Properties of the PriA Helicase Domain and Its Role in Binding PriA to Specific DNA Structures*

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PriA protein is one of seven proteins (including PriB, PriC, DnaT, DnaB, DnaC, and DnaG) that make up the restart primosome, an apparatus that promotes assembly of replisomes at recombination intermediates and stalled replication forks (for reviews, see Refs. 1–4). PriA plays a critical function in restarting DNA replication after fork arrest by binding to forked DNA structures to promote the assembly of the primosome and consequently the replisome (5–8). DNA binding by PriA begins the assembly of the multiprotein primosome complex (9), including the loading of the major repliative helicase DnaB at the fork. PriA protein possesses 3′ to 5′ helicase activity (10, 11), which can serve to unwind any duplex on the lagging-strand arm of the fork and load DnaB onto the lagging-strand template. Once bound, DnaB translocates 5′ to 3′ (12) along the template to unwind the parental duplex, anchors the dimeric DNA polymerase III holoenzyme to the fork for catalysis of concurrent leading- and lagging-strand synthesis (13), and attracts primase (DnaG) needed to initiate multiple cycles of lagging-strand synthesis (14).

PriA belongs to the Superfamily 2 of helicases (15), based on homology of several conserved amino acid motifs (I–VI; Fig. 1A). Proteolysis analysis with trypsin has indicated hypersensitive cleavage sites at Arg-198 and Lys-518 that separate PriA into three domains (16). The N-terminal 181 residues have been found to bind to D-loop structures although with 20-fold reduced affinity than the binding of full-length PriA (16). This indicates that the Met–1–Arg–198 domain functions in DNA binding (Fig. 1A) and that the remaining portion (Leu-199–Gly-732) of PriA may function to increase DNA binding affinity and specificity. The Leu-199–Gly-732 segment may be considered the helicase domain (HD) since it includes all of the helicase motifs as well as a zinc finger motif implicated in helicase action; the latter motif most likely functions in interactions PriA establishes with DNA to translocate 3′ to 5′ (Fig. 1B). Although certain inactivating mutations in motif I (Walker A box) (18), which most likely functions in ATP binding and/ or hydrolysis, and the zinc finger motif do not significantly reduce DNA binding (19), replacement of the conserved residues Arg-512, Arg-517, Glu-527, and His-529 with alamines resulted in a mutant protein unable to bind D-loop DNA (16), further confirming the important role of the HD of PriA in allowing stable binding to D-loop DNA.

The contribution of the HD domain to DNA binding raises questions as to its helicase activity and DNA binding property when it is separated from the DBD. Full-length PriA has been characterized to have two modes of DNA binding (20). In one mode PriA binds D-loop, and it has been hypothesized that PriA recognizes DNA at three strand junctions. In the second mode PriA binds to duplex DNA with a protruding 3′ single strand; this mode has been hypothesized to reflect the intrinsic ability of a 3′ to 5′ helicase to recognize such a DNA substrate (20, 21). Both the D-loop and the duplex with the single-stranded 3′ tail have been found to be substrates of PriA helicase action (5). Helicase action on the lagging-strand side of forked substrates and the basal mode of helicase action on the minimal substrate (duplex with the 3′ single-stranded tail) do not have distinct properties (22). Unwinding of the minimal substrate by PriA is inhibited by the presence of single-strand binding protein (SSB). In contrast, binding of SSB to the single-stranded leading-strand template of the fork greatly stimulates unwinding of duplex DNA on the lagging-strand arm by PriA. Moreover, the bound SSB on the fork inhibits unwinding of the parental duplex, helicase action that would result from translocation of PriA along the leading-strand template. Thus, bound SSB generally inhibits basal mode of helicase on duplexes with the 3′ single-stranded extension; however, it can

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1 Timothy C. Mueser, personal communication.

2 The abbreviations used are: HD, PriA helicase domain; DBD, PriA DNA binding domain; DTT, dithiothreitol; nt, nucleotides; PAS, primosome assembly site; SSB, single-stranded binding protein.
The results are representative of at least two experiments.

The putative zinc finger motif implicated in helicase activity (see the Introduction) divided into two parts by a trypsin cleavage site at Arg-198. Residues on the C-terminal side include the conserved motifs (I–IV) of ATP binding by purified HD, HD-K230R, and PriA. Binding of [γ-32P]ATP was measured by a nitrocellulose filter binding assay. Reactions were allowed to proceed at 37 °C for 4 min. The proteins were cleaved at the trypsin site at Arg-198. Residues on the N-terminal side have DNA binding activity on its own whereas the HD plays an important role together with the DBD in this action. However, it has very low DNA binding affinity and, thus, has very low activity. Nevertheless, in full-length PriA, this residue at the N terminus of the HD proteins. The sequence of all constructed plasmids was verified by sequencing.

For purification, intein-tagged DBD and HD proteins were expressed in E. coli BL21 (DE3) (Novagen) transformed with the overproducing plasmid vectors. Cells were grown in 500 ml of LB broth containing 100 µg/ml ampicillin at 30 °C to an A600 of 0.5. Isopropyl-β-D-thiogalactopyranoside (0.4 mM) was added, and the culture was incubated with shaking overnight at 16 °C. Cells were harvested and resuspended in 25 ml of buffer A (20 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA) with 0.1% Tween 20, and they were lysed by sonication and cleared by centrifugation for 1 h at 13,000 × g. Purification of the proteins was based on the protocol suggested by the IMPACT system manufacturer and was carried out at 4 °C unless otherwise indicated. The lysate was applied to a chitin column (2-ml bed volume; New England Biolabs) in buffer column. The column was washed with 25 ml of buffer A containing 1 mM NaCl. The column was quickly flushed with 6 ml of buffer A containing 50 mM dithiothreitol (DTT) and then left at room temperature for 4 h to allow intein cleavage. DBD and HD proteins were eluted with buffer A, and the eluted fraction was dialyzed overnight against buffer B (50 mM imidazole-HCl, pH 6.7, 5 mM DTT, 1 mM EDTA, and 10% w/v sucrose). Further purification was based on a previously described PriA purification procedure (23). The dialyzed protein fraction was applied to a Bio-Rex 70 column (1-ml bed volume) equilibrated in buffer B, and the column was washed with 5 ml of buffer C (buffer B containing 40 mM ammonium sulfate). Protein was eluted by an 8-ml linear gradient from 100 to 800 mM NaCl in buffer C. DBD protein was eluted at 0.25 mM NaCl whereas HD and HD-K230R were found in the flow-through fractions. Proteins (Fig. 1B) were dialyzed against storage buffer (50 mM imidazole-HCl, pH 6.7, 5 mM DTT, 0.1 mM EDTA, 40 mM ammonium sulfate, 40% glycerol) overnight, frozen in liquid nitrogen, and stored at −80 °C. The typical yield was about 1 mg for the DBD and 0.2 mg for HD (or HD-K230R) from 500 ml of culture.

Protein of comparable specific activity was obtained whether intein cleavage was carried out at 4 °C or room temperature, but the highest yield was obtained at room temperature. DBD proteins cleaved at the two temperatures, for example, bound to forked DNA substrates with comparable dissociation constants, measured by electrophoretic mobility shift assays described below. Moreover, the binding affinity of DBD for forked substrates presented in this work is comparable with the affinity of PriA N-terminal segments (residues 1–181 and 1–193) for D-loops previously described (16). The activity of the HD protein was further evaluated by comparing its ATPase, helicase, and ATP binding activities with that of PriA as described under "Results."

The affinity of PriA, PriA-K230R, and HD proteins in the assay were previously described (6). Protein concentrations were determined by the method of Bradford (24). Protein concentrations of PriA, HD, and HD-K230R were indicated in equivalents of protein monomers. SDS-PAGE analysis was performed under nonreducing conditions.

ATPase and Helicase Assays—ATP hydrolysis was measured as previously described (6) in reaction mixtures containing SSB and denatured pG2215 DNA. The plasmid pG2215 includes the two-primosome assembly site (PAS) sequences of plasmid pBR322 (25). Reaction mixtures (15 µl) contained 50 mM HEPES-K, pH 8.0, 10 mM magnesium acetate, 1 mM DTT, 100 mM potassium glutamate, 100 µg/ml salmon sperm albumin, 10 µM [γ-32P]ATP, 0.13 mM linearized and denatured pG2215 DNA (in equivalents of plasmid molecules), 1 µM SSB, and the indicated concentrations of PriA, HD, or DBD. Reactions were allowed to proceed at 37 °C and stopped at the indicated...
times by the addition of 200 mM EDTA to 40 mM final concentration. A 5-μl portion of each reaction mixture was spotted on a polyethyleneimine-cellulose thin layer chromatography plate (J. T. Baker Inc.), which was developed in 0.5 M LiCl, 4.6% v/v formic acid. The plate was dried and subjected to phosphorimaging. Substrates for PriA helicase and DNA binding were constructed and purified as previously described (6, 22). Oligonucleotides S1, S2, S3, S4, M1, M2, M3, and M4 (6, 22) were used to construct the various substrates (Fig. 2). In each substrate one oligonucleotide was radiolabeled with $^{32}$P to a specific activity of 2 × 10$^6$ cpm/pmol using [γ-$^{32}$P]ATP and T4 polynucleotide kinase. Substrates were assembled by annealing the respective oligonucleotides and were then purified by resolution on non-denaturing polyacrylamide gels. Any partially annealed products were distinguished by mobility on the polyacrylamide gel. The mobility of the desired substrates and partially annealed products was established in reconstruction experiments, in which various combinations of the oligonucleotides were annealed to the radiolabeled oligonucleotide and resolved by electrophoresis. The oligonucleotide composition of DNA substrates was also confirmed by radiolabeling all oligonucleotides in the purified substrate and then separating them on a denaturing polyacrylamide gel. Helicase reactions were carried out as previously described (6) in reaction mixtures (20 μl) containing 20 mM Tris-HCl, pH 7.5, 5.4 mM MgCl$_2$, 1 mM DTT, 100 μg/ml bovine serum albumin, 2 mM ATP, 0.8 mM DNA substrates, 12 mM SSB when present and the indicated concentrations of PriA, HD, or DBD. Products were treated with SDS and proteinase K and resolved on 10% polyacrylamide gels (30:1 acrylamide:bisacrylamide) in TBE (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3; 10 V/cm for 2 h). The extent of unwinding was quantified using a PhosphorImager.

Electrophoretic Mobility Shift Assay—Mobility shift analyses were conducted as described (22) in reaction mixtures (15 μl) containing 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM DTT, 6% v/v glycerol, 100 μg/ml bovine serum albumin, 0.8 mM DNA substrates, 12 mM SSB where indicated, and PriA, DBD, or HD proteins as indicated. When reaction mixtures included SSB, it was incubated with substrates for 5 min on ice before the addition of PriA, DBD, or HD, and then incubation was continued for 15 min on ice. The reaction mixtures were then directly loaded onto a 6% polyacrylamide gel (80:1 acrylamide: bisacrylamide) in 0.5× TBE and 2.4% glycerol. Electrophoresis was conducted at room temperature at 5 V/cm for 2 h. To quantify the level of DNA binding using phosphorimaging, the radioactivity in the free, unbound substrate and the total activity in the shifted forms were quantified for each lane. The ratio of bound DNA substrate to total substrate was calculated.

ATP Binding Assay—Binding of [α-$^{32}$P]ATP to HD or PriA protein was measured by retention on nitrocellulose filters. Reaction mixtures (20 μl) contained reaction buffer (25 mM Tris-HCl, pH 7.5, 3 mM MgCl$_2$, 50 mM NaCl, 5 mM DTT, and 100 μg/ml bovine serum albumin), 100 μM [α-$^{32}$P]ATP (1.1 Ci/mmol), and 250–600 nM HD or PriA. 100 μM ATP was found to be saturating for binding by HD. Reaction mixtures were incubated at 0 °C for 10 min and passed through nitrocellulose filters (Millipore, white HAWP, 0.45-μm pore size, and 25-mm diameter) presoaked in reaction buffer. Filters were then washed with 10 ml of reaction buffer (prechilled to 0 °C) at a flow rate of 10 ml/min, dried, and counted in liquid scintillation fluid. The background from the reaction mixture containing no HD or PriA protein was subtracted from all measurements. Protein concentrations for this analysis were determined from the calculated molar extinction coefficient for HD and PriA as previously described (26).

RESULTS

Hypersensitive trypsin cleavage site at Arg-198 divides PriA protein into two domains (Fig. 1A), the DBD and HD. To purify these parts of the PriA protein, DBD protein was expressed with an intein tag attached at the C terminus, and HD was expressed with the intein tag at the N terminus. These self-cleaving tags contain a chitin binding domain, and they are cleaved off and removed during affinity purification. Any minor contaminating bands were further removed by chromatography on a Bio-Rex 70 column. HD protein with a mutation in the Walker A box (HD-K230R) was purified in an analogous fashion. The final preparations of DBD, HD, and HD-K230R (Fig. 1B) were greater than 95% pure.

HD Has Low Levels of DNA-independent ATPase Activity—PriA protein has ATPase activity that can be activated by the presence of single-stranded DNA that contains a PAS site, a sequence to which PriA binds even in the presence of SSB (27, 28). In reactions mixtures containing 5 nM PriA protein, ATPase activity could be readily activated by denatured DNA that contains the two PAS sites of pBR322 (25) in the presence of SSB. However, ATPase activity of HD present at 5 nM could not be detected even in the presence of DNA, indistinguishable from the reaction with PriA and no DNA (Fig. 3A). When present at 10-fold higher levels (50 nM), HD exhibited ATPase activity that was significantly higher than the same concentration of PriA without DNA (Fig. 3B). Notably, this ATPase activity of HD could be elicited in the absence of DNA, and the presence of DNA did not increase the level ATPase activity (Fig. 3B). On the other hand, HD-K230R, which bears a mutation in motif I rendering PriA deficient in ATPase and helicase activity (29), had no detectable ATPase activity when present at the high concentration (Fig. 3B).

In our standard assay SSB together with denatured DNA was added to reaction mixtures to activate the ATPase; SSB could conceivably inhibit interactions of HD with DNA. However, the addition of denatured DNA without SSB also did not promote an increase in ATPase activity (data not shown). The DBD protein, even when it was added in excess over HD, could not further activate the ATPase activity of HD in the presence of DNA (Fig. 3C). These results indicate that HD severed from the DBD retains intrinsic ATPase activity; however, its ATPase activity was not further activated by the presence of DNA. This is consistent with the idea that HD can function as a stand-alone ATPase and that not all HD activity is subject to the inhibitory effects of the DBD.

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The DBD of HD also has ATPase function. In the absence of DNA, HD ATPase function can be further increased by DNA, its relatively low ATPase activity may reflect an inability to translocate processively along single-stranded DNA, a process that would be fueled by extensive ATP hydrolysis. Because the lack of the DBD in HD protein results in a gain of function (i.e. DNA-independent ATP binding and hydrolysis), low ATPase activity attributed to misfolding or denaturation of HD due to the lack of the DBD segment is a less likely possibility even though some inactivation of HD molecules by this mechanism cannot be ruled out.

**HD Can Catalyze the Basal Mode of PriA Helicase**—We previously determined that there are two modes of PriA helicase action on the model fork Substrate C[-5] (22). This substrate (Table I; 3'-OH ends are indicated by half-arrows) has a single-stranded leading-strand arm and a duplex lagging-strand arm. PriA unwinds both duplexes of this fork. The [-5] designation for the substrate indicates a 5-base gap between the lagging strand and the fork (see Fig. 2); a gap of at least two nucleotides is required to unwind the lagging-strand arm of this fork (22). We had hypothesized that unwinding of the parental duplex of the fork, where PriA would translocate 3' to 5' on the leading-strand template, represents a basal mode of helicase action; in this mode the basic features of a minimal substrate, a duplex with a 3' single-stranded tail, are recognized (22). Like the unwinding of the minimal substrate W, this mode of unwinding Substrate C[-5] is diminished by the presence of SSB (Table I). Unwinding of the lagging-strand arm of the fork is hypothesized to be the result of fork-specific binding, which orients the helicase to translocate along the lagging-strand template. In contrast to its effect on unwinding the parental duplex, SSB increases unwinding of the lagging-strand arm of Substrate C[-5] (Table I).

In the absence of SSB, HD had basal helicase activity, but very high levels of the protein were required for activity comparable with PriA (HD was present at 26 times the concentration of PriA for the experiments shown in Table I). HD was able to promote unwinding of the parental duplex of Substrate C[-5] but was unable to unwind the lagging-strand arm of the fork. No products corresponding to a fork with two single-stranded arms were formed (Table I). A small amount of single-stranded S2 DNA was formed as product. However, this most likely corresponds to two reactions, which are unwinding of the parental duplex of Substrate C[-5] to first form the minimal substrate W and then the unwinding of substrate W to yield single-stranded S2. HD was very inefficient in unwinding the parental duplex of substrate F[-5] (Table I), a forked substrate with duplex DNA on the leading-strand arm and a 5-base gap adjacent to the fork. In contrast, PriA was able to unwind the parental duplex of substrates F[-5] at a significant level although not as efficiently as it unwound the parental duplex of Substrate C[-5]. These results indicate that HD can catalyze
the basal mode of helicase but is unable to catalyze the hypothesized fork-specific mode. The lack of the DBD reduces its basal helicase activity, most likely by reducing its affinity for DNA. The larger segment of 3'-single-stranded DNA present on Substrates C[-5] and W but not on F[-5] may be necessary for HD to bind to and translocate along the leading-strand template.

SSB Stimulates the Basal Helicase Mode of HD but Inhibits That of PriA—One way that SSB could inhibit the basal mode of PriA helicase would be to prevent the binding of its HD moiety to duplexes with a 3'-single-stranded extension. However, SSB increases the HD basal mode of helicase action on Substrates C[-5] and W while inhibiting this mode of helicase action by PriA (Table I). On substrate W, the level of unwinding increases linearly with HD concentration in the presence or absence of SSB, the activity being about 2-fold higher in its presence (Fig. 4, A and B). In contrast, at high concentrations of HD required to elicit helicase action (50 nM and higher), PriA is essentially inactive on substrate W in the absence of SSB, it did promote a very low level of unwinding in the presence of SSB. Both HD and HD-K230R were able to promote this level of unwinding in the absence of ATP when SSB was present (data not shown). These results indicate that HD can destabilize the duplex of Substrate W without ATP.

The HD and DBD of Full-length PriA Together Promote Its Stable Binding to Duplexes with 5'- or 3'-Single-stranded Extension That Is Bound by SSB—Because both the DBD and HD of PriA are needed for high affinity binding to D-loops, we wished to examine further how the presence of the HD in PriA increases its affinity and specificity for forked DNA structures and whether HD by itself has considerable affinity for specific DNA substrates. Our purified DBD binds to substrate A, a forked DNA structure, but with lower affinity than PriA (Fig. 5, A and B). At low concentrations (e.g. 10–50 nM) PriA is more active than HD when SSB is absent (Fig. 4A). These results indicate that SSB can facilitate the basal mode of helicase by HD. They suggest that SSB may not inhibit the PriA basal mode of helicase simply by preventing binding of the minimal substrate by the HD moiety.

Control experiments with HD-K230R indicate that the HD ATPase activity is indeed involved in helicase action (Fig. 4, A and B). Although HD-K230R promoted little to no unwinding of Substrate W in the absence of SSB, it did promote a very low level of unwinding in the presence of SSB. Both HD and HD-K230R were able to promote this level of unwinding in the absence of ATP when SSB was present (data not shown). These results indicate the HD can destabilize the duplex of Substrate W without ATP.

TABLE I

**Comparison of the helicase activity of PriA and HD**

| Substrate/enzyme | Total substrate consumed (fmol) | Labeled products (fmol) |
|------------------|--------------------------------|------------------------|
|                  |                                |                        |
| **A. In the absence of SSB:** |
| 1) C[-5] S[1] S[2] S[3(5)] | PriA | 6.0 ± 1.0 |
| HD | 0.9 ± 0.2 |
| 2) W S[2] S[3(5)] | PriA | 1.0 ± 0.2 |
| HD | 1.1 ± 0.2 |
| 3) F[-5] S[1] S[2] S[4] | PriA | 0.9 ± 0.3 |
| HD | < 0.1 |
| **B. In the presence of SSB:** |
| 1) C[-5] S[1] S[2] S[3(5)] | PriA | 9.1 ± 0.8 |
| HD | 1.8 ± 0.3 |
| 2) W S[2] S[3(5)] | PriA | 0.2 ± 0.0 |
| HD | 2.8 ± 0.3 |
| 3) F[-5] S[1] S[2] S[4] | PriA | 1.0 ± 0.2 |
| HD | 0.1 ± 0.0 |

*Substrates were constructed from the indicated oligonucleotides; the 5'-32P label is indicated with an asterisk. The size of any gap at the fork as illustrated in Fig. 2 is indicated in brackets.

*Helicase assays were performed as described under “Experimental Procedures” using 16 fmol of substrate and 13 nM PriA or 340 nM HD. Values are the average of three or more independent trials ± S.D.
replication (6, 7). PriA bound with only a modest decrease in affinity to Substrate Y, a fork with two single-stranded arms (Fig. 5, C and E). In contrast, DBD bound to Substrate Y with very low affinity (Fig. 5 D). Although some DNA in complexes could be detected at very high DBD concentrations (Fig. 5E, 160 nM DBD), such complexes did not clearly resolve as discrete bands and were only evident as a smear by mobility shift assays. These results are consistent with the previous finding that the DBD contains a binding pocket that recognizes 3'-OH end provided by the leading strand of a fork (the M4 strand of substrate A; see Fig. 2); such a 3'-OH end greatly increases the affinity of PriA for forked DNA substrates (31). On the other hand, HD had very little affinity for both Substrates A and Y (Fig. 5E). Protein-DNA complexes were not even evident as a smear at 275 nM HD.

Mobility shift experiments also indicated that SSB does not inhibit binding of PriA to the minimal Substrate W. Binding of PriA to this substrate produced a shifted band (Fig. 6A), consistent with previous studies (20). Binding was also evident as a smear present above the free substrate, indicating unstable or nonspecific binding of PriA. Substrate W is a relatively large substrate with a duplex of 65 bp and a single strand of 35 nucleotides, designed to have a single strand large enough to accommodate stable binding of SSB and a stable duplex for helicase assays. This may increase the amount of nonspecific PriA binding at very high protein concentrations. The presence of SSB produced one major shifted band and at least one additional minor band (Fig. 6B), and binding of PriA to SSB-bound substrate produced two discrete bands (open arrows).

**Fig. 4.** Effect of SSB on helicase action by PriA and HD on a minimal substrate. The indicated helicase reaction with Substrate W was allowed to proceed in the presence (B) or absence (A) of SSB at the indicated concentrations of PriA, HD, or HD-K230R. Reactions were allowed to proceed at 30 °C for 15 min. All values are the average of at least two independent determinations.

**Fig. 5.** Binding of PriA, DBD, and HD to forked DNA substrates as detected by mobility shift assays. Reaction mixtures contained the indicated concentrations of PriA, DBD, or HD and either Substrate A (A and B) or Substrate Y (C and D). Solid arrows indicate the origins of the polyacrylamide gel, and the open arrows indicate band shifts produced in the presence of PriA. Total substrate entering into protein-DNA complexes was quantified (E).
concentrations in the 50–100 nM range (Fig. 6B). In contrast, those complexes formed in the absence of SSB predominantly produced a smear at high PriA concentrations. These results indicate that SSB stabilizes binding of PriA to substrate W. When total PriA binding to the substrate is measured regardless of whether a discrete band or smear is produced (Fig. 6C), the dissociation constant for PriA binding to the Substrate W-SSB complex was significantly lower than that for its binding to the naked substrate. These results indicate that SSB does not inhibit PriA helicase action on substrate W by inhibiting its binding.

Although we were able to detect PriA helicase action on substrate W, we were unable to detect by mobility shift analysis any significant binding of HD to this substrate, even when protein concentrations were very high and SSB was present (Fig. 6, D and E). In addition, the DBD also bound poorly to substrate W. Some DNA binding was detected at very high DBD concentrations, and this binding was only apparent as a smear (Fig. 6D). These results indicate that neither the DBD nor HD alone has the capacity to form a tight complex with substrate W, stabilized by the presence of SSB.

We also examined the effect of SSB on the binding of PriA to a DNA duplex with a 5′ single-stranded tail. It has been determined that PriA has little affinity for these substrates (20). Because PriA binds to duplexes with a 3′ single-stranded tail, one possibility is that binding is enhanced by recognition of a bend at the transition between duplex and single-stranded DNA, a bend that may be enhanced by the binding of SSB. We therefore wished to determine whether a duplex with a 5′ single-stranded extension could also provide a determinant for PriA if such a substrate is bound by SSB. In the absence of SSB, PriA bound poorly to Substrate X, a 35-bp duplex with a 30-nucleotide 5′-stranded tail. A very diffuse shifted band arose especially at very high PriA concentrations although PriA binding was mostly apparent as a smear above the position of the free substrate (Fig. 7A). The presence of SSB promoted formation of PriA-Substrate X complexes that formed discrete bands (Fig. 7B). In contrast, PriA could not form a complex with single-stranded oligonucleotides that have bound SSB (Fig. 7C). Although binding of SSB to single-stranded oligonucleotide S1 produced one major shifted band and one minor band of lower mobility (suggesting two tetramers bound in the minor...
species), the minor SSB-DNA band was not detected at high PriA concentrations, with almost all radioactivity of the oligonucleotides shifted to the lower position of the major band. The binding of the second SSB tetramer may be weak, and its binding may be prevented in the presence of very high PriA concentrations even though PriA clearly does not enter into a stable complex with the DNA. In addition, PriA does not bind to duplex DNA (data not shown) or naked single-stranded DNA (Fig. 7C) as previously demonstrated (6, 20). These results indicate that PriA can bind to substrates with a transition from duplex to single-stranded DNA when SSB is present no matter the polarity of the protruding single strand. The overall results support the hypothesis that PriA binds with high affinity to a bend in the DNA enhanced by SSB binding at the duplex-single-strand interface. As was observed with Substrate W, DBD protein had little affinity for Substrate X in the presence or absence of SSB (Fig. 7, A and B).

**Binding of PriA-K230R to Substrate W Can Inhibit Helicase Action by the HD Protein**—In reaction mixtures containing Substrate W and SSB, PriA-K230R effectively shut down helicase action by the HD protein (Fig. 8A). More than 10-fold higher concentrations of the DBD protein were required to inhibit HD helicase activity. These results confirm that full-length PriA binds with high affinity to minimal substrates with bound SSB and that this mode of binding of an individual PriA

![Graph showing inhibition of HD helicase action by PriA-K230R](image)

**Fig. 8. Inhibition of HD helicase action on a minimal helicase substrate by PriA-K230R.** A, the analysis was carried out in standard reaction mixtures that included 32P-labeled Substrate W (16 fmol), SSB, and the indicated amounts of PriA-K230R or DBD. The 15-min incubation at 30 °C was started by the addition of 340 nM HD. When no PriA-K230R or DBD was added, 2.8 fmol of the substrate was unwound, and this was set to 100%. B, hypothesized binding pockets contributing to stable binding of a forked DNA substrate to PriA. C, model for binding of a duplex with a 3' single-stranded tail by PriA. The mode of substrate binding determines whether the duplex portion is accessible to the HD, allowing the helicase to be active on that substrate.
molecule to Substrate W can prevent action by other helicase molecules as well as its own HD. The potential to inhibit other helicase molecules provides a possible explanation why PriA, even at very high concentrations, cannot unwind much more than 1 fmol of a total of 16 fmol of Substrate W when SSB is absent (Fig. 4A). As hypothesized under “Discussion,” PriA may be able to bind the minimal substrate in two orientations in the absence of SSB (Fig. 8C). One mode allows the helicase to be active, whereas the second mode, favored in the presence of SSB, may prevent helicase action. If PriA can act as both helicase and inhibitor on the minimal substrate, the inhibitory activity may prevent increased helicase action at higher PriA concentrations.

**DISCUSSION**

The major cellular function of PriA is to promote reassembly of the replisome after stalling of replication forks. A critical part of this function is the recognition of forked DNA structures associated with D-loop and stalled replication forks at which DNA replication would be restarted. PriA has an associated 3’ to 5’ helicase activity (10, 11) that can function to promote duplex opening and the subsequent loading of DnaB onto the lagging-strand template of the fork (6). The importance of this function was first exemplified by the important role PriA helicase function plays in the initiation of phage Mu DNA replication by transposition (6). Strand exchange catalyzed by the Mu transposase creates at each Mu end a fork structure (analogous to Substrate A; Fig. 2), which has a double-stranded lagging-strand arm (30), and a duplex opening would be needed at this fork to load DnaB. Mutants with priA300 mutation, which encodes PriA-K230R with an inactive helicase, have essentially a wild-type phenotype (29, 32), unlike priA knockout strains, which exhibit characteristics of low viability, high sensitivity to DNA damaging agents, and a constantly induced SOS system (33, 34). The PriA-K230R retains the ability to promote assembly of a primosome (29). However, the priA300 in combination with the priB knockout mutation, which also has little effect by itself on the cell phenotype, produces a severe negative phenotype much like that of priA knockout strains (32). Moreover, there is evidence that the PriA helicase plays a critical role in a RecG-dependent pathway for replication restart, a major alternative to the restart mechanism requiring RuvABC and RecBCD proteins (35–37). That is, the PriA helicase is not essential, apparently due to existence of overlapping mechanisms for restart, especially those that involve replisome assembly at a D-loop where duplex opening for DnaB loading would not be necessary. However, the PriA helicase role becomes critical as alternative restart mechanisms are knocked out.

How PriA binds to its DNA substrates is critical to its function. Replication restart requires a primosome to be assembled on the lagging-strand arm of the fork. We hypothesize that the cooperation of the DBD and HD in full-length PriA allows recognition of two bends at the fork, one at the transition between the parental duplex and the lagging-strand arm of the fork and the other at the transition between the parental duplex and the leading-strand arm (Fig. 8B). Thus, the tightest binding would be apparent when both bends of the fork as well as the 3’-OH of the leading strand are recognized. PriA has been characterized to have two binding modes (20). One mode is exemplified by its binding to a fork structure formed at a three-strand junction where one strand (either the leading or lagging-strand template) forms a sharp bend. PriA has been demonstrated to have a 30-fold preference for substrates with the bend on the lagging-strand template (20). The bend indeed appears to be a critical determinant for PriA, but in light of new evidence that the DBD has a binding pocket for a 3’-OH end (31), the preference for this substrate may also be due to the presence of the leading-strand 3’-OH. PriA forms discrete band shifts with DNA duplexes that have either a 5’ or 3’ single-stranded tail that is bound by SSB. Because SSB does not promote stable binding of PriA to single strands, we suspect that SSB stabilizes PriA binding by creating or enhancing a bend at the transition between duplex and single-stranded DNA. There is no direct evidence that SSB bound to single-stranded DNA protruding from duplex DNA can stabilize a bend in DNA. However, single-stranded DNA wraps around the SSB tetramer to form a compact structure (38), and conceivably, a bend in the DNA may result at the transition between such a complex and duplex DNA. PriA is able to bind with high affinity to forks with nonhomologous single-stranded arms, even in the absence of SSB.

The junctions between duplex and single-stranded DNA would be flexible enough to form a bend. A bend formed on each strand may have a cooperative effect of stabilizing PriA binding even if such a bend exists only transiently in the absence of SSB. In contrast, PriA binds with very low affinity to bubble DNA (i.e., lacking the invading DNA strand of D-loops) (20). Because the PriA DBD contains a site involved in recognizing the 3’-OH end of the leading strand (i.e., primer for leading-strand synthesis), lack of such a 3’-OH end in the bubble DNA may greatly decrease binding affinity (31). In addition, PriA may be unable to bind to bubbles because the single-stranded arms of the two forks may not be flexible enough to stably form the required bend. Nurse et al. (20) indicate that the single-stranded segments of the bubble would be tangled about each other due to the free rotation of duplex regions, and this may not only limit access of PriA to the single strands but also constrain their orientation with respect to duplex DNA.

The potential bend in the minimal substrate W may play an important role in promoting PriA binding even in the absence of SSB. Indeed, the intrinsic ability of 3’ to 5’ helicases to recognize 3’ single strands protruding from duplex DNA may contribute to this mode of binding (20), but this determinant alone may not be sufficient for stable binding. Although HD protein has ATPase and helicase activity by itself, it cannot form a stable complex with Substrate W. When SSB is absent, the potential bend in the substrate as well as the intrinsic affinity of the helicase for a duplex with a 3’ single-stranded extension may together contribute to stable PriA binding. On a duplex with a 5’ single-stranded extension, the bend at the duplex-single strand interface may contribute solely to PriA binding, and thus, SSB may play a more critical role for promoting stable association of PriA with this substrate.

Our results suggest that SSB promotes PriA binding in a specific orientation at the transition between duplex and single-stranded DNA. We determined that SSB enhances helicase action on the lagging-strand arm of Substrate C-[5] and that it inhibits helicase action while enhancing PriA binding to Substrate W. When Substrate W is bound by PriA in the absence of SSB, it may occupy either that site normally occupied by the bent leading-strand template or the lagging-strand template (Fig. 8, B and C). When bound at the lagging-strand site, the duplex may be unwound. In this configuration the single strand of the substrate occupies the site normally bound to the parental duplex. In contrast, when the substrate is bound to the leading-strand site, the helicase may be oriented to translocate along the lagging-strand arm on the opposite strand, an arm missing in Substrate W. In this orientation the duplex of Substrate W would be bound at the site for the parental duplex. SSB bound to the single strand may not only enhance PriA binding but also prevent the single strand from occupying the parental duplex binding site.
Having pockets for DNA binding able to recognize a bend in both the leading- and lagging-strand templates has potential advantages for PriA function. PriA would encounter a variety of DNA structures in its role in restarting DNA replication. Among DNA structures of D-loops and arrested replication forks, one or both arms can be single-stranded. At D-loops the helix formed by the annealed invading strand (leading-strand arm) and the adjacent parental duplex may tend to align such that the most pronounced bend is at the transition between the parental duplex and the displaced strand (the lagging-strand arm). In the absence of a bend on the leading-strand template, the recognition of the 3′-OH end of the invading strand and the 5′-OH of the leading strand, the duplex of the lagging-strand arm may tend to align with the parental duplex. It has much higher affinity for Substrate A with two single-stranded arms, structures. It may serve to correctly align PriA on such forks.

The DBD does appear to have an affinity for forked DNA structures. It has much higher affinity for Substrate A with two duplex arms than to Substrate Y with two single-stranded arms, and it has even less affinity, if any, for Substrates W and X. Thus, the cooperation of the DBD and HD appears to increase affinity for some determinant at the transition of duplex and single-stranded DNA enhanced by SSB. Previous analysis has indicated the importance of the alignment of DBD with HD for high affinity binding to D-loops, and mutations that affect this binding in both the DBD and HD have been characterized (16, 31). These results are consistent with both the DBD and HD contributing to recognition of a sharp bend between the parental duplex and one or both arms of the fork, helping to provide specificity of PriA for forked DNA structures. PriA loads the DnaB helicase on the same DNA strand on which PriA helicase translocates (6), and the proper alignment of PriA at the fork would be vital for assembling a productive replisome.

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Properties of the PriA Helicase Domain and Its Role in Binding PriA to Specific DNA Structures
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