PriB Stimulates PriA Helicase via an Interaction with Single-stranded DNA*

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The frequency with which replication forks break down in all organisms requires that specific mechanisms ensure completion of genome duplication. In Escherichia coli a major pathway for reloading of the replicative apparatus at sites of fork breakdown is dependent on PriA helicase. PriA acts in conjunction with PriB and DnaT to effect loading of the replicative helicase DnaB back onto the lagging strand template, either at stalled fork structures or at recombination intermediates. Here we showed that PriB stimulates PriA helicase, acting to increase the apparent processivity of PriA. This stimulation correlates with the ability of PriB to form a ternary complex with PriA and DNA structures containing single-stranded DNA, suggesting that the known single-stranded DNA binding function of PriB facilitates unwinding by PriA helicase. This enhanced apparent processivity of PriA might play an important role in generating single-stranded DNA at stalled replication forks upon which to load DnaB. However, stimulation of PriA by PriB is not DNA structure-specific, demonstrating that targeting of stalled forks and recombination intermediates during replication restart likely resides with PriA alone.

Genome duplication is rapid, accurate, and highly processive. However, the DNA replication machinery of all organisms may stall at a frequency that demands the presence of replication repair systems (1, 2). If the replication machinery dissociates from the DNA upon stalling, then the replication machinery must also be reassembled back onto the chromosome (3).

In Escherichia coli there are multiple overlapping mechanisms of replication fork reassembly (4). These reassembly pathways are initiated either by PriC or by PriA helicase (5, 6). Cells lacking PriA have low cell viability, reduced recombination, and extreme sensitivity to DNA-damaging agents, demonstrating the frequency with which replication forks stall and also the importance of a PriA-catalyzed mechanism for replication fork reassembly (7–10). PriA binds preferentially to three-way branched DNA structures possessing a leading strand in vitro, reflecting the structures thought to occur at recombination intermediates and at some stalled replication forks in vivo (11, 12). Recognition of such DNA structures is then followed by loading of the replicative helicase, DnaB, back onto the lagging strand template (13, 14). DnaB reloading is thought to be the key step in restarting replication, allowing reassembly of both leading and lagging strand polymerases back onto the chromosome and resumption ofpriming of lagging strand synthesis by DnaG (15). However, PriA cannot facilitate DnaB reloading by itself. Binding of PriA to branched DNA structures possessing a leading strand at the branch point is followed by recruitment of PriB and subsequently of DnaT (14, 16). This PriA-PriB-DnaT complex can then promote reloading of DnaB onto the lagging strand template (5).

DnaB can bind to single-stranded DNA (ssDNA)2 without the assistance of PriA (17). Indeed, the helicase loader, DnaC, greatly increases binding of ssDNA by DnaB from a DnaB-DnaC complex (18, 19). However, two factors present in vivo result in a requirement for the facilitation by other enzymes of loading of DnaB from a DnaB-DnaC complex. First, DnaB cannot bind to ssDNA bound by single-stranded binding protein (SSB) (17). This inhibition can be overcome by the PriA-PriB-DnaT complex allowing loading of DnaB onto an SSB-coated single-stranded lagging strand template (5, 13). This inhibition of DnaB loading by SSB and the ability to overcome this inhibition by PriA at branched DNA structures likely provide a regulatory mechanism to ensure DnaB loading occurs only where necessary (20, 21). Second, unwinding of the parental strands at a replication fork requires loading of DnaB onto the single-stranded lagging strand template (17, 22). D-loops formed by recombination are thought to be one means by which structures are produced onto which PriA can load DnaB (II, 23), and the strand destined to become the lagging strand template at D-loops is single-stranded. However, at stalled fork structures rather than D-loops the lagging strand template may be double-stranded (24). Unwinding of this lagging strand duplex may require the 3′- to 5′-helicase function of PriA (13, 24).

Thus PriA can form a complex with PriB and DnaT to promote loading of DnaB onto ssDNA in the presence of SSB. PriA can also unwind any dsDNA present on the lagging strand arm to uncover ssDNA required for binding of DnaB. How these two processes are achieved in the context of the PriA-PriB-DnaT complex is not well understood. PriB has been shown to stabilize binding of PriA to a hairpin structure flanked by ssDNA ends (the primosome assembly sequence of the bacteriophage φX174) (16). PriB also interacts with naked ssDNA or with ssDNA bound by SSB (25) and may also modulate PriA helicase function (26). However, the requirement for PriB in loading of DnaB can be circumvented by high concentrations of DnaT (16). This implies that PriB facilitates interaction between PriA and DnaT. It also suggests that PriA and/or DnaT interact directly with the DnaB-DnaC complex during loading of DnaB, whereas PriB acts only to stabilize PriA and DnaT binding on the DNA.

A recent clue to the function of PriB during replication restart came...
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with the elucidation of the crystal structure of E. coli PriB. PriB forms a dimer with two oligonucleotide/oligosaccharide folds, one in each monomer (27–29). Moreover, the structure of subunits within PriB shows striking similarities with the subunit structure of SSB. Indeed, the priB and ssb genes may have arisen by a gene duplication event (30). Thus, PriB and SSB may bind ssDNA in a similar manner, as suggested by mutational analysis of aromatic and basic residues in which the affinity of PriB for ssDNA was reduced (27). However, the quaternary structures of dimeric PriB and tetrameric SSB differ markedly. One consequence of this difference is that there is a large surface-exposed face on the PriB dimer available potentially for interaction with other proteins (27). Thus PriB might function by simultaneous binding of ssDNA and of partner proteins such as PriA and DnaT.

In this study we have observed that PriB stimulated PriA helicase. This stimulation of PriA was manifested as an increase in apparent processivity. The stimulation was not DNA structure-specific, suggesting that, unlike PriA, PriB did not bind preferentially to branched DNA. PriB formed a stable higher order complex with PriA in bandshift assays only in the presence of ssDNA. The importance of ssDNA binding in PriB function was underlined by the reduction in stimulation of PriA helicase by mutations in PriB which decreased the ability of PriB to form stable PriA-PriB complexes on ssDNA. Taken together, these data suggest that PriB may act as a processivity factor for PriA helicase by interacting simultaneously with both PriA and ssDNA. Such an activity may facilitate reloading of the replication machinery by PriA at stalled replication forks by promoting exposure of ssDNA on the lagging strand template for loading of DnaB.

MATERIALS AND METHODS

DNA Substrates—DNA substrates were constructed by annealing complementary oligonucleotides, one of which in each structure was labeled with [γ-32P]ATP at the 5’ end and purified by gel electrophoresis (31). Sequences of the oligonucleotides written 5’ to 3’ are as follows: 1) GTCGATCTCTAGAGCTAGCTTACGCTGACTGCTGAGAGTATGGA; 2) GCCGATCTCTACACACTGCGCTGCTGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 3) TAGCAATGTATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 4) ATCACTGGCAGTATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 5) AACGTAATGTAATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 6) TAGCAATGTATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 7) ACGTAGGGCGAGGAAACGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 8) TAGCAATGTATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 9) AACGTAATGTAATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 10) TACGATATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 11) CTGTAGAGTTATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA; 12) TAGCAATGTATCGCTATACGTGTTTTCGCGCTGCTGCTGATTAGCCGATGCTGCTGAGAGTATGGA.

DNA Unwinding and Binding Assays—Junction dissociation was assayed at 37 °C in 40 mM HEPES-HCl (pH 8.0), 10 mM MgCl2, 0.1 mM dithiothreitol, and 0.1 mg/ml bovine serum albumin. 1 nM junction DNA was used together with the indicated concentrations of proteins. Unwinding reactions were preincubated at 37 °C for 5 min, together with SSB and/or PriB if present, prior to the addition of PriA to a final volume of 10 μl, and incubation was continued at 37 °C for a further 10 min. Reactions were stopped by the addition of 2.5 μl of stop buffer (100 mM Tris-HCl (pH 7.5), 5% SDS, 200 mM EDTA, and 10 mM MgCl2) and incubation at 37 °C for 20 min. The amount of junction dissociation was analyzed by electrophoresis through 7% polyacrylamide gels in 10 mM Tris borate, 2 mM EDTA and quantified using a PhosphorImager.

DNA binding assays were performed by incubation of 1 nM of the DNA substrate with the stated concentration(s) of protein(s) in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 6% glycerol. Incubation was performed on ice for 15 min, and samples were run on a 10% polyacrylamide gel in 1x TBE buffer at 300 V for 30 min. Gels were phosphorimaged and bands were quantified using a PhosphorImager.

FIGURE 1. PriB stimulates PriA helicase activity. A, unwinding of fork 1 by 8 nM PriA in the absence of PriB (lane 2) and in the presence of 0.5, 1, 5, 20, and 100 nM PriB dimers (lanes 3–7). Lane 1 contains no protein, and lane 8 contains 100 nM PriB dimers only. Features of fork 1 are shown illustrating the duplexes equivalent to 25 bp leading and 70 bp lagging strand arms and the 25-bp parental duplex. Arrows represent the 3’ ends of oligonucleotides, and the circle represents a 5’ 32P label. B, unwinding of partial duplex 1 by PriA and PriB. Lanes are as described in A. C, levels of unwinding of fork 1 (closed circles) and partial duplex 1 (open circles) by 8 nM PriA in the presence of increasing amounts of PriB. Data shown are the means of four independent experiments, and error bars represent means ± S.D.
The reactions were then electrophoresed through 4% polyacrylamide gels in 50 mM Tris borate, 2 mM EDTA, dried, and analyzed by PhosphorImager analysis and autoradiography.

**ATPase Assays**—The rate of hydrolysis of ATP by PriA was monitored in the presence and absence of PriB essentially as described (34), except that the buffer used was 40 mM HEPES-HCl (pH 8.0), 10 mM MgCl₂, 10 mM dithiothreitol, 2 mM ATP and 0.1 mg ml⁻¹ bovine serum albumin. The concentrations of PriA and of PriB dimers used were 5 and 20 nM, respectively, and the concentration of fork 6 was 100 nM.

**RESULTS**

**PriA Helicase Activity Is Stimulated by PriB**—Given that PriB interacts with ssDNA (25, 27–29) and with PriA bound to ssDNA (16, 35), we sought to analyze the effects of PriB on PriA helicase function at forked DNA substrates. Addition of PriA alone to fork 1, a model replication fork substrate having 25-bp leading and 70-bp lagging strand duplex arms, failed to generate significant levels of unwound product (Fig. 1A, lane 2). PriA helicase is targeted specifically to the lagging strand in such structures (24), but PriA possesses limited processivity of 40 bp or less (21, 36, 37). Lack of unwinding of fork 1 by PriA is consistent with this limited processivity. However, titration of PriB into reactions containing PriA and fork 1 revealed that unwinding of the 70-bp lagging strand duplex was elevated by up to 9-fold (Fig. 1A, lanes 3–7). This unwinding required PriA (Fig. 1A, lane 8), suggesting that PriB facilitated unwinding by PriA of the 70-bp duplex.

PriA, although demonstrating a higher binding affinity for and greater helicase activity on forked DNA substrates, can also unwind unbranched partial duplex DNA substrates (11, 12). The ability of PriB to stimulate unwinding by PriA of a partial duplex substrate containing the same 70-bp duplex of fork 1 was assessed (Fig. 1B). Increasing concentrations of PriB stimulated PriA-catalyzed unwinding of this unbranched structure to the same extent as fork 1 (Fig. 1C). Thus stimulation of PriA helicase by PriB was not DNA structure-specific.

There may be several different mechanisms for the reloading of DnaB at stalled forks and recombination intermediates (4, 38). One such mechanism requires PriA, PriB, and DnaT, whereas a second mechanism utilizes PriC only (5). We therefore tested the ability of PriC and DnaT to stimulate PriA-catalyzed unwinding of fork 1. Although PriB stimulated unwinding of the lagging strand by PriA (Fig. 2, lane 3), no stimulation was observed upon addition of PriC or DnaT (Fig. 2, lanes 4 and 5, respectively). Furthermore, addition of PriC or DnaT to reactions containing both PriA and PriB had no effect on the levels of unwinding of fork 1 (data not shown). We conclude that stimulation of PriA helicase is specific to PriB. Furthermore, PriB failed to stimulate Rep heli-

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**FIGURE 2.** Neither PriC nor DnaT stimulates PriA helicase. Unwinding of fork 1 by 8 nM PriA was monitored in the presence or absence of 100 nM of PriB dimers, PriC monomers, or DnaT trimers as indicated. DNA substrate and products of unwinding are indicated.

**FIGURE 3.** Stimulation of PriA helicase by PriB can occur in the presence of SSB at branched but not at unbranched DNA structures. Unwinding of fork 1 (A) or of partial duplex 1 (B) by PriA in the presence or absence of PriB and/or SSB. C and D, quantification of levels of unwinding of fork 1 and partial duplex 1, respectively, by PriA with PriB and SSB. PriA was present at 8 nM, PriB at 30 nM dimers, and SSB at 30 nM tetramers as indicated. Data are reported in triplicate, and error bars represent mean ± S.D.
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FIGURE 4. Unwinding by PriA of duplexes of varying length in branched and unbranched DNA structures. A, structures of the forked DNA and the partial duplex DNA used. The forks had parental and leading strand duplexes of 25 bp, although the lengths of the lagging strand duplexes ranged from 25 to 60 bp. The partial duplexes were constructed by using the two oligonucleotides from the forked DNAs that formed the lagging strand duplex and so had a 3’ ssDNA tail of 25 bases and duplex regions of 25–60 bp. B, levels of unwinding by PriA of fork 2 (25-bp lagging strand duplex; closed circles), fork 3 (40-bp lagging strand duplex; open squares), fork 4 (50-bp lagging strand duplex; closed triangles), and fork 5 (60-bp lagging strand duplex; open diamonds). C, levels of unwinding by PriA of partial duplex 2 (25-bp duplex; closed circles), duplex 3 (40-bp duplex; open squares), duplex 4 (50-bp duplex; closed triangles) and duplex 5 (60-bp duplex; open diamonds). Note the different scales of the ordinates in B and C.

FIGURE 5. Stimulation of PriA-catalyzed unwinding of duplexes of varying length by PriB. A, structures of the DNA substrates used. B, unwinding of forks 2, 3, 4, and 5 containing lagging strand duplexes of 25–60 bp in the presence of 5 nM PriA and/or 100 nM PriB dimers. C, unwinding of fork 2 by 0.2 nM PriA and/or 100 nM PriB dimers. D, unwinding of partial duplexes 2, 3, 4, and 5 containing duplexes of 25–60 bp in the presence of 5 nM PriA and/or 100 nM PriB dimers.

case on a variety of DNA substrates (data not shown). Rep, like PriA, is a low processivity DNA helicase whose activity is directed at the nascent lagging strand of stalled replication forks to effect reloading of DnaB (6). Thus PriB stimulation of replication restart helicases is limited to PriA.

Given that most protein-DNA transactions in vivo take place in the presence of SSB, the effect of SSB on PriB stimulation of PriA helicase was also tested. Moreover, SSB is known to stimulate PriA helicase on branched DNA structures, and on unbranched DNA SSB inhibits PriA helicase (20, 21). On fork 1, the presence of either PriB or SSB stimulated unwinding of the 70-bp lagging strand duplex by PriA (Fig. 3, A and C). Addition of both PriB and SSB to PriA led to a greater degree of stimulation of unwinding of fork 1 than with either PriB or SSB only (Fig. 3C). The stimulation by PriB and SSB appeared therefore to be additive. On duplex 1 PriB stimulated PriA (Fig. 3B, lanes 2 and 3). However, SSB inhibited unwinding of partial duplex 1 by PriA only (Fig. 3B, lanes 2 and 4), and SSB also inhibited the stimulation of PriA by PriB (Fig. 3, B, compare lanes 3 and 5 and D). Thus PriB could stimulate PriA helicase on branched DNA in the presence of SSB. However, SSB inhibited unwinding of unbranched DNA by PriA regardless of the presence of PriB.

PriB Increases the Apparent Processivity of PriA—Because the data in Fig. 1 suggested that PriB might increase the apparent processivity of PriA helicase, the processivity of PriA at branched and unbranched DNA substrates was analyzed further. Using forked DNA structures containing lagging strand duplexes of 25–60 bp (Fig. 4A) there was a marked decrease in PriA-catalyzed unwinding upon increasing the lagging strand duplex from 25 to 40 bp, whereas unwinding of 50- and 60-bp duplexes was negligible (Fig. 4B).

Analysis of PriA processivity on the equivalent partial duplex DNA structures revealed a similar pattern (Fig. 4C). However, the absolute levels of unwinding were lower on the partial duplexes compared with the equivalent forked DNA substrates (Fig. 4, B and C). Furthermore, unwinding of the partial duplexes did not increase upon moving from 5 to 25 nM PriA, suggesting that binding of PriA was not the limiting factor at 25 nM PriA (Fig. 4C). If binding of PriA to the partial duplexes was not limiting, then lowered levels of unwinding of partial duplexes as compared with forks would be due to the lower activity of PriA helicase on partial duplex substrates as compared with forked DNA.

The ability of PriB to stimulate PriA-catalyzed unwinding of 25–60-bp duplexes was tested. Using forked DNA substrates and 5 nM PriA, stimulation of unwinding of the lagging strand duplex by addition of PriB was detected with duplex lengths of 40–60 bp (Fig. 5B, lanes 5–16, and TABLE ONE). However, the level of unwinding of the 25-bp lagging strand duplex was so high with 5 nM PriA in the absence of PriB that any stimulation was precluded (Fig. 5B, lanes 1–4, and TABLE ONE). The concentration of PriA was reduced therefore to 0.2 nM, and although unwinding of the 25-bp duplex was stimulated by PriB (Fig. 5C, lanes 2 and 3), the effect was small compared with the stimulation observed with lagging strand duplexes of 40–60 bp (TABLE ONE). We concluded that addition of PriB led to enhancement of the ability of PriA to unwind lagging strand duplexes of 40 bp or greater.

Analysis of the effects of PriB on the equivalent partial duplex structures revealed significant stimulation of PriA-catalyzed unwinding of all duplexes from 25 to 60 bp (Fig. 5D and TABLE ONE). The 3-fold stim-
ulation by PriB of PriA-catalyzed unwinding of the 25-bp partial duplex contrasts with that seen for the identical duplex in the context of fork 2 (TABLE ONE). This difference in stimulation correlates with the proposed lower processivity of PriA on partial duplex substrates as opposed to the forked DNA suggested above. However, the degree of stimulation of PriA helicase by PriB on partial duplexes did still show some dependence on duplex length. Addition of PriB gave ~3-, 6-, 9-, and 14-fold stimulation of PriA-catalyzed unwinding of the 25, 40, 50, and 60-bp duplexes, respectively (TABLE ONE). Therefore, the degree of stimulation correlated again with the lower processivity of PriA as the duplex length increased from 25 to 60 bp (Fig. 4C).

Thus PriB increased the apparent processivity of PriA helicase. Furthermore, this increase was apparent on both branched and non-branched DNA structures, supporting the conclusion that stimulation of PriA by PriB was not DNA structure-specific.

**PriB Forms a Stable Complex with PriA in the Presence of ssDNA**—PriB has been shown to form a stable complex with PriA on the primosome assembly site, a stem-loop structure within single-stranded bacteriophage ϕX174 DNA (35). PriB has also been shown to bind ssDNA (25). Gel shift assays were therefore used to probe the DNA structural requirements for formation of a PriA-PriB-DNA complex. PriA bound to fork 3, having a 40-bp lagging strand duplex, to form a single retarded complex (Fig. 6A, lane 2, complex I). PriB alone did not form a complex with fork 3, as expected given the lack of ssDNA in fork 3 (Fig. 6A, lanes 6–8). Addition of PriB to fork 6 and PriA did result in formation of a second complex (Fig. 6A, lanes 11–13, complex III). Given the dependence of PriA helicase by PriB on partial duplexes did still show some dependence on duplex length. Addition of PriB gave ~3-, 6-, 9-, and 14-fold stimulation of PriA-catalyzed unwinding of the 25, 40, 50, and 60-bp duplexes, respectively (TABLE ONE). Therefore, the degree of stimulation correlated again with the lower processivity of PriA as the duplex length increased from 25 to 60 bp (Fig. 4C).

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ence of complex III formation on both PriA and PriB, we concluded that this complex represented a stable association of both proteins with fork 6. Furthermore, at all concentrations of PriB tested, only a single PriA-PriB complex was detected (Fig. 6A, lanes 11–13, and data not shown). This lack of detectable complexes migrating between complex II and III suggested that complex III contained PriA and a single PriB dimer.

A similar gel shift experiment was also performed with a 40-base oligonucleotide. The lower affinity of PriA for ssDNA as opposed to forked DNA (12) necessitated using a 10-fold higher PriA concentration than that used with the forked DNA structures in Fig. 6A. At this PriA concentration, a PriA-DNA complex was detected with this oligonucleotide (Fig. 6B, lane 2, complex IV). No stable complex with the oligonucleotide was detected with PriB only (Fig. 6B, lanes 6–8). However, in the presence of both PriA and PriB, a second shifted complex was seen (Fig. 6B, lanes 3–5, complex V). We concluded that formation of a stable PriA-PriB-DNA complex requires that the DNA contain ssDNA but does not require that the DNA be branched. Moreover, although we obtained a complex between PriA and a 25-base oligonucleotide, we failed to observe a more slowly migrating complex upon addition of PriB to this 25-mer-PriA complex (data not shown). Thus formation of a PriA-PriB-DNA complex required between 25 and 40 bases of ssDNA.

Formation of a stable PriA-PriB complex on DNA containing ssDNA suggested that PriB might modulate PriA ATPase activity on such structures. Therefore, PriA-catalyzed ATP hydrolysis was analyzed on fork 6 with and without PriB. However, no difference in the rates of ATP hydrolysis by PriA in the presence and absence of PriB could be detected (Fig. 6C). More detailed analysis of PriA ATPase catalysis revealed that PriB had no significant effect on the $k_{\text{cat}}$ or $K_m$ values with respect to ATP (data not shown). However, PriB did reduce the $K_m$ values with respect to DNA from 2.0 ± 0.8 to 0.6 ± 0.2 nM for fork 6 (data not shown). Thus, although PriA and PriB could form a stable complex on DNA structures containing ssDNA, this association had little effect on ATP hydrolysis by PriA.

**Stimulation of PriA by PriB Requires Binding of PriB to ssDNA**—We tested whether the binding of ssDNA by PriB played a role in stimulation of PriA helicase. Four histidine-tagged PriB mutant proteins with reduced affinity for ssDNA (27) were analyzed for stimulation of PriA helicase on fork 3, which possessed a 40-bp lagging strand duplex. Two mutants, F42A and K82A, gave similar levels of stimulation as histidine-tagged wild type PriB on fork 3 (Fig. 7). PriB W47A gave reduced stimulation of PriA at low PriB concentrations, but no significant reduction was observed at higher PriB concentrations. The fourth mutant tested bore two mutations, W47A and K82A. PriB W47A,K82A displayed significantly reduced stimulation of PriA at all concentrations tested (Fig. 7 and data not shown). Similar patterns of stimulation were observed using fork 1, possessing a 70-base lagging strand as opposed to the 40-base lagging strand of fork 3 (data not shown).

The apparent dissociation constants for the wild type PriB, the single mutants F42A, W47A, and K82A, and the W47A,K82A double mutant were estimated as 35, 73, 112, 218, and 427 nM for a 30-mer oligonucleotide in the absence of PriA (27). The reduced affinity of the single mutants did not appear to impact significantly upon the ability of PriB to stimulate PriA helicase. These data argue against ssDNA binding by PriB playing a major role in stimulation of PriA helicase. However, the greatly reduced stimulation of PriA by PriB W47A,K82A did correlate with the high apparent $K_d$ of this mutant PriB for ssDNA (27). One explanation for this apparent discrepancy is that interaction of PriB with PriA bound to DNA might increase the local concentration of PriB near the DNA substrate. Such a local increase in PriB concentration might therefore ameliorate the effects of reduced affinity for ssDNA displayed by the mutant PriB proteins in the absence of PriA. Therefore, the ability of the PriB mutant proteins to form complexes with PriA bound to DNA was analyzed. Each PriB single mutant in the presence of PriA formed a complex with fork 6 with a reduced electrophoretic mobility as compared with the complex formed with PriA and fork 6 only (Fig. 8, complexes II and I, respectively). We concluded that the F42A, W47A, and K82A PriB single mutants retained the ability to form a PriA-PriB-DNA complex. It is noteworthy, however, that the tendency for complex II to show a faster migrating smear positively correlates with the defect of each mutant for ssDNA binding, as reported previously (27). The smearing of complex II might reflect a faster off rate for the PriB-ssDNA interaction, thus destabilizing the complex. In contrast, no stable complex II was detected with PriB W47A,K82A (Fig. 8). However, some smearing of complex I was seen in the presence of PriB W47A,K82A, suggesting that a ternary complex was formed but was unstable during the electrophoretic separation. Both wild type PriB and PriB K82A also formed low levels of a complex with fork 6 in the absence of PriA (complex III in Fig. 8), likely reflecting low affinity binding of PriB to DNA in the absence of PriA.

Thus the stimulation of PriA by PriB correlated with the ability of PriB to form a stable PriA-PriB-DNA complex. Moreover, formation of this ternary complex correlated with the inherent affinity of PriB for
ssDNA. Binding of ssDNA by PriB within a PriA-PriB-DNA complex appears to be required therefore for stimulation of PriA helicase.

**DISCUSSION**

PriB is known to bind ssDNA (25, 27, 28), to interact with PriA to form a PriA-PriB-DNA complex (35), and to facilitate interaction between PriA and DnaT (16). The data presented here demonstrate that PriB also stimulates PriA-catalyzed unwinding of duplex DNA (Fig. 1). This stimulation resulted in an apparent increase in the processivity of PriA helicase (Fig. 5 and TABLE ONE). A stable PriA-PriB-DNA complex was required for maximal stimulation of PriA by PriB (Figs. 7 and 8), and formation of this stable ternary complex required DNA substrates possessing ssDNA (Fig. 6).

How might PriB enhance the apparent processivity of PriA helicase? Another protein capable of binding ssDNA, SSB, increases the apparent processivity of PriA by two mechanisms. First, the acidic C terminus of SSB interacts directly with PriA and effects stimulation of PriA-catalyzed unwinding (21). Second, SSB binding of ssDNA intermediates of PriA-catalyzed unwinding increases the apparent processivity of PriA (21, 36, 37). Trapping of partially unwound intermediates of DNA unwinding reactions by single strand DNA-binding proteins is known to enhance the apparent processivity of more distributive helicases (39), preventing their reannealing upon dissociation of the helicase and allowing binding of a second helicase molecule to complete unwinding.

Although 4 of the 10 C-terminal residues of PriB are acidic (40), this C terminus does not form a flexible, structurally disordered domain in the crystal structure of PriB, as found in SSB (41). Therefore, the PriB C terminus might not act as a protein interaction domain. It is therefore tempting to speculate that PriB increases the apparent processivity of PriA by prevention of reannealing of partially unwound intermediates. In this model the limited processivity of PriA (Fig. 4) would result in dissociation of PriA prior to completion of unwinding. PriB bound to the unwound intermediates would prevent their reannealing and allow a second PriA molecule to bind to the DNA substrate, translocate, and initiate a second unwinding cycle to complete unwinding of the remaining dsDNA. This model predicts that PriB would remain bound to ssDNA in the absence of PriA and that it is this activity of PriB that enhances the apparent processivity of PriA. However, the affinity of PriB for ssDNA is enhanced significantly when the DNA substrate is bound also by PriA (Figs. 6 and 8). The corollary of this observation is that PriB might not remain associated with ssDNA upon dissociation of PriA, arguing against PriB merely preventing reannealing of ssDNA in a manner analogous to SSB. Instead, simultaneous contact between PriB and ssDNA and PriB and PriA might be needed for stimulation of PriA helicase. This idea is supported by the inability of PriB to stimulate another low processivity helicase involved in replication restart, Rep (6) (data not shown). Stimulation within a ternary PriA-PriB-DNA complex might be achieved via PriB interacting with PriA to effect a conformational alteration in PriA. Such a conformational change might decrease the rate of dissociation of PriA from DNA during the unwinding cycle.

Previous data that indicated PriB stabilized binding of PriA to DNA (16) also raise another possibility: upon dissociation of PriA from partially unwound DNA substrates, interaction of PriA with PriB bound to ssDNA exposed by PriA catalysis might tether the dissociated PriA near the DNA substrate. Such an interaction would lead to an increase in the rate of reassociation of PriA molecules with partially unwound intermediates resulting in an apparent increase in processivity. However, although the level of bound single-stranded oligonucleotide was enhanced upon addition of PriB to PriA (Fig. 6B, compare lanes 2 and 5), no such enhancement was seen on the forked DNA containing ssDNA (Fig. 6A, compare lanes 10 and 13). Identification of PriB mutants that display high affinity for ssDNA but cannot interact with PriA are needed to help resolve the mechanism of PriB-directed stimulation of PriA.

A region of ssDNA was needed for stable formation of a PriA-PriB-DNA complex (Fig. 6). This indicated that stimulation of PriA helicase by PriB might occur only after partial unwinding of any duplex lagging strand DNA by PriA has exposed ssDNA for PriB binding. Although this observation does not distinguish between the models of PriA stimulation discussed above, it does indicate that any stimulation of PriA helicase at forks bearing a dsDNA lagging strand arm must occur after initiation of unwinding by PriA. Specific targeting of stalled forks for replication fork reloading would therefore appear to reside solely with the DNA binding specificity of PriA rather than PriB. This was supported by the absence of DNA structure specificity of PriB stimulation of PriA (Figs. 1 and 5).

If PriB plays no role in the DNA structure specificity of replication restart, what might the function of PriB-directed stimulation of PriA helicase be in vivo? The stimulation of PriA by SSB has been suggested to ensure that sufficient ssDNA is exposed on the lagging strand template of stalled forks for PriA-directed loading of DnaB to occur (21). PriB stimulation of PriA might perform a similar task. However, genetic studies have suggested that the helicase function of PriA is important for PriC-dependent but not PriB-dependent replication restart (42). It might be that the PriA/PriC pathway restarts replication primarily from stalled forks, giving rise to a requirement for PriA helicase activity when the lagging strand arm is duplex, while the PriA/PriB pathway might restart from both forks and D-loops. When restarting replication from D-loops is an option, PriA helicase activity would presumably not be required, although it probably would be required when PriA/PriB is mediating restart from a fork with a duplex lagging strand arm. This could explain how PriA helicase activity appears to be dispensable in the context of a bacterium competent to restart replication from D-loops. Could the stimulation of PriA helicase be related therefore to the ability of PriB to enhance interaction between PriA and DnaT (16)? One model proposed above for the increase in apparent processivity of PriA invokes a PriB-induced conformational change in PriA. It is therefore possible that any PriB-induced conformational shift in PriA both stabilizes an interaction between PriA and DnaT and increases the processivity of PriA.

The stimulation of PriA helicase by both PriB and SSB highlights a more general point concerning helicase catalysis. Many helicases when studied in isolation display rather low processivity, suggesting that the extent of unwinding reactions is limited in vivo. The case of PriA illustrates that helicases operate frequently as part of complex multiprotein machines in which networks of protein-protein contacts play a vital role in the correct functioning of the machine. Care should therefore be taken when drawing conclusions concerning the in vivo function of helicases from in vitro studies of helicases in isolation.

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