DNA helicases are motor proteins that couple the chemical energy of nucleoside triphosphate hydrolysis to the mechanical functions required for DNA unwinding. Studies of several helicases have identified strand-separating “pin” structures that are positioned to intercept incoming dsDNA and promote strand separation during helicase translocation. However, pin structures vary among helicases and it remains unclear whether they confer a conserved unwinding mechanism. Here, we tested the biochemical and cellular roles of a putative pin element within the *Escherichia coli* PriA DNA helicase. PriA orchestrates replication restart in bacteria by unwinding the lagging-strand arm of abandoned DNA replication forks and reloading the replicative helicase with the help of protein partners that combine with PriA to form what is referred to as a primosome complex. Using *in vitro* protein–DNA cross-linking, we localized the putative pin (a β-hairpin within a zinc-binding domain in PriA) near the ssDNA–dsDNA junction of the lagging strand in a PriA–DNA replication fork complex. Removal of residues at the tip of the β-hairpin eliminated PriA DNA unwinding, interaction with the primosome protein PriB, and cellular function. We isolated a spontaneous intragenic suppressor mutant of the *priA* β-hairpin deletion mutant in which 22 codons around the deletion site were duplicated. This suppressor variant and an Ala-substituted β-hairpin PriA variant displayed wildtype levels of DNA unwinding and PriB binding *in vitro*. These results suggest essential but sequence nonspecific roles for the PriA pin element and coupling of PriA DNA unwinding to its interaction with PriB.

Cellular DNA replication complexes often encounter obstacles such as DNA lesions or protein–DNA complexes that block replication fork progression, leading to genome instability (1, 2). In bacteria, these encounters can cause replication fork collapse, leaving abandoned replication forks that are lethal if left unresolved (3, 4). Bacteria use conserved “DNA replication restart” pathways to reinitiate DNA replication at prematurely terminated replication sites (5–7). The PriA DNA helicase is the primary protein that orchestrates replication restart in bacteria. PriA recognizes abandoned DNA replication forks by binding them in a structure–specific manner, remodels the DNA around the fork junction, and recruits other DNA replication restart proteins to form a “primosome” complex that reloads the replicative helicase onto the lagging-strand DNA template (5). The replicative helicase can then assemble the rest of the DNA replication machinery to allow replication to continue (8). Primosome assembly in *Escherichia coli* can proceed through two PriA-mediated pathways: the “PriA–PriB” pathway involving PriA, PriB, and DnaT or the “PriA–PriC” pathway, which relies on PriA, PriC, and DnaT (5, 6, 9). PriC can mediate a third, PriA-independent, DNA replication restart pathway in *priA*-null strains, although cells that exclusively rely on this pathway display several defects including SOS induction, cell filamentation, and chromosome partitioning (9–11).

PriA is a 3′-5′ SF2 SUP2 DEH helicase that remodels DNA replication fork structures using its 3′-5′ helicase activity to preferentially unwind the lagging arm if it is double strand or by modulating the DNA-binding modes of SSB if ssDNA gaps are present (12, 13). These activities expose ssDNA on the lagging arm, which is thought to be required for replicative helicase loading.

PriA is comprised of a central motor domain surrounded by four accessory domains that contribute to PriA activity (Fig. 1A). The motor domain is defined by two RecA-like lobes with evolutionarily conserved sequence motifs that link ATP hydrolysis to DNA translocation and unwinding. Outside of the helicase core, the N-terminal 3′-binding domain binds the leading arm of replication forks, with specificity for the 3′ end of the nascent leading strand (14, 15). A winged helix domain is tethered to the 3′-binding domain and the helicase core by structurally-dynamic linkers. The winged helix domain interacts with the parental duplex (14). Within the second helicase lobe, PriA contains a CRR that coordinates two zinc ions through eight invariant Cys residues. The CRR domain is thought to serve a role in DNA unwinding and interaction with the primosomal protein PriB (16). Finally, a C-terminal domain forms a structural core by interfacing with each of the other domains (except for the loosely-associated winged helix domain). The
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C-terminal domain also engages the leading strand DNA and is hypothesized to interact with the replication fork branch site (13, 14). In addition to its DNA-binding functions, PriA interacts with other proteins, including SSB and PriB (12, 17–19). Binding to either protein stimulates PriA DNA unwinding (12, 17–19).

Despite our advanced understanding of the structure of PriA, the mechanisms by which PriA unwinds DNA remain ill-defined. An aromatic-rich loop/helicase motif IIA that interacts with DNA near the fork junction has been shown to be critical for coupling DNA binding to ATP hydrolysis and DNA unwinding (20). It is not clear, however, how this activity is linked to strand separation. Based on its location adjacent to the helicase core and prior mutagenesis studies, the CRR domain has been hypothesized to serve as a DNA-unwinding domain in PriA (13, 16, 21–23). The CRR domain is in a similar location to unwinding structures found in other SF2 helicases and mutations of the Cys residues within the CRR domain result in a loss of helicase activity, but not DNA binding or DNA-dependent ATPase functions (16). A prominent β-hairpin is located between the two zinc-coordinating Cys clusters within the CRR (Fig. 1A) (13). The β-hairpin extends into the putative DNA-binding tract of the helicase core, placing it in the predicted path of dsDNA substrate. β-Hairpin DNA strand-separation elements (termed “pins” for short) have been identified in multiple SF1 and SF2 helicases and are thought to serve as physical barriers that split the two strands of dsDNA (for review, see Refs. 24 and 25). In several mechanistic models, the bilobed helicase core translocates on ssDNA, forcing the incoming dsDNA against the pin element. Pin elements often encode residues that interact with the terminal bp of the dsDNA at ssDNA–dsDNA junctions (26–32). However, the structures and positions of pins within helicase primary structures can vary, and it remains unclear whether they convey a conserved mechanism of DNA unwinding among helicases.

In this study, we examined the PriA CRR β-hairpin as a putative DNA strand-separation pin. Consistent with a pin-like function, cross-linking studies mapped the CRR β-hairpin to the ssDNA–dsDNA junction of the lagging strand within a PriA–replication fork complex. Deletion of the tip of the β-hairpin led to a loss in helicase activity but not DNA binding or DNA-dependent ATP hydrolysis functions. The β-hairpin deletion variant also lost the ability to interact with the PriB primosome protein in vitro. An E. coli strain in which the priA gene was mutated to encode the PriA β-hairpin deletion variant displayed genomic stress and chromosome partitioning defects that resembled those of a priA-null strain. Interestingly, a spontaneous intragenic suppressor of the β-hairpin deletion mutant was isolated in which 22 codons around the original deletion site were duplicated. The suppressor variant and an Ala-substituted β-hairpin PriA variant displayed DNA unwinding and PriB interaction functions in vitro that were indistinguishable from WT PriA. Together these results support a role for the PriA CRR β-hairpin as a pin structure that is essential for DNA unwinding, protein partner (PriB) interaction, and cellular function. Surprisingly, the sequence of the PriA pin was not critical to its function, suggesting that it functions as a simple physical wedge to aid DNA unwinding and that this wedge/DNA interface is critical for subsequent PriB binding.

Results

PriA–DNA fork cross-linking maps the CRR β-hairpin to the location of DNA strand separation

To identify structures that could be important for PriA DNA unwinding, the Klebsiella pneumoniae and E. coli PriA helicase structures (13, 14) were superimposed with 3′–5′ SF2 helicases for which structures with DNA substrates have been determined. These included Hel308 (28) and several RecQ family helicases (30–32). Each of these proteins have strand-separation pin elements that map to similar locations relative to the helicase domain despite being located in different domains (helicase lobe 2 and C-terminal winged helix domain; Fig. S1). NS3 helicase was also overlaid, although not bound to a similar ssDNA–dsDNA structure, because its putative strand-separation pin has been identified (28, 33). As has been noted (13, 24, 34), the overlay showed that a β-hairpin from the PriA CRR (residues 452–462 in E. coli PriA (Fig. 1A)) was located at a similar position to pins in other helicases, implicating it as a putative strand-separation pin. Using the position of the DNA from the bacterial RecQ (32) and RecQ1 (30), a model of PriA bound to a helicase substrate was built (Fig. 1). Because PriA operates on abandoned replication forks and preferentially unwinds the nascent lagging strand (12), the DNA-binding model predicts that the CRR β-hairpin would specifically interact with replication fork DNA near the ssDNA–dsDNA junction of the lagging strand arm.

We took a site-specific cross-linking approach to map the location of the CRR β-hairpin within a PriA–replication fork complex. E. coli PriA variants were created with the non-natural amino acid Bpa incorporated at 1 of 4 residues at the tip of the β-hairpin (PriA Q456Bpa, A457Bpa, Q458Bpa, and H459Bpa). Each purified variant was incubated with a synthetic DNA replication fork, then exposed to UV light and assayed for the presence of covalent protein–DNA cross-links by a denaturing EMSA, as has been done previously to map other DNA-binding interfaces in PriA (14, 20). A previously published PriA E492Bpa variant, which also cross-links with the template lagging strand (20) and is positioned near the CRR β-hairpin, was analyzed for comparison. Four DNA fork types were analyzed with each variant, where each of the four oligonucleotides of the DNA fork were individually radiolabeled. If a Bpa side chain is in close proximity to DNA and in an appropriate orientation, UV exposure will generate a covalent protein–DNA cross-link (35–38). The covalently-linked PriA–DNA complexes migrate more slowly than replication fork DNA in protein-denaturing electrophoresis experiments. Consistent with the CRR being near DNA, each PriA Bpa variant produced significant levels of PriA–DNA cross-linking in the EMSA analysis (left four lanes for each variant in Fig. 2). We mapped the specific strand(s) to which the PriA variants cross-linked by carrying out the EMSA on heat-denatured samples of the cross-linked PriA–DNA complexes, which separates noncovalently linked DNA molecules (right four lanes for each variant in Fig. 2). The cross-links all appeared to be specifically with the template-lagging strand.
We next used primer extension to map the specific DNA sites on the template-lagging strand to which Bpa residues cross-linked. In this assay, a labeled primer was annealed to the template-lagging strand 1–7 nucleotides downstream from the position of the first bp of the lagging strand. These data align well with the model shown in Figs. 1A and 3B, which places the CRR β-hairpin in close proximity to the template-lagging strand in the PriA–DNA fork complex, near the ssDNA–dsDNA junction of the DNA replication fork.

**The CRR β-hairpin is essential for PriA DNA unwinding**

To test whether the CRR β-hairpin is required for *E. coli* PriA DNA helicase activity, we created a deletion variant in which the loop residues from the tip of the β-hairpin were removed (PriA ΔβH) (Fig. 1B). The deletion removed 5 residues, leaving 1 His in the place of the 2 His residues that mark the ends of the hairpin β-strands. We hypothesized that, if the CRR β-hairpin is involved in DNA unwinding, this variant should be defective in unwinding DNA but retain DNA-binding and ATPase activities, as has been seen for other SF1 and SF2 strand-separation pins (33, 40, 41). Consistent with our prediction, PriA ΔβH had no detectable helicase activity on either a 4-strand or 2-strand DNA fork substrate that WT PriA unwinds robustly (Fig. 4A). These data matched the results seen with an ATPase-dead variant in which an essential Lys residue in helicase motif I was changed to an Arg (PriA K230R). Inclusion of higher concentrations of PriA (up to 50 nM) or longer incubation times (up to 90 min) did not alter the result. Because SSB and PriB are known to stimulate PriA helicase activity (12, 17–19), we also tested whether their inclusion would allow DNA unwinding by PriA ΔβH. Neither SSB nor PriB stimulated PriA ΔβH DNA unwinding (Fig. S2).

PriA ΔβH DNA-binding and DNA-dependent ATP hydrolysis activities were also tested to determine whether the variant maintained other core biochemical activities of PriA. In an equilibrium fluorescence anisotropy DNA-binding assay, PriA ΔβH bound forked DNA with high affinity (Fig. 4B). The disso-
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Figure 2. PriA–DNA fork cross-linking localizes the CRR β-hairpin to the DNA interface with the template-lagging strand. PriA–Bpa variants (3.5 nm) were incubated with a synthetic 4-strand DNA fork (1 nm; depicted in top right) that had each of the four oligonucleotides individually 5'-32P-labeled and exposed to UV. These samples were SDS denatured and resolved by PAGE (first set of four lanes for each PriA variant: 1) template-lagging strand labeled; 2) template-leading strand labeled; 3) nascent lagging strand labeled; 4) nascent leading strand labeled). These samples were additionally heat-denatured in the presence of trap oligonucleotide (excess unlabeled version of the 32P-labeled oligonucleotide) for better resolution of the single oligonucleotides. Taken together, these data suggest that the CRR PriA. In DNA-dependent ATPase activity, PriA was similar to the 8.8 KD association constant (Kd) of PriA ΔβH was 17.3 ± 0.7 nm, which was similar to the 8.8 ± 0.6 nm apparent Kd measured for WT PriA. In DNA-dependent ATPase activity, PriA ΔβH was indistinguishable from WT PriA, in both maximal ATPase rates (kmax) and dT28 required for 50% maximal stimulation (KD) (Fig. 4C). Taken together, these data suggest that the CRR β-hairpin is essential for PriA coupling of DNA unwinding to DNA-binding and ATP hydrolysis. These observations support the idea that the CRR β-hairpin acts as a strand-separation pin in PriA.

The CRR β-hairpin is essential for PriA function in vivo

We next analyzed the consequences of the β-hairpin tip deletion in vivo by creating an E. coli strain in which the priA gene was mutated to encode PriA ΔβH (priAΔβH). We predicted that the priAΔβH strain would behave like a priA300 mutant, which encodes the ATPase- and helicase-dead PriA K230R protein in vivo. The priA300 mutation has no phenotype in otherwise WT E. coli backgrounds, whereas priA-null strains display a highly induced SOS DNA-damage response and defects in chromosome partitioning, characterized by elongated nucleoids and cells (42–46). These results have been interpreted to mean that PriA helicase activity is not essential for the majority of DNA replication restart events required in normal growing conditions. However, priA300 cells are sensitized to mutations that affect other genome maintenance pathways in E. coli (21, 47–49) and PriA helicase activity is required for a number of phage and plasmid replication systems and following certain DNA repair pathways (5, 6).

E. coli cells with the priAΔβH allele were analyzed by microscopy and the phenotypes were compared with isogenic strains carrying the WT priA or priA-null alleles (priA2::kan). Two reporter systems were used to measure the level of SOS induction and to visualize nucleoids. SOS induction was quantified in live cells by expressing gfp linked to the sulA SOS-inducible promoter (46, 50). Nucleoids were visualized by observing the location of a fusion protein comprising HupA nucleoid-binding protein and mCherry (51). In unexpected contrast to priA300 cells, priAΔβH cells displayed phenotypes that were similar to priA-null cells (Fig. 5). These included significant SOS induction, nucleoid irregularities (Par phenotype), and elongated cells (quantified through increased cell area). These phenotypes indicate that priAΔβH cells have high levels of DNA damage and are defective for chromosome partitioning, presumably due to inability of cells to complete DNA replication at levels near that observed with priA-null cells. The data suggest that the CRR β-hairpin is essential for PriA cellular function.

To further test whether the priAΔβH allele is an inactive priA allele, we tested for synthetic lethality with a priC-null mutation. PriC can mediate an alternative replication restart pathway that allows E. coli cells to survive in the absence of a func-
tional PriA, and a priA priC double mutation is synthetically lethal due to the essential nature of DNA replication restart (7, 9). A transduction experiment was carried out to determine whether the priA/H9004/H9252 allele could be combined with a priC-null allele that is lethal in combination with a priA-null allele. Consistent with an inactive priA allele (9), introducing priA/H9004/H9252 into a priC303::kan strain nonselectively by P1 transduction resulted in zero double mutants when 32 transductants were screened. In contrast, transduction of priA/H9004/H9252 into a priC/H11001 strain revealed a 57% co-transduction frequency (16 of 28). This analysis indicates that the priA/H9004/H9252 and priC303::kan mutations are synthetically lethal as would be expected if priA/H9004/H9252 behaves as a priA-null mutant. Additionally, priA/H9004/H9252 cells could be complemented by a plasmid borne copy of WT priA, indicating it is a recessive allele (Fig. S3). These results are consistent with the deletion of the β-hairpin inactivating an essential PriA function.

The CRR β-hairpin also serves a role in PriB interaction with the PriA–DNA complex

E. coli have two PriA-mediated DNA replication restart pathways: a PriA–PriB and a PriA–PriC pathway (5, 7). Because the priAΔβH mutant behaves like a priA-null mutation, it appears that deletion of the tip of the CRR β-hairpin has ablated essential PriA functions required in both the PriA–PriB and PriA–PriC pathways. PriA ATPase/helicase activity is required for the PriA–PriC pathway, whereas the PriA–PriB pathway remains functional without PriA ATPase activity (9, 21). Therefore, the essential role of the CRR β-hairpin in DNA unwinding explains a nonfunctional PriA–PriC pathway in priA/H9004/H9252 cells, but a second function of the CRR β-hairpin is needed to explain a nonfunctional PriA–PriB pathway.

Mutations of the Cys residues within the PriA CRR affect both helicase activity and interaction with PriB (16, 21–23). Therefore, we tested whether the CRR β-hairpin serves a role in PriB interaction. In EMSAs, PriA binding slows the migration of a replication fork DNA structure and PriB binds with high affinity to the PriA–DNA complex, leading to formation of a slower migrating (super-shifted) PriA–PriB–DNA species (Fig. 6)(17, 23). At high PriB concentrations, PriB alone incompletely shifts the DNA to a distinct band that is faster migrating than the PriA–DNA or PriA–PriB–DNA complexes (Fig. 6), indicating that the PriB supershift is due to PriB binding to the PriA–DNA complex. When PriAΔβH is used in the EMSA, no PriB supershift was observed, indicating that PriB is not able to form a stable interaction with the PriA ΔβH–DNA complex. We note that in these assays, a significant fraction of the DNA...
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Figure 4. The CRR β-hairpin is required for the PriA DNA unwinding mechanism. A, PAGE resolved helicase products from incubation of the indicated PriA variants (0.1, 1, and 10 nM) with a synthetic DNA fork (1 nM; shorter version of that used in Figs. 2 and 3; 60-bp duplex and 38-nucleotide arms) composed of four strands (left) or two strands (right). Initial substrates are depicted in black to left of the gel panel and helicase products are depicted in gray. Red star notes location of the 5'-32P label. The two gel panels are from one experiment of at least three replicates. B, DNA-binding assay following the increase in anisotropy upon increasing levels of PriA binding to a 5'-fluorescein-labeled two-strand DNA fork (1 nM; same fork as in A with green star noting location of 5'-fluorescein). Mean ± S.D. (error bars) of three replicates are fit to a single-site specific model (see “Experimental procedures”). Error bars are obscured by data point when not visible. Parameters of fit ± S.D. are listed in the inset. C, PriA DNA-dependent ATP hydrolysis rates upon incubation with increasing amounts of ssDNA (dT28). Data are mean ± S.D. (error bars) of three replicates. Data were fit (solid line) to obtain parameters ± S.D. listed in the inset.

Figure 5. The CRR β-hairpin is required for PriA function in vivo. A, microscopy images of some of the strains used in this study (Table S2). Phase-contrast (left column) shows the cell area. GFP fluorescence (second column) was used as a measure of SOS induction, as a fusion reported of the gfp gene to the sulA promoter was used. mCherry fluorescence (right column) was used to visualize the nucleoid through a translational fusion of the hupA gene to mcherry. Scale bar is 10 μm. B, quantification of microscopy images similar to those in A. Relative fluorescence intensity (RFI) of green fluorescent protein (GFP) signal. WT nucleoids are indicated as positive “+” for chromosome partitioning, whereas irregular/elongated nucleoids are indicated as negative “−” for chromosome partitioning. Average cell area (number of pixels) was quantified as a measure for elongated cells. Data are mean ± S.D.
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Figure 6. The CRR β-hairpin serves a role in PriB interaction with the PriA–DNA complex. Native PAGE of EMSA of the synthetic DNA fork (1 nm; depicted to left of gel) used in Fig. 4 except nascent leading strand was included and nascent lagging strand excluded) incubated with PriA variant (2 nm) and increasing concentrations of PriB (0, 10, 40, and 160 nm monomers). First lane is DNA fork alone. Second lane is PriB (160 nm) without PriA. Representative gel of three replicates.

was not bound by PriA ΔβH and that this was observed under increased PriA concentrations as well. However, this experiment focused on PriB interaction with the PriA–DNA complex. Unlike PriB in the presence of other PriA variants in this study (described below), in the presence of PriA ΔβH, PriB only bound DNA alone, forming the low affinity PriB–DNA shift without binding/shifting any PriA–DNA complexes. Thus, the PriA CRR β-hairpin appears to be essential for interaction with PriB in vitro, which could explain PriA ΔβH inactivity in the PriA–PriB pathway.

A priAΔβH intragenic suppressor mutation restores the CRR β-hairpin functions

Spontaneous suppressors of the rich media growth defects of priAΔβH cells were isolated. Previously, suppressors of priA-null rich media growth defects have mapped to the helicase loader, dnaC in E. coli; these suppressors are thought to allow bypass of replication restart by relaxing restrictions on replication reinitiation (5, 6). Indeed, several expected dnaC mutations were found among the priAΔβH suppressor strains but, interestingly, one strain that lacked a dnaC mutation was isolated. This suppressor mutation was mapped by P1-transduction to the priA gene and sequencing of the entire priA gene revealed a duplication event that resulted in a 22-codon insertion near the site of the original β-hairpin deletion (priAsupΔβH) (Fig. 1B). In a fresh genetic background, the priAsupΔβH allele showed no difference from the WT phenotype (Fig. 5B).

To begin to test the functional impact of the priAsupΔβH suppressor, we analyzed whether the function of the PriA–PriB and/or PriA–PriC pathways were restored in vivo with the suppressor mutation. These two pathways were analyzed by individually deleting priB or priC and determining if the presence of the priAsupΔβH allele led to a priA+/priA-null phenotype. The PriA–PriB and PriA–PriC pathways are thought to be genetically redundant and priB or priC can individually be deleted without apparent consequence in the presence of WT priA (53). To analyze the function of the PriA–PriB pathway, we combined priAsupΔβH with a priC-null mutation. This double mutant had only slightly increased SOS levels from the WT strain, suggesting that the PriA–PriC pathway could be ablated and that the cells were nearly unaffected due to the presence of a functional PriA–PriB pathway (Fig. 5B).

In contrast, when we analyzed the function of the PriA–PriC pathway by combining priAsupΔβH with a priB deletion, the double mutant had significantly increased SOS levels, nucleoid irregularities (Par−), and increased cell area, at levels similar to the priAΔβH strain (Fig. 5B). This indicates that the PriA–PriB pathway is required in priAsupΔβH cells due to a nonfunctional PriA–PriC pathway. This situation might be expected if the suppressor mutation restored an essential function of the CRR β-hairpin in the PriA–PriB pathway but did not restore PriA helicase activity, because the results with priAsupΔβH are similar to what is seen with the ATPase-dead priA300 mutation (21).

To determine the extent to which the suppressor mutation restored the PriA CRR β-hairpin functions, we purified the priAΔβH suppressor PriA protein (PriA SupΔβH) and tested its ability to interact with PriB and to unwind DNA in vitro. Because in vivo double mutant analysis of this suppressor indicated a functional PriA–PriB pathway and nonfunctional PriA–PriC pathway, we predicted that the 22-residue insertion would restore the PriA–PriB interaction but not PriA helicase activity. Analysis of PriB interaction by EMSA showed that the PriA SupΔβH–DNA complex was able to form a complex with PriB in a manner that was indistinguishable from WT PriA (Fig. 6). Thus, it appears that the suppressor supports complex formation with PriB.

We next tested the helicase activity of PriA SupΔβH. In contrast to our expectation that the variant would lack helicase activity like PriA300, PriA SupΔβH exhibited helicase activity on two different substrates (Fig. 7). On a four-strand DNA fork, PriA SupΔβH unwound the lagging strand at significant levels, although the maximal unwinding was reduced 2.9-fold from that observed with WT PriA. PriA SupΔβH was also able to be stimulated by SSB and PriB, yielding PriA SupΔβH activity levels near that of WT PriA. PriA SupΔβH also unwound a simple, two-strand DNA fork at WT PriA levels, with or without the presence of PriB (Fig. 7). These results show substantial helicase activity with the suppressor mutation in contrast to what was predicted from in vivo analysis, suggesting that full PriA helicase activity could be required for the PriA–PriC pathway in vivo. Notably, the PriA–PriC pathway was analyzed in a priB
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**Figure 7.** The CRR β-hairpin serves a physical role in DNA unwinding without sequence requirement. Quantification of PAGE of helicase products, as described in the legend to Fig. 4A, from incubating PriA (0.075, 0.15, 0.3, 0.6, 1.2, and 2.4 nM PriA) with a 1 nm 4-strand DNA replication fork (top), which PriA variants specifically unwound the nascent lagging strand or two-strand DNA fork (bottom). Inset depicts substrate (black) and product (gray). Fraction unwound was determined by the quantification of the product band over the total quantification of the lane. Data were fit (left panel, solid lines). Right panels quantified PriA (1.2 nM) helicase activity under SSB (250 nM monomers) or PriB (10 nM monomers) stimulation for the 4-strand (top) or 2-strand DNA fork (bottom). Data are mean ± S.D. of three replicates.

deletion strain that would therefore lack PriB-stimulated levels of PriA helicase activity, which could exaggerate the lowered helicase activity levels in this specific experiment. Alternatively, these results could suggest that the suppressor mutant (and original deletion mutant) lack some other, yet unknown, function of PriA that is essential for the PriA–PriC pathway.

The PriA strand-separation pin serves a sequence-independent role

The considerable in vitro activity of the PriA $\text{SUP}_{\text{DH}}$ variant suggests that the CRR β-hairpin could serve a physical role in DNA unwinding and PriB interaction, without sequence requirements. This predicts that Ala substitutions within the loop would have little effect on the in vitro helicase activity and DNA–PriA–PriB interaction. To test this hypothesis, we substituted all of the residues of CRR β-hairpin tip (residues 456–460 in *E. coli* PriA; Fig. 1B) for Ala and tested the resulting variant (PriA $\text{H5Ala}$) in vitro. Consistent with a sequence-independent role of the β-hairpin in these two PriA functions, PriA $\text{H5Ala}$ exhibited helicase levels that were indistinguishable from WT PriA (Fig. 7) and formed a DNA fork–PriA–PriB supershift at low PriB concentrations similar to WT PriA (Fig. 6). We further asked if this variant was competent for PriA function in vivo. Consistent with a sequence-independent role of the CRR β-hairpin in PriA function, a strain in which the WT $\text{priA}$ allele was replaced with the PriA $\text{H5Ala}$ allele ($\text{priA}_{\text{H5Ala}}$) showed WT phenotypes for SOS induction, nucleoid partitioning, and cell area (Table S1). When we made the $\text{priA}_{\text{H5Ala}}$ $\text{priC}$ and $\text{priA}_{\text{H5Ala}}$ $\text{priB}$ double mutations, we similarly observed normal SOS levels, nucleoids, and cell area, indicating that both PriA pathways are functional with this variant (Table S1). These results differed from what we observed with $\text{priA}_{\text{SUP}_{\text{DH}}}$, which was nonfunctional for the PriA–PriC pathway. This difference is consistent with a requirement for full PriA helicase activity in the PriA–PriC pathway, because PriA $\text{H5Ala}$ unwound DNA at WT PriA levels in our in vitro helicase assays in the absence of PriB, whereas PriA $\text{SUP}_{\text{DH}}$ had reduced helicase activity on the lagging arm (Fig. 7).

Overall, the results with $\text{H5Ala}$ and $\text{SUP}_{\text{DH}}$ PriA variants strongly suggested that the CRR β-hairpin functions without a strict sequence requirement and that the sequence of the PriA element tip would not be well-conserved evolutionarily. We therefore examined the sequence conservation of the PriA β-hairpin across diverse bacterial PriA sequences (Fig. S4). This analysis showed low sequence conservation of the β-hairpin residues relative to the surrounding sequence and identified one PriA sequence (from *Thiomicrospira milos*-T2) that has 13 additional residues in the middle of this hairpin. Thus it appears that a significant sequence variation can be tolerated in the CRR β-hairpin.

In contrast to the hairpin loop and C-terminal border His, the His at the N-terminal end of the β-strand hairpin tip (His-455 in *E. coli* PriA) is highly conserved. This His coordinates a water that is additionally coordinated by residues within helicase lobe 2 and the CTD, which could make the residue important for stabilization of the CRR/β-hairpin (14). To additionally test the sequence requirement at this location, we purified and tested the activity of a PriA variant that changed His-455 to Ala along with residues 456–460 in *E. coli* (PriA $\text{H6Ala}$; Fig. 1B). In EMSAs, PriA $\text{H6Ala}$–DNA fork complexes shifted with increasing PriB levels to the ternary complex but at significantly reduced levels from PriA $\text{H5Ala}$ or WT PriA (Fig. 6). Interestingly, this variant had no detectable helicase activity except under PriB stimulation (Fig. 7). In the presence of PriB and the four-strand DNA fork, PriA $\text{H6Ala}$ unwound the lagging strand at WT PriA levels. These results suggest that this N-ter-
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Figure 8. Model of CRR β-hairpin function within the PriA–PriB replication restart pathway. A, PriA domains are colored as described in the legend to Fig. 14. (1) An abandoned DNA replication fork is created. (2) PriA binds and interacts with the three arms of a DNA replication fork (14) positioning the CRR β-hairpin (black outlined triangle) near the incoming dsDNA. (3) Helicase lobe 1 and 2 domain movements (20, 56) and PriA affinity for fork junctions may result in lobe 2 binding DNA in an extended form and “pulling” the DNA across the CRR β-hairpin. (4) We propose PriB (dimer; magenta) interacts near the CRR β-hairpin/DNA interface. (5) At this location, PriB could bind ssDNA created by PriA helicase activity and stimulate PriA helicase activity through this interaction and/or stabilization of the CRR β-hairpin wedge. (6) PriB hands off its site on PriA and the DNA to DnaT (trimer; cyan) (23, 57, 80, 81). (7) The PriA–DnaT complex hands off the ssDNA site at the fork junction to the replicative helicase DnaB (hexamer; red), with the help of the replicative helicase loader DnaC (yellow) (52, 64). B, (1b) on forks with ssDNA gaps where SSB (tetramer; gray) is bound, (2b) PriA interacts with the SSB tail (light red; binding site on opposite face of PriA, yellow), with the help of the replicative helicase loader DnaC (yellow) (52, 64, 80, 81). (3b) The role of the CRR β-hairpin in unwinding the template lagging arm could additionally function to promote exclusion of proteins from the DNA, such as SSB. (4–7) PriB binding and possible β-hairpin stabilization/helicase stimulation could further promote this process and facilitate ssDNA hand-off and restart pathway progression.

Discussion

We have examined the roles of a β-hairpin in DNA strand-separation and primosome-assembly activities of PriA. In vitro protein–DNA cross-linking experiments indicated that the CRR β-hairpin localizes to the ssDNA–dsDNA junction of the lagging strand in a PriA–DNA replication fork complex, consistent with its hypothesized role as a strand-separation pin. A PriA variant in which the tip of the CRR β-hairpin was deleted (PriA ΔβH) failed to unwind DNA but was able to bind DNA and hydrolyze ATP in a DNA-dependent fashion. The variant also failed to interact with PriB within the PriA ΔβH–DNA complex in vitro. Mutation of the priA gene to encode PriA ΔβH led to phenotypes that closely resembled those of priA-null cells. An intragenic suppressor that duplicated 22 codons near the deletion site restored PriA DNA-unwinding activity and interaction with PriB in vitro, suggesting that a surprisingly diverse structure could substitute for the CRR β-hairpin in PriA. This notion was further tested with a PriA variant in which residues at the tip of the β-hairpin were substituted for Ala (PriA βH5Ala); this variant retained PriA DNA-unwinding, PriB interaction levels, and cellular activity. In addition to supporting a role for the CRR domain β-hairpin as a DNA-unwinding pin, these results suggest an essential but nonsequence-specific role for the pin in coupling DNA remodeling of abandoned DNA replication forks to protein–protein interactions in DNA replication restart.

The PriAβpa–DNA fork cross-link results (Figs. 2 and 3) coupled with the similarity of PriA to other helicases allows construction of a model of PriA–DNA fork binding that positions the CRR β-hairpin as a strand-separation pin that interacts with the lagging strand of a replication fork (Fig. 8). This correlates with the known specificity for PriA in unwinding the nascent lagging strand within abandoned replication forks and with prior studies that have assigned an overall arrangement of the three DNA arms bound to domains within PriA (14). The model is, furthermore, consistent with the loss of helicase activity observed upon deletion of the CRR β-hairpin (Fig. 4). Similar observations have been made for pins in other helicases (33, 40, 41), suggesting that the CRR β-hairpin serves as a strand-
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separating pin in PriA. Our studies showing that both PriA βH5Aia and SupΔBH had high levels of helicase activity and were able to interact with PriB (Figs. 6 and 7) suggest that a physical wedge is required at the β-hairpin site, but that the sequence or length of the pin is not important. These results fit with a “snow-plow” model for helicase unwinding in which the bilobed helicase core “pulls”/translocates on the tracking ssDNA strand and forces the dsDNA junction against the strand-separation pin (54). This pin could serve as a steric barrier to dsDNA, facilitating fraying of the lagging strand as the motor translocates.

The lack of apparent sequence requirement for the PriA pin distinguishes it from related elements in many other helicases. Sequence changes within the tips of pins in other helicases reduce DNA unwinding function (SF1: UvrD/PcrA, Dda; SF2: NS3, RecQ1/BLM (27, 31, 33, 40, 41, 55)). In these helicases, the tips of strand-separation pins often include aromatic and/or polar residues that interact with the DNA bases in a manner that is thought to promote strand separation (26–32). Although the structure (a β-hairpin) and types of residues found within the PriA pin appear to be similar to those found in other helicases, the PriA helicase/pin mechanism differs in its lack of a sequence requirement. We hypothesize that the PriA unwinding mechanism relies on steric exclusion upon thermal fraying of the DNA bases, whereas the pin mechanism of other helicases may additionally rely upon pin residue–DNA interactions to facilitate DNA unwinding. Intriguingly, convergent evolution may have selected for a strand-separation pin, considering the source of the PriA strand-separation pin within a domain not found in other helicases. One noted exception to these comparisons is the bacterial RecQ β-hairpin, in which deletion of this loop or Ala substitution of the His, which interacts with the displaced strand in the Cronobacter sakazakii RecQ (32) did not reduce helicase activity of E. coli RecQ (41), suggesting an altered strand-separation mechanism (32).

In our model of PriA helicase activity, the N-terminal domains of PriA would likely remain bound at the fork junction (inferred from the high affinity of PriA for DNA fork structures), whereas the helicase core acts on the lagging DNA strand, resulting in PriA “pulling in” that arm (Fig. 8). This action may occur through the movement of the helicase lobes with respect to each other, as proposed for other bilobed helicases (56). Consistent with this model, we have observed a bimodal distribution of helicase lobe 2 (E492_H11001) cross-links on the template-lagging strand that is enhanced by the presence of ATP analogs (20). During the resulting translocation of the helicase core on the template-lagging strand, the CRR β-hairpin promotes separation of the nascent lagging strand. We have not observed PriA cross-links to the nascent lagging strand and for model clarity we have drawn the unwound DNA as free. However, PriA surface electrostatics include a basic patch near the modeled excluded nascent lagging strand that could potentially interact with the excluded strand (Fig. S5). Moreover, SSB, which associates directly with and stimulates PriA (18), could bind that excluded strand to enhance DNA unwinding.

The results in this study support a coupling between PriA DNA unwinding and PriB interaction with functional implications. The spontaneous intragenic suppressor of PriA ΔBH restored surprisingly robust levels of both helicase activity and PriB interaction in vitro (Figs. 6 and 7). These results suggested a sequence-independent role of the PriA β-hairpin in PriB interaction, as with DNA unwinding, which was further supported by the functional nature of the Ala-substituted β-hairpin variant (PriA βH5Aia) (Figs. 5–7). Considering that protein–protein interactions are rarely sequence-independent and PriB is thought to interact with PriA at the protein–DNA interface (57), we propose that the DNA unwinding mechanism is required for generating the interface required for PriB binding. PriB could interact with the PriA–DNA interface near the β-hairpin to stimulate PriA activities. Alternatively, PriB may stimulate PriA by interacting with the PriA-generated ssDNA present on the DNA-binding face of PriA, where the CRR β-hairpin is located, and additionally serving as a steric exclusion strand separation block. These models are not mutually exclusive.

Consistent with both of these models of PriB interaction with PriA, the N-terminal β-hairpin tip bordering His in PriA appears to be required for β-hairpin structure stabilization, however, loss of this His can be suppressed by the addition of PriB (Fig. 7). In our model of PriA helicase activity (Fig. 8), DNA pulled in by PriA would be in close proximity to PriB bound near the DNA–β-hairpin interface. As a SSB paralog that binds ssDNA (58–60), PriB could interact with this unwound DNA, “wrapping” the DNA similarly to SSB. In the replication restart model, PriB would then hand this DNA to DnAT, which also binds ssDNA (61). The DnAT–PriA–DNA complex could then utilize the ssDNA site to facilitate replicative helicase (DnAb in E. coli) loading. Considering that the ssDNA site size of PriA on the gapped DNA used in this study is 5 nucleotides and the ssDNA site sizes of PriB, DnAT, and DnAb are 8–12 (62, 63), ~25 (61), and ~20 nucleotides (65), respectively, PriA helicase activity and PriB may function together to create and protect the ssDNA for DnAT binding (and ultimately DnAb binding). Additionally, the β-hairpin and PriB could function in displacement of proteins, such as SSB, from the template-lagging strand (Fig. 8B). Helicases that use steric occlusion on a paired nucleic acid strand have the intrinsic potential to displace nucleic acid-binding proteins from the tracking strand as well (66). This work, therefore, provides insights into why PriA helicase activity and PriB may be required for certain DNA replication restart reactions, whereas ATPase-dead PriA or PriC are competent for others (6).

Experimental procedures

Protein purification

PriA proteins were purified as described previously (20), with the exception of PriA SupΔBH, which could not be expressed from a pET15 plasmid backbone and was expressed from pBAD/HisB. For PriA SupΔBH expression, E. coli C41 overexpression cells were transformed with pBAD-PriASupΔBH. Autoinducing media (67) supplemented with ampicillin and 0.3% arabinose was inoculated with transductant colonies and grown to an OD600 of 1.0. Cells were harvested and PriA protein purified as described previously. Briefly, cells were lysed via sonication and protein was purified using Ni²⁺ affinity, ion
exchange (SPFFF), and size exclusion (S300) chromatography. PriA_Bpa variants were constructed and purified as described previously (20). PriB and SSB were purified as described previously (58, 68).

Protein–DNA cross-linking and analysis

Cross-linking and analysis of protein–DNA cross-links (gel shifts and primer extension) was performed as described previously (20). The DNA fork structure was fully ds except for a 5-nucleotide ssDNA gap before the 5’ end of the nascent lagging strand, which is the ssDNA site size of PriA on gapped DNAs (69) and is commonly used for PriA because it is required for high PriA helicase levels (12) (Fig. 2). Briefly, the cross-linking fork was constructed by annealing oTW140, oTW141, and oTW168 with oTW143 (20) in 10 mM Tris-HCl, pH 7.5, 7 mM magnesium chloride, and 200 mM sodium chloride at 95 °C for 10 min, 70 °C for 60 min, and slowly cooling to room temperature. Annealed forks were purified by 7.5% PAGE in 1× TBE before cutting out the appropriately sized band and electroelution. All PriA variants used in cross-linking assays bound the DNA substrate near WT PriA levels (data not shown). 1 nM purified DNA cross-linking fork (5’-32P-labeled on 1 of the 4 oligonucleotides) was incubated with 3.5 nM PriA variant (or 2 nM unlabeled fork and 7 nM PriA for primer extension) in 50 mM Tris-HCl, pH 8.0, 0.1 mg/ml of BSA, 5 mM EDTA, 6% glycerol, and 50 mM sodium chloride for 30 min. Samples were exposed to UV for a total of 30 min. For gel shift analysis, samples were then diluted 1:1 with 2× SDS-loading buffer. Half of each sample was removed and boiled for 10 min in the presence of trap oligonucleotide (unlabeled version of the labeled oligonucleotide). All samples were resolved through 5% PAGE in 1× TBE, before fixing, drying, and exposing to a PhosphorImager screen and imaging on a Typhoon FLA 9000 (GE Healthcare). For primer extension analysis, cross-linked samples were added as the template in a primer extension reaction with Tag polymerase and a 5’-32P-labeled primer (oTW144 or oTW206 (20)) for 20 thermal cycles. Samples were resolved through 8% urea, 12% PAGE in 1× TBE before exposing the gel and imaging as above.

DNA-binding fluorescence anisotropy assay

PriA (0.1 nM to 5 μM) was incubated with 1 nM DNA fork (1b-98) and 5’ fluorescein-3L-98 (10, 11) incubated 1:1 at 95 °C for 10 min, 70 °C for 60 min, and slowly cooled to room temperature, in 20 mM HEPES-HCl, pH 7.0, 5% glycerol, 75 mM potassium glutamate, 0.1 μg/ml of BSA, and 10 mM DTT at 30 min at room temperature. Fluorescence anisotropy was measured using a Beacon 2000 and fit to a single site-specific model using GraphPad Prism. Data are the mean ± S.D. of three replicates. The data were modeled best with a Hill coefficient of ∼2–3, consistent with the positive cooperativity of PriA–dsDNA interactions (70).

ATPase assay

PriA (50 nM) was incubated with 0.1–5,000 nM dT28 in 20 mM HEPES-HCl, pH 8.0, 50 mM sodium chloride, 1 mM β-mercaptoethanol, 5 mM magnesium chloride, 0.1 mg/ml of BSA, 2 mM phosphoenolpyruvate, 3 units/ml of proteinase kinase, 4.5 units/ml of lactate dehydrogenase, and 1.33 mM NADH for 10 min at room temperature, before adding 1 mM ATP and monitoring the change in absorbance at 340 nm for 60 min at room temperature. Data were analyzed as described previously (32).

Helicase assay

5’-32P-Labeled 3L-98 was annealed with 1b-98 (for the two-strand fork) or 1b-98, 11b-38, and B-33 (for the four-strand fork) (10, 11), gel purified through 5% PAGE, an appropriately sized gel band was cut out, and electroeluted. Purified fork (1 nM) was incubated with PriA (0.075–2.4 nM) in 50 mM HEPES-HCl, pH 8.0, 40 mg/liter of BSA, 2 mM DTT, 2 mM ATP, and 4 mM magnesium acetate for 30 min at 37 °C. For the indicated reactions, SSB was preincubated with the DNA at 250 nM (monomers) or PriB at 10 nM (monomers). Reactions were stopped by the addition of 20 mM EDTA, 0.5% SDS, 0.2 mg/ml of proteinase K, and 2.5 ng/µl of trap oligonucleotide (unlabeled version of the 32P-labeled 3L-98) and incubated at 37 °C for 30 min. Samples were resolved in DNA-loading buffer through 10% PAGE and gels were fixed, dried, and imaged as above. ImageQuant (GE Healthcare) was used to quantify bands and the fraction unwound was determined by the quantification of the unwound band over the total for that lane.

PriB–PriA–DNA fork EMSAs

PriB interaction was analyzed in vitro as described previously (17). A three-strand DNA fork was used, where 5’-32P-labeled 3L-98, 1b-98, and 11b-38 were annealed and purified as above. 1 nM purified fork was incubated with 2 nM PriA and 0–160 nM PriB (monomers) in 50 mM Tris-HCl, pH 8.0, 0.1 mg/ml of BSA, 2 mM DTT, 5 mM EDTA, and 6% glycerol for 15 min on ice. Samples were resolved through 4% PAGE in 50 mM Tris and 2 mM EDTA. Gels were fixed, dried, and exposed to film.

Strains and media

All bacterial strains are derivatives of E. coli K-12 and are described in Table S2. As previously used with priA mutant strains, the strains analyzed in this study carried the sulB103 allele of ftsZ to prevent the action of the SOS cell division inhibitor sulA (71). The protocol for P1 transduction has been described previously (72). All P1 transductions were selected on 2% agar plates made with either Luria broth or 56/2 minimal media (supplemented with 0.2% glucose, 0.001% thiamine and specified amino acids, and containing the appropriate antibiotics) (72). Kanamycin was used at 50 μg/ml, chloramphenicol at 25 μg/ml, ampicillin at 50 μg/ml, and tetracycline at 10 μg/ml. All transductants were grown at 37 °C and purified on the same type of media on which they were selected. To place priAΔB (ΔpriA339; Table S2) onto the chromosome in place of the WT priA gene, we first prepared a strain that had both priA334:galK dnuC809,820 on the chromosome (SS10411). We made priA334:galK by inserting the galK cassette at position about 1400 bp into the gene (approximate site of the deletion to be made) (73). priA334:galK was then transferred to SS9574 to make SS10411. We then cloned the priAΔB into the BamHI–SalI restriction sites of pK03 (74) using PCR with prSJS1363 (GAT CAC TAC TAC ACG CTG CAT CAG CCG...
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CAG CAC CAT CTG CGC TAC CAC TGT GAC CTG TGT ACA ATT AAT CAT CGG CA and pSJS1364 (GGA CCA GGT GCG TGG AAC CGC AGG AAG GGC ACT GGC GCG GCA CGG GCT GAC TGT CAG GCC TGT CCT CTG) on pET15-Pri\( \Delta \)BH and called this plasmid pMAX7. As per the protocol of Link et al. (74), Pri\( \Delta \)BH was placed on the chromosome in a two-step procedure: first selecting for co-integrate formation and then allowing recombination to resolve the duplication of priA alleles, removing priA334:galK and retaining priA\( \Delta \)BH. The sequence of this allele was checked by DNA sequencing.

We placed priA \( \beta \)H5Ala (priA352::cat; Table S2) onto the chromosome in the presence of the normal priA gene. To do this priA \( \beta \)H5Ala was transferred to a plasmid called pSJS1625 that had the \( \Delta \)priA333 allele (14) and \(~\)250 bp upstream and 500 bp downstream of priA. It additionally had the CAT gene inserted just after the priA stop codon. pSJS1625 was restricted with EcoRV, PstI, and Eagl and the fragments of DNA were used to transform SS9276 (with pKD46). SS9276 had the priA gene from codon 270 to the TAA stop codon substituted with the kanamycin-resistance gene and contained dnaC809,820. A linear transformation protocol using exo-bet plasmid pKD46 (75) and electroporation of the DNA fragments was used to select CAT resistance transformants on LB-CAT plates and then screened for Kan\(^5\). One strain, SS11972, was saved and determined to have the priA \( \beta \)H5Ala allele on the chromosome. This priA \( \beta \)H5Ala allele was then checked by DNA sequencing.

Preparation and analysis of cells for microscopy

The cells for SOS expression were prepared, imaged, and computer analyzed as previously described (76). Briefly, cells were grown in minimal media to mid-log phase and then around 1 ml was spun down. The cells were then resuspended in \(~\)100 \( \mu \)l and 2 \( \mu \)l of that suspension was added to a 2\% agarose slab. A coverslip was then applied on top of the cells. Images (phase-contrast and fluorescent) were taken for at least 100 fields of view (three on three different days) for each strain. These images were analyzed by a combination of OpenLabs v5.5, Oufit (77), I-Vision (BioVision, Inc.), SuperSegger (78), and Matlab R2016 programs (Improvision, Inc., (79) and Mathworks Inc.). The relative fluorescence intensity for each strain was normalized to the average fluorescence intensity of the background. Typically, between 800 and 2000 cells are counted for each strain. Oufit is an open-source software designed for the analysis of microscope images (77). Programs written in Matlab R2016 (Mathworks, Inc.) were used to analyze the binary and fluorescent images to produce the data in Fig. 5B. Statistical analysis of the data were performed using \( \chi \)-square test of homogeneity.

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