), and this binding was still detected even when the 3′-end of the ligand was phosphorylated, although the affinity was considerably decreased (K_D, 4.2 µM, Fig. 5, C and D). The PriA-(1–105) polypeptide, on the other hand, did not show significant binding to AFM under the same condition (Fig. 5, C and D).

We also compared the DNA binding of PriA-(1–193) and PriA-(1–105) in gel shift assay. In this assay, we used A-fork [3′]...
The presence of the 106–193 segment specifically facilitates binding to the Y-fork structure.

We then purified a polypeptide derived from PriA-(109–181), and conducted gel shift assays with a 3’-extension or 5’-extension substrate. We observed efficient mobility shift of the DNA substrates in the presence of PriA-(109–181) (supplemental Fig. S4), indicating that the PriA-(109–181) is capable of binding to DNA on its own. These results support the above speculation that PriA-(1–181) contains an additional domain that facilitates the recognition of forked structures or junction from single strand to duplex present on arrested fork structures. It should be noted, however, that the affinity of PriA-(1–105) or PriA-(109–181) to DNA is much lower than that of PriA-(1–181) or PriA-(1–193), suggesting that the coordination of the two DNA binding domains facilitates the binding to a forked structure (see Fig. 5E, and supplemental Figs. S1 and S4).

**DISCUSSION**

*3’ Terminus-dependent Bipartite Interaction of PriA with an Arrested Fork Structure—PriA is a helicase with unique DNA binding specificity.* A number of studies have been conducted on binding and helicase actions of PriA on various DNAs. It binds to structures mimicking D-loop or arrested replication forks in vitro (11–13, 22, 23, 27, 32). It was concluded that PriA binds to fork structures with a single-stranded gap on the lagging strand template and that it efficiently displaces the lagging strand arm fragment (12, 13, 32, 33). It was also suggested that PriA recognizes bent DNA at the fork (12). We reported the presence of a structure-specific DNA binding domain within the N-terminal 181-amino acid segment of PriA as well as involvement of the C-terminal helicase domain in specific and stable interaction of PriA with arrested fork structures (22). We also reported the presence of a 3’ terminus binding pocket within the N-terminal DNA binding domain and showed that it plays a critical role in specific binding of PriA to the arrested fork structures
carrying a hybridizing nascent leading strand as well as in the biological activities of PriA (24).

In this report, we attempted to clarify the modes of binding of PriA on various DNA substrates and their effect on its ATPase activity. Our results of nuclease footprinting/protection analyses indicate that PriA interacts with two separate surfaces of arrested fork structures carrying a nascent leading strand with a 3′-end at the branch point. One protection spanned over the 22-bp segment centering on the branch point (Fig. 1; 12 and 10 bp on the unreplicated arm and leading strand arm, respectively). Similar protection over the single to double strand DNA transition point was detected on a D-loop structure (15). The other protection was detected on the 7-bp segment (−22 to −16) within the unreplicated arm. Consistent with this, exonuclease III protection assays indicated the 3′-extent of the binding of the PriA-(1–732) to A-fork [3′] at +8 and −26 (Fig. 1C). On the other hand, the N-terminal DNA binding domain polypeptide carrying the 3′ terminus binding pocket protected a limited segment over the branch point from DNase I attack and the 3′-extent of the binding was mapped around −4, supporting our proposal that the N-terminal DNA binding domain mainly recognizes the branch point carrying the 3′ terminus. However, protection by the N-terminal DNA binding domain alone is weak and requires concentrations higher than that for the full-length protein. This is consistent with our previous conclusion that the helicase domain plays an important role in high affinity and stable binding of PriA to forked DNAs (22).

FIGURE 6. Modes of binding and helicase actions of PriA protein: possible modes of binding and helicase actions of PriA on various arrested replication fork structures. We propose that PriA binds to various fork structures in distinct modes, which affect its helicase action. The direction of translocation is indicated by the blue arrows. PriA binds to A-fork [3′] through interaction of the 3′ terminus binding pocket with the 3′ terminus (mode I). This interaction directs the position of the helicase domain in one orientation, which is inert for fork unwinding. The presence of a 3′ terminus prevents the binding of PriA in other modes. A-fork [5′] is bound by PriA in two modes, one of which is similar to mode I (mode II) and the other in which the helicase domain is placed on the leading strand arm (mode III), leading to unwinding of the lagging strand arm and unreplicated arm, respectively. PriA binds to A-fork [3′, 5′] in two modes through interaction of the 3′ terminus binding pocket with the 3′ terminus (modes IV and VI). In mode IV, PriA can displace the nascent lagging strand. In mode V, PriA cannot translocate on or unwind the unreplicated or leading strand arm. PriA binds to Y-fork in a mode similar to mode III and unwinds the fork (mode VI). See text for details.

‘3′’ Terminus-independent Mode of PriA Binding to the Arrested Fork Structures—In contrast, on the A-fork [5′], only the 3′ terminus-independent mode of binding occurs. This may be manifested by low affinity and extensive and weak interaction of PriA with the lagging strand template as well as with the unreplicated duplex arm (Fig. 1E). This “loose” interaction leads to unreplicated duplex unwinding as well as to displacement of the nascent lagging strand (27) (Fig. 6, modes II and III). PriA may bind to the 3′-extension in a similar manner, displacing the hybridizing DNA (11, 27).

This 3′ terminus-independent mode of binding also operates when PriA interacts with Y-fork. PriA may interact with the single-stranded arms as well as with the unreplicated duplex arm, but this interaction appears to be very weak judged from the nuclease protection assays (Fig. 1, F and G). The exonuclease III assays show blocks around −5 (on the unreplicated arm), suggesting the interaction at the fork branch point (supplemental Fig. S5 and Fig. 6, mode VI). PriA is able to displace the Y-fork presumably through translocation on the leading strand template (11, 27, 32). Under this condition on the Y-fork, the helicase domain of PriA would be oriented in a direction opposite to that on the A-fork [3′] (Figs. 1, B and F, and 6, compare modes I and VI). This particular mode of binding would also occur with A-fork [5′] (Figs. 1E and 6, mode III), facilitating the displacement of the unreplicated duplex (27, 32), but does not occur with A-fork [3′] or A-fork [3′, 5′], because PriA cannot displace the unreplicated duplex on these substrates (27).
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PriA binds to A-fork [3', 5'] with affinity similar to A-fork [3'], suggesting that the binding is dependent on a 3’ terminus. The protection on the unreplicated arm was less intense and somewhat more extensive protection was observed on the leading strand arm at higher concentrations of PriA in A-fork [3', 5'] than observed in A-fork [3']. The hypersensitive site appearing at +10 on the leading strand arm in A-fork [3'] was not detected on A-fork [3’, 5’] (Fig. 1, B and D), consistent with less localized binding of PriA on the unreplicated arm. In contrast, significant protection was observed on the lagging strand arm on A-fork [3’, 5’] (Fig. 1E). P1 nuclease footprinting on A-fork [3'] indicated very little protection on the lagging strand arm (Fig. 1E), indicating that PriA does not interact with the single-stranded lagging strand arm of A-fork [3’]. These results show that the mode of interaction of PriA with A-fork [3', 5'] is not identical to that with A-fork [3’]. We speculate that PriA interacts with A-fork [3’, 5’] in two different orientations in a manner dependent on a 3’ terminus (Figs. 1, D and E, 6, modes IV and V). The high affinity binding of PriA to a fork requires interaction of the helicase domain with the unreplicated duplex segment as well. We predict that, on A-fork [3’, 5’], the unreplicated arm and lagging strand arm may be equivalent relative to the 3’ terminus, generating two different complexes depending on which duplex arm the helicase domain interacts. Thus, the footprints on A-fork [3’, 5’] are the sum of these two different interactions (Fig. 1, D and E).

A Second DNA Recognition Module within the N-terminal DNA Binding Domain—Because the N-terminal 193-amino acid polypeptide can bind to A-fork [3’-P] or 3’-extension (supplemental Fig. S6), the domain required for 3’ terminus-independent binding is expected to be contained within this polypeptide. Although the N-terminal 105-amino acid polypeptide binds to the 3’-end in the BlAcore assay, it cannot bind to the A-fork [3’] mimic with a phosphorylated 3’-end with appreciable binding constant (Fig. 5, C and D). In contrast, the N-terminal 181-amino acid polypeptide can bind to this ligand even when its 3’-end is phosphorylated. In gel shift assays, PriA-(1–193) can bind to A-fork [3’] as well as to Y-fork, whereas PriA-(1–105) binds to A-fork [3’] but not to Y-fork (Fig. 5E). Thus, the segment 106–181 may likely contain a domain required for 3’ terminus-independent recognition.

This second recognition domain within the N-terminal DNA binding domain is likely to recognize the fork structure or duplex to single-stranded DNA junction present in the fork or in the 3’-extension structures. The ability of the N-terminal polypeptide to bind to the 3’-phosphorylated 3’-extension structure (which does not contain a free 3’-end except at the duplex end; supplemental Fig. S6) indicates that the additional domain may recognize the junction from duplex to single strand. Consistent with this, the PriA footprints are detected mainly over the single to double strand transition area on the 3’-extension substrate as well (Fig. 2). We also showed that the isolated polypeptide of PriA-(109–181) can bind to extension structures in vitro (supplemental Fig. S4). These results suggest an intriguing possibility that the recognition of the arrested fork structure by PriA is achieved by concurrent actions of two DNA recognition modules in the N-terminal DNA binding domain, one recognizing the 3’ terminus of the leading strand and the other the single to duplex junction.

Possible Modes of ATPase Activation by PriA Protein—PriA hydrolyzes ATP in a manner dependent on its binding to DNA (7). The helicase domain alone exhibits low but constitutive levels of ATPase activity, which is not further activated by addition of DNA (Figs. 3 and 4). Thus, the N-terminal DNA binding domain suppresses the basal ATP hydrolysis activity of the helicase domain, as reported previously (23). DNA binding at the N-terminal segment may induce an allosteric conformational change, activating the ATP hydrolysis activity.

Non-structured single-stranded DNA can also activate ATP hydrolysis by PriA in the absence of single-stranded DNA-binding protein (29). This activation is largely dependent on the presence of a 3’ terminus (Fig. 4D), as predicted from the absence of any other structural feature. This indicates that interaction of a 3’ terminus with the 3’ terminus binding pocket may be sufficient for stimulation of PriA ATP hydrolysis. However, the requirement of the minimum of 40 to 50 nucleotides for significant ATP hydrolysis suggests that PriA may need to interact not only with the 3’-end but also with other portions of DNA to be able to hydrolyze ATP. The extended single-stranded DNA of 50 nucleotides is about 30 nm long, approximately the length of nine turns of double helix. The footprinting and protection data indicate that the DNA helicase domain makes contact with about 20-base pair segments (two turns of double helix) of unreplicated duplex DNA of an arrested fork. We speculate that single-stranded DNA may need to adopt a compact structure, which enables its interaction not only with the 3’ terminus binding pocket but also with the helicase domain.

On the other hand, A-fork [3’] can stimulate ATP hydrolysis even in the absence of 3’-recognition (Fig. 3, B–D), suggesting that DNA binding through the putative additional domain alone can also activate PriA ATPase. We propose that the N-terminal DNA binding domain (through either of the two recognition modules) facilitates the interaction of the DNA helicase domain with the effector DNA by first recognizing and binding to it. This bipartite interaction of effector DNA with PriA (through the N-terminal DNA binding domain and helicase domain) would cause conformational change of the helicase domain, which now relieves the inhibition and permits efficient hydrolysis of ATP. Because the isolated helicase domain completely lacks DNA binding activities (Ref. 23 and data not shown), the “activation” of DNA binding capacity of the helicase domain, caused by the initial interaction at the N-terminal DNA binding domain, would be crucial for ATPase activation of PriA.

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