DNA Structure Specificity Conferred on a Replicative Helicase by Its Loader*

Received for publication, October 3, 2009, and in revised form, October 29, 2009. Published, JBC Papers in Press, October 30, 2009, DOI 10.1074/jbc.M109.072520

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Prokaryotic and eukaryotic replicative helicases can translocate along single-stranded and double-stranded DNA, with the central cavity of these multimeric ring helicases being able to accommodate both forms of DNA. Translocation by such helicases along single-stranded DNA results in the unwinding of forked DNA by steric exclusion and appears critical in unwinding of parental strands at the replication fork, whereas translocation over double-stranded DNA has no well-defined role. We have found that the accessory factor, DnaC, that promotes loading of the Escherichia coli replicative helicase DnaB onto single-stranded DNA may also act to confer DNA structure specificity on DnaB helicase. When present in excess, DnaC inhibits DnaB translocation over double-stranded DNA but not over single-stranded DNA. Inhibition of DnaB translocation over double-stranded DNA requires the ATP-bound form of DnaC, and this inhibition is relieved during translocation over single-stranded DNA indicating that stimulation of DnaC ATPase is responsible for this DNA structure specificity. These findings demonstrate that DnaC may provide the DNA structure specificity lacking in DnaB, limiting DnaB translocation to bona fide replication forks. The ability of other replicative helicases to translocate along single-stranded and double-stranded DNA raises the possibility that analogous regulatory mechanisms exist in other organisms.

A central feature of DNA replication is separation of the two strands of a double helix, allowing each parent strand to act as a template to direct synthesis of a new complementary strand. This separation is achieved by DNA helicases, enzymes that couple the energy derived from the hydrolysis of nucleoside triphosphates to translocation along the DNA and associated disruption of hydrogen bonding between the parental DNA strands (1). Helicases required for strand separation during replication are generally multimeric ring proteins with DnaB being the most well-studied member of this class of enzymes (2). This toroidal structure might be expected to catalyze unwinding at a replication fork (4). Unwinding is effected by a steric exclusion mechanism in which the strand not initially bound within the DnaB ring is excluded from the ring (6, 7). However, steric exclusion is promoted by the structure of the DNA along which DnaB translocates and is not an inherent property of DnaB itself (6). DnaB can accommodate two and even three DNA strands within its hexameric ring even though it can initially load only onto ssDNA (8, 9) (see also Fig. 1Cii). In such instances, catalysis by DnaB results in translocation only, allowing the helicase ring to slide over double-stranded DNA (dsDNA) without significant disruption of base pairing (10).

Generation of ssDNA for binding of DnaB is effected by DnaA at the chromosomal origin of replication oriC (11–13) and by PriA or PriC plus Rep at recombination intermediates and stalled replication forks (14–16). At oriC, translocation of DnaB upon loading onto ssDNA leads to each hexamer encountering forked DNA structures at opposite ends of oriC (17). The forked nature of the DNA leads to the strand not passing through the DnaB ring being excluded from the ring, the outcome being unwinding of the two parental DNA strands.

DnaB can load onto ssDNA without the aid of other proteins in vitro (4), but this process is promoted by DnaC in vitro resulting in stimulation of DnaB helicase activity (18). Indeed, promotion by DnaC is essential for DnaA-directed loading of DnaB at oriC (19–21). DnaC is a monomer in solution but associates with DnaB in a 1:1 stoichiometry, forming primarily a DnaB6-DnaC6 complex (21–24). How DnaC acts to promote binding of DnaB onto ssDNA is still not understood but DnaC can bind both ssDNA and adenine nucleotides (25–29) and, in the presence of both DnaB and ssDNA, DnaC can hydrolyze ATP (30). However, the ATP-bound form of DnaC is not essential for promotion of DnaB loading onto ssDNA as both ADP-bound and nucleotide-free DnaC also promote DnaB helicase activity (30). DnaC ATP is, though, essential for DnaC-directed loading of DnaB at oriC possibly via DnaC ATP-dependent expansion of the ssDNA generated by DnaA at oriC (30). However, DnaC interacts with DnaA, an interaction that depends upon both
proteins being in an ATP-bound state (29). The essential requirement for DnaC-ATP at oriC might reflect therefore the need for a DnaA-ATP, DnaC-ATP interaction rather than expansion of the ssDNA bubble at oriC. However, DnaC-ATP also inhibits DnaB helicase activity (18), relief of which requires hydrolysis of ATP by DnaC, stimulated by the presence of both DnaB and ssDNA (30). Neither DnaC-ADP nor nucleotide-free DnaC inhibits DnaB helicase function (30), possibly caused by enhanced dissociation of DnaC from the DnaB-ssDNA complex (22, 23, 25). However, the intrinsic affinity of DnaC-ADP for DnaB is similar to that of DnaC-ATP (24, 28). Any enhancement of dissociation of DnaC-ADP from DnaB may therefore be driven by the decreased affinity for ssDNA displayed by DnaC-ADP as compared with DnaC-ATP (28). Regardless of the exact mechanism of DnaC-dependent loading of DnaB, the end result of DnaC function is facilitated assembly of active DnaB onto ssDNA.

The above biochemical views of DnaC imply that DnaC acts only to load DnaB onto ssDNA and subsequently plays no part in replisome function. Indeed, if the concentration of DnaC exceeds that of DnaB in vitro then the helicase function of DnaB is inhibited, as is the DnaA-directed replication of oriC-containing plasmids (18, 31). A 10-fold increase in DnaC concentration in vivo also results in a slowing of chromosomal duplication (32). DnaC and DnaB might therefore be in a dynamic equilibrium in which the continual association of DnaC-ATP with DnaB inhibits DnaB helicase, while the DnaB- and ssDNA-stimulated hydrolysis of ATP by DnaC results in dissociation of DnaC from DnaB (25, 30). Excess DnaC-ATP would lead to an increased rate of association with, and inhibition of, DnaB.

We have analyzed the interplay between DnaC and DnaB on a range of DNA substrates and have discovered that the inhibition of DnaB helicase by excess DnaC is dependent on the structure of the DNA substrate. We find that in the presence of ATP excess DnaC is a potent inhibitor of DnaB translocation over dsDNA but translocation of DnaB over ssDNA is not inhibited. This differential inhibition is dependent on the nucleotide-bound state of DnaC. DnaC-ADP or nucleotide-free DnaC do not inhibit DnaB regardless of the DNA structure. In contrast, DnaC bound to the non-hydrolyzable ATP analog ATPγS inhibits DnaB translocation over both ssDNA and dsDNA. Given that ssDNA but not dsDNA stimulates ATP hydrolysis by DnaC (30), our data indicate that continual association of DnaC-ATP with DnaB is not inhibitory to DnaB helicase as long as ssDNA is available for stimulation of DnaC ATPase. Consequently DnaC may act as a DNA structure-selective modulator of DnaB helicase, promoting translocation of DnaB along ssDNA but inhibiting translocation along dsDNA. DnaC might therefore limit DnaB helicase activity, and thus replisome function, to forked DNA structures.

MATERIALS AND METHODS

DNAs and Proteins—DnaB and DnaC were purified as described (33). Streptavidin was purchased from Sigma. All protein concentrations refer to the monomer.

DNA substrates (Table 1) were constructed using oligonucleotides, one of which in each structure was labeled with [γ-32P]ATP at the 5′-end and purified by gel electrophoresis (34). Sequences of the oligonucleotides are indicated in supplemental Table S1. Oligonucleotides were obtained from Eurogentec. All DNA concentrations refer to the concentration of the DNA structure rather than of nucleotide equivalents.

Helicase Assays—Assays on substrates 1–7 were performed at 30 °C with 1 nM DNA substrate using reaction volumes of 10 μl in 40 mM HEPES-HCl (pH 8.0), 10 mM magnesium acetate, 10 mM dithiothreitol, 2 mM ATP (except as indicated otherwise in the experiments in Fig. 4). 0.1 mg/ml bovine serum albumin. CTP, ADP, and ATPγS were also included where indicated at the specified concentrations. Reactions were preincubated at 30 °C for 2 min prior to addition of DnaB and DnaC to the indicated final concentrations. Where indicated, streptavidin was included during this 2-min incubation. When DnaB and DnaC were both present, the two proteins were mixed on ice prior to addition to the reaction. Reactions were incubated for 4 min at 30 °C then were terminated by addition of 2.5 μl of stop buffer (100 mM Tris-HCl, pH 7.5, 5% SDS, 200 mM EDTA, and 10 mg ml−1 proteinase K) followed by 2.5 μl of 0.25% bromphenol blue in 30% glycerol. Rates of dissociation of substrates 1–6 were performed as above, except that the reaction volume was increased to 70 μl to allow removal of 10 μl aliquots and addition of stop buffer, at the stated intervals. Dissociation of the Holliday junction substrates (8–11) was performed as above except that reactions were performed at 37 °C for 16 min, and addition of stop buffer was followed by incubation at 37 °C for 10 min. The stop buffer also contained 25 μM of unlabeled oligonucleotide 12 for substrates 10 and 11 to inhibit the reannealing of labeled oligonucleotide 12 with other products of unwinding.

The amount of DNA structure dissociation was analyzed by electrophoresis through 10% polyacrylamide/TBE gels for 90 min at 190 V on 16-cm gels. Quantification was performed using a phosphorimager. All assays were performed 2–4 times, and the means of data were used for graphical presentation.

RESULTS

Effects of DnaC on DnaB Helicase Are Dependent on the Structure of the DNA Substrate—The ATP- but not the ADP-bound form of DnaC can inhibit DnaB helicase, whereas single-stranded and not double-stranded DNA stimulates DnaC to hydrolyze bound ATP (18, 30). DnaB can translocate along both single-stranded and double-stranded DNA (6), suggesting that stimulation of ATP hydrolysis by DnaC within a DnaC-DnaB-DNA complex may depend upon the nature of the DNA along which DnaB is translocating. To test this idea, we designed substrates to analyze movement of DnaB along both single-stranded and double-stranded DNA. These substrates exploited previous work demonstrating that DnaB can load onto ssDNA and subsequently translocate over dsDNA (6, 10). Substrate 1 was a forked DNA substrate possessing a 38-base 3′ arm and a 67-base 5′ arm (Table 1). This substrate could be unwound by DnaB, a 5′-3′ translocase (4), to generate single-stranded DNA products (Fig. 1A, lane 2). Thus, DnaB could load onto the 67-base 5′ single-stranded DNA arm and translocate in a 5′ to 3′ direction to effect unwinding of the fork (Fig. 1, C). Placement of a 28-base pair duplex DNA region in the path of the translocating DnaB did not prevent unwinding of
the fork (Fig. 1B, lane 2) but the 28-base pair duplex region was not unwound (supplemental Fig. S1, B–D). We conclude that on substrate 2, DnaB bound the single-stranded 5' arm and translocated subsequently over the duplex DNA (Fig. 1Cii) consistent with previous work (6).

Displacement of the labeled oligonucleotide from substrates 1 and 2 therefore monitored the movement of DnaB along the single-stranded and double-stranded regions engineered to be present on the 5’ arm of each substrate. To determine the effect of DnaC on DnaB translocation along double-stranded and single-stranded DNA, the effect of increasing concentrations of DnaC on unwinding of substrates 1 and 2 by DnaB was monitored. Addition of DnaC up to a ratio of DnaC:B::1:1 stimulated displacement of the labeled oligonucleotide by DnaB in both substrates (Fig. 1, A and B, lanes 1–4). When the ratio of DnaC to DnaB was increased to 2:1 or more, unwinding of the labeled oligonucleotide by DnaB in substrate 1 was still stimulated by the presence of excess DnaC (Fig. 1A, lanes 5–7 and Fig. 2D).

However, stimulation of unwinding of the labeled oligonucleotide in substrate 2 was abolished when the ratio of DnaC:B exceeded 1:1 (Fig. 1, B, lanes 5–7 and D). Monitoring of the rates of unwinding of substrates 1 and 2 by DnaB at increasing DnaC:B ratios confirmed that excess DnaC had little effect on the stimulation of DnaB on substrate 1 but actually inhibited DnaB on substrate 2 (Fig. 2, A and B). Stimulation of DnaB helicase by DnaC depended therefore on the structure of the DNA substrate.

DnaC Inhibits Movement of DnaB along dsDNA but Not ssDNA—The effect of addition of DnaC on DnaB helicase activity differed markedly on DNA substrates 1 and 2. The duplex region present on the 5’ arm of substrate 2, but absent in substrate 1, suggested that the requirement for DnaB to translocate over dsDNA in substrate 2 to catalyze unwinding of the labeled oligonucleotide may have been responsible for this difference. However, the two substrates also differed in the amount of ssDNA available for binding of DnaB to effect unwinding. Substrate 1 had 67 bases whereas substrate 2 had 39 bases (Table 1). To establish if the size of the ssDNA target for DnaB binding

### TABLE 1

| Substrate structure | Substrate name | Oligonucleotides |
|---------------------|----------------|------------------|
| ![structure1](image1.png) | 1 | 1, 2 |
| ![structure2](image2.png) | 2 | 1, 2, 3 |
| ![structure3](image3.png) | 3 | 2, 3 |
| ![structure4](image4.png) | 4 | 1, 2, 4 |
| ![structure5](image5.png) | 5 | 1, 5 |
| ![structure6](image6.png) | 6 | 1, 2, 6 |
| ![structure7](image7.png) | 7 | 2, 6 |
| ![structure8](image8.png) | 8 | 7, 8, 9, 10 |
| ![structure9](image9.png) | 9 | 7, 8, 11 |
| ![structure10](image10.png) | 10 | 8, 9, 10, 12 |
| ![structure11](image11.png) | 11 | 8, 9, 11, 12 |

**FIGURE 1.** Stimulation of DnaB by excess DnaC depends on the structure of the DNA along which DnaB is translocating. Substrates 1 and 2 (A and B, respectively) were incubated with 150 nM DnaB and 0, 75, 150, 300, 600, or 1200 nM DnaC (lanes 2–7) for 4 min at 30 °C. Lane 1 contained neither DnaB nor C whereas lane 8 contained 1200 nM DnaC only. Shaded circles represent positions of the 5’ 32P label. C, models of DnaB translocation along substrates 1 and 2. D, quantification of the extent of unwinding by DnaB of substrates 1 and 2 at increasing DnaC:B ratios. Error bars represent S.D.
was responsible for the differential inhibition of DnaB by excess DnaC, a third substrate was analyzed, substrate 5 (Table 1) that had the same ssDNA binding site size as substrate 2 but lacked a duplex region over which DnaB had to translocate to unwind the labeled oligonucleotide. Analysis of the rates of unwinding of substrate 5 by DnaB in the presence of increasing concentrations of DnaC demonstrated that stimulation was observed at all concentrations of DnaC tested (Fig. 2C). This stimulation was observed on substrate 5 even in the presence of an 8-fold excess of DnaC over DnaB (Fig. 2C). Differences in the ssDNA binding site size for DnaB in substrates 1 and 2 could not account therefore for the differential effects of excess DnaC.

If the requirement for DnaB to translocate over the dsDNA in substrate 2 did account for the inhibition by excess DnaC, then prevention of DnaB from sliding over this duplex should prevent inhibition by excess DnaC. Addition of a 3′ ssDNA arm to an unbranched duplex region inhibits DnaB translocation over the duplex by steric exclusion of the 3′ arm from the central cavity of the DnaB hexamer (6). The dsDNA in substrate 2 was converted therefore into a forked duplex (substrate 4). Analysis of the effects of DnaC on DnaB-catalyzed unwinding of substrate 4 revealed that unwinding by DnaB was stimulated at all concentrations of DnaC tested, in contrast to substrate 2 (Fig. 2, compare B and D). Thus, the inhibition of DnaB-catalyzed unwinding of substrate 2 by excess DnaC could be relieved by replacement of the unbranched duplex region with a forked duplex.

These data are consistent with a model in which inhibition of DnaB by excess DnaC occurs only when DnaB translocates over dsDNA, as in the case of substrate 2. However, the ssDNA-dsDNA junction present in substrate 2, and encountered first by translocating DnaB, lacked a 3′ ssDNA arm. Lack of this feature might therefore have impacted directly on the interaction of DnaC with DnaB, possibly via interaction between DnaC and the 3′ ssDNA arm, rather than exerting an effect indirectly by preventing DnaB translocation over dsDNA. To test this possibility we employed a DNA structure identical to substrate 2 except for the presence of a deoxyribose-biotin moiety at the 3′ terminus of the ssDNA-dsDNA junction (substrate 6 in Fig. 3 and Table 1). Although hexameric ring helicases such as DnaB unwind forked DNA structures by steric exclusion of the 3′ ssDNA arm, the presence of streptavidin bound to a biotinylated 3′ DNA end also results in exclusion of the DNA end from the central cavity of the helicase and unwinding by steric exclusion (35, 36). As expected, this 3′ terminal biotin moiety...
allowed unwinding of the partial duplex by DnaB in the presence but not the absence of streptavidin (supplemental Fig. S2). We next compared the effects of DnaC upon unwinding of substrate 6 by DnaB. In the absence of streptavidin, unwinding of substrate 6 by DnaB was stimulated by an equimolar concentration of DnaC, but this stimulation was reduced in the presence of excess DnaC (Fig. 3A). This pattern was similar to the effects of DnaC upon unwinding of substrate 2 by DnaB although the negative impact of excess DnaC was reduced on substrate 6 (compare Fig. 3, A and C). In contrast, addition of streptavidin resulted in similar levels of stimulation of DnaB-catalyzed unwinding of substrate 6 by both an equimolar concentration and an 8-fold excess of DnaC (Fig. 3B). Thus, in the presence of streptavidin, the effects of DnaC on unwinding of substrate 6 by DnaB reflected the patterns seen previously with substrates 1, 4, and 5 (compare Figs. 2, A, C, and D and 3B). This marked difference in the effect of streptavidin on unwinding of substrate 6 was not seen on substrate 2 (Fig. 3, A and D) demonstrating that maintenance of stimulation of DnaB by excess DnaC was a specific consequence of the streptavidin-biotin interaction. These data demonstrate that the inhibitory effects of excess DnaC on unwinding of substrate 2 by DnaB were not due specifically to the lack of a 3′ ssDNA arm at the ssDNA-dsDNA junction. The data in Figs. 1–3 demonstrate that inhibition of DnaB by DnaC is dictated by the structure of the DNA substrate. They also demonstrate that the primary determinant of inhibition by DnaC is whether DnaB must translocate over dsDNA.

Note that the assays in Figs. 1 and 2 were performed at 30 °C, not 37 °C, to facilitate the measurement of rates of unwinding. Similar overall patterns of stimulation and inhibition of DnaB by DnaC were observed at 37 °C (data not shown) but initial rates of unwinding were too rapid to measure accurately. Note also that this current study employed 10 mM magnesium acetate and 2 mM ATP. However, reaction conditions employed previously to investigate DnaB translocation along dsDNA (8, 10) were also tested and similar patterns of stimulation and inhibition noted (data not shown).

The Differential Effects of DnaC on DnaB Require ATP—DnaB-catalyzed unwinding of an unbranched partial duplex DNA substrate is stimulated by equimolar concentrations of DnaC/ATP, DnaC/ADP, or nucleotide-free DnaC but only DnaC/ATP inhibits when in excess over DnaB (30). We tested therefore whether ATP binding and/or hydrolysis by DnaC played any role in conferring DNA structure specificity on DnaB helicase activity by comparing the effects of different nucleotide states of DnaC on unwinding of substrates 2 and 5 by DnaB. We exploited the ability of DnaB to hydrolyze CTP to promote DNA unwinding (30, 37, 38). In contrast, while the Kᵦ of ATP, ADP, and ATPγS for DnaC is 3–8 × 10⁻⁹ M, the affinity of CTP for DnaC is at least 3–4 orders of magnitude lower (27). Consequently, in the presence of high concentrations of CTP but low concentrations of or no adenine nucleotides, DnaB would be bound predominantly to CTP while DnaC would be bound to the adenine nucleotide or nucleotide-free (30).

In the presence of 2 mM CTP, stimulation of DnaB helicase was observed on both substrates 2 and 5 with an equimolar concentration of DnaC, reflecting the stimulation seen with 2 mM ATP (Fig. 4, A and B, compare columns 5 and 6 with 1 and 2). However, with 2 mM CTP, stimulation of DnaB-catalyzed unwinding of substrate 2 was maintained even when excess DnaC was present, in contrast to the inhibition seen with 2 mM ATP (Fig. 4A, compare columns 8 and 4). On substrate 5, stimulation of DnaB by DnaC occurred at all concentrations of DnaC tested in the presence of 2 mM CTP, reflecting the pattern seen with 2 mM ATP (Fig. 4B, compare columns 5–8 with 1–4). Given that, in the presence of 2 mM CTP but no adenine nucleotide, DnaC would be nucleotide-free (27, 30), these data indicate that the nucleotide-free state of DnaC did not inhibit unwinding of substrate 2 by DnaB and so did not confer DNA structure specificity on DnaB (Fig. 4, compare columns 5–8 in A with those in B). In contrast, when reactions contained both 2 mM CTP and 40 μM ATP DNA structure-specific inhibition of DnaB by DnaC was restored (Fig. 4, compare columns 9–12 in A with those in B). Under such conditions, DnaB would bind primarily to CTP and DnaC to ATP, confirming that the nucleotide-free and nucleotide-bound forms of DnaC had very different impacts on unwinding of substrate 2, but not 5, by DnaB.

We then tested whether it was the ATP- or ADP-bound form of DnaC that was responsible for the negative effects of DnaC on unwinding of substrate 2 versus 5 by DnaB. In 2 mM CTP and 40 μM ADP significant stimulation of DnaB-cata-
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lyzed unwinding of substrates 2 and 5 was observed at all tested concentrations of DnaC (Fig. 4, A and B, columns 13–16). Thus the ADP-bound state of DnaC did not inhibit unwinding of substrate 2, or 5, by DnaB. In contrast, with 2 mM CTP and 40 μM ATPyS (a non-hydrolyzable analogue of ATP), whereas there was significant unwinding of substrates 2 and 5 by DnaB in the absence of DnaC, unwinding was inhibited at all concentrations of DnaC tested (Fig. 4, A and B, columns 17–20). Thus hydrolysis of ATP was a prerequisite for stimulation of DnaB by any concentration of DnaC regardless of the structure of the DNA substrate.

These data demonstrate that the ADP-bound and nucleotide-free forms of DnaC stimulate DnaB helicase regardless of the structure of the DNA substrate. They also demonstrate that DnaCATP is inhibitory to DnaB, regardless of the structure of the DNA substrate unless the bound ATP can be hydrolyzed. The corollary of these observations is that, on substrate 5, inhibition by DnaCATP is likely alleviated by hydrolysis of the ATP bound to DnaC in a DNA structure-dependent manner. Note also that patterns of stimulation and inhibition observed on substrate 5 with the above five combinations of nucleotides were also seen with substrate 1 (data not shown).

Conferral of DNA Structure Specificity on DnaB Is a General Property of DnaC—The above data indicate that excess DnaC inhibits DnaB translocation along dsDNA but not ssDNA. The only proposed physiological role for translocation of DnaB along dsDNA is the branch migration of Holliday junction structures during recombination (10). We tested therefore whether the DNA structure specificity conferred on DnaB by DnaC was also seen on synthetic Holliday junction substrates.

On Holliday junctions containing a 5’ ssDNA extension, DnaB can bind and translocate along this ssDNA and subsequently continue translocation along the dsDNA of the Holliday junction to unwind the structure (10, Fig. 5, Di). In the absence of DnaC, unwinding of substrate 10 by DnaB generated the expected forked duplex product (Fig. 5A, product 2, lanes 1–6). Addition of an equimolar concentration of DnaC stimulated unwinding (Fig. 5A, lanes 7–12 and Ci). However, unwinding of this substrate was severely inhibited in the presence of excess DnaC (Fig. 5, A, lanes 13–18 and Ci). Because substrate 10 can only be unwound by binding of DnaB to the ssDNA arm and subsequent translocation over the dsDNA (10) (see also supplemental Fig. S3), these data indicate that excess DnaC inhibits such a reaction.

This stimulation and inhibition at different concentrations of DnaC on substrate 10 reflected the pattern seen with substrate 2 (Fig. 2B). Conversion of the ssDNA-dsDNA junction encountered by DnaB on substrate 2 to a forked DNA structure alleviated inhibition by excess DnaC (Fig. 2D). We tested therefore whether conversion of the ssDNA-dsDNA junction encountered by DnaB on substrate 10 into a forked structure also relieved inhibition by DnaC. On this Holliday junction (substrate 11) unwinding by DnaB in the absence of DnaC generated primarily a forked DNA product rather than the expected labeled ssDNA (Fig. 5B, lanes 1–6, product 1), implying that both ssDNA arms of substrate 11 could be accommodated within the central cavity of DnaB (Fig. 5Diiii). Note, though, that both ssDNA arms were (dT)30 precluding stable formation of duplex DNA between the two ssDNA arms. Addition of equimolar amounts of DnaC stimulated unwinding (Fig. 5Cii) but also resulted in the generation of labeled ssDNA as the main product instead of the forked DNA product (Fig. 5, B, lanes 7–12, product 3 versus 1 and Di). Thus DnaC inhibited the reaction shown in Fig. 5Diiii but stimulated that shown in Fig. 5Dii. With excess DnaC, DnaB-catalyzed unwinding of substrate 11 was still stimulated (Fig. 5, B, lanes 13–18 and Cii) in contrast to the inhibition seen on substrate 10. These differential effects of DnaC on DnaB helicase activity were caused by the ssDNA arms present in substrates 10 and 11 as no unwinding of a Holliday junction bearing either no ssDNA arms or only a 3’ ssDNA extension was detected regardless of the presence or absence of DnaC (supplemental Fig. S3). The effects of DnaC on unwinding of substrates 10 and 11 by DnaB reflected therefore the patterns observed previously with substrates 2 and 4 (Fig. 2, B and D).

DISCUSSION

The above data demonstrate that DnaC can confer DNA structure specificity on DnaB helicase. This structure specificity is related to the nature of the DNA along which DnaB is translocating, with inhibition seen only on substrates that require DnaB to translocate along dsDNA. Moreover, the differential effects of DnaC on DnaB helicase requires DnaCATP, indicating that nucleotide binding and hydrolysis by DnaC confers this DNA structure specificity.

How might the ATP-bound form of DnaC specifically prevent DnaB translocation over dsDNA? The inhibitory effect of DnaCATP• on DnaB helicase, shown previously to inhibit DnaB function (18), was observed regardless of the structure of the DNA substrate (Fig. 4, A and B, columns 17–20) indicating that blockage of DnaC ATPase activity inhibits DnaB translocation along not only dsDNA but also ssDNA. This finding implies that the lack of inhibition by excess DnaCATP during DnaB translocation over ssDNA should be viewed as relief of inhibition. This relief of inhibition is consistent with binding of ssDNA by DnaC (26, 28) and with the ability of ssDNA but not dsDNA to stimulate DnaC ATPase activity when DnaC is bound to DnaB (30). Thus repeated association of DnaCATP with DnaB translocating along ssDNA would result in hydrolysis of ATP by DnaC and relief of inhibition of DnaB movement (Fig. 6A). DnaC interacts with the leading edge of DnaB (24, 39) implying that ssDNA bound by DnaC must be immediately ahead of DnaB. Whether this relief of inhibition is achieved by reduced affinity of DnaCADP for DnaB-ssDNA (28) or by the ability of DnaB to translocate even when bound by DnaCADP is currently not clear. Upon encountering forked DNA, DnaB translocation would continue to generate ssDNA at the fork and would also result in stimulation of DnaC ATPase (Fig. 6B). Note that although a 3’ ssDNA arm at the fork relieved DnaC-dependent inhibition of DnaB (Fig. 2D) replacement of this ssDNA arm with a streptavidin-biotin complex also provided relief of inhibition (Fig. 3B). The strand along which DnaB translocates would therefore more likely interact with DnaC to promote ATP hydrolysis rather than the displaced 3’ strand (Fig. 6B, step 5), a model that implies ssDNA immediately ahead of DnaB at a fork is accessible to DnaC. In contrast, upon trans-
FIGURE 5. Inhibition of DnaB-catalyzed unwinding of ssDNA tailed Holliday junctions by excess DnaC. A, time courses of unwinding of substrate 10 by 600 nM DnaB in the absence of DnaC (lanes 1–6), with 600 nM DnaB and DnaC (lanes 7–12) and with 600 nM DnaB plus 2400 nM DnaC (lanes 13–18). Samples were taken at 0, 1, 2, 4, 8, and 16 min, as indicated in each time course. Shaded circles indicate positions of the 5′ 32P label. B, time courses of unwinding of substrate 11. Concentrations of DnaB and DnaC, and time points, are as described in A. C, quantification of total levels of unwinding of substrates 10 (i) and 11 (ii) as a function of time by DnaB only (black squares), equimolar DnaB and DnaC (open squares), and 4-fold excess of DnaC over DnaB (open circles). Concentrations of DnaB and DnaC are as described in A. D, schematic of DnaB-catalyzed unwinding of substrates 10 (i) and 11 (ii and iii).
location of DnaB from ssDNA onto dsDNA, upon association of DnaC ATP with DnaB no ssDNA would be available to stimulate DnaC ATPase, resulting in inhibition of DnaB translocation over dsDNA (Fig. 6C, step 6).

The above model implies continual binding of DnaC to DnaB as DnaB translocates along ssDNA or dsDNA. The possible need for repeated binding of DnaC may reflect our observation that inhibition of DnaB by DnaC occurred only in the presence of excess DnaC regardless of the nature of the DNA substrate (Figs. 1–5), consistent with a dynamic equilibrium between binding of DnaB and DnaC (25). Thus after DnaC-facilitated loading of DnaB onto ssDNA and dissociation of the initial DnaC upon hydrolysis of ATP (25) the probability of DnaC ATP binding to translocating DnaB is increased in the presence of excess DnaC regardless of the DNA substrate. However, only in the absence of stimulation of DnaC ATPase activity might this result in inhibition of DnaB helicase (Fig. 6C).

The DNA structure-specific inhibition of DnaB helicase activity by excess DnaC appears to contrast with previous studies which concluded that DnaC was generally inhibitory to DnaB helicase (18, 30). However, the inhibition of DnaB by DnaC observed in (30) employed an unbranched partial duplex DNA substrate formed by annealing a fully complementary oligonucleotide to M13 ssDNA. Unwinding of such a substrate by DnaB presumably requires transient formation of a forked DNA structure to facilitate unwinding by steric exclusion (6, 7, 35). Most of the time, though, such a structure would remain fully annealed and DnaB would translocate over the dsDNA within the partial duplex region, a reaction that we have shown results in blockage of DnaB translocation in the presence of excess DnaC ATP (Fig. 4). The DNA substrate in (18) also used M13 ssDNA but employed a partially complementary oligonucleotide that formed a forked DNA structure, unwinding of which by DnaB was inhibited by excess DnaC. We have tested M13 substrates formed using partially and fully complementary oligonucleotides and have found that excess DnaC inhibited DnaB-catalyzed unwinding of unbranched partial duplex but not forked substrates (data not shown), in contrast to the previous study (18) but consistent with the data in Figs. 1–5.

If excess DnaC can indeed confer DNA structure specificity on DnaB, is DnaC present in excess of DnaB in vivo? Intracellular levels of DnaB monomer have been estimated as 60–120 per cell based on protein purification yields (40, 41), while Western blotting detected ~100 molecules of DnaC per cell (32). However, estimation of the ratio of DnaC to DnaB requires that the concentration of both proteins be measured within the same cell extract and, to date, we have been unable to obtain accurate estimates of intracellular DnaC concentration by quantitative Western blotting (data not shown). The inhibitory effect of excess DnaC on DNA replication in vitro (30, 31) might imply that the concentration of DnaC must not exceed that of DnaB in vivo. However, this inhibitory effect might reflect competition between DnaC and other components of the replisome for binding to DnaB. The inhibition observed in vitro might therefore be a consequence of this putative replisome component(s) being present at an artificially low concentration as compared with the concentration in vivo, a possibility we are currently investigating.

Why might DNA structure specificity need to be imposed on DnaB? The function of DnaB at the replication fork is to translocate along the lagging strand template and, as a consequence, disrupt the parental duplex. This function requires translocation of DnaB along ssDNA. In contrast, although DnaB can translocate over dsDNA after initial binding onto ssDNA (6), it is difficult to envisage such a capacity being of any benefit at the replication fork. Translocation over dsDNA has been suggested to allow DnaB to function in branch migration of Holliday junctions during homologous recombination but, other than the capacity of DnaB to perform this reaction in vitro (10), there is no evidence for such a role in vivo. The ability of such ring helicases to accommodate more than one DNA strand within the central cavity might therefore be an unavoidable consequence of the translocation mechanisms employed by such enzymes and may be something that needs to be inhibited to

![Figure 6](https://example.com/fig6.png)  
**Figure 6.** A potential mechanism to explain the effects of DnaC on DnaB translocation along single-stranded DNA (A), forked DNA (B), and partial duplex DNA (C). Note that relief of inhibition of DnaB upon hydrolysis of ATP bound to DnaC (steps 2 and 3, and 6 and 7) could occur because of reduced affinity of DnaC ATP for DnaB-ssDNA and/or because of the ability of DnaB to translocate along ssDNA when bound by DnaC ADP.
avoid unscheduled DNA transactions in vivo. DnaC might therefore provide not only the means to facilitate loading of DnaB onto ssDNA but also to restrict DnaB translocation to ssDNA. Similar considerations might apply to eukaryotic replicative helicases, given their shared ability to translocate over dsDNA in addition to ssDNA (42).

Acknowledgment—We thank Ken Marians for supplying the DnaB overexpression plasmid.

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