DnaC, the indispensable companion of DnaB helicase, controls the accessibility of DnaB helicase by primase

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Former studies relying on hydrogen/deuterium exchange analysis suggest that DnaC bound to DnaB alters the conformation of the N-terminal domain (NTD) of DnaB to impair the ability of this DNA helicase to interact with primase. Supporting this idea, the work described herein based on biosensor experiments and enzyme-linked immunosorbent assays shows that the DnaB-DnaC complex binds poorly to primase in comparison with DnaB alone. Using a structural model of DnaB complexed with the C-terminal domain of primase, we found that Ile-85 is located at the interface in the NTD of DnaB that contacts primase. An alanine substitution for Ile-85 specifically interfered with this interaction and impeded DnaB function in DNA replication, but not its activity as a DNA helicase or its ability to bind to ssDNA. By comparison, substitutions of Asn for Ile-136 (I136N) and Thr for Ile-142 (I142T) in a subdomain previously located at the interface in the NTD of DnaB that contacts primase. In view of these findings, we propose that DnaC controls the ability of DnaB to interact with primase by modifying the conformation of the NTD of DnaB.

Like its counterparts in other bacteria, DnaB of Escherichia coli is the DNA helicase that unwinds the parental DNA so that it can be copied during DNA replication (reviewed in Refs. 1–4). Its function in the stages of DNA replication requires its interaction with specific proteins. At the stage of initiation, DnaB must interact with DnaC, forming a stable complex. DnaB alone is inadequate. DnaB in this complex then interacts with DnaA assembled as a self-oligomer at oriC, which loads the DnaB-DnaA complex onto each DNA strand in an AT-rich region unwound by DnaA (5–9). During the transition from initiation to the elongation stage of DNA replication, DnaB interacts with primase, which leads to the release of DnaC from DnaB and its activation as a DNA helicase (10). At the elongation stage, primer formation by primase for DNA synthesis appears to require an interaction between primase and DnaB bound to the unwound lagging strand of the parental DNA (reviewed in Refs. 11–16). The frequency of this interaction that is transient in E. coli determines how often primers for Okazaki fragment synthesis are made (17–20). Whereas models suggest that three primase molecules are able to interact with DnaB as the helicase unwinds the parental DNA, the number of DnaB-bound primase molecules that are sufficient for primer synthesis has not been established. Of interest, DnaB also interacts with the σ subunit of the clamp loader (DnaX complex), which leads to a 20-fold increase in the rate of replication fork movement that stems from the synergy between DnaB as it unwinds the parental duplex DNA and DNA polymerase III holoenzyme as it copies the unwound parental DNA (21). At the termination stage, one of the proposed models to prevent replication fork movement from progressing beyond the terminus region, which would otherwise cause overreplication, involves an interaction of DnaB with Tus bound to a Ter site in an orientation that blocks the replisome (22–26).

Structural and biochemical studies have revealed that DnaB is a toroid composed of six identical subunits (27–30). Each DnaB protomer of the DnaB ring has a RecA-like fold contained in its larger C-terminal domain (CTD)3 to which DnaC binds (19, 30–33) and a smaller N-terminal domain (NTD) composed of two subdomains named the globular head (or the globular helical bundle) and the helical hairpin formed by two α-helices (Fig. 1A) (19, 28, 34–39). The NTDs of the DnaB protomers form an N-terminal collar as a trimer of dimers. Driven by nucleotide hydrolysis during unwinding, the lagging strand DNA template is thought to pass through the DnaB toroid in the 5’ → 3’ direction, whereas the other DNA strand is excluded (29, 40, 41).

The structures of DnaB of hyperthermophiles (some complexed to ssDNA) have been determined by X-ray crystallography, cryo-electron microscopy, and electron microscopy of negatively stained samples (19, 28, 35, 37, 38, 42, 43). On the basis of the crystal structures of Geobacillus stearothermophilus DnaB (GstDnaB) in which the helicase is either a closed ring.

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3 The abbreviations used are: CTD, C-terminal domain; NTD, N-terminal domain; GstDnaB, G. stearothermophilus DnaB; HDX, hydrogen/deuterium exchange; ATPγS, adenosine 5’-O-(thiotriphosphate); IPTG, isopropyl 1-thio-β-D-galactopyranoside; HBD, helicase-binding domain; PDB, Protein Data Bank.
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or open spiral (19,35), the channel in the interior of the N-terminal collar is wide or dilated (Fig. 1A). To form the dilated N-terminal collar, the helical hairpin and globular head domain of one DnaB protomer interact pairwise with the respective subdomain in pairs of protomers of the DnaB hexamer. For the closed ring form of *Aquifex aeolicus* DnaB, image analysis of negatively stained samples obtained by electron microscopy revealed a narrow inner channel within the N-terminal collar (42). For this constricted form, which was observed in equal abundance as the dilated form in the presence of ATP or ATP analogues, one set of globular heads are positioned on the outside of the N-terminal collar with the other set in the interior (Fig. 1A). For either dilated or constricted conformations, the interactions between the helical hairpins of pairs of DnaB protomers are preserved. These conformations of the N-terminal collar correlate with electron microscopic studies of *E. coli* DnaB in which this domain has either 3- or 6-fold symmetry (44).

Compared with these structures, 3D electron microscopic reconstructions of *E. coli* DnaB in a complex with DnaC suggests that this helicase is a spiral, but the NTD is constricted (43). In contrast, the crystal structure of GstDnaB complexed with the CTD of the cognate primase revealed that this DNA helicase is a closed ring, but its NTD is dilated (19). Compared with these static X-ray structures, the helicase presumably engages in a larger set of conformations during its catalytic cycle.

This structure of GstDnaB bound to primase also describes the interacting surfaces of these proteins (19). Related studies of *E. coli* primase demonstrated that truncations or amino acid substitutions within 16 residues from its C terminus blocked or altered its ability to associate with DnaB (18,45,46), supporting the crystal structure of GstDnaB bound to the CTD of primase. However, the mutational study of *E. coli* primase should have but did not suggest that primase interacts with the second globular head of DnaB. Moreover, in contrast with the stability of the complex containing GstDnaB and the cognate primase (16,47–49), the *E. coli* proteins do not form a stable complex but instead only have a weak affinity for each other (1.4 or 8.5 μM) (50,51). It has been suggested that the low negative charge distribution of the NTD of *E. coli* DnaB is the reason for its weaker binding to primase (36). Alternatively, *E. coli* primase may make contact with DnaB primarily through one but not both of its globular heads.

Other experiments on *E. coli* DnaB demonstrated that an Asn substitution for Ile-136 or a Thr substitution for Ile-142 (referred herein as the I136N or I142T mutants to denote the position of the amino acid substitution relative to the first residue of DnaB) in the helical hairpin does not disrupt the ATPase activity of DnaB, but negatively affects general priming (14), an assay that requires a physical interaction between primase and ssDNA-bound DnaB for primer formation (46,52–54). A separate study of the corresponding substitutions in GstDnaB showed that the mutant proteins are impaired in forming a complex with primase (16). Together, these findings suggest that the wild-type residues, which are conserved, contact primase directly.

More recent experiments of differential hydrogen/deuterium exchange (HDX (55)), combined with mass spectrometry and molecular modeling, identified sites in the CTD of DnaB and in the NTD of DnaC that interact in the formation of the DnaB-DnaC complex (33). This method measures the status of amide hydrogens of a protein that are solvent-exposed compared with buried residues. The ability of amide hydrogens to exchange is also affected by the binding of a protein or small molecule, which led to the identification of segments of DnaB and DnaC that interact, or by structural changes. Other evidence showed that the DnaB ring spontaneously opens and closes and that DnaC upon binding appears to trap DnaB as an open ring. The open ring conformation is evidently necessary for the DnaB-DnaC complex to load at the unwound region of oriC. This study also found that the binding of DnaC to DnaB causes a dramatic reduction in exchange of specific DnaB peptides (residues 122–134, 135–141, and 158–162) of the helical hairpin (residues 120–168). Apparently, this binding event induces a conformational change of the NTD that results in occlusion of the helical hairpin. The possibility that this structural change inhibits the binding of primase to DnaB has not been tested experimentally.

Noting the alternate conformations of the NTD described above, the underlying purpose of this study was to test the model that the altered conformation of DnaB’s NTD caused by its association with DnaC affects the binding of primase. Evidence from biosensor experiments, ELISAs, and hydrogen/deuterium exchange substantiates this model. Supporting biochemical assays showed that an I85A substitution in the globular head of DnaB specifically impairs the interaction between the helicase and primase. Other results showed that I136N and I142T substitutions alter the conformation of the helical hairpin and/or its pairing with this subdomain in the companion DnaB protomer to interfere with the binding of DnaB to ssDNA and to primase. Hence, primer formation and subsequent DNA replication are inhibited.

Results

The I85A, I136N, and I142T substitutions impair the ability of DnaB to interact physically with primase

We prepared a homology model of *E. coli* DnaB bound to the CTD of primase using the X-ray structure of GstDnaB complexed to the CTD of the cognate primase (19) and determined that specific amino acids in the globular head, including Ile-85, a conserved residue among DnaB homologues, are in close proximity (3.8–4.7 Å) to the CTD of primase (Fig. 1 (B–E) and supplemental Fig. S1). However, in contrast with Ile-85, our structural model shows little if any interaction (>8 Å) between primase and Ile-136 or Ile-142 in the helical hairpin of each DnaB protomer in the DnaB hexamer. These observations suggest that Ile-85 contacts primase, but Ile-136 and Ile-142 do not. A reservation with this conclusion is that the X-ray structure on which the model was built depicts only one conformation and does not exclude the possibility that Ile-136 or Ile-142 interacts directly with primase at some point of helicase function.

To better understand how DnaB interacts with primase, we performed biosensor assays in which we immobilized primase...
after biotinylation to streptavidin-coated biosensors. As shown in Fig. 2, we observed the concentration-dependent binding of DnaB to immobilized primase. With mutant forms of DnaB bearing an I85A substitution, to test our structural model, and I136N and I142T substitutions, as implicated in studies of GstDnaB (16), they were dramatically impaired in interacting with primase compared with wild-type DnaB. Apparently, the individual substitutions influence this essential interaction.

These biosensor experiments, which test the model of Fig. 1 on how DnaB interacts with primase, were performed in the absence of DNA based on the following studies. In work by others (50, 54), the affinity \( K_d \) of \( E. coli \) DnaB for immobilized primase was only 2-fold better than the affinity of primase for DnaB that had been immobilized by its interaction with ssDNA (1.4 and 2.8 \( \mu \)M, respectively). These results strongly suggest that ssDNA minimally influences the ability of DnaB to interact...
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Figure 2. The mutant DnaBs are defective in interacting with primase.
Biosensor assays were performed with DnaB or the mutants at the indicated concentrations by incubation at 25 °C with immobilized primase as described under “Experimental procedures.” Data analysis was performed with Octet Data Analysis software (Pall ForteBio).

with primase. In support of this idea, the closed ring form of Geobacillus kaustophilus helicase bound to ssDNA has a shape and dimension similar to those of the GstDnaB-primase complex (19, 37). Evidently, DnaB bound to ssDNA is in a conformation that is suitable for interaction with primase. Moreover, the firm binding of GstDnaB to primase in the absence of ssDNA (16, 47–49) suggests that the weaker affinity of the E. coli counterparts described above is due to another reason and not the absence or lack of ssDNA.

Because HDX analysis of the DnaB-DnaC complex in comparison with DnaB indicates that the binding of DnaC to DnaB occludes the helical hairpin region (33), we speculated that this change may affect the binding of primase. To test this idea, we performed biosensor assays in which we measured the interaction of wild-type DnaB or the DnaB-DnaC complex with primase that was immobilized on the sensor surface. Whereas both DnaB and the DnaB-DnaC complex bound to primase in a concentration-dependent manner, the rate of binding of DnaB was substantially faster than that of the DnaB-DnaC complex (Fig. 3A). In control ELISA experiments,4 we compared the level of binding to DnaB of wild-type primase and a truncated form of primase lacking its C-terminal 16 residues. This region is known to be required for primase to interact with DnaB (50, 54). Compared with wild-type primase, the marginal binding of the mutant primase to DnaB verifies that this part of the CTD of primase is needed for its interaction with DnaB.

As an independent method, ELISA was used to measure the interaction between DnaB and primase. Compared with DnaB, the DnaB-DnaC complex was markedly reduced in binding to primase (Fig. 3B). As a control, DnaB was incubated together with a truncated form of DnaC (DnaCΔ51), which lacks key residues within the first 51 N-terminal residues that interact directly with DnaB (33, 56). Under conditions that would support assembly of the DnaB-DnaC complex (32), the presence of this mutant did not diminish the binding of DnaB to primase. Thus, DnaC bound to DnaB impairs its ability to interact with primase. In reactions with only DnaCΔ51 or wild-type DnaC, their marginal reactivity to the antibody documents its specificity for DnaB. Considering that the interaction of primase with DnaB in the DnaB-DnaC complex at oriC and primer formation leads to the release of DnaC from DnaB (10), these observations indicate that DnaC and primase have opposing effects on the binding of the other to DnaB.

The I136N and I142T substitutions cause an altered conformation of the helical hairpin and/or affect its pairing with this subdomain in the companion DnaB protomer

We performed HDX analysis to obtain insight into why the mutant forms of DnaB were defective in interacting with primase. Focusing on peptides that form the helical hairpin, those containing residues 122–134 and 135–141 located in helix 5 and peptide 158–162 in helix 6 (Fig. 1E) showed substantially

4 M. M. Felczak and J. M. Kaguni, unpublished data.
greater rates of exchange of the I142T mutant in comparison with wild-type DnaB (Fig. 4). Despite our inability to detect peptide 135–141 for the I136N mutant, the exchange behavior of the other peptides was similar to that of the I142T mutant. These results indicate that the I136N and I142T substitutions lead to a considerable conformational change of the helical hairpin and/or its interaction with this subdomain in the partnering DnaB protomer. In contrast, the moderately lower rates of exchange of these peptides in the I85A mutant compared with DnaB suggest that this substitution only causes a small decrease in flexibility of its helical hairpin.

For wild-type DnaB and the mutants, we also found that the peptide containing residues 76–84 displayed a similar rate of exchange, which suggests that the substitutions, including I85A, do not substantially alter the conformation of this part of DnaB. Likewise, the exchange rates were essentially identical for peptides of these proteins within the N-terminal globular domain (residues 92–97), the linker helix (residues 187–196), the Walker A region (residues 227–240), and peptides that interact directly with DnaC in forming the DnaB-DnaC complex (residues 295–304 and 431–435; supplemental Fig. S2). Except for the helical hairpin of the I136N and I142T mutants, the behavior of these representative peptides strongly suggests that the substitutions do not disrupt the global conformation of DnaB. Moreover, the results support the idea that the I85A substitution interferes with the direct binding of DnaB to primase.

As an independent method, we examined the mutants by gel-permeation chromatography (Superose 12 HR 10/30). The elution volumes of 9.75 ± 0.06 ml for the I136N mutant and 9.70 ± 0.04 ml for the I142T mutant in comparison with 10.11 ± 0.07 ml for wild-type DnaB indicate their somewhat distorted conformation (Table 1). These results confirm an earlier study, which showed that mutants bearing the I136N and I142T substitutions assemble as hexamers, but gel filtration experiments revealed that they have larger Stokes radii than wild-type DnaB (14). In contrast, the I85A mutant eluted slightly later at 10.25 ± 0.05 ml, suggesting a more closely packed structure.

In companion gel-permeation chromatography experiments, we combined the mutants or wild-type DnaB with DnaC at a ratio of two DnaC monomers per DnaB protomer to measure the formation of the DnaB-DnaC complex. Consistent with the elution volume expected for the DnaB-DnaC complex for which elution volume is a log function of molecular weight, material was observed that eluted slightly earlier than wild-type DnaB or the mutants when chromatographed in the absence of DnaC (Table 1). A second later-eluting peak was at the position expected for DnaC, which was confirmed in Coomassie Blue–stained SDS-polyacrylamide gels using purified DnaC and DnaB as markers. For the earlier eluting peak, quantitative densitometric analysis of the stained gels showed that it contained both DnaB and DnaC at a relative ratio of 1.78, which agrees with the reported ratio of 1.81 for the DnaB6-DnaC6 complex (57). Compared with the predicted masses of DnaB (52,390 Da) and DnaC (27,953 Da), the expected ratio is 1.87. The lower observed ratios are consistent with reports that the dye preferentially stains basic proteins like DnaC (58, 59).

### Table 1

| Protein         | Elution volume (ml) | No. of experiments |
|-----------------|---------------------|--------------------|
| DnaB            | 10.11 ± 0.07        | 3                  |
| I136N           | 9.75 ± 0.06         | 2                  |
| I142T           | 9.70 ± 0.04         | 2                  |
| I85A            | 10.25 ± 0.05        | 2                  |
| DnaB-DnaC       | 10.06               | 1                  |
| I136N-DnaC      | 9.54                | 1                  |
| I142T-DnaC      | 9.61                | 1                  |
| I85A-DnaC       | 10.02               | 1                  |

### The mutant DnaBs are defective in DNA replication

As the mutants poorly interact with primase (Fig. 2), a prediction is that they should be impaired in DNA replication of a supercoiled oriC-containing plasmid (M13oriC2LB5) and general priming. We confirmed this expectation under conditions in which wild-type DnaB was active (Fig. 5). For the I136N and I142T mutants, the results of general priming assay confirm a previous study but with poly(dT) as a template (14).

I85A is active as a DNA helicase but I136N and I142T are inactive in DNA unwinding

We also measured the activity of the mutants in the formation of a highly negatively supercoiled oriC-containing plasmid,
termed Form I* (56, 60). This assay measures the DnaA-mediated loading of DnaB complexed with DnaC at oriC (61), ssDNA binding by DnaB (62), and its activity as a DNA helicase that unwinds the plasmid after helicase loading (2, 40). The latter requires ATP binding and its hydrolysis. In the presence of DNA gyrase, which removes the positive superhelicity that accumulates in the duplex portion of the plasmid, a highly negatively supercoiled DNA results, which can be detected by agarose gel electrophoresis. Upon comparison of the mutants with DnaB, we found that I85A was fairly active (Fig. 6), which greatly contrasts with its inactivity in general priming and in DNA replication of the oriC-containing plasmid (Fig. 5). However, the others were only marginally active (Fig. 6). Because this assay does not require primase, these mutants may unwind the plasmid DNA more slowly, pause more frequently, and/or dissociate from the DNA more readily. For the latter, the results raise the possibility that the substitutions impair ssDNA binding, which is required for both oriC-dependent DNA replication, Form I* formation, and general priming.

I85A retains its ability to bind to ssDNA, but I136N and I142T are defective in ssDNA binding

To address whether the substitutions affect ssDNA binding, we modified the conditions of a gel mobility shift assay, which included ADP in reactions (63), and used a radiolabeled ssDNA fragment of 40 nucleotides. With SSB as a control, a saturating level led to the formation of a discrete complex (Fig. 7, A and B). With wild-type DnaB, the complexes formed were more abundant upon inclusion of ATPγS instead of ADP (Fig. 7, A and C), confirming a previous study in which ssDNA binding was measured by fluorescence anisotropy (64). Compared with the mobility of the complex formed with SSB (76 kDa as a tetramer), the predominant DnaB-ssDNA complex had a lower mobility that presumably reflects the larger size of DnaB (314 kDa as a hexamer). As the DNA site size of DnaB is 20 nucleotides (29, 65), two DnaB hexamers may have bound to the ssDNA above the most abundant complex. By comparison, the complex at the approximate position of the SSB-ssDNA complex may contain a monomer of DnaB (52 kDa) bound to the ssDNA. Quantifying the amount of free ssDNA, we found that I85A was functional, albeit less so than wild-type DnaB (Fig. 7, B and D). In contrast, I142T was substantially less active, whereas I136N oddly bound to a fraction of the ssDNA at lower but not higher protein levels (Fig. 7, A–D). Moreover, the aberrant DNA binding did not lead to the formation of a discrete complex.
The mutants are active in ATP hydrolysis

In separate experiments, we confirmed that ssDNA enhances the ATPase activity of wild-type DnaB (supplemental Fig. S3), but the level of stimulation was not as great as reported by others (42, 66, 67), even with different preparations and under various reaction conditions. Whereas the mutants were reasonably active in ATP hydrolysis, the inclusion of ssDNA in reactions stimulated the ATPase activity of I85A but not of I136N and I142T. Hence, the mutants apparently are able to bind ATP and presumably ATPγS. Moreover, the inability of ssDNA to stimulate ATP hydrolysis by I136N and I142T corresponds with their defect in binding to ssDNA. For I85A, these findings together with those described above support a direct role of Ile-85 in the interaction of DnaB with primase. Another possibility is that the I85A substitution alters the conformation of DnaB so that primase cannot bind.

The mutant DnaBs do not support viability

To show that the mutants are functionally defective in vivo, we measured the ability of the dnaB alleles carried in a plasmid to complement the temperature-sensitive phenotype of E. coli RM84 (relevant genotype: dnaB22(Ts)). Compared with the plasmid carrying the dnaB+ gene that conferred growth to the mutant strain at nonpermissive temperature (Table 2), plasmids carrying the dnaB alleles were defective. For the I136N and I142T substitutions, these observations confirm earlier results in which the corresponding mutations in the S. typhimurium dnaB gene were analyzed (68).

The complementation assay was performed with isogenic derivatives of pET11a carrying the dnaB alleles downstream from the bacteriophage T7 gene 10 promoter. As the host strain (E. coli RM84) lacks T7 RNA polymerase, we presume that transcription from an upstream promoter is responsible for expression of the respective dnaB alleles. For the plasmid encoding the wild-type dnaB gene, its ability to complement the temperature-sensitive phenotype of E. coli RM84 indicates that induced expression from the T7 RNA polymerase promoter is not required. Of interest, immunoblot analysis of whole-cell lysates prepared from this strain bearing the individual plasmids and grown at 30 °C failed to demonstrate a gene dosage effect of an increased DnaB level compared with chromosomally encoded DnaB in the strain carrying the empty vector.5 These observations indicate that the plasmid-encoded

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level of DnaB is far less than that encoded by the chromosomal dnaB locus.

As it is possible that the mutant proteins failed to complement the temperature-sensitive phenotype of RM84 due to their proteolytic instability, we addressed this issue by performing immunoblot analysis. Because we were unable to distinguish DnaB that was chromosomally encoded from plasmid-encoded DnaB using RM84, we turned to a strain (E. coli HMS174 (DE3) (pLysS)) that conditionally expresses T7 RNA polymerase. In control experiments with this strain bearing a plasmid (pET11a-dnaB) that permits the overproduction of DnaB, the experimental approach involved separating a culture grown at 42 °C to mid-log phase into four portions organized as two pairs (supplemental Fig. S4A). Chloramphenicol, which inhibits protein synthesis, was added to portion I and II of one pair. IPTG was added to only portion II, and samples were removed for analysis at the times indicated. To portion IV of the second pair, IPTG was added followed by chloramphenicol after 1 h of induced expression. Samples were removed from this subculture at various times after the addition of chloramphenicol. Portion III of the culture was not induced with IPTG, but chloramphenicol was added after the 1-h time span of induced expression in the other subculture of this set. A sample was removed from portion III at the time of antibiotic addition. Analysis of the second pair showed that the addition of IPTG leads to the expected overproduction of DnaB and that DnaB is relatively stable for 60 min after the addition of chloramphenicol. Considering that each sample was adjusted to an equivalent turbidity (A$_{595 \text{ nm}}$), the indicated volumes were chosen on the basis that pilot experiments showed these amounts to be within the response range for immunochromatographic detection. Taking into account the 15-fold lower sample volume of the culture that overproduced DnaB, the lesser abundance of DnaB in the former pair of samples shows that the treatment with chloramphenicol inhibited overproduction of DnaB. For this set, the level of DnaB detected presumably represents that encoded by the chromosomal dnaB locus. In these experiments, the rabbit antiserum also reacted with a polypeptide at a position above DnaB and less well with other smaller polypeptides.

Companion experiments were performed with HMS174 (DE3) (pLysS) bearing the empty vector (pET11a) or its derivatives carrying the dnaB alleles. Following the approach for the second set, IPTG was added where indicated to cultures at a mid-log phase of growth at 42 °C followed by the addition of chloramphenicol 1 h later. Samples were removed at that time and thereafter and adjusted to equivalent turbidities, and appropriate volumes were analyzed by immunoblotting. The experiment reaffirms that the addition of IPTG leads to an elevated level of DnaB and that it is stable (supplemental Fig. S4B). By comparison, I85A was overproduced, but to a lower extent than wild-type DnaB as judged by scanning densitometry (supplemental Fig. S4C), whereas the induced levels of the I136N and I142T mutants were comparable with DnaB (supplemental Fig. S4, D and E). In experiments to investigate the lesser overproduction of I85A, we found that the plasmid encoding it was less abundant per culture density than the isogenic plasmid carrying wild-type dnaB.5 Other results described below suggest that the mutant plasmid is toxic. Hence, cells are apparently selected that have a lower plasmid copy number or lack the plasmid despite the presence of ampicillin in the culture medium. Moreover as described earlier, we found that the plasmid-encoded level of DnaB in this strain at 30 °C is far less than that encoded by the chromosomal dnaB locus.5 Taking these results together, the maintenance of the mutant proteins after the addition of chloramphenicol strongly suggests that their inability to complement the temperature sensitivity of RM84 is not the result of their proteolytic degradation. However, we cannot exclude the possibility that loss of the mutant plasmids at 42 °C accounts for their failure in complementation.

**Discussion**

The X-ray structure of *G. stearothermophilus* DnaB complexed with the CTD of the cognate primase (19) and our model of the homologous *E. coli* complex derived from this structure suggest that primase makes direct contact with the globular head domain but has little if any contact (>8 Å) with the helical hairpin (Fig. 1, D and E). These models correlate with studies showing that DnaB from several bacteria is able to bind to two or three molecules of primase (16, 19, 69) and with another report suggesting a model in which one primase molecule bound to DnaB interacts with another molecule of primase to regulate primer length during primer synthesis (70).

The structural model places Ile-85 in close proximity (3.8–4.7 Å) with the CTD of primase (Fig. 1, D and E), which correlates with the specific defect of the I85A mutant in interacting with primase. The corresponding dnaB allele in a plasmid was unable to complement a dnaB(Ts) mutant at nonpermissive temperature. Other experiments showed that the I85A mutant is defective in interacting with primase in biosensor experiments, in general priming, and in an in vitro system of DNA replication with an oriC-containing plasmid. However, I85A retains its ATPase activity and its ability to interact with ssDNA. Separate evidence based on gel-permeation chromatography strongly suggests that the mutant assembles as a hexamer and apparently retains the ability to interact with DnaC in formation of the DnaB-DnaC complex. Despite a more compact structure of I85A as suggested by its somewhat later elution by gel-permeation chromatography relative to wild-type DnaB, the essentially identical exchange rates for a peptide (residues 76–84) of the I85A mutant and wild-type DnaB suggest that the substitution, which is next to this peptide, only slightly alters this region of DnaB. These results taken together strongly suggest that Ile-85 is at the interface between DnaB and primase, but we cannot exclude the possibility that the I85A substitution alters the conformation of DnaB so that primase cannot bind.

On the basis of a homology model prepared using the corresponding crystal structure from *G. stearothermophilus* (19), Lewis et al. (71) suggested that Phe-103 of DnaB is at the interface between *E. coli* DnaB and the CTD of primase. Although our homology model supports this idea, we found that an alanine substitution for this residue marginally affected the ability of the allele carried in a plasmid (pET11a) to complement a temperature-sensitive dnaB mutant (*E. coli* RM84 assayed as described in Table 1).4 Similar results were obtained with alleles encoding L97A and D98A substitutions. If Phe-103 is at the
interface, it apparently does not participate in the interaction between DnaB and primase.

In comparison, HDX experiments revealed that the binding of DnaC to DnaB caused a severe reduction in the exchange rate for DnaB peptides (residues 122–134, 135–141, and 158–162) that reside in the helical hairpin (residues 120–168) (33), which indicates a substantial conformational change of the NTD. If so, an expectation is that DnaB, but not I136N, I142T, or the DnaB-DnaC complex formed with wild-type DnaB, should be able to interact with immobilized primase. Confirming this prediction, ELISA showed greater binding of wild-type DnaB to immobilized primase compared with the DnaB-DnaC complex. As an independent method, biosensor experiments demonstrated a faster rate of binding by DnaB to immobilized primase relative to the DnaB-DnaC complex. Biosensor assays also showed that mutants bearing amino acid substitutions in the helical hairpin (I136N and I142T) are defective in interacting with primase, which correlates with HDX analysis that indicated an altered conformation of helical hairpin of the mutants and/or its pairwise arrangement with this subdomain in the partnering DnaB protomer. As these mutants are impaired in binding to ssDNA, the amino acid substitutions perturb the conformation of the helical hairpin to affect the ability of DnaB to interact with primase and ssDNA.

The change in conformation of DnaB induced by the binding of DnaC to DnaB, which may affect the arrangement of the globular heads to inhibit the association of primase, strongly suggests that DnaC governs the interaction of DnaB with primase. However, it is unknown whether the binding of only one DnaC monomer to DnaB is enough to cause this conformational change. The paradox is that the interaction of primase with DnaB after loading of the DnaB-DnaC complex at oriC has the opposing effect, leading to the dissociation of DnaC and activation of DnaB as a DNA helicase (10). As DnaC and primase make contact with separate domains of DnaB, their individual binding apparently transduces an adverse conformational change to the adjoining domain to affect binding of the other protein. This process may explain why excess primase is toxic in E. coli (72); its association with DnaB presumably interferes with binding of DnaC to the helicase to impede DNA replication. A mechanistic understanding of how the interaction of DnaC or primase with DnaB interferes with the binding of the other is a fascinating and critical issue.

The Berger laboratory constructed a mutant DnaB bearing S36R and I85R substitutions in the globular head (42). Its constricted structure was ascribed to charge repulsion by these residues that correlated with impaired activity of the mutant in general priming. Whereas these results suggest that primase is unable to interact with the constricted form of DnaB (42), our results substantiate the position of Ile-85 at the interface between DnaB and primase. Thus, it is uncertain whether the S36R/I85R mutant is unable to interact with primase because it is in the constricted form.

The I136N and I142T alleles carried in a multicopy plasmid and presumably expressed from the natural dnaB promoter were identified by their dominant-negative phenotypes (68). Considering that DnaB is a hexamer, the growth interference conferred by these mutations suggests that the presence of a mutant subunit together with wild-type subunits in the hexamer disrupts DnaB function. However, the colony size and frequency of transformation at 30 °C of HMS174 (DE3) (pLysS) bearing the derivatives of pET11a were comparable. Of interest, when the I136N and I142T alleles were placed downstream of the T7 gene 10 promoter in pET3c, which lacks the gene for Lac repressor and its binding site that is in the T7 promoter region of pET11a, the colony size of the plasmid-borne I136N allele in E. coli RM84 was comparable with that of the strain carrying either the empty vector or this plasmid encoding the dnaB gene (supplemental Fig. S5). In contrast, colonies of RM84 harboring the I142T allele in pET3c were smaller and heterogeneous in size. Despite several attempts, we were unable to introduce the I85A mutation by site-directed mutagenesis into pET3c-dnaB, which is at odds with its trouble-free assembly in pET11a-dnaB. We presume that the absence of Lac repressor in pET3c leads to an increased abundance of I85A, which is toxic. These results with I142T and I85A support the idea that the association of one or more mutant subunits with wild-type DnaB protomers in the intact enzyme inhibits the activity of DnaB.

**Experimental procedures**

**Proteins**

DnaB, DnaC, and the indicated mutants were purified essentially as described (10, 56, 73) and quantified by the dye-binding method (74) and also by SDS-PAGE after staining with Coomassie Blue using bovine serum albumin as a standard. DnaC∆51 lacks the first 51 N-terminal residues of DnaC and fails to form the DnaB-DnaC complex due to its inability to interact with DnaB (56). Other replication proteins were purified as described (73, 75).

**Plasmids encoding dnaB alleles**

A gift from Dr. Kenneth Marians at Sloan Kettering Institute, the plasmid named pET3c-dnaB contains the E. coli dnaB gene inserted into plasmid pET3c (Novagen) (15). By site-directed mutagenesis (QuikChange, Agilent Technologies), the I136N and I142T substitutions were introduced into the dnaB coding region of pET3c-dnaB and verified by DNA sequence analysis. The wild-type dnaB gene was removed from pET3c-dnaB by cleavage with NdeI and BamHI and inserted downstream from the bacteriophage T7 promoter in pET11a that had been digested with NdeI and BamHI to form pET11a-dnaB. By site-directed mutagenesis, the I85A, I136N, and I142T substitutions were constructed, forming plasmids named pET11a-I85A, pET11a-I136N, and pET11a-I142T, respectively. To confirm the introduction of the mutations, DNA sequence analysis of wild-type dnaB and the alleles was performed.

**Differential hydrogen/deuterium exchange assays**

Hydrogen/deuterium exchange analysis was performed as described (33). After a 10-fold dilution at room temperature of DnaB (3 μg) from buffer T2 (25 mM HEPES-KOH, pH 8.0, 20 mM NaCl, 10% glycerol, 5 mM MgCl₂, 1 mM ATP, and 2 mM DTT) into this buffer but made with D₂O, samples (0.1 ml) were incubated for the times indicated and added to an equal volume.
Protein dynamics of DnaB, DnaC, and primase

of ice-cold 1% formic acid to inhibit back-exchange. Samples were then immediately subjected to on-line digestion for 2 min on a pepsin column on ice. The peptides were then separated on a reverse-phase C18 analytical column and analyzed by electro-spray ionization-MS and identified as described (33). To assess the relative level of back-exchange after dilution, samples were incubated overnight at room temperature in the above buffer but made with D2O to permit extensive deuteration of susceptible amide protons, followed by the analysis of peptic fragments by mass spectrometry. Because samples were analyzed under essentially identical conditions, the results were not corrected for back-exchange, which ranged from 30 to 50% depending on the individual peptide. Under similar conditions, the overall sequence coverage of DnaB peptides identified was about 90% (33).

Biosensor analysis

E. coli primase (DnaG) was biotinylated via amine coupling in 50 mM HEPES-KOH, pH 7.5, 0.18 mM NaCl, 10% glycerol, and 5 mM DTT using “No Weigh” NHS-PEG4-Biotin at an equimolar coupling ratio of biotin to protein following the manufacturer’s instructions (Thermo Scientific). The modified protein, which should have the biotin attached predominantly to its N terminus via the 29-Å PEG spacer, was separated from free biotin by filtration through a ZEBA desalt spin column (Thermo Scientific) equilibrated in Buffer E (20 mM HEPES-KOH, pH 7.4, 0.1 mM NaCl, 5 mM MgCl2, 5% glycerol, 0.005% Tween 20, 2 mM DTT, and 0.1 mM ATP). Wild-type DnaB or its mutant derivatives purified as described above were transferred into Buffer E by gel-permeation chromatography on a Superose 12 HR 10/30 column (GE Healthcare) equilibrated in Buffer E. Likewise, DnaB and the mutants were assembled into the DnaB-DnaC complex by their incubation for 10 min on ice at molar ratio of 2 DnaC monomers/DnaB protomer of hexameric DnaB in 33 mM HEPES-KOH, pH 7.6, 10% glycerol, 0.33 mM NaCl, 10 mM MgCl2, 0.32 mM EDTA, 2 mM DTT, and 0.1 mM ATP followed by gel-permeation chromatography in Buffer E (Superose 12 HR 10/30).

Using an Octet RED96 system (Pall ForteBio), biotinylated primase (0.5 μM) in Buffer E was immobilized onto streptavidin-coated biosensors for 1 min (0.35 nm) at 30 °C. After 2 min in Buffer E to establish a stable baseline, the biosensors were incubated with serial dilutions of the indicated proteins for 5 min, followed by incubation for 5 min in buffer without protein. Data analysis was performed with Octet Data Analysis software.

ELISA

Primase (0.8 μg/well) was immobilized on microtiter plates in 100 μl of Buffer A (50 mM HEPES-KOH, pH 7.4, 5 mM MgCl2) for 1 h at 37 °C. After removing unbound protein with three washes of Buffer A supplemented with 0.005% Tween 20 and 2% nonfat milk, the indicated proteins in Buffer A supplemented with 0.1 mM NaCl, 2 mM DTT, and 2 mM ATP were added in triplicate, followed by incubation for 1 h at room temperature. The wells were then washed at this step and beyond with Buffer A supplemented with 2% nonfat milk, 2 mM DTT, and 2 mM ATP, which was also used as the diluent for rabbit antisera that recognizes DnaB and for goat anti-rabbit antibody conjugated to horseradish peroxidase. This DnaB antisera was also used in supplemental Fig. S4, which revealed an immunoreactive polypeptide slightly larger than DnaB (52 kDa), three or four smaller polypeptides, and material near the bottom of the gel. After incubation overnight with an appropriate dilution of DnaB antisera at 4 °C, the wells were washed and then incubated at room temperature for 1 h with goat anti-rabbit antibody. Immune complexes were then detected colorimetrically at 490 nm.

Assays of in vitro DNA replication

General priming reactions (25 μl) contained M13-A site ssDNA (66 ng, 27.6 fmol (76)), primase (10 ng, 6 nM), DNA polymerase III* (90 ng, 7 nM), β clamp (11.4 ng, 6 nM), and the indicated amounts of wild-type DnaB or the indicated mutant derivatives in buffer containing 40 mM HEPES-KOH, pH 7.6, 20 mM Tris-HCl, pH 7.6, 0.08 mg/ml BSA, 4% sucrose, 4 mM DTT, 10 mM magnesium acetate, 2 mM ammonium sulfate, 0.08 mM dATP, dCTP, dGTP, and [methyl-3H]dTPP, 0.2 mM GTP, UTP, CTP, and 2 mM ATP essentially as described (54). Incubation was for 10 min at 30 °C. After quenching the reaction by trichloroacetic acid precipitation, the insoluble radioactive material was captured on glass fiber filters, and radioactivity incorporated was measured by liquid scintillation spectrometry.

To measure DNA replication of an oric-containing plasmid, reactions (25 μl) were assembled in the buffer described above. Reactions also contained 40 mM phosphocreatine, creatine kinase (100 μg/ml), M13oriC2LB5 supercoiled DNA (200 ng, 46 fmol), DNA polymerase III* (90 ng, 7 nM), β clamp (11.4 ng, 6 nM), SSB (260 ng, 140 nM as a tetramer), the α dimer of HU (10 ng, 0.2 nM), DNA gypse A subunit (370 ng, 150 nM), DNA gypse B subunit (520 ng, 230 nM), DnaC (56 ng, 80 nM), primase (10 ng, 6 nM), DnaA (110 ng, 84 nM), and the indicated amounts of wild-type DnaB or the indicated mutants as described (77). After incubation for 20 min at 30 °C, DNA replication was measured as described above.

Gel electrophoretic mobility shift assay

Modifying the conditions of Atkinson et al. (63), reactions (10 μl) containing the indicated amounts of mutant or wild-type DnaB or SSB (6 ng, 8 nM) and a 5’32P-labeled ssDNA (10 fmol or 1 nM, GATAATAATTCCCGCGATGCCTGCTAAA-TCACTGTTTTTCG) were assembled in 50 mM HEPES-KOH, pH 8, 10 mM magnesium acetate, 10 mM DTT, 10 μM ATPγS or ADP as indicated, and 50 μg/ml bovine serum albumin followed by incubation at 37 °C for 10 min. After the addition of glycerol to 10% (v/v) to the reactions, protein-DNA complexes were separated from the ssDNA by electrophoresis at 160 V for 90 min in a gel (6.5%, 60:1 acrylamide/bisacrylamide) cast in 90 mM Tris borate, 10 mM magnesium acetate, and 10 μM ADP. The electrophoresis buffer contained 45 mM Tris borate, 5 mM magnesium acetate, and 10 μM ADP.

Complementation of E. coli RM84

Electrocompetent E. coli RM84 (dnaB22(Ts) F− Strr (λ112), (78)) was transformed with pET11a-dnaB or its derivatives encoding missense dnaB mutations and incubated in LB broth.
for 1 h at 30 °C. After serial dilution, bacteria were plated on LB agar containing 100 μg/ml ampicillin followed by incubation at 30 °C or 42 °C for 24 or 16 h, respectively. The relative plating efficiency is calculated as the ratio of the number of colonies observed at 42 °C compared with 30 °C.

Author contributions—M. M. F. performed the molecular modeling of the DnaB-DnaC complex. M. M. F. also performed the biosensor experiments, ELISA, assays of DNA replication, and gel-permeation chromatography. S. C. performed the hydrogen/deuterium exchange experiments and analyzed the data. S. C. also performed the experiments of gel mobility shift with ssDNA, ATP hydrolysis, immunoblotting, and plating of plasmid-bearing strains. All authors discussed and interpreted the results. J. M. K., S. C., and M. M. F. wrote the manuscript.

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