Supplementary Discussion

Based on enhanced binding of PriB variant Q45A to FITC-DnaT in the absence of ssDNA, we expected that DnaT would have a greater ability to compete with ssDNA for binding to PriB:Q45A compared to wild type PriB. However, PriB:Q45A shows wild type levels of competition between fluoro-ssDNA and DnaT and has a $K_{d,app}$ of 352.2 +/- 54.4 nM (Figure 3B and Table 1). One possible explanation is that the PriB Q45A variant presents a surface to DnaT that bears resemblance to ssDNA-bound PriB at this region of the protein. Indeed, the co-crystal structure of the PriB:ssDNA complex shows a change in position of the Q45 sidechain in the PriB:ssDNA complex relative to apo PriB (Huang et al., 2006). If the Q45 sidechain must be relocated from its position in apo PriB to achieve higher affinity binding to DnaT, then mutation of Q45 to alanine might promote PriB's interaction with DnaT by eliminating the sidechain altogether. Thus, residue Q45 might serve an important role in negatively regulating the PriB:DnaT interaction such that DnaT binds preferentially to ssDNA-bound PriB. Whether this mechanism operates in cells is not clear since the Q45A mutation does not produce a readily detectable phenotype in *E. coli* by plasmid complementation (Supplementary Table 3).

Since the residues on the surface of PriB analogous to the homotetramerization surface of SSB do not seem to play a role in PriA, DnaT, or ssDNA binding, what function do they confer? One possibility is that they serve as a binding platform

for heterologous proteins other than PriA or DnaT. At least one other protein, SSB, has been reported to physically interact with PriB (Low et al., 1982), and there may be others awaiting discovery. While no other binding partners were identified for PriB in a recent, genome-wide tandem-affinity-purification study (Butland et al., 2005), our results indicate that protein:protein interactions involving PriB can be largely DNA-dependent and might not be observed under conditions that could remove DNA from nucleoprotein complexes. A second possible function for these residues, given that *priB* is thought to have arisen by gene duplication of *ssb* (Ponomarev et al., 2003), is that they have been selected for over time because they destabilized homotetramerization of the product of the ancestral *priB* gene, giving rise to a stable PriB homodimer that was subsequently adapted for a new role in the cell. If this were the case, one would expect that mutation of these residues to create a SSB-like surface could promote formation of a PriB homotetramer and disrupt PriB function in replication restart pathways. Clearly, further investigation will be required to unravel the functional role of this surface of PriB.

Supplementary Experimental Procedures

Cloning *priA* variants, *priB* variants, and *dnaT*. *E. coli priA* variants were amplified from strain K12 genomic DNA by polymerase chain reaction (PCR) using oligonucleotide primers oML100 and oML101 (intact *priA*), oML100 and oML141 (*priA* DBD), and oML101 and oML143 (*priA* HD) (Supplementary Table

1). The PCR-amplified products were individually cloned into the pET28b expression vector (Novagen) using NdeI and BamHI restriction sites. The *priA:K230A* variant was constructed using the Stratagene Quikchange site-directed mutagenesis kit using oligonucleotide primers oML221 and oML222 (Supplementary Table 1) as previously described (Lopper et al., 2004). The resulting plasmids, pML100 (intact *priA*), pML118 (*priA* DBD), pML120 (*priA* HD), and pML152 (*priA:K230A*) encode *priA* variants with a 5' six-Histidine tag and thrombin cleavage site. The *priB* gene of *E. coli* was cloned as previously described (Lopper et al., 2004). Variants of *priB* were constructed using the Stratagene Quikchange site-directed mutagenesis kit using appropriate oligonucleotide primers (Supplementary Table 1) as previously described (Lopper et al., 2004). The *dnaT* gene of *E. coli* was amplified from strain K12 genomic DNA by PCR using oligonucleotide primers oML106 and oML107 (Supplementary Table 1). The PCR-amplified product was cloned into pET28b using NdeI and BamHI restriction sites. The resulting plasmid, pML103, encodes *dnaT* with a 5' six-Histidine tag and thrombin cleavage site.

Purification of PriA variants, PriB variants, and DnaT. PriA was purified from cultures of BL21(DE3) *E. coli* harboring plasmid pML100 grown at 37°C in Lauria-Bertani (LB) medium supplemented with 50 ug·mL^{-1} kanamycin. Over-expression of *priA* was induced with 1 mM Isopropyl β-D-thiogalactopyranoside (IPTG) and cells were harvested after 4 hours of growth at 37°C by centrifugation at 5,000 x *g*. Cells were lysed in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M

NaCl, 0.1 M glucose, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride (PMSF) by sonication on ice. Lysates were clarified by centrifugation at 26,000 x *g* and His-tagged PriA was bound to nickel-NTA agarose (Qiagen). The nickel-NTA agarose beads were washed with lysis buffer and bound proteins were eluted in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. PriA was further purified through a Sephacryl S-300 size exclusion column (GE Healthcare) in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. PriA fractions were pooled, concentrated to approximately 30 $g \cdot L^{-1}$, and stored at -80°C. PriA:K230A was purified in the same manner as wild type PriA protein.

PriA DBD was purified from cultures of BL21(DE3) *E. coli* harboring plasmid pML118 grown at 37°C in LB medium supplemented with 50 $ug \cdot mL^{-1}$ kanamycin. Over-expression of *priA* DBD was induced with 1 mM IPTG and cells were harvested after 4 hours of growth at 37°C by centrifugation at 5,000 x *g*. Cells were lysed in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 0.1 M glucose, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF by sonication on ice. Lysates were clarified by centrifugation at 26,000 x *g* and His-tagged PriA DBD was bound to nickel-NTA agarose. The nickel-NTA agarose beads were washed with lysis buffer and bound proteins were eluted in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. PriA DBD was further purified through a Sephacryl S-300 size exclusion column in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. PriA DBD

fractions were pooled, concentrated to approximately 10 g·L^{-1}, and stored at -80°C.

PriA HD was purified from cultures of BL21(DE3) *E. coli* harboring plasmid pML120 grown at 37°C in LB medium supplemented with 50 ug·mL^{-1} kanamycin. Over-expression of *priA* HD was induced with 0.5 mM IPTG and cells were harvested after 4 hours of growth at 37°C by centrifugation at 5,000 x *g*. Cells were lysed in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 0.1 M glucose, 10 mM imidazole, 1 mM β-mercaptoethanol, 1 mM PMSF by sonication on ice. Lysates were clarified by centrifugation at 26,000 x *g* and His-tagged PriA HD was bound to nickel-NTA agarose. The nickel-NTA agarose beads were washed with lysis buffer and bound proteins were eluted in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol. PriA HD was further purified through a Sephacryl S-300 size exclusion column in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. PriA HD fractions were pooled, concentrated to approximately 1 g·L^{-1}, and stored at -80°C.

Purification of PriB variants was as previously described (Lopper et al., 2004).

DnaT was purified from cultures of BL21(DE3) *E. coli* harboring plasmid pML103 grown at 37°C in LB medium supplemented with 50 ug·mL^{-1} kanamycin. Over-expression of *dnaT* was induced with 0.5 mM IPTG and cells were harvested

after 4 hours of growth at 37°C by centrifugation at 5,000 x *g*. Cells were lysed in 10 mM Tris·HCl pH 8.5, 10% (v/v) glycerol, 6 M guanidine·HCl, 250 mM NaCl, 1 mM β-mercaptoethanol by sonication on ice and lysates were clarified by centrifugation at 26,000 x *g*. His-tagged DnaT was bound to nickel-NTA agarose. The nickel-NTA agarose beads were washed with lysis buffer and bound proteins were eluted in 10 mM Tris·HCl pH 8.5, 10% (v/v) glycerol, 6 M guanidine·HCl, 300 mM imidizole, 250 mM NaCl, 1 mM β-mercaptoethanol. DnaT was refolded by extensive dialysis against 10 mM Tris·HCl pH 8.5, 10% (v/v) glycerol, 50 mM NaCl, 1 mM β-mercaptoethanol. DnaT was concentrated to approximately 3 $g \cdot L^{-1}$ and stored at -80°C.

FITC-labeling PriB and DnaT. PriB and DnaT were individually suspended in 1 M sodium bicarbonate pH 9 at 3 $g \cdot L^{-1}$ and 0.1 $g \cdot L^{-1}$, respectively. Proteins were incubated with a 10- to 12-fold molar excess of FITC for 1 hour at 4°C in the dark. Residual reactive FITC was quenched with 67 mM hydroxylamine pH 7-8 for 1 hour at 4°C in the dark. FITC-labeled proteins were purified with nickel-NTA agarose beads (Qiagen). Bound FITC-PriB was eluted in 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 100 mM imidazole, 1 mM β-mercaptoethanol and extensively dialyzed against 10 mM Hepes pH 7, 10% (v/v) glycerol, 0.5 M NaCl, 1 mM β-mercaptoethanol. Bound FITC-DnaT was eluted in 10 mM Tris·HCl pH 8.5, 10% (v/v) glycerol, 50 mM NaCl, 100 mM imidazole, 1 mM β-mercaptoethanol and extensively dialyzed against 10 mM Tris·HCl pH 8.5, 10% (v/v) glycerol, 50 mM NaCl, 1 mM β-mercaptoethanol. FITC-labeled proteins

were aliquoted and stored at -80°C. The degree of labeling is 0.3 FITC per PriB dimer and 0.6 FITC per DnaT monomer.

DNAs. PhiX174 virion ssDNA was purchased from New England Biolabs. Single-stranded DNA oligonucleotides (30-mer, 5'-GCG TGG GTA ATT GTG CTT CAA TGG ACT GAC; 18-mer, 5'-AAG CAC AAT TAC CCA CGC) with fluorescein covalently linked at their 3' ends were synthesized by MWG Biotech. Single-stranded DNA oligonucleotides used in the DnaB loading assay were purchased from Integrated DNA Technologies, Inc. and were annealed to create the forked DNA substrate as previously described (Heller and Marians, 2005).

Limited proteolysis. Purified PriA (1.3 μM) was incubated with 0.5 μM PhiX174 virion ssDNA in 10 mM Tris-HCl pH 8, 33 mM NaCl, 1 mM EDTA for 10 minutes on ice. Trypsin (0.013 μM) was added and the reactions were incubated at room temperature for 2, 10, or 20 minutes. Reactions were stopped by addition of SDS-PAGE sample buffer and the products were resolved through a 12.5% polyacrylamide gel and visualized with Coomassie Brilliant Blue.

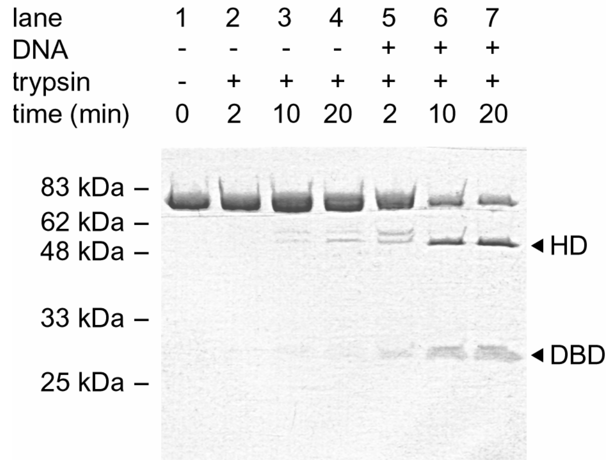
DnaB loading assay. Reactions were performed as previously described (Heller and Marians, 2005) except that 13 nM PriA:K230A and 60 nM DnaT variants were used.

Structure determination of PriB:E39A. Crystallization of PriB:E39A was performed as previously described for wild type PriB (Lopper et al., 2004). The crystal structure of PriB:E39A was solved to 2.25 Å resolution by molecular replacement with the program Amore (Navaza, 1994) using wild type PriB as a model (Protein Data Bank accession code 1TXY). Following molecular replacement, model building was performed manually with the program O (Jones et al., 1991) and the model was improved by rounds of refinement with the program Refmac (Winn et al., 2001). The structure was refined to a final R-factor of 24.7% and an R_{free} of 27.6% with good bond geometries. No residues fall into disallowed regions of Ramachandran space (Supplementary Table 2).

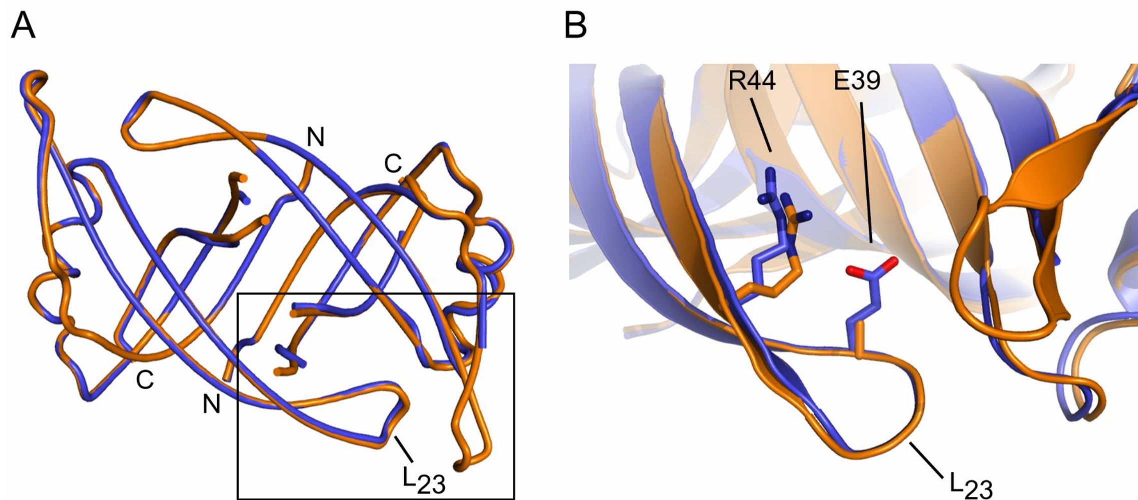
Coordinates. Coordinates have been deposited in the Protein Data Bank (accession code 2PNH).

Plasmid complementation assay. Plasmid complementation assays with *priB* variants were performed as previously described (Boonsombat et al., 2006).

Supplementary Figures



Supplementary Figure 1. Limited proteolysis of PriA. Purified PriA was treated with trypsin in the presence and absence of DNA as described in Supplementary Experimental Procedures. The products were separated by SDS-PAGE and visualized with Coomassie Brilliant Blue staining. Molecular weight estimates from SDS-PAGE are indicated and bands corresponding to the DNA binding domain (DBD) and helicase domain (HD) are marked with arrows.



Supplementary Figure 2. PriB residues E39 and R44 lie in a binding pocket. (A) Wild type PriB (Protein Databank accession code 1TXY, colored blue) and

the E39A variant (Protein Databank accession code 2PNH, colored orange) align with an RMSD of 0.8 Å across all polypeptide backbone atoms. The box marks the region depicted in (B). (B) Residues E39 and R44 lie in a shallow pocket between adjacent monomers. PriB variants are colored as in (A) and E39, A39, and R44 sidechains are shown as sticks with oxygen atoms colored red and nitrogen atoms colored dark blue.

Supplementary Tables

Supplementary Table 1. Oligonucleotide primers used to clone *E. coli* primosome protein variants.

Gene	Plasmid	oligonucleotides (5' - 3')	
priA	pML100	oML100	GCGTATTCCATATGCCCGTTGCCCACGTTGCCTTG
		oML101	GTCACGGATCCTTAACCCTCAATCGGATCAAC
priA DBD	pML118	oML100	GCGTATTCCATATGCCCGTTGCCCACGTTGCCTTG
		oML141	GTCACGGATCCTTATCGCAACCGCTCACCAGAAACGGC
priA HD	pML120	oML143	GCGTATTCCATATGTTGAATACCGAACAGGCCACCGCCG
		oML101	GTCACGGATCCTTAACCCTCAATCGGATCAAC
priA:K230A	pML152	oML221	GTTACCGGTTCCGGTGCAACGGAGGTTTATCTC
		oML222	GAGATAAACCTCCGTTGCACCGGAACCGGTAAC
dnaT	pML103	oML106	GCGTATTCCATATGTCTTCCAGAGTTTTGACCCCG
		oML107	GTCACGGATCCTTACCCTCTGAATCCTGGTGG
priB:E32A	pML132	oML123	CACTGCCAGTTCGTGCTTGCGCATCGTTCTGTGCAGG
		oML124	CCTGCACAGAACGATGCGCAAGCACGAACTGGCAGTG
priB:E38A	pML133	oML125	GCATCGTTCTGTGCAGGCGGAAGCCGGCTTTCACC
		oML126	GGTGAAAGCCGGCTTCCGCCTGCACAGAACGATGC
priB:E39A	pML134	oML127	CATCGTTCTGTGCAGGAGGCAGCCGGCTTTCACCGGC
		oML128	GCCGGTGAAAGCCGGCTGCCTCCTGCACAGAACGATG
priB:H43A	pML146	oML195	GAGGAAGCCGGCTTTGCCCGGCAGGCGTGGTGTC
		oML196	GACACCACGCCTGCCGGGCAAAGCCGGCTTCCTC
priB:R44A	pML145	oML193	GAAGCCGGCTTTCACGCGCAGGCGTGGTGTCAAATG
		oML194	CATTTGACACCACGCCTGCGCGTGAAAGCCGGCTTC
priB:Q45A	pML144	oML191	GCCGGCTTTCACCGGGCGGCGTGGTGTCAAATG
		oML192	CATTTGACACCACGCCGCCCGGTGAAAGCCGGC
priB:K84A	pML112	oML119	ATTTCATGCCACAAGGCAGCGAACGGACTGAGC
		oML120	GCTCAGTCCGTTCGCTGCCTTGTGGCATGAAAT
priB:K89A	pML114	oML121	GCAAAGAACGGACTGAGCGCAATGGTTTTGCATGCCG

		oML122	CGGCATGCAAAACCATTGCGCTCAGTCCGTTCTTTGC
priB:E95A	pML135	oML131	CAAAATGGTTTTGCATGCCGCGCAGATTGAATTGATAG
		oML132	CTATCAATTCAATCTGCGCGGCATGCAAAACCATTTTG
priB:E98A	pML136	oML133	GCATGCCGAGCAGATTGCATTGATAGATTCTGGAG
		oML134	CTCCAGAATCTATCAATGCAATCTGCTCGGCATGC
priB:D101A	pML137	oML135	GCAGATTGAATTGATAGCTTCTGGAGACTAGGGATCC
		oML136	GGATCCCTAGTCTCCAGAAGCTATCAATTCAATCTGC
priB:D104A	pML138	oML137	GAATTGATAGATTCTGGAGCCTAGGGATCCGAATTCG
		oML138	CGAATTCGGATCCCTAGGCTCCAGAATCTATCAATTC

Supplementary Table 2. X-ray crystallographic data collection, molecular replacement, and structure refinement.

Dataset	PriB:E39A
Data Collection	
Space group	$P2_12_12_1$
a (Å)	49.98
b (Å)	60.69
c (Å)	66.27
Resolution (Å)	20.0 – 2.25 (2.33 – 2.25)
R_{sym} (%)*	8.3 (43.8)
$I/\sigma(I)$	28.1 (2.7)
Completeness (%)	92.6 (75.1)
Redundancy	5.2 (3.4)
Refinement	
Resolution (Å)	20.0 – 2.25 (2.33 – 2.25)
R/R_{free}**	24.7/27.6
Number of atoms	
Protein	1472
Water	43
B-factors	
Protein	66.2
Water	57.9
Root mean square deviations	
Bond lengths (Å)	0.009
Bond Angles (°)	1.175
Ramachandran statistics	
% core region	95.7
% allowed region	4.3
% generously or disallowed regions	0

$^{*}R_{sym} = \Sigma\Sigma_j \mid I_j - \langle I \rangle \mid / \Sigma I_j$, where Ij is the intensity measurement for reflection j and $\langle I \rangle$ is the mean intensity for multiply recorded reflections.

$^{**}R_{work/free} = \Sigma|| F_{obs} | - | F_{calc} || / | F_{obs} |$, where the working and free R factors are calculated using the working and free reflection sets, respectively. The free reflections (5% of the total) were held aside throughout refinement.

Supplementary Table 3. Plasmid complementation of *priB* variants.

Strain	*priA*	*priB*	*priB* plasmid[a]	SOS induction ratio[b]	average cell size (μm)	% filament cells[c]	cells counted
SS5515	+	+	-	1.03	2.12	0	2061
SS4315	+	+	*priB*	1.46	2.20	0	1837
SS4317	+	+	E39A	1.41	2.57	0	1993
SS5523	+	+	R44A	1.01	1.93	0	2253
SS5519	+	+	Q45A	1.23	1.89	0	1951
SS5527	+	+	H43A	1.08	1.84	0	2253
SS5516	*300*	+	-	1.56	2.32	0	2087
SS4307	*300*	+	*priB*	1.27	2.22	0	2068
SS4309	*300*	+	E39A	1.70	2.33	0	2136
SS5524	*300*	+	R44A	1.48	2.05	0	1776
SS5520	*300*	+	Q45A	1.39	2.02	0	2192
SS5528	*300*	+	H43A	1.51	1.78	0	2133
SS5531	+	*302*	-	1.67	2.41	1	1494
SS5532	+	*302*	*priB*	1.09	2.31	0	1867
SS5534	+	*302*	E39A	1.72	2.38	0	1829
SS5537	+	*302*	R44A	2.02	2.42	0	2239
SS5536	+	*302*	Q45A	1.72	1.98	0	1799
SS5538	+	*302*	H43A	1.62	1.94	0	1882
SS5518	*300*	*302*	-	10.90	3.98	17	1463
SS3465	*300*	*302*	*priB*	1.60	2.16	0	2065
SS3480	*300*	*302*	E39A	7.21	3.37	9	1927
SS5526	*300*	*302*	R44A	Lethal			
SS5522	*300*	*302*	Q45A	1.79	1.96	0	1955

SS5530	*300*	*302*	H43A	1.32	2.04	0	1989

[a](-) indicates the presence of empty pET28b vector. All priB plasmids are derivatives of pET28b containing *priB* or a *priB* mutation as indicated.

[b]The induction ratio is the average relative intensity of the entire population of cells. The relative intensity is calculated by dividing the mean pixel value of a cell by the normal pixel value of a wild-type cell (JC13509) not containing $\Delta att\lambda$::*sulAp*Ω*gfp-mut2*.

[c]Cells larger than 6 µm.

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