PriC-mediated DNA Replication Restart Requires PriC Complex Formation with the Single-stranded DNA-binding Protein

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Received for publication, April 16, 2013. Published, JBC Papers in Press, April 29, 2013, DOI 10.1074/jbc.M113.478156

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Background: PriC can reload the DnaB helicase onto single-stranded DNA-binding protein (SSB)-coated DNA, which is the first step in DNA replication restart.

Results: PriC directly interacts with SSB and alters its mode of DNA binding.

Conclusion: PriC/SSB complex formation is essential for PriC-mediated DnaB loading.

Significance: Learning how DNA replication restart occurs is critical for understanding bacterial genome maintenance mechanisms.

Frequent collisions between cellular DNA replication complexes (replisomes) and obstacles such as damaged DNA or frozen protein complexes make DNA replication fork progression surprisingly sporadic. These collisions can lead to the ejection of replisomes prior to completion of replication, which, if left unrepaired, results in bacterial cell death. As such, bacteria have evolved DNA replication restart mechanisms that function to reload replisomes onto abandoned DNA replication forks. Here, we define a direct interaction between PriC, a key *Escherichia coli* DNA replication restart protein, and the single-stranded DNA-binding protein (SSB), a protein that is ubiquitously associated with DNA replication forks. PriC/SSB complex formation requires evolutionarily conserved residues from both proteins, including a pair of Arg residues from PriC and the C terminus of SSB. In vitro, disruption of the PriC/SSB interface by sequence changes in either protein blocks the first step of DNA replication restart, reloading of the replicative DnaB helicase onto an abandoned replication fork. Consistent with the critical role of PriC/SSB complex formation in DNA replication restart, PriC variants that cannot bind SSB are non-functional in vivo. Single-molecule experiments demonstrate that PriC binding to SSB alters SSB/DNA complexes, exposing single-stranded DNA and creating a platform for other proteins to bind. These data lead to a model in which PriC interaction with SSB remolds SSB/DNA structures at abandoned DNA replication forks to create a DNA structure that is competent for DnaB loading.

DNA replication in bacteria is initiated by sequence-specific recognition and unwinding of the origin of replication, oriC (1, 2). The melted oriC structure is rapidly bound by single-stranded (ss) DNA-binding protein (SSB)3 tetramers that must be displaced to allow loading of replicative helicases (DnaB in *Escherichia coli*, which is in complex with the DnaC helicase loader when it is not bound to DNA) onto each of the two single strands of DNA. Once DnaB is loaded, it mediates protein interactions that assemble functional DNA replication complexes, or replisomes, at each of the two replication forks. The replisomes then move bi-directionally away from oriC and, under ideal conditions, replicate the circular chromosome until they converge at the replication termination site.

However, replisomes frequently encounter obstructions such as impassable DNA damage or frozen protein complexes that can arrest replication and/or eject the replisome from the template prior to completion of replication, which makes genome duplication a sporadic process. The latter event produces an abandoned replication fork or a D-loop structure (resulting from recombinational repair (3)) at which DNA replication must be reinitiated to complete genome duplication (4, 5). Replisome dissociation and reloading occurs with surprising frequency in bacteria with nearly every replisome assembled at the origin requiring reloading at least once during replication (6).

To mediate the risk posed by premature replisomal disassembly, bacteria have evolved DNA replication restart mechanisms that reload replisomes onto abandoned DNA replication forks in a structure-specific, but sequence-independent, manner. Along with the processes of DNA replication, recombination, and repair, DNA replication restart is an essential, albeit more poorly understood, genome maintenance process in bac-

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1 Supported in part by National Institutes of Health Training Grant GM07215 (to J. L. K. and S. J. S.) and GM065367 (to T. H.), National Science Foundation Grant 0822613 (to T. H.). T. H. is an employee of the Howard Hughes Medical Institute. J. L. K. is cofounder of Replisoma, Inc.

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3 The abbreviations used are: SSB, ssDNA-binding protein; nt, nucleotide; smFRET, single-molecule fluorescence resonance energy transfer; TAP, tandem affinity purification; ITC, isothermal titration calorimetry; SD, synthetic dextrose; SSB-Ct, C terminus of SSB.
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teria (6). E. coli encodes multiple redundant DNA replication restart pathways, each of which links abandoned replication fork binding to reloading of the DnaB helicase from the DnaB/DnaC complex onto SSB-bound ssDNA at the stalled fork (7–9). As is the case at oriC, a major challenge in DNA replication restart is modulation of the SSB/DNA structure to allow DnaB to bind the lagging-strand template; once DnaB is reloaded, it can direct reassembly of the mature replisome (10, 11). The mechanisms by which SSB barriers to DnaB loading are bypassed are currently unknown.

In E. coli, DNA replication restart is driven by the replication restart proteins, which include PriA DNA helicase, PriB, PriC, DnaT, and Rep DNA helicase (12, 13). Genetic experiments have defined three replication restart pathways that depend on distinct subsets of the replication restart proteins (PriA/PriB/DnaT, PriC/Rep, and PriA/PriC) (7–9). Combinations of gene deletions that inactivate all three pathways (e.g. priC priB) are lethal, emphasizing the essential nature of DNA replication restart (7, 13). Biochemical reconstitution experiments have demonstrated that the PriA/PriB/DnaT system preferentially operates on stalled forks with a leading strand 3’ OH that is 0–6 nucleotides (nt) from the fork junction (such as a D-loop structure), whereas PriC functions on stalled forks with larger leading strand gaps of >5 nt (14–18), although more recent experiments have shown that PriA can also function on substrates with larger gaps and therefore may have significant functional overlap with PriC (19). In the PriA/PriB/DnaT system, PriA recognizes the abandoned fork or D-loop through a combination of structure-specific DNA binding and direct interaction with SSB on the lagging strand (15, 16, 20–22). The PriA/SSB complex is formed by PriA binding to the evolutionarily conserved C terminus of SSB (SSB-Ct), which is a structurally dynamic element (22, 23). After PriA associates with the proper substrate, PriB and DnaT dock onto PriA to form a complex that can then load DnaB onto the lagging-strand ssDNA template (24, 25). In contrast to the elaborate multiprotein PriA/PriB/DnaT complex, PriC is able to catalyze DnaB reloading onto DNA replication fork structures on its own (14) and although it is not well conserved across bacterial species, this property makes PriC an excellent minimal model system for probing DnaB reloading pathways.

To better understand the mechanisms of DNA replication restart, we have investigated PriC activities with SSB/DNA structures. PriC interacts directly with SSB through two conserved Arg residues that are required for binding to the SSB-Ct. PriC/SSB complex formation is essential for in vivo PriC activity and PriC-mediated DnaB loading onto a synthetic DNA replication fork prebound with SSB in vitro. Single-molecule (sm) fluorescence resonance energy transfer (FRET) studies demonstrate that PriC binding to SSB alters SSB/DNA complexes in a manner that preferentially stabilizes a highly cooperative binding mode of SSB over that of a less cooperative mode and in the process exposes ssDNA, making it available for additional protein binding. These data support a model in which PriC binding to SSB helps to exclude SSB from a region of ssDNA onto which DnaB can be loaded.

EXPERIMENTAL PROCEDURES

Tandem Affinity Purification (TAP)—E. coli strain MG1655 (DE3 lysogen) transformed with a plasmid encoding a fusion protein with an N-terminal dual affinity tag (protein A and calmodulin peptide binding domains) followed by a tobacco etch virus protease cleavage site and E. coli SSB (pTAP-SSB) was grown at 37 °C in Luria Broth (LB) medium supplemented with 50 μg/ml of ampicillin to mid-log phase (A600 = 0.5). TAP-SSB expression was induced with 2 μM isopropyl β-D-thiogalactopyranoside and the culture was grown for an additional 3 h. Cells were harvested by centrifugation; all subsequent steps were conducted at 4 °C. The cell pellet was resuspended in Nonidet P-40 buffer (25 mM dibasic sodium phosphate, 25 mM monobasic sodium phosphate, 150 mM NaCl, 2 mM EDTA, 50 mM sodium fluoride, 4 μg/liter of leupeptin, 0.1 mM sodium vanadate, 19.5 mg/liter of benzamidine, 8.7 mg/liter of phenylmethylsulfonyl fluoride (PMSF), 1% Nonidet P-40 substitute), and lysed by sonication. Soluble lysate was incubated at 4 °C for 1 h with IgG-Sepharose beads. Beads were washed with 3 volumes of equilibrium buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40 substitute). The beads were then washed and resuspended in cleavage buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM dithiothreitol (DTT)). The protein A domain was cleaved by addition of tobacco etch virus protease over 12 h at 4 °C. The eluent was incubated with 300 μl of calmodulin affinity resin (Stratagene) in calmodulin binding buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% Nonidet P-40, 0.5 mM magnesium acetate, 1 mM imidazole, 10 mM 2-mercaptoethanol, 5 mM calcium chloride) for 1 h. Resin was washed with calmodulin binding buffer, and TAP-SSB was eluted first with a high salt buffer (calmodulin binding buffer adjusted to 1.5 M NaCl) and then with elution buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.02% Nonidet P-40, 1 mM magnesium acetate, 10 mM imidazole, 10 mM 2-mercaptoethanol, 20 mM EGTA). Eluted protein samples were precipitated with trichloroacetic acid (25% w/v), pelleted by centrifugation, and washed three times with 80% acetone. The protein pellets were subjected to MALDI-TOF mass spectrometry for identification of peptides (University of Wisconsin Mass Spectrometry facility).

Yeast Two-hybrid Assay—Plasmids expressing Gal4 fusion proteins were generated by cloning priC and ssb into pGBD and pGAD vector backbones, which fuse the Gal4 binding domain (pGBD) and activation domain (pGAD) to the N terminus of the proteins to be tested (26). Plasmid pairs of interest were transformed into a pl69-4a Saccharomyces cerevisiae strain (26) and grown on Leu- and Trp-deficient synthetic dextrose (SD) plates. Transformants were inoculated into SD Leu- and Trp-deficient liquid media and grown at 30 °C overnight. Cultures were diluted to an A600 = 0.5 in 2% glucose. Serial 10-fold dilutions were made in 2% glucose. These were spot plated onto SD His- and Ade-deficient SD plates and grown at 30 °C.

Protein Purification—SSB was purified as described previously (27). E. coli DnaB and DnaC were gifts from James Berger. BL21 (DE3 lysogen) E. coli transformed with pET11a-priC, a T7-inducible overexpression plasmid that encodes E. coli PriC (pSW005), were grown in LB media supplemented with 100
µg/ml of ampicillin and 25 µg/ml of chloramphenicol at 37 °C. Mid-log phase cultures (A600 = 0.6) were induced to express PriC by the addition of isopropyl β-D-thiogalactopyranoside to 1 mM. After 3 h of additional growth, cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Hepes-HCl (pH 7.0), 100 mM NaCl, 10% sucrose, 5 mM EDTA, 15 mM spermidine HCl, 2 mM PMSF, 2 mM benzamidine, 1× protease inhibitor tablet (Roche Applied Science)) and lysed by sonication on ice. Lysates were clarified by centrifugation and solid ammonium sulfate was added to a final concentration of 0.2 g/ml. Precipitated material was pelleted by centrifugation, resuspended in resuspension buffer (10 mM Hepes-HCl (pH 7.0), 1 M NaCl, 10% glycerol, 2.5 mM EDTA, 1 mM DTT), and dialyzed against Buffer A (10 mM Hepes-HCl (pH 7.0), 0.35 M NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT) at 4 °C. The dialyzed protein was loaded onto a SPFF ion-exchange column (GE Healthcare) equilibrated in Buffer A and PriC was eluted using a NaCl gradient from 0.35 to 1 M. Fractions containing pure PriC were combined and dialyzed at 4 °C against Buffer B (10 mM Hepes-HCl (pH 7.0), 0.75 M NaCl, 10% glycerol, 1 mM EDTA, 1 mM DTT), then further purified by size exclusion chromatography (Sephacryl S-100 column, GE Healthcare). Fractions containing pure PriC were combined and dialyzed against PriC storage buffer (10 mM Hepes-HCl (pH 7.0), 0.5 M NaCl, 10 mM EDTA, 50% glycerol). PriC variants R121A and R155A were purified identically to wild-type PriC (plasmids pSW006 and pSW007, respectively).

**Isothermal Titrination Calorimetry (ITC)—**PriC variants were dialyzed at 4 °C against a buffer containing 10 mM Hepes-HCl (pH 7.0), 100 mM NaCl, 3% glycerol. Protein was concentrated to 5–10 µM (determined by absorbance at 280 nm). SSB-Ct peptide was dissolved in the same buffer to a concentration of 200–300 µM. SSB-Ct peptide injections (1 × 1, 2 × 2, 5 × 4, and 29 × 8 µl) were performed at 25 °C. The data were fit with a single site binding model using Origin software (Microcal). Peptide sequences were: SSB-Ct, Trp-Met-Asp-Phe-Asp-Asp-Ile-Pro-Phe; SSB113-Ct, Trp-Met-Asp-Phe-Asp-Asp-Ile-Pro-Pro-Phe; and SSBΔF-Ct, Trp-Met-Asp-Phe-Asp-Asp-Ile-Pro-Pro-Phe.

**DnaB Loading Assays—**Reactions were performed as previously described (14) with the following modifications: reactions were carried out at pH 7.0 and the final concentrations of components (when included) were: 67 mM SSB, 60 mM DnaB, 400 mM DnaC, and 20 mM PriC (and PriC variants). Briefly, a forked DNA substrate (60 nt duplex, 38 nt 5’ and 3’ overhangs) was made by annealing oligonucleotides 3L-98 (5’-GACTACTACAGTTCCGGAGCTCGCCGCGAGATCCATTAGC-CCTTTATCGTATTGCGTCGCTAGTGCGCTACGGCCGCACCGCAGGCGACCCGCACGGCGGACCGC-TGTCTACGCCGCAACGAGTCCGAGAATCTAAAGGGGTCTGACGACAGGGGACGACCAATTTCAACCAAG-ACAATTCAACCGCCAGCAACCGTA-3’). The 3L-98 oligonucleotide was 32P-labeled using T4 polynucleotide kinase prior to annealing. This fork substrate was preincubated with SSB (or SSB variants) for 3 min at 25 °C (in reactions containing no SSB, preincubation consisted of buffer only). Reactions were started by the addition of DnaB, DnaC, and PriC (when included) and incubated at 37 °C for 25 min. Unwinding was determined by the addition of 20 mM EDTA, 0.5% SDS, 0.2 mg/ml of proteinase K, and 2.5 ng/µl of oligonucleotide 3L-98 (final concentrations) and incubated at 37 °C for 30 min. Samples were resolved on a 10% native polyacrylamide gel. The gel was then fixed in 10% methanol, 7% acetic acid, and 5% glycerol, dried, and exposed to a phosphorimager screen and imaged on a Typhoon FLA 9000. Band intensities were quantified using ImageQuant (GE Healthcare) and percent unwinding was determined by dividing the intensity of the single strand product band by the total intensity in the lane.

**Single Molecule FRET Assays—**All smFRET experiments were performed at 22 ± 1 °C using a total internal reflection fluorescence microscope (28). Sample assembly, data acquisition, and FRET efficiency calculations were performed as described previously (28). Briefly, 50–100 pm of the partial duplex DNA substrates ((dT)_69) were immobilized on a quartz slide surface coated with polyethylene glycol (mPEG-SC, Laysan Bio) to eliminate nonspecific surface adsorption of proteins. The immobilization was mediated by biotin-Neutravidin binding between biotinylated DNA, Neutravidin (Pierce), and biotinylated polymer (Bio-PEG-SC, Laysan Bio). All measurements were performed in an imaging buffer containing 50 mM Hepes (pH 7.0), 100 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, 5% (v/v) glycerol, 0.5% (w/v) d-glucose, 165 units/ml of glucose oxidase, 2170 units/ml of catalase, and 3 µM Trolox with the indicated protein concentrations. Single-molecule FRET time traces were recorded with a time resolution of 30 ms and the single molecule FRET efficiency histograms were generated by averaging 300 ms from >5000 molecules each.

**Co-transduction Tests of priC Mutants—**All bacterial strains are derivatives of E. coli K12 and are described in supplemental Table S1. The protocol for P1 transduction has been described previously (29). All P1 transductions were selected on 2% agar plates made with LB. Selection with antibiotics used both 100 µg/ml of ampicillin (to maintain the plasmid in each recipient) and 10 µg/ml of tetracycline (the selectable marker in each donor). Transductants were grown at 37 °C and purified on the same type of media on which they were selected.

SSS513 (zif-599::Tn10 del(priB)302) is the donor in crosses that were used to determine whether the recipient strain had priC activity. The recipient strains had a priC303::kan insertion mutation and a plasmid with different priC alleles (supplemental Table S2). Tet” transductants were selected on LB agar plates supplemented with ampicillin and tetracycline at 37 °C, then purified and patch plated on the same media at 37 °C. PCR using primers pSS278 (5’-CAACGTAGCCGTAAATTCG-3’) and pSS279 (5’-GACCGATTACTGATGGCG-3’) were used to determine the state of the priB allele. The wild-type allele produces a ~600 bp fragment, whereas the del(priB)302 allele produces a ~300 bp fragment. In some cases, further PCR analysis was carried out using internal priB primers pSS1319 (5’-GTCTGGTTGGTGTCCCGCACCCT-3’) and pSS1320 (5’-GCTCGTCCGTTTTGGCTCGTCCCGCACCCTGTC-3’) to determine whether the transduction events were accompanied by gene duplication. In cases where gene duplication occurred, these were scored as not bringing in the del(priB)302 gene because its survival depends on the presence of the priB” gene. Similar transductions were conducted using...
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We next used the two-hybrid assay to test whether the SSB-Ct, which serves as the binding site for all known SSB interaction partners (23), was necessary for interaction with PriC. A mutation was introduced to the pGAD-SSB plasmid that altered the penultimate Pro to a Ser, replicating a well defined temperature-sensitive variant of SSB (SSB113) from *E. coli* (31, 32). This sequence change weakens or abolishes interactions between SSB and several of its characterized binding partners (23) but does not prevent the SSB/SSB interaction (Fig. 1B). As predicted for the SSB-Ct being necessary for interaction with PriC, strains transformed with pGBD-PriC and pGAD-SSB113 failed to grow on selective media (Fig. 1B). A second mutation in which the C-terminal-most Phe was deleted (SSBΔF) was also introduced to the pGAD-SSB plasmid. Deletion or alteration of this terminal residue blocks binding to SSB protein partners (33). Consistent with a similar effect in PriC/SSB, a strain transformed with pGBD-PriC and pGAD-SSBΔF failed to grow on selective media (data not shown), however, SSBΔF also failed to self-interact indicating that the deletion variant is not suitable for two-hybrid analysis (Fig. 1B). Because purified SSB C-terminal deletion variants maintain their DNA-binding and oligomerization properties *in vitro* (34), it is not clear why this variant failed to self-interact in the two-hybrid assay.

To determine whether the SSB-Ct element is sufficient for binding PriC and to more quantitatively assess the PriC/SSB-Ct interaction in a purified system, an ITC-based binding assay was established. In this experiment, an SSB-Ct peptide comprising the final 9 residues of *E. coli* SSB with an N-terminal Trp for quantification (Trp-Met-Asp-Phe-Asp-Asp-Ile-Pro-Phe) was titrated into a solution of purified *E. coli* PriC and the heat generated upon binding was measured. The data fit well to a single-site binding model, which showed that PriC binds to the SSB-Ct peptide in a 1:1 complex with an apparent dissociation constant (*K_d*) of 3.7 ± 0.7 μM (Fig. 1C). This *K_d* is con-
The PriC/SSB Interaction Is Necessary for PriC-mediated DnaB Loading in Vitro—As interactions with SSB have been shown to play functional roles in the activities of several SSB protein partners (23), we hypothesized that the PriC/SSB interaction would be important for PriC-mediated DnaB loading. To test this hypothesis, we used an established assay that measures PriC-mediated loading of the DnaB helicase from the DnaB/SSB complex onto a synthetic replication fork structure that was prebound by SSB (14). DnaB loading was quantified in this system by measuring the appearance of ssDNA generated by DnaB helicase activity using gel electrophoresis. We confirmed that SSB blocked loading of DnaB onto the replication fork structure in vitro (Fig. 2, compare lanes 3 and 4) and that inclusion of PriC facilitated DnaB loading onto an SSB-coated fork to levels comparable with those of spontaneous DnaB loading onto a non-SSB coated fork as had been observed previously (14) (Fig. 2, compare lanes 3 and 7). To test the importance of the PriC/SSB interaction in DnaB loading, SSB113 and SSBΔF were then substituted for wild-type SSB in the assay. PriC-mediated DnaB loading was reduced 2–3-fold with SSB113 relative to that observed with wild-type SSB (Fig. 2, lane 9) and to background levels measured in the absence of PriC with SSBΔF (Fig. 2, lane 10). These results point to an important role for the PriC/SSB interaction in DnaB loading; however, the SSB113 result was more modest than expected given the lack of interaction of PriC with either SSB113 in the two-hybrid assay or the SSB113-Ct peptide in ITC. This difference could indicate that SSB113 retains a weakened affinity for PriC in the context of a reconstituted DNA replication restart reaction, but that the complex is too unstable to be detected in isolated PriC/SSB interaction assays.

Identification of the SSB-binding Site on PriC—High resolution structures of several different SSB-Ct-bound proteins have defined similar electrostatic surfaces in each of the protein partners that are used for binding the SSB-Ct (35–39). These SSB-Ct binding sites typically include a hydrophobic pocket that envelops the side chain of the C-terminal-most Phe, a “basic lip” residue that coordinates the α-carboxyl group of the C terminus, and a “basic ridge” surface that coordinates the Asp residues in the SSB-Ct. The basic lip is most often an evolutionarily conserved Arg residue and neutralizing mutations in this residue generally eliminate SSB-Ct binding (35–39). Neutralizing mutations of Arg residues in the basic ridge regions of SSB interacting proteins can similarly destabilize interactions with SSB.

Using these observations as a guide, we carried out a screen to identify Arg residues in PriC that, when changed to Ala, lost the ability to interact with SSB. Of the 25 Arg residues in E. coli PriC, only five were found to be invariant across PriC sequences from multiple bacterial species. The codons for each of these five residues were individually mutated to code for Ala in the pGBD-PriC plasmid and screened for interaction with SSB in the two-hybrid assay. PriC-GBD fusion proteins with changes to Arg-88, Arg-96, or Arg-158 each retained the ability to support growth on His- and Ade-deficient media, indicating that the mutations did not eliminate the PriC/SSB interaction (Fig. 3A). However, Ala substitutions of Arg-121 and Arg-155 produced PriC-GBD fusion proteins that failed to support growth on selective media, consistent with these residues being required for interaction with SSB.

To further examine the roles of these residues in binding to SSB, PriC R121A and R155A variants were purified and tested in SSB-Ct binding and DnaB loading activity assays. As predicted from the two-hybrid results, both PriC variants failed to interact with the SSB-Ct peptide in ITC binding experiments (supplemental Fig. S2). When these variants were substituted for PriC in the DnaB-loading assay, neither variant was able to stimulate DnaB loading to levels above that observed in the absence of PriC (Fig. 3B). Both variants behaved identically to wild-type PriC during purification and in limited proteolysis experiments that probe the low-resolution structure; thus the variants appeared to be properly folded. These data indicate that Arg-121 and Arg-155 are essential for SSB binding and provide further evidence that the PriC/SSB interaction is necessary for DnaB loading activity.

The SSB-Ct Peptide Blocks PriC-mediated DnaB Loading in Vitro—Because PriC can bind directly to the SSB-Ct, we next tested the consequences of including SSB-Ct peptides in the PriC-mediated DnaB loading assay. If SSB-Ct binding simply activates PriC to mediate DnaB loading, inclusion of the wild-type SSB-Ct peptide in the assay would be expected to increase DnaB loading efficiency. Alternatively, if SSB binding allows PriC to modulate the SSB/DNA structure and/or recruits PriC to the replication fork, then inclusion of the peptide would be...
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expected to compete with full-length SSB for binding to PriC, resulting in reduced DnaB loading.

When the SSB-Ct peptide was titrated into the DnaB loading reactions, a dose-dependent decrease in DnaB loading was observed (Fig. 4A). The strong inhibition of DnaB loading was only seen at peptide concentrations greater than the $K_d$ for the SSB-Ct/PriC interaction. In contrast, when the SSB113-Ct peptide was titrated into the reaction, inhibition required higher concentrations than with the wild-type SSB-Ct peptide (Fig. 4, A and B, compare 30 μM peptide lanes). This is consistent with the SSB113-Ct peptide binding to PriC with a reduced affinity, as described earlier. When the SSB/H9004F-Ct peptide was titrated into the DnaB loading reaction, no effect on loading efficiency was detected, indicating that the observed effect on DnaB loading is dependent on the presence of a functional interaction between PriC and the SSB-Ct (Fig. 4C). These results rule out a model in which SSB-Ct binding alone activates PriC and are consistent with models wherein the interaction allows PriC to modulate the SSB/DNA structure and/or localizes PriC to stalled replication forks.

PriC Modulates the Structure of SSB/DNA Complexes—We next used an smFRET assay to test whether the PriC interaction with SSB modulates the SSB/DNA structure. *E. coli* SSB can bind ssDNA in two major modes: a highly cooperative mode in which 35 nt are bound per tetramer (SSB$_{35}$) and a less cooperative mode that binds 65 nt per tetramer (SSB$_{65}$) (40). These modes can be distinguished from one another using an established smFRET assay in which SSB is bound to a partial duplex DNA substrate containing a 77-nt ssDNA overhang (referred to hereafter as (dT)$_{69}$H$_{11001}$8) that includes a fluorescence donor/acceptor pair (Cy5 and Cy3) that are separated by 69 nt (Fig. 5A) (28, 41). In the absence of SSB, the labeled DNA molecule is unconstrained, which leads to a very low FRET efficiency between Cy3 and Cy5 (efficiency $\sim 0.1$, supplemental Fig. S3). When a single SSB tetramer is bound to the DNA in the SSB$_{65}$ mode, the Cy3 and Cy5 fluorophores are brought in close proximity, yielding a high FRET state (efficiency $\sim 0.4$). FRET fluctuations were observed for this high FRET state due to the rapid diffusion of an SSB tetramer on (dT)$_{69}$H$_{11001}$8, as seen previously (28, 42). In contrast, when two SSB tetramers are bound to the DNA in the SSB$_{35}$ mode, an intermediate FRET state is observed (efficiency $\sim 0.2$), because Cy3 and Cy5 are farther apart than in the SSB$_{65}$ mode but closer together than in the SSB-free substrate (Fig. 5, A and B).

To test for PriC effects on SSB/DNA complexes, smFRET solution conditions were used in which individual complexes could readily transition between SSB$_{35}$ and SSB$_{65}$ binding modes (Fig. 5, B and C). When wild-type PriC was added under these conditions, the fraction of SSB molecules bound in the SSB$_{35}$ binding mode was strongly induced, whereas the SSB$_{65}$ mode was dramatically reduced (Fig. 5, B and D). This effect was only observed when free SSB was available in solution, indi-

FIGURE 3. PriC Arg-121 and Arg-151 are required for interaction with SSB and DnaB loading. A, two-hybrid protein interaction assay with PriC variants. Yeast transformed with the indicated plasmids were spot plated in a dilution series on selective media that requires protein interactions for growth. B, DnaB loading assays with PriC variants as described in the legend to Fig. 2. All lanes are from the same gel.

FIGURE 4. DnaB loading is inhibited by SSB-Ct peptide binding to PriC. SSB-Ct peptides (A, wild-type SSB-Ct; B, SSB113-Ct; and C, SSBΔF-Ct) were titrated into PriC-mediated DnaB loading reactions at the indicated concentrations. Reactions included SSB, DnaB, DnaC, PriC, and ATP (as described in the legend to Fig. 2).
cating that the addition of PriC resulted in recruitment of an additional SSB tetramer to the DNA (supplemental Fig. S4). To test whether this effect required PriC/SSB complex formation, the PriC R155A variant was substituted in the smFRET assay. Unlike the FRET shift observed with wild-type PriC, the PriC R155A variant had no effect on the distribution between the two SSB binding modes. These results support a model in which PriC binding to SSB induces structural changes in the SSB/DNA complex that stabilize the more highly cooperative SSB_{35} DNA binding mode.

Two alternative explanations for the PriC-dependent FRET efficiency changes were also considered. These were: 1) that the PriC-induced FRET efficiency change was due to PriC displacing SSB and binding to the ssDNA in a manner that led to a FRET efficiency similar to that of the SSB_{65} binding mode and 2) that the shift was due to formation of a PriC/SSB/DNA ternary complex. Although PriC does bind ssDNA on its own (43), the FRET efficiency signature of PriC binding differed from either of the SSB binding modes (supplemental Fig. S3, efficiency ~0.3), which ruled out the first possibility. Notably, DNA binding by the PriC R155A variant led to the same FRET efficiency signature as wild-type PriC, showing that Arg-155 is not required for PriC DNA binding (supplemental Fig. S3) and demonstrating that the variant is properly folded. In addition, the low FRET state was observed only when free SSB was available in solution (supplemental Fig. S4); therefore the observed shift could not be due to ssDNA/SSB/PriC ternary complex formation where a prebound SSB tetramer is joined by PriC. Instead, the simplest explanation is that PriC binding to SSB stabilizes the SSB_{35} mode relative to the SSB_{65} mode, which reduces the effective DNA binding site size of a preloaded SSB tetramer, making additional ssDNA segments available for the binding of another SSB tetramer. In the context of an abandoned DNA replication fork, this modulation could be important in establishing an ssB-free ssDNA region that will serve as the loading site for DnaB.

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There is a 1 in 5,000 chance of this result occurring by chance. From this we conclude that PriC R155A is non-functional in vivo.

Analysis of the PriC R121A variant was more complicated. Initial analysis of transduction into this strain revealed co-transduction of del priB)302 in 3/47 TetR colonies. The p value for a χ² test was 0.03. This value is just in the range that suggests that it was likely that this priC allele was not able to complement the priC chromosomal mutation. It was possible, however, that the priC chromosomal allele somehow altered the ability of the cell to undergo recombination, thus altering the co-transduction frequency. It was also possible that these transductants were not the result of simple inheritance of the del(priB)302 mutation and are the result of a more complicated process. To test these ideas, we first assessed whether the priC allele on the plasmid altered the co-transduction frequency relative to wild-type. Table 1 shows that by measuring the nonselective linkage to argE, the co-transduction frequency was not affected by the presence of the mutant priC allele. Second, we examined the three transductants with the del(priB)302 allele using PCR primers internal to the priB gene. We found that two of the transductants had wild-type priB genes in addition to the mutant. Hence, these are likely the result of a gene duplication event. The spontaneous frequency of gene duplication in enteric bacteria is ~10⁻³ to 10⁻⁵/cell/division (44). Thus, the probability of finding a gene duplication coupled to transduction (about 10⁻¹/endpoint) is the product of both events and would be quite rare. We therefore suspect that the third transductant showing only the priB deletion is also the recipient of another rare event, the acquisition of a spontaneous suppressor mutation allowing growth. This is supported by the observation that mutations in dnaC are known to suppress the growth defects of priBC double mutations (13).

We conclude that neither priC mutant has the ability to complement the priC chromosomal mutation. These data indicate that PriC/SSB complex formation is essential for PriC-mediated DNA replication restart in vivo.

DISCUSSION

In this study, we have examined the mechanisms by which the PriC DNA replication restart protein facilitates DnaB helicase loading onto SSB-coated DNA substrates. PriC can catalyze DnaB loading without the assistance of other replication restart proteins in vitro (14), and therefore provides a minimal model system for probing the biochemical and cellular requirements for driving DNA replication restart. Here, we have identified and characterized a direct physical interaction between PriC and SSB. Using protein variants that weaken the PriC/SSB complex and SSB-Ct peptides that act as inhibitors, we have shown that PriC/SSB complex formation is essential for PriC-mediated DNA replication restart in vitro. Similarly, mutations in priC that abrogate binding to SSB failed to complement a priC deletion in E. coli, indicating that the interaction is essential for PriC function in vivo as well. smFRET experiments showed that PriC binding to SSB modulates the structure of SSB/DNA complexes by preferentially stabilizing the highly cooperative SSB₃₅ mode of SSB₆₅. This observation suggests that PriC-mediated modulation of SSB/DNA complexes at abandoned DNA replication forks could be important for establishing a DnaB-loading site on the lagging strand.

Taken together with previously published results, our data support an overall model in which PriC functions by coordinating interactions with protein and DNA components present at abandoned replication forks (Fig. 6). The first step in any DNA replication restart system is recognition of an abandoned replication fork structure (Fig. 6, step 1). Our model posits that PriC, which can bind to both ssDNA (supplemental Fig. S3 and Ref. 43) and SSB, will interact with ssDNA at the leading strand gap and with SSB on the lagging strand. Consistent with the requirement for a ~5 nt leading strand gap for PriC-mediated replication restart (14), preliminary experiments from our group support a ~5 nt site size for PriC binding. PriC binding to abandoned forks with larger leading-strand gaps may also be facilitated by its interaction with SSB, which is likely to localize PriC to DNA replication forks in cells. In the event that the lagging strand DNA is double-stranded (i.e. the 5’ end of an Okazaki fragment is present at the replication fork junction) and SSB is therefore absent, the helicase activity of either Rep (in the PriC/Rep pathway) or PriA (in the PriA/PriC pathway) would be required to generate ssDNA to which SSB would then bind (45).

The second step in replication restart function requires remodeling of the lagging strand to allow DnaB to be reloaded (Fig. 6, step 2). When SSB coats the ssDNA of the lagging strand, it prevents DnaB loading (14) (Fig. 2). However, we have shown that the interaction between SSB and PriC preferentially stabilizes the highly cooperative SSB₃₅ binding mode over the less cooperative SSB₆₅ binding mode. Interestingly, this shift correlates with the effects of removing the SSB-Ct from SSB, which also stabilizes the SSB₃₅ mode relative to the SSB₆₅ mode (41). Thus SSB binding by PriC mirrors the effects of removing the SSB-Ct. This activity could lead to a condensation of SSB tetramers on the lagging strand, which could transiently expose small tracts of ssDNA onto which DnaB can bind (DnaB has a binding site size of 10 nt (46)). It is also possible that PriC could bind to this newly exposed ssDNA and help to prevent SSB from reoccupying the site prior to DnaB loading. Interestingly, PriA also interacts with SSB; this interaction has been shown to

### TABLE 1

Co-transduction analysis to determine if the plasmid-borne priC allele can complement the priC303::kan

| Recipient strain (priC303::kan) | del(priB)302 linkage to zjf-599::Tn10 | argE<sup>+</sup> linkage to btuB3191::Tn10 |
|---------------------------------|--------------------------------------|------------------------------------------|
| SS3800 (pACYC177)              | 0/56 (0%)                            | 46/64 (72%)                              |
| SS9702 (priC<sup>C</sup>)      | 14/56 (25%)                          | 46/64 (72%)                              |
| SS9705 (priC R121A)            | 7/47 (2%)                            | 45/64 (71%)                              |
| SS9704 (priC R155A)            | 0/56 (0%)                            | 48/64 (74%)                              |

<sup>4</sup> The one transductant is likely to contain a suppressor mutation and therefore is not indicative of priC complementation. See text for full explanation.

<sup>4</sup> S. R. Wessel and J. L. Keck, unpublished observation.
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localize PriA to DNA replication sites and to stimulate its helicase activity (21, 22). It will be interesting to determine whether PriA binding to SSB also preferentially stabilizes the SSB₃₅ binding mode in a manner that is functionally conserved with PriC.

The final step in DNA replication restart is to recruit the DnaB helicase, bound to the DnaC helicase loader, to the abandoned replication fork structure and enable its reloading onto the stalled fork (Fig. 6, steps 3 and 4). This step is not informed by our current study. However, an interaction between PriC and DnaB has been identified in a proteome-wide study of E. coli protein interactions (47). A direct interaction between PriC and DnaB (or the DnaB/DnaC complex) is therefore plausible and it could be important in PriC-mediated DnaB loading. Further experiments will be needed to address this important step in DNA replication restart.

Taken together, our model suggests that the minimal requirements for a DNA replication restart mechanism include: 1) DNA substrate binding; 2) SSB binding and structural modulation; and 3) helicase binding. Although proteins with significant sequence homology to PriC are not widely distributed among diverse bacteria, there may be unidentified functionally homologous proteins that mediate replication restart pathways that are complementary and/or redundant with the more broadly conserved PriA pathway. Our finding that PriC requires interaction with SSB for DnaB loading suggests that SSB binding partners lacking assigned functions in other bacteria would be good candidates as possible DNA replication restart proteins. The requirement for interaction with SSB in addition to the other minimal requirements described here may provide starting points for identifying DNA replication restart proteins that catalyze pathways that function in diverse bacterial species.

Acknowledgments—We thank Matthew Lopper (University of Dayton) and members of the Keck lab for critical review of the manuscript.

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