Identification of a Region of Escherichia coli DnaB Required for Functional Interaction with DnaG at the Replication Fork*

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Pearl Chang‡ and Kenneth J. Marians‡§

From the ‡Molecular Biology Graduate Program, Weill Graduate School of Medical Sciences of Cornell University and the §Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

The fundamental activities of the replicative primosomes of Escherichia coli are provided by DnaB, the replication fork DNA helicase, and DnaG, the Okazaki fragment primase. As we have demonstrated previously, DnaG is recruited to the replication fork via a transient protein-protein interaction with DnaB. Here, using site-directed amino acid mutagenesis, we have defined the region on DnaB required for this protein-protein interaction. Mutations in this region of DnaB affect the DnaB-DnaG interaction during both general priming and X174 complementary strand DNA synthesis, as well as at replication forks reconstituted in rolling circle DNA replication reactions. The behavior of the purified mutant DnaB proteins in the various replication systems suggests that access to the DnaG binding pocket on DnaB may be restricted at the replication fork.

In bacteria, the DNA unwinding and Okazaki fragment-priming functions at the replication fork are provided by a primosome, a multienzyme conglomerate that moves progressively along the lagging-strand template (1). There are two primosomes in Escherichia coli, one that forms in a DnaA-directed fashion at the chromosomal origin, oriC, and one that forms at recombination intermediates to restart stalled or aborted replication forks (2). The replicative primosome formed at oriC requires DnaB, DnaC, and DnaG for assembly, whereas the replication restart primosome (formerly the φX174-type primosome (3)), which can form at D loops (4), requires PriA, PriB, DnaT, and possibly PriC, in addition to the former three proteins, for assembly (3).

Primosomes provide both the DNA unwinding and Okazaki fragment-priming functions of the replisome. In the case of each of the bacterial primosomes, these activities are provided by DnaB and DnaG, respectively. To form a replication fork, DnaB must be placed onto single-stranded (ss) DNA that is coated with the single-stranded DNA-binding protein (SSB). Whereas DnaB itself can bind to naked ssDNA, it is prevented from doing so in vivo because it is found in a stoichiometric complex with DnaC (5). DnaC, which has a cryptic ssDNA binding activity that is activated when it is complexed with DnaB (6), can transfer DnaB to naked ssDNA but not to SSB-coated DNA. This mechanism presumably prevents promiscuous loading of DnaB to any region of the chromosome that happens to become single-stranded. Thus, DnaB must be directed to specific regions of the DNA by the action of other proteins that somehow manage to create an SSB-free region of ssDNA. At oriC this is accomplished by a protein-protein interaction between DnaA and DnaB (7). During replication fork reactivation, PriA identifies the site for restart primosome loading (2, 3, 8), and it is probably a protein-protein interaction between DnaT and DnaB that mediates transfer of DnaB to SSB-coated DNA (9).

Initial studies demonstrated that DnaG, which had been identified as a primase (10, 11), was not present in restart primosomes formed in the absence of DNA synthesis and isolated by gel filtration bound to φX174 ss(c)DNA. For primer synthesis to occur, DnaG had to be added back to those protein-DNA complexes (12, 13). We showed that this was also the case at active replication forks, i.e. DnaG did not remain permanently associated with the replication fork; rather, a new molecule of DnaG was recruited from solution to synthesize the primer for each new Okazaki fragment (14). This distributive action, with respect to the cycle of Okazaki fragment synthesis, of DnaG at the replication fork acts to regulate the size of the nascent lagging-strand fragments. Thus, Okazaki fragment size is inversely related to the concentration of DnaG in the reaction mixture (14, 15).

Using partial proteolysis to resolve DnaG into independent domains, we demonstrated that the C-terminal 16 kDa of the protein were not required for primer synthesis but were required for DnaG activity in any replication assay that also required DnaB (16). Because the isolated C-terminal fragment of DnaG could compete with the intact protein at the replication fork and cause Okazaki fragment size to be altered, we concluded that this domain mediated a protein-protein interaction between DnaB and DnaG that acted to recruit DnaG to the replication fork. Subsequent studies indicated that the C-terminal 16 amino acids of DnaG were crucial to the interaction with DnaB (17). For example, at identical concentrations, DnaG Q576A directs the synthesis of Okazaki fragments that are at least 15-fold longer in size than those directed by the wild type protein (18).

Here we report the isolation of reciprocal mutations in DnaB that specifically affect the DnaB-DnaG interaction at the replication fork. As was the case with the mutant DnaG proteins, the mutant DnaB proteins direct the synthesis of larger Okazaki fragments at the replication fork than the wild type protein. These mutations lie in the N-terminal region of DnaB, mapping very close together in the crystal structure (19), and do not affect the ability of the mutant proteins to act as replication fork DNA helicases. Interestingly, the mutant proteins display a different spectrum of activities in a number of DNA replication systems that utilize DnaB, suggesting that the...
DnaB-DnaG interaction at the replication fork is further modulated by another factor.

**MATERIALS AND METHODS**

**Reagents, DNAs, Enzymes, and Replication Proteins—**Restriction enzymes were from Amersham Pharmacia Biotech. pET15b plasmid DNA was from Novagen. Oligonucleotides were from Integrated DNA Technologies. Bacteriophage φ29-7M and φ229-As6 ss(c)DNAs (20), as well as αX174 viral DNA, were prepared as described previously (21). PriA, PriB, PriC, DnaT, DnaC, and DnaG were purified as described (22). Subunits of the DNA polymerase III holoenzyme (Pol III HE) were purified as described (23, 27). DnaB activity. As an example, titrations comparing the activity of wild type, E32A, E32K, and Y105A mutant DnaBs is shown in Fig. 1. Multiple experiments demonstrated that the N-His tag had essentially no effect on DnaB activity. As an example, titrations comparing the activity of wild type DnaB and N-His DnaB E32A (a mutant DnaB with activities indistinguishable from wild type) see “Results” in the rolling circle DNA replication assay are shown in Fig. 2.

**Construction of Mutated dnaB Alleles and Isolation of the Mutant Proteins—**The precise dnaB open reading frame was removed from pET3c-dnaB (22) by digestion with NdeI and BamHI and inserted into NdeI- and BamHI-digested pET15b to give pET15b-dnaB. This results in the addition of 20 amino acids onto the N terminus of DnaB. This tag includes a hexahistidine sequence and a thrombin cleavage site. Mutant alleles encoding the E32A, E32K, and Y105A amino acid substitutions in DnaB were engineered according to the Stratagene Quick Change technique as per the manufacturer’s instructions. Mutant alleles were completely sequenced before use.

For purification, BL21(DE3)pLysS (Novagen) carrying either a wild type or mutant pET21a-dnaB plasmid was grown in 12 liters of LB broth supplemented with 0.4% glucose and 0.5 mg/ml ampicillin to A600 = 0.4. Isopropyl-1-thio-β-D-galactopyranoside was then added to 0.4 mM, and the synthesis of the target protein was induced for 2 h. The cells were harvested and resuspended in 50 mM Tris-HCl (pH 8 at 4 °C) and 10% glycerol. DnaB was eluted from the column with a 10-column volume gradient of 10–300 mM imidazole-HCl (pH 8) in the same buffer. The fraction (0.5 ml) containing DnaB were pooled and dialyzed overnight against ATP agarose buffer (50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM PMSF, 50 mM NaCl, and 20% glycerol). The purified DnaB was then washed with two column volumes of 50 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM PMSF, and 10% sucrose. The column was loaded with a total volume of 50 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM PMSF, 10 mM imidazole-HCl (pH 8.0), and 10% glycerol. DnaB was eluted from the column with a 10-column volume gradient of 10–300 mM imidazole-HCl (pH 8) in the same buffer. Fractions (0.5 ml) containing DnaB were pooled and dialyzed overnight against ATP agarose buffer (50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM PMSF, 50 mM NaCl, and 20% glycerol). The purified DnaB was then washed with two column volumes of 50 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM PMSF, 10 mM imidazole-HCl (pH 8.0), and 10% glycerol. DnaB was eluted from the column with a 10-column volume gradient of 10–300 mM imidazole-HCl (pH 8) in the same buffer. Fractions (0.5 ml) containing DnaB were pooled and dialyzed overnight against ATP agarose buffer (50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM PMSF, 50 mM NaCl, and 20% glycerol). The purified DnaB was then washed with two column volumes of 50 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM PMSF, 10 mM imidazole-HCl (pH 8.0), and 10% glycerol. DnaB was eluted from the column with a 10-column volume gradient of 10–300 mM imidazole-HCl (pH 8) in the same buffer. Fractions (0.5 ml) containing DnaB were pooled and dialyzed overnight against ATP agarose buffer (50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM PMSF, 50 mM NaCl, and 20% glycerol). The purified DnaB was then washed with two column volumes of 50 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM PMSF, 10 mM imidazole-HCl (pH 8.0), and 10% glycerol. DnaB was eluted from the column with a 10-column volume gradient of 10–300 mM imidazole-HCl (pH 8) in the same buffer. Fractions (0.5 ml) containing DnaB were pooled and dialyzed overnight against ATP agarose buffer (50 mM Tris-HCl (pH 7.5 at 4 °C), 1 mM EDTA, 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM PMSF, 50 mM NaCl, and 20% glycerol). The purified DnaB was then washed with two column volumes of 50 mM Tris-HCl (pH 7.5 at 4 °C), 50 mM NaCl, 1 mM PMSF, 10 mM imidazole-HCl (pH 8.0), and 10% glycerol).

**RESULTS**

**DnaG Binding Pocket on DnaB**

The protein provide the DNA unwinding necessary for replication fork propagation, it also serves to attract DnaG to the replication fork via a protein-protein interaction. In addition, another protein-protein interaction between DnaB and the τ subunit of the DNA Pol III HE literally cements the replisome together, stimulating the helicase activity of DnaB (28) and

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2 M. Olson, J. Carter, H. G. Dallmann, and C. S. McHenry, personal communication.

**FIG. 1. SDS-PAGE analysis of the wild type and mutant DnaB proteins.** 1 μg of the wild type (lane 1), E32A (lane 2), E32K (lane 3), and Y105A (lane 4) DnaB proteins was analyzed by SDS-PAGE through a 10% gel. The gel was stained with Coomassie Brilliant Blue, and the image was recorded using a Bio-Rad Gel Doc imaging system. The faint bands present in all lanes represent proteolytic products corresponding to fragments 1 and 2 (26).
defining which of the two polymerase cores in the holoenzyme becomes the leading-strand polymerase (29, 30). Thus, understanding replication fork function requires observation of the effects of disrupting these interactions. To define the regions on DnaB that are involved in these important protein-protein interactions, we have subjected \textit{dnaB} to alanine scanning and charge reversal mutagenesis. The mutated proteins are expressed and purified by a combination of nickel-nitrilotriacetic acid-agarose and ATP-agarose affinity chromatography, and the initial screening of their biochemical phenotype was performed using rolling circle DNA replication.

Rolling circle DNA replication is established on a tailed form II DNA template by the addition of the replication restart primosomal proteins (including the mutant DnaB under consideration), SSB, and the DNA Pol III HE. We use this system as the initial screen because, as we have documented previously (14, 20), it accurately mimics the behavior of the cellular DNA replication fork. Moreover, the products of rolling circle DNA replication are cleanly resolved by alkaline agarose gel electrophoresis into a large leading-strand population that barely enters the gel and a population of Okazaki fragments that is typically centered about 1.5–2.5 kilobases in length. Thus, mutant DnaBs affected in the functions described above can therefore easily be identified as a result of the predicted effect on the products of the reaction.

When incorporated into the replisome, DnaB proteins that have become modified in their ability to interact with DnaG should exhibit, at identical concentrations of primase, a population of Okazaki fragments of altered size compared with those made at replication forks reconstituted with the wild-type protein. This is because, as described above, Okazaki fragment size is controlled by the cycle of DnaG binding to and dissociating from DnaB at the replication fork. Thus, any change in the affinity of this interaction will result in a change in the average size of the population of Okazaki fragments synthesized.

We have identified two single amino acid substitutions in DnaB that fulfill these predictions. These mutant DnaB proteins were culled from a set of mutant proteins engineered by substituting charged amino acid residues that were conserved among DnaB proteins on the assumption that these residues were more likely to reside on the surface of the protein, and thus altering them might affect protein-protein interactions.

When wild type DnaB was used to reconstitute rolling circle replication, Okazaki fragment size reached its minimum as a function of DnaG concentration between 100 and 200 nM (Fig. 3). In fact, the average size of Okazaki fragments synthesized...
sized at these two concentrations was nearly identical. Reduction of the DnaG concentration below 100 nM resulted in a large increase in Okazaki fragment size, such that at 25 nM, it was not possible to determine the average size of the fragments because the population of lagging-strand products had merged with the population of leading-strand products.

Replication forks reconstituted with DnaB E32A produced Okazaki fragment populations that were identical to those made in the presence of the wild type protein (Fig. 3); however, those containing DnaB E32K consistently produced Okazaki fragments that were about 3-fold longer than those synthesized by replication forks containing either the wild type or E32A DnaB (Fig. 3). Okazaki fragments produced by replication forks containing DnaB Y105A were even longer (Fig. 3). Note that in Fig. 3, the lowest concentration of DnaG in the titration of DnaB Y105A is nearly 90% greater than the highest value in the titration for either the wild type, E32A, or E32K DnaBs. And even at 3 μM DnaG, Okazaki fragments synthesized by replication forks containing DnaB Y105A are still larger than those synthesized by replication forks containing the wild type protein with DnaG at 100 nM. A conservative estimate is that at equivalent concentrations of DnaG, the Okazaki fragments synthesized by replication forks containing DnaB Y105A are at least 15-fold larger than those synthesized by replication forks containing the wild type DnaB.

Given that DnaB is also the replication fork DNA helicase, the observed variation in Okazaki fragment size as a function of the DnaB present at the fork could arise for one of two reasons. It could be, as described above, that the mutations actually affected the affinity of the protein-protein interaction between DnaB and DnaG. On the other hand, it could also be that the mutations affected the rate of replication fork progression. The size of an Okazaki fragment is essentially the distance on the lagging-strand template between two successful DnaG-primed initiation events by the lagging-strand polymerase. Because in the rolling circle system the nascent leading strand is the lagging-strand template, Okazaki fragment size can also be made to vary at a fixed concentration of DnaG by altering the rate at which the lagging-strand template is generated, i.e. by altering the rate of DnaB-catalyzed unwinding at the replication fork. Although we considered this explanation unlikely because in this scenario DnaB Y105A would have to have at least a 15-fold greater rate of DNA unwinding at the replication fork than the wild-type protein, we compared the rate of replication fork progression for the wild type and mutant proteins directly.

The rate of replication fork progression sustained by replicosomes containing either the wild type or mutant DnaBs was assessed by sampling rolling circle replication reactions in 10-s intervals from the start of the incubation and analyzing the products by alkaline agarose gel electrophoresis (Fig. 4). The change in the length of the longest leading-strand present is a direct measure of the rate of replication fork movement. As evident in Fig. 4, the size of the nascent leading-strand was identical at each time point for the wild type and three mutant DnaBs. We thus conclude that the E32A, E32K, and Y105A amino acid substitutions have not affected, in any gross manner, the ability of that particular DnaB to act as the replication fork DNA helicase. Thus, the variation in Okazaki fragment size observed with replication forks containing the mutant DnaB proteins is very likely the result of an alteration of the affinity of the interaction between the mutated DnaB and DnaG.

The Mutant DnaB Proteins Behave Differently in Single-stranded DNA Priming Systems than They Do at the Replication Fork—The results described above suggested that the interaction between DnaG and DnaB Y105A was more severely altered than the interaction between DnaG and DnaB E32K. If this were the case, it should also hold true in the general priming reaction where only DnaB and DnaG are present with the HE. In this reaction, DnaB binds to the protein-free ss(c)DNA and then serves to attract DnaG to synthesize a primer that is then elongated by the HE. Alterations in the affinity of the interaction between DnaB and DnaG can therefore be directly read out from the dose response curve of DnaG concentration.

Surprisingly, both the E32K and Y105A DnaBs behaved identically in the general priming reaction (Fig. 5). At subsaturating levels of primase, about 2–2.5-fold higher concentrations of DnaG were required to support an equivalent amount of nucleotide incorporation as wild type DnaB when these two mutant proteins were present in the assay. As expected, DnaB E32A did not exhibit any defect in this assay; if anything, it might have been somewhat more active than the wild type.

The general priming data would have predicted that both DnaB E32K and DnaB Y105A would show similar defects at replication forks. However, although at the same concentration of DnaG the lagging-strand products formed in the presence of either mutant protein are clearly larger than those formed in the presence of the wild type, the Okazaki fragments formed by the DnaB Y105A forks are much larger than those formed by the DnaB E32K forks. We considered that this apparent difference might be because there are probably more proteins present on the DNA at replication forks formed in the rolling circle system, which utilizes all the restart primosomal proteins, than in the general priming system, which utilizes only DnaB and DnaG. We therefore compared the activity of the mutant protein during synthesis of the complementary strand of 4X174 ss(c)DNA.

In this assay, SSB-coated 4X viral DNA is converted to the replicative form by the formation of a restart primosome at the primosome assembly site. The primosome catalyzes primer

**Fig. 4.** The E32K and Y105A amino acid substitutions in DnaB do not affect the rate of replication fork progression. Standard rolling circle replication reactions containing the indicated DnaB proteins were incubated at 30 °C. Aliquots (2 μl) were withdrawn at the indicated times from the start of the incubation, and the reactions were quenched by rapid mixing with 50 mM EDTA (10 μl). DNA products were analyzed by alkaline agarose gel electrophoresis as described under "Materials and Methods." WT, wild type; kb, kilobases.
synthesis, and the primer is elongated by the HE to form the complementary strand. Once again, both the E32K and Y105A DnaBs required higher concentrations of primase to sustain the same level of nucleotide incorporation as the wild-type protein (Fig. 6). In this case, the defect exhibited by DnaB Y105A was somewhat greater than that exhibited by DnaB E32K. Thus, it was possible that the presence of other primosomal proteins at the replication fork might alter the interaction between DnaB and DnaG and exacerbate the effect of the Y105A amino acid substitution. This predicts that the E32K and Y105A DnaBs should behave identically at replication forks reconstituted in the presence of only DnaB, DnaC, and DnaG.

The E32K and Y105A DnaBs Maintain Their Differential Defects in Replication Forks Formed Only with DnaB and DnaG—Typically, we use all the restart primosomal proteins to form replication forks in the rolling circle system. This is because loading of DnaB to DNA by DnaC is relatively inefficient. Auxiliary proteins are required to maximize the process. At oriC, this is accomplished by DnaA, which has been shown to interact with DnaB (31). Effectively, the combination of PriA, PriB, DnaT, and possibly PriC (32, 33) act as the equivalent of DnaA at the primosome assembly site on ϕX viral DNA and at recombination intermediates (8). However, replication forks can be formed in the rolling circle system in the absence of PriA, PriB, PriC, and DnaT if the concentration of DnaB and DnaC is increased 15–20-fold (20). In addition, the reaction has to be staged somewhat differently because DnaC cannot load DnaB to SSB-coated DNA. Thus, DnaB and DnaC are exposed to the TFII template first for a short period of time, and then SSB is added.

Interestingly, the dramatic difference between the Y105A and E32K DnaBs was maintained at replication forks formed in the absence of PriA, PriB, PriC, and DnaT (Fig. 7). Replication forks formed in the presence of the E32K protein consistently gave Okazaki fragments that were, at equivalent concentrations of DnaG, about 2–3-fold longer than those synthesized at replication forks formed in the presence of DnaB E32A (which is essentially identical to wild type). On the other hand, at the DnaG concentrations shown, Okazaki fragments produced by replication forks formed in the presence of DnaB Y105A were very long and could barely be distinguished from the leading-strand DNA.

To prove that Okazaki fragments were, in fact, being made at replication forks formed in the presence of DnaB Y105A, the ability of a restriction enzyme to digest the DNA products formed was examined. BamHI will only digest the rolling circle DNA product if both leading- and lagging-strand DNA had been synthesized, producing a duplex DNA tail. This was the case when DNA made by replication forks containing wild-type DnaB was treated with BamHI (Fig. 8, lanes 1 and 2). In the absence of primase, so no Okazaki fragments could be synthesized, DNA made by replication forks containing DnaB Y105A became progressively more sensitive to BamHI digestion, being essentially completely digested at 200 nM primase (Fig. 8, lanes 3 and 4). As the concentration of primase was increased, DNA made by replication forks containing DnaB Y105A became progressively more resistant to BamHI treatment (Fig. 8, lanes 3 and 4). Thus, it is clear that even though they could not be distinguished from leading-strand DNA under these conditions, Okazaki fragments were being made.

These data therefore suggest that either the architecture of the replication fork itself or some interaction between DnaB and a polymerase subunit restricts or modifies access to the DnaG binding pocket on DnaB that is defined by the E32A and Y105A amino acid substitutions.

**DISCUSSION**

The interaction between DnaB and DnaG is of crucial importance to the replisome. These two proteins form the core of the replicative primosome, providing both the DNA unwinding function, via the 5'→3' DNA helicase activity of DnaB, and the Okazaki fragment priming function, via the oligoribonucleotide synthetase activity of DnaG, necessary for proper replication fork propagation.

In E. coli, the DnaB-DnaG interaction is transient (1). At first glance, this seems to create an inefficiency at the replication fork. Because Okazaki fragments are an average of about 2 kilobases in length and the speed of replication fork propagation is nearly 1000 nucleotides/s, a new primer for lagging-
strand DNA synthesis must be manufactured at least once every 2 s. Thus, it would seem reasonable to expect that the primase would remain permanently associated with the replication fork, waiting to synthesize a new primer as soon as it was needed. However, this is not the case. At the *E. coli* replication fork, DnaG acts distributively with respect to a cycle of Okazaki fragment synthesis (14). That is, a molecule of DnaG associates with the replication fork via a protein-protein interaction with DnaB (17), synthesizes a primer and then leaves the fork to be replaced by a different molecule of DnaG that will synthesize the next primer.

The cyclical association of DnaG with the replication fork proved to be a regulatory feature governing the size of Okazaki fragments (15, 18). This is because the size of an Okazaki fragment synthesis reactions containing either the wild type (WT) or mutant DnaB proteins and the indicated concentrations of DnaG were incubated and analyzed as described under “Materials and Methods”. The right-hand panel is an exploded view of the left-hand panel. □, wild type DnaB; ○, DnaB E32A; ◦, DnaB E32K; △, DnaB Y105A.

**FIG. 6.** Activity of the wild type and mutant DnaB proteins in φX174 complementary strand synthesis. Standard φX174 complementary strand synthesis reactions containing either the wild type (WT) or mutant DnaB proteins and the indicated concentrations of DnaG were incubated and analyzed as described under “Materials and Methods”. The right-hand panel is an exploded view of the left-hand panel. □, wild type DnaB; ○, DnaB E32A; ◦, DnaB E32K; △, DnaB Y105A.

**FIG. 7.** The E32K and Y105A mutant DnaB proteins maintain their differential defects at replication forks formed only with DnaB and DnaG. Rolling circle replication reactions containing the TFII, SSB, the Pol III HE, DnaC, the indicated DnaB, and varying concentrations of DnaG (increasing 2-fold from left to right) were incubated, processed, and analyzed as described under “Materials and Methods.” kb, kilobases.
fragment is determined by the distance between two successful initiation events by the lagging-strand polymerase on the lagging-strand template and the frequency of primer synthesis is governed by the cycle of association/dissociation of DnaG with DnaB (18). Thus, a complete understanding of replisome function requires a thorough understanding of the dynamics of this protein-protein interaction.

We have previously reported our determination that the C-terminal 16 amino acids of DnaG were crucial for the functional interaction between DnaG and DnaB at the replication fork (17). We demonstrated that single amino acid substitutions in this region affected the period of the Okazaki fragment clock, leading to the synthesis of Okazaki fragments of altered size compared with those directed by the wild-type enzyme. Here we have used directed single amino acid substitutions to localize the reciprocal region of DnaB.

Two mutant DnaB proteins were described that exhibited, when incorporated into replication forks, an alteration in the size of Okazaki fragments synthesized, and protection of DnaC from inactivation by NEM, suggested that fragment 3 was also the site of binding to both DnaG and DnaC.

Previous studies have addressed assignment of the various activities of DnaB to particular regions of the protein. Nakayama et al. (36) demonstrated, using partial proteolysis, that DnaB was composed of a N-terminal domain of about 12 kDa, named fragment 3, corresponding roughly to amino acid residues 14–136, a C-terminal domain of about 33 kDa, named fragment 2, corresponding roughly to amino acid residues 172–470, and a linker region between these two domains. Electron microscopic examination of the structure of DnaB confirm that a single protomer of the DnaB hexamer appears with two globular domains, one large and one small, connected by a hinge (37, 38).

Fragment 2 appears to provide the primary hexamerization contacts, with fragment 3 providing additional stabilization via dimer contacts. Indeed, as described above, the crystal structure of what is essentially fragment 3 is that of a dimer (Fig. 9).

Fragment 2 is responsible for DNA binding and ATPase activity, whereas both fragments 2 and 3 are required for helicase activity (39, 40). Based on the relative activity of the DnaB fragments in general priming, dX174 complementary strand synthesis, and protection of DnaC from inactivation by NEM, the initial structure-function studies of Nakayama et al. (36) suggested that fragment 3 was also the site of binding to both DnaG and DnaC.

Previous studies have yielded some information on the region of DnaB involved in the interaction with DnaG. In an investigation of the role of the linker region, Stordal and Maurer (41) found that purified Salmonella typhimurium mutant DnaB proteins carrying the I135N, I141T, and L156P amino acid substitutions were all defective in the general priming reaction. Unfortunately, none of these amino acid residues are present in the fragment 3 crystal structure of Fass et al. (19), so their proximity to the region defined in this report cannot be assessed. However, given that hinge regions are, by definition, very flexible, it is certainly possible that these amino acid residues are involved in determining the DnaG-binding pocket as well.

Lu et al. (35) assessed the ability of in vitro translated, truncated derivatives of DnaB to be retained by an N-terminal glutathione S-transferase-DnaG chimera bound to a glutathione affinity resin and concluded that the region between amino acid residues 211 and 256, which falls in fragment 2, was important for binding of DnaG. Based on these data, these authors constructed three double mutants, D212A/D213A, K216A/K217A, and D253A/K254A, and assayed their ability to interact with DnaB by enzyme-linked immunosorbent assay. Only the former two mutant DnaB proteins exhibited a decreased ability to sustain primer synthesis on M13 ss(c)DNA in the presence of DnaG.

Given the lack of a crystal structure of the entire DnaB molecule, it is, of course, difficult to determine whether the region encompassing amino acid residues 212–217 is anywhere
The two mutant DnaB proteins. This is because the differential defect was maintained in replication forks reconstituted in the presence of only the mutant DnaB, DnaC, and DnaG. These observations suggest that, at the replication fork, access to the DnaG binding pocket on DnaB that includes Glu^{32} and Tyr^{105} is restricted either by interaction with either SSB or by one of the subunits of the polymerase or that a protein-protein interaction between either SSB or a polymerase subunit and DnaB alters the affinity of the interaction between DnaB and DnaG.

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Pearl Chang and Kenneth J. Marians

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