Published in final edited form as:
Mol Cell. 2010 January 15; 37(1): 90. doi:10.1016/j.molcel.2009.12.031.

Primase Directs the Release of DnaC from DnaB

Magdalena Makowska-Grzyska and **Jon M. Kaguni***
Department of Biochemistry and Molecular Biology Michigan State University, East Lansing, Michigan 48824-1319

Summary

An AAA+ ATPase, DnaC delivers DnaB helicase at the *E. coli* chromosomal origin by a poorly understood process. This report shows that mutant proteins bearing alanine substitutions for two conserved arginines in a motif named box VII are defective in DNA replication, but this deficiency does not arise from impaired interactions with ATP, DnaB or single-stranded DNA. Despite their ability to deliver DnaB to the chromosomal origin to form the prepriming complex, this intermediate is inactive. Quantitative analysis of the prepriming complex suggests that the DnaB-DnaC complex contains three DnaC monomers per DnaB hexamer, and that the interaction of primase with DnaB and primer formation triggers the release of DnaC but not the mutants from DnaB. The interaction of primase with DnaB, and the release of DnaC mark discrete events in the transition from initiation to the elongation stage of DNA replication.

Keywords

DnaC; DnaB; primase; initiation

Introduction

In a process that is tightly coupled with cell growth (Nielsen and Lobner-Olesen, 2008), DnaA initiates DNA replication from the *E. coli* replication origin, *oriC*, by promoting the assembly of the enzymatic machinery that duplicates the bacterial chromosome. Biochemically, the process that leads to replisome assembly can be separated into discrete steps (Kaguni, 2006). Bound to ATP, DnaA first binds to the DnaA boxes, I- and τ-sites within *oriC* (Leonard and Grimwade, 2009). Aided by HU or IHF, DnaA then unwinds an AT-rich region carrying three 13-mer motifs (Bramhill and Kornberg, 1988), and loads one DnaB hexamer from the DnaB-DnaC complex onto each of the separated strands within *oriC* to form the prepriming complex (Carr and Kaguni, 2001; Fang et al., 1999). After DnaC dissociates from DnaB, the helicase translocates on the parental template DNA and interacts via its N-terminal domain with primase, leading to the formation of primers (Tougu and Marians, 1996b; Wu et al., 1992). Several recent studies provide insight into how DnaB and primase coordinate their functions (Bailey et al., 2007a; Corn et al., 2008). A dimeric DNA polymerase III holoenzyme at each replication fork then extends the primers to duplicate the chromosome (McHenry, 2003; Pomerantz and O'Donnell, 2007). Comparing the mechanism of *E. coli* DNA replication with

*Corresponding author: Mailing address: Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824-1319, Phone: (517) 353-6721, Fax: (517) 353-9334, kaguni@msu.edu.

that of eukaryotes, it is remarkable that organisms in separate kingdoms utilize similar biochemical mechanisms (Nielsen and Lobner-Olesen, 2008; Robinson and Bell, 2005).

DnaB is the replicative helicase for the *E. coli* chromosome and for many bacterial plasmids. Six identical DnaB protomers assemble into a ring-like structure through which the single-stranded DNA template is thought to pass during DNA unwinding (Lo et al., 2009; Yu et al., 1996). For DnaB to load at *oriC*, it must be complexed to DnaC, which regulates the helicase function of DnaB (Davey et al., 2002; Wahle et al., 1989b). Whereas DnaB can bind to naked ssDNA (Biswas et al., 2004; Davey et al., 2002), the helicase cannot bind to the unwound region of *oriC* without DnaC (Sutton et al., 1998). X-ray crystallography of a gram-positive DnaB and cryo-electron microscopy of *E. coli* DnaB reveal that each DnaB protomer has a larger C-terminal domain containing a RecA-like fold, and a smaller N-terminal domain (Bailey et al., 2007a; San Martin et al., 1998). DnaC bound to the larger domain, possibly as a maximal set of three dimers (Galletto et al., 2003), forms the DnaB-DnaC complex. The N-terminal region of DnaC is necessary for it to interact with DnaB (Ludlam et al., 2001).

DnaC is a member of the AAA+ family of ATPases that contain specific amino acid sequence motifs proposed to coordinate enzyme function with ATP binding and its hydrolysis (Koonin, 1993). For DnaC, ATP mediates several important functions. First, ATP increases the affinity of DnaC for ssDNA, which stimulates DnaC's ATPase activity synergistically with DnaB (Biswas et al., 2004; Davey et al., 2002). Second, ATP or ATPγS, an ATP analogue that is poorly hydrolyzed, stabilizes DnaC complexed to DnaB to arrest DnaB as a DNA helicase (Allen and Kornberg, 1991; Wahle et al., 1989b). In support that ATP binding is essential for DnaC function, mutant DnaCs that are defective in ATP binding due to substitutions in the Walker A box, a nucleotide binding motif, do not inhibit DnaB in vivo, and fail to deliver DnaB to *oriC* in vitro (Davey et al., 2002; Ludlam et al., 2001). The conundrum is that nucleotide binding apparently is not required for DnaC to interact with DnaB (Biswas and Biswas-Fiss, 2006; Davey et al., 2002; Galletto et al., 2003). Also, the comparable affinity of DnaC for DnaB in the presence of ATP or ADP suggests that ATP hydrolysis by DnaC is not required for its dissociation from DnaB (Davey et al., 2002; Galletto et al., 2003). These contradictory observations, which overlook the possible effect of *oriC* on ATP metabolism by DnaC, raise several questions. Why does DnaC require ATP to deliver DnaB to *oriC*, and what induces the dissociation of DnaC from DnaB?

Of the AAA+ motifs of DnaC, the box VII motif contains two conserved arginines. Based on the X-ray crystallographic structure of *Aquifex aeolicus* DnaC (residues 43-235) bound to ADP, one of the arginines of *E. coli* DnaC is proposed to interact with the γ phosphate of ATP bound in the pocket formed either between adjacent monomers of a DnaC oligomer (Mott et al., 2008), or between neighboring DnaC protomers in the DnaB-DnaC complex. This residue called the “arginine finger” is thought to promote and to coordinate ATP hydrolysis with a conformational change (Erzberger and Berger, 2006). If ATP hydrolysis by DnaC causes it to dissociate from DnaB at *oriC*, a prediction is that substitution of this arginine with a nonfunctional residue should lead to DnaC remaining complexed to DnaB, blocking DnaB function to inhibit initiation. Because substitution of the arginine finger residue of HslU/ClpY (Song et al., 2000), or of a conserved arginine in the box VII motif of DnaA obstructs formation of a self-oligomer (Felczak and Kaguni, 2004), the second arginine at residue 216 of DnaC may similarly act in the oligomerization of DnaC monomers in the DnaB-DnaC complex.

We tested these predictions, and showed that substitution of these conserved arginines with alanine substantially impairs DnaC activity in DNA replication in vivo and in vitro. Compared with wild type DnaC, the inactivity of the mutant DnaC proteins in DNA replication is not explained by defects in binding to ATP as measured in a crosslinking assay, or to ssDNA or DnaB as measured respectively by surface plasmon resonance or a protein overlay assay. We

show that, like the wild type enzyme, both mutant DnaCs are able to deliver DnaB to *oriC* and are retained there. We also demonstrate that primase induces the release of DnaC, but not of the mutant DnaCs from DnaB in the prepriming complex whereas DNA polymerase III holoenzyme is only partially effective. Moreover, ATPγS stabilizes the mutant DnaC bearing an alanine substitution for arginine 216 in the prepriming complex whereas ATP does not, suggesting that aberrant ATP hydrolysis by this mutant facilitates its dissociation from DnaB.

Results

The conserved arginines in the box VII motif are necessary for DnaC function in vivo

With derivatives of pACYC184 that encode alanine substitutions for arginine 216 and arginine 220, which reside in the box VII motif of DnaC (Table S1, supplemental data; Figure 1), we used a plasmid exchange assay to show that the *dnaC* alleles are defective in vivo. This assay relies on a strain that lacks almost the entire *dnaC* gene, which is complemented by a *dnaC*-containing plasmid that requires IPTG for its maintenance (Hupert-Kocurek et al., 2007). When this plasmid is lost on media lacking IPTG, we can measure *dnaC* function encoded by another plasmid. With this method, we observed that a pACYC184 derivative carrying the $dnaC^+$ gene but not the empty vector (pACYC184) maintained the viability of isogenic Δ*dnaC* strains that encoded either the $recA^+$ or $recA::kan^R$ allele (Table S2, supplemental data). Hence, viability requires the *dnaC* gene, but does not depend on integration of the *dnaC* plasmid into the bacterial chromosome. In comparison, plasmids encoding the mutant DnaCs (hereafter named R216A and R220A) failed to complement these strains when the resident plasmid was lost. We then performed immunoblot analysis of whole cell lysates prepared from *E. coli* MC1061 bearing the plasmid-encoded *dnaC* alleles to confirm that their steady-state levels were sufficient for complementation (data not shown). Whereas the chromosomally-encoded level of wild type DnaC was undetectable in several strains (MC1061, MG1655 and their derivatives; 5×10^8 cells), the abundance of plasmid-encoded R216A and R220A at roughly 3×10^3 molecules per cell was comparable to the level that maintains viability of the Δ*dnaC* strains by the IPTG-dependent $dnaC^+$ plasmid *(pAM34dnaC)*. As the mutant DnaCs were not proteolytically unstable, these results show that the mutant DnaCs are inactive in this genetic assay. The inactivity of R220A is consistent with a previous study (Mott et al., 2008).

However, the inactivity of R216A encoded by pACYC184 contrasts with the mutant protein's ability to complement the temperature sensitivity of a *dnaC2* mutant when this allele was downstream from the *araBAD* promoter in a separate plasmid (Mott et al., 2008). Hence, we constructed plasmids that bear the box VII mutations under *araBAD* promoter control (*pR216AdnaC* and *pR220AdnaC*), and compared them with the isogenic $dnaC^+$ plasmid ($pINC_{SSD}$) in complementation of a *dnaC2* strain under similar uninduced conditions. Table S3 (supplemental data) shows that the $dnaC^+$ allele but not the allele encoding R220A under control of the *araBAD* promoter was active in complementation. In contrast, R216A complemented the *dnaC2* strain at nonpermissive temperature if the allele was downstream from the *araBAD* promoter, but not if it was carried in pACYC184. These results, which suggest that R216A is partially active, correlate with biochemical studies described below showing that R216A is modestly active in DNA replication.

By UV crosslinking, R216A and R220A are active in ATP binding

To gain more insight into the specific functions of these arginine residues, we examined the properties of the purified R216A and R220A DnaC mutants (Figure S1, supplemental data) in several biochemical assays. As earlier studies showed that DnaC binds to ATP, albeit weakly (Biswas et al., 2004; Davey et al., 2002; Galletto et al., 2003; Wahle et al., 1989a), we measured the ability of the mutant DnaCs to interact with ATP. The poor affinity of DnaC for ATP obstructed our efforts to measure ATP binding by the nitrocellulose filter retention method.

Relying on UV crosslinking, which showed that DnaC is able to bind to ATP (Biswas and Biswas, 1987; Ludlam et al., 2001), we observed similar levels of crosslinked [α-^{32}P] ATP with wild type DnaC and with the mutant proteins but not with bovine serum albumin (BSA) (Figure 2A). As a control, the inclusion of an excess of unlabeled ATP abolished the crosslinking of [α-^{32}P] ATP to wild type DnaC. These observations suggest that R216A and R220A are active in ATP binding. The results with R220A support a recent study, which showed that an alanine substitution of the corresponding amino acid (lysine 210) of *Aquifex aeolicus* DnaC did not affect ATP binding (Mott et al., 2008).

R216A and R220A can interact with DnaB

Previously, we developed a genetic assay to identify mutant DnaC proteins that are defective in interacting with DnaB (Ludlam et al., 2001). We also showed biochemically that specific amino acid substitutions, including an L11Q substitution near the N-terminus of DnaC, abrogate this interaction, thus identifying an N-terminal domain that is necessary to interact with DnaB. However, other amino acid substitutions mapped to the Walker A box of DnaC, suggesting that ATP stabilizes the DnaB-DnaC complex, which is consistent with the conclusion of an earlier study (Wahle et al., 1989a). Hence, we examined the R216A and R220A for their ability to interact with DnaB because of the proposed role of residues in the box VII motif of DnaC in ATP binding and ATP hydrolysis. Using a protein overlay method, we showed that wild type DnaC, R216A and R220A were similar in their ability to bind to DnaB, and that ATP stabilized this interaction (Figure 2B, C). As controls, we failed to detect an interaction between the L11Q-substituted protein or BSA and DnaB. These results indicate that the alanine substitutions do not impair the interaction of DnaC with DnaB. In support, biosensor results confirmed that DnaC^{+}, R216A and R220A interacted with DnaB, and suggest that the affinity of DnaC^{+} compared with the mutants for DnaB is about two-fold stronger (data not shown). Although the effect of ATP on the mutant DnaCs was not tested, ATP (150 μM) increased the affinity of wild type DnaC for DnaB by about two-fold. In summary, both methods show that the inactivity of the mutant proteins in vivo (Table S2, supplemental data) does not stem from a defect in interacting with DnaB.

R216A and R220A can interact with ssDNA

The interaction of DnaC with single-stranded DNA (ssDNA) has been demonstrated by UV crosslinking, fluorescence polarization, and surface plasmon resonance (Biswas et al., 2004; Davey et al., 2002; Learn et al., 1997; Mott et al., 2008). ATP enhances this interaction (Biswas et al., 2004; Davey et al., 2002), but the mechanism by which DnaC binds ssDNA and how ATP stimulates this process are uncertain. Mott *et al*, suggested that the assembly of DnaC monomers into a helical structure may play a role in ssDNA binding (Mott et al., 2008). If so, mutation of residues involved in stabilizing the interaction between the monomers, such as arginine 216 or perhaps arginine 220, may disrupt ssDNA binding. To test this possibility, we compared the binding of the mutant proteins with wild type DnaC to an immobilized oligonucleotide by surface plasmon resonance in buffer lacking or supplemented with ATP. We calculated the dissociation constants based on the rates of association and dissociation derived from the binding isotherms. As shown in Figure 3, ATP stimulated ssDNA binding by wild type DnaC by about 2.4-fold, which is comparable to results of an independent study (Biswas et al., 2004). ATP also enhanced ssDNA binding by R220A, but the magnitude of this effect (1.7-fold) was not as great. In contrast, ATP only marginally stimulated ssDNA binding by R216A. Despite these differences, the ability of the mutant DnaCs to bind to ssDNA does not explain their inactivity in vivo.

The capacity of R220A to bind to ssDNA contrasts with defective ssDNA binding by a mutant DnaC of *A. aeolicus* (Mott et al., 2008). Measured by fluorescence polarization, substitution of the corresponding “arginine” finger residue (lysine 210) with alanine disrupted binding to

poly-dT_{25}. As this mutant DnaC also lacked the first 42 amino acids of the native protein, the defect in ssDNA binding suggests the requirement for the missing N-terminal region.

R216A and R220A are defective in DNA replication in vitro

Although R216A and R220A can interact with ATP, DnaB and ssDNA, we expected these mutant proteins to be defective in DNA replication in vitro because of their apparent inactivity in vivo. In a reconstituted system dependent on a supercoiled *oriC*-containing plasmid, one DnaB hexamer is loaded onto each of the separated strands within *oriC* (Carr and Kaguni, 2001; Fang et al., 1999). This loading process may be concerted because fork movement is bidirectional even when DnaB is at a subsaturating level (Baker et al., 1987). By comparison, DNA replication of a ssDNA that carries a DnaA box in a hairpin structure involves the binding of a single DnaB hexamer next to the DnaA box (Carr and Kaguni, 2002). Because it is conceivable that one or both substitutions specifically impair the concerted loading of DnaB at *oriC*, we examined the mutant proteins in DNA replication of both the *oriC* plasmid and the ssDNA. With the *oriC*-containing plasmid, R220A was essentially inert whereas R216A reproducibly supported a marginal level of DNA synthesis (Figure 4A). With the ssDNA, R216A supported a limited level of DNA synthesis that was slightly greater than the level observed with R220A (Figure 4B). These results indicate that the alanine substitutions for these arginine residues substantially reduce the activity of DnaC in both replication systems.

R216A and R220A are able to deliver DnaB to *oriC*

The O'Donnell laboratory reported that a mutant DnaC bearing an arginine substitution for the conserved lysine in the Walker A box was inert in ATP binding (Davey et al., 2002). Despite its ability to form a complex with DnaB, the mutant DnaC's failure to guide the helicase to *oriC* suggests the requirement for ATP binding. Because of the proposed role of arginine 220 and perhaps arginine 216 in ATP binding and its hydrolysis, we considered the possibility that our mutant DnaCs may fail to deliver DnaB to *oriC*. As an assay, we measured the assembly of the prepriming complex, which forms by the binding of DnaA to *oriC* in a supercoiled plasmid followed by the HU-stimulated unwinding of a region within *oriC* (Chodavarapu et al., 2008). At the step of unwinding, DnaA complexed to ATP or ATPγS but not ADP is active (Sekimizu et al., 1987). DnaA then mediates the binding of DnaB from the DnaB-DnaC complex to the resultant unwound region to form the prepriming complex (Marszalek and Kaguni, 1994).

We assembled the prepriming complex with wild type DnaC or the mutants and other necessary components, and then separated the unbound proteins from the prepriming complex by gel filtration chromatography (see *Experimental Procedures*). In several essentially identical experiments, such as that of Figure 5A, we showed that the isolated complex assembled with wild type DnaC was active upon addition of primase, DNA polymerase III holoenzyme, DNA gyrase, and other reagents that are required during the subsequent stage of DNA synthesis, recovering 70-90% of the activity in the sample before isolation. Hence, this complex is a stable intermediate of the initiation process. In contrast, the isolated complex assembled with R220A was essentially inactive whereas that formed with R216A had only about 15% of the activity of the isolated complex assembled with wild type DnaC.

We then quantified the amount of the *oriC* plasmid in the isolated complex by agarose gel electrophoresis (data not shown). In parallel, we determined the amounts of DnaA, DnaB and DnaC by quantitative immunoblot analysis, and calculated the stoichiometry of these proteins relative to the *oriC* plasmid (Figure 5B, C). In the prepriming complex assembled with wild type DnaC, we observed nine DnaA monomers and eleven DnaB monomers per *oriC* plasmid, which substantiate previous results of ten DnaA monomers (Carr and Kaguni, 2001; Chodavarapu et al., 2008; Felczak and Kaguni, 2004; Felczak et al., 2005), and two DnaB

hexamers bound to *oriC* (Carr and Kaguni, 2001; Fang et al., 1999). Previous work showed that the loading of DnaB requires DnaC (Funnell et al., 1987; Davey et al., 2002). In agreement, we determined that the omission of DnaC under conditions that otherwise assemble the prepriming complex (as in Figure 5) led to the absence of DnaB on the *oriC*-containing plasmid (data not shown). We also showed that DnaB was not retained on the DNA when we replaced the *oriC*-containing plasmid (M13*oriC*2LB5) with the plasmid vector (M13ΔE101). Hence, DnaB requires DnaC and the *oriC* sequence for the helicase to load at *oriC* in a supercoiled plasmid.

For the prepriming complex assembled with R216A or R220A, the stoichiometry of DnaA and DnaB corresponds within experimental error with the results obtained with wild type DnaC (Figure 5). Thus, despite the marginal activity of R216A and inactivity of R220A in *oriC* plasmid replication, both mutant DnaCs are able to deliver two DnaB hexamers to *oriC*. We conclude that their biochemical defect is at a step after the delivery of DnaB.

Interestingly, we calculated about six DnaC monomers in the prepriming complex assembled with wild type DnaC and 5 mM ATP (Figure 5B, C; see below). The inclusion of all four ribonucleotides or subsets at 0.5 mM each in combination with 5 mM ATP did not lead to the dissociation of DnaC (data not shown). When we replaced the *oriC* plasmid with the empty vector (M13ΔE101) as a control, the ratio of 0.7 DnaC monomers per DNA indicates that nonspecific binding of DnaC to DNA is negligible. The simplest explanation for these results is that each DnaB hexamer at *oriC* is bound by three DnaC monomers, but other ratios cannot be excluded. Relative to the plasmid, we also calculated a ratio of 5.3 R220A monomers, which is similar to that for DnaC, or 2.6 R216A monomers. That R216A is able to interact with DnaB (Figure 2), and assembles two DnaB hexamers at *oriC* suggests that some other defect leads to its lower ratio per *oriC* plasmid. We excluded the possibility that impaired ATP binding explains its lower stoichiometry because ATP binding by DnaC is not required for its interaction with DnaB (Biswas and Biswas-Fiss, 2006; Davey et al., 2002; Galletto et al., 2003). Moreover, R216A (and R220A) are active in ATP binding (Figure 2). Because DnaC is reported to hydrolyze ATP weakly in the presence of ssDNA (Davey et al., 2002), we considered the possibility that R216A aberrantly hydrolyzes ATP, which causes it to partially dissociate from DnaB at *oriC*. However, we were unable to measure ATP hydrolysis by wild type DnaC under the conditions described. Our observations are consistent with other studies that failed to detect an ATPase activity for DnaC (Biswas and Biswas, 1987), but these experimental conditions may not contain the proper effector necessary to elicit ATPase activity.

Inasmuch as DnaC can interact with DnaB in the absence of ATP, ATP binding by DnaC is required to deliver the helicase to *oriC* (Davey et al., 2002). Also, ATP hydrolysis by DnaC is speculated to promote its release (Biswas et al., 2004; Wahle et al., 1989a). Because ATPγS stabilizes DnaC complexed to DnaB (Wahle et al., 1989b), we reasoned that this poorly hydrolysable nucleotide analog should block the dissociation of DnaC from DnaB at *oriC*. Alternatively if a factor is necessary to facilitate the release of DnaC and is absent in the reaction to assemble the prepriming complex, the ratio of DnaB to DnaC will be similar to that obtained with ATP. Figure 6A shows that the ratios of wild type DnaC, R216A, and R220A in the prepriming complex assembled and isolated with ATPγS are comparable; DnaC was retained at about 6 monomers per *oriC* plasmid. DnaB at about 9-11 monomers is consistent with two DnaB hexamers bound to *oriC*, validating the results of Figure 5 and previous results (Carr and Kaguni, 2001; Fang et al., 1999). Compared with Figure 5C, we measured slightly lower ratios for DnaA, which presumably reflect experimental variation. Nevertheless, these results strongly suggest that each DnaB hexamer is complexed with three DnaC monomers, and that aberrant ATP hydrolysis by R216A leads to its lower ratio in the prepriming complex assembled with ATP. However, the mutant DnaC is still able to deliver DnaB at *oriC*.

Primer formation by primase induces the dissociation of DnaC from DnaB in the prepriming complex

DnaC attenuates the ATPase and helicase activity of DnaB (Allen and Kornberg, 1991; Wahle et al., 1989b). That DnaC persists in the prepriming complex suggests a mechanism that induces its dissociation so that DnaB can unwind the parental DNA to advance the replication fork. If so, the mutant DnaCs may remain complexed to DnaB because they fail to respond to the dissociating signal.

After the delivery of DnaB at *oriC*, primase interacts with DnaB as the helicase translocates on the parental DNA, and forms primers for leading and lagging strand DNA synthesis (Tougu and Marians, 1996a; Wu et al., 1992). The primers are only 10-12 nucleotides long (Kitani et al., 1985; Yoda and Okazaki, 1991), and the template sequence used to initiate and direct primer synthesis is not very specific in vitro (Bhattacharyya and Griep, 2000). These observations suggest that the binding of primase to DnaB in the prepriming complex may induce DnaC to dissociate. To test this possibility, we assembled the prepriming complex but also included primase, and found that DnaC (5.2 DnaC monomers per *oriC* plasmid) was still retained (Figure 6B). As the reaction contained ATP, the capacity of DnaB or DnaC to hydrolyze ATP is insufficient to liberate DnaC. Interestingly, we failed to detect DnaC in the isolated complex assembled as above but supplemented with both primase and either all four ribonucleotides (Figure 6B) or only ATP, CTP and UTP that should support the synthesis of primers of shorter average length (data not shown). As described above, the inclusion of all four ribonucleotides but without primase during assembly did not lead to the dissociation of DnaC. Because DNA polymerase III holoenzyme via its τ subunit interacts with DnaB (Kim et al., 1996), this DNA polymerase may also induce the release of DnaC from the prepriming complex. We found that this enzyme was only partially effective based on the ratio of 1.9 DnaC molecules per *oriC* plasmid (Figure 6B). These results suggest that the interaction of primase with the N-terminal region of DnaB and primer synthesis induce a conformational change that promotes the release of DnaC from the larger C-terminal region of DnaB where it was once bound.

Primer formation by primase fails to liberate R216A and R220A from the prepriming complex

We then examined the mutant DnaCs in reactions supplemented with primase, ribonucleotides to support primer formation, and the DNA polymerase. We reasoned that the retention of a mutant DnaC at the same level as when primase and the DNA polymerase were absent would indicate that the mutant resists both proteins. We found that wild type DnaC was not retained (Figure 6C), but R216A and R220A remained at about the same stoichiometry as when these components were omitted (Figure 5C). These results with wild type DnaC agree with those of Figure 6B and an earlier study in which less than one DnaC molecule was retained in prepriming complex assembled under similar conditions (Fang et al., 1999). The failure of primase to induce the release of R220A or R216A indicates that their complete dissociation from DnaB is necessary in the transition from the initiation to the elongation stage of DNA replication, and provides an explanation for their inactivity in DNA replication.

Discussion

Arginine 216 of DnaC stabilizes the interaction of DnaC with DnaB

DnaC has two conserved arginines in the box VII motif (Figure 1). Based on studies of mutant proteins of HslU/ClpY and DnaA (Felczak and Kaguni, 2004;Song et al., 2000), we speculated that the R216A substitution may disrupt the interaction between DnaC protomers to impede the assembly of the DnaB-DnaC complex. Alternatively, this substitution may weaken the association between mutant DnaC molecules in the DnaB-DnaC complex after helicase loading at *oriC*. In the first case, the mutant DnaC should fail to deliver DnaB into the prepriming complex. In the second, R216A in the prepriming complex may be unstable. Our results show

that R216A delivers DnaB to *oriC* to exclude the first possibility. Supporting the second possibility, the partial dissociation of R216A and not wild type DnaC from the prepriming complex assembled with ATP suggests that arginine 216 stabilizes DnaC molecules that are complexed with DnaB. Interestingly, R216A was retained at a ratio comparable with wild type DnaC in the presence of ATPγS but at a lower ratio with ATP, suggesting that this mutant improperly hydrolyzes ATP that leads to its partial dissociation. As the isolated complex assembled with DnaC and not with R216A is fully active in DNA replication, these results also suggest that after R216A delivers DnaB to *oriC*, the few molecules of R216A that remain bound to DnaB inhibits the helicase.

In response to primase, arginine 220 of DnaC coordinates the release of DnaC from DnaB

Based on the crystal structure of the ATP binding region of *Aquifex aeolicus* DnaC complexed to ADP with BeF_3, a widely used substitute for the γ phosphate of ATP (Petsko, 2000), homology modeling predicts that arginine 220 of *E. coli* DnaC is the arginine finger residue. In AAA+ proteins, the arginine finger is proposed to promote ATP hydrolysis and to coordinate a conformational change during this process. Regardless of whether the prepriming complex was assembled with ATP, or ATPγS (Figure 5, 6), R220A was retained at a level comparable with $DnaC^+$. With all four ribonucleotides, or with ATP, CTP and UTP to support primer formation, primase induced the release of $DnaC^+$ but not R220A. In contrast, ATP as the sole ribonucleotide was insufficient. These observations suggest that arginine 220 acts to transduce the signal generated by the interaction of primase with DnaB and primer formation, which leads to the release of DnaC from DnaB, and implicates ATP hydrolysis by DnaC in the dissociation process. That DNA polymerase III holoenzyme, which also interacts with DnaB via its τ subunit (Kim et al., 1996), caused the partial release of DnaC suggests a separate pathway to favor that DnaB remains uninhibited by DnaC at the replication fork.

DnaC at oriC

Previous studies have attempted to detect DnaC in the prepriming complex with varying results. The Kornberg laboratory visualized proteins in the *oriC* prepriming complex by electron microscopy by antibody labeling and Protein A conjugated to colloidal gold, and failed to detect DnaC (Funnell et al., 1987). However, other proteins or DNA may have occluded DnaC from the antibody. Our finding corroborates previous studies, which found that DnaC is retained in the prepriming complex (Marszalek and Kaguni, 1994; Wold et al., 1998).

Different forms of the DnaB-DnaC complex

Our observations of about six DnaC monomers and two DnaB hexamers bound to *oriC* suggest that each DnaB hexamer is complexed to three DnaC monomers. This conclusion appears at odds with several studies that document the formation of the $DnaB_6$-$DnaC_6$ complex (Galletto et al., 2003; Kobori and Kornberg, 1982b). Despite the maximum capacity of six DnaC molecules in the DnaB-DnaC complex, biophysical studies show fewer DnaC molecules in complexes that are predicted to exist in vivo (Galletto et al., 2003). Our results suggest that the $DnaB_6$-$DnaC_3$ complex is the active form at *oriC*.

Interestingly, the N-terminal domain of DnaC, which interacts with DnaB (Ludlam et al., 2001), is similar in amino acid sequence with the corresponding region of P protein encoded by bacteriophage λ (Nakayama et al., 1987). This region of λ P protein presumably interacts with DnaB in forming the DnaB-λ P protein complex in a process whereby bacteriophage λ appropriates DnaB helicase to the λ replication origin. Thus, λ P protein is the viral analogue to DnaC, but it outcompetes with DnaC in binding to DnaB and also displaces DnaC from the DnaB-DnaC complex (Mallory et al., 1990). Like the $DnaB_6$-$DnaC_3$ complex at *oriC*, three λ P monomers interact with DnaB to form the $DnaB_6$-$[\lambda\ P\ protein]_3$ complex. These

observations, which add support to the validity of the $DnaB_6$-$DnaC_3$ complex, suggest a common mechanism at the step of helicase loading at the respective replication origins.

A model of the switch from the stage of initiation to elongation of DNA replication

In the hexamer of *E. coli* DnaB, each protomer (470 residues) has two domains joined by a linker that may function as a flexible hinge (Miles et al., 1997; Nakayama et al., 1984). The N-terminal domain (residues 15 to 126) is required for helicase activity, and the structure of this domain has been solved by NMR and X-ray crystallography (Fass et al., 1999; Weigelt et al., 1999). The X-ray crystallographic structure of hexameric *Bacillus stearothermophilus* DnaB, or monomeric *Thermus aquaticus* DnaB has been solved also (Bailey et al., 2007a; Bailey et al., 2007b). During fork movement, the N-terminal domain faces away from the replication fork (Jezewska et al., 1998a; Jezewska et al., 1998b; Jezewska et al., 1998c) and transiently interacts with primase during primer formation (Bird et al., 2000; Chang and Marians, 2000; Oakley et al., 2005; Tougu and Marians, 1996a; Tougu and Marians, 1996b). The larger C-terminal domain (residues 172 to 470) is a hexamer containing a RecA-like fold, and bears the ATPase and DNA-binding sites (Bird et al., 2000; Nakayama et al., 1984). DnaC appears to bind to the larger domain (Barcena et al., 2001; Galletto et al., 2003). In the dynamic interactions that establish the replication fork machinery at *oriC*, the results of our study support a model that the interaction of primase with the N-terminal domain of DnaB induces the dissociation of DnaC from the C-terminal domain of DnaB.

The results of our study support the following model (Figure 7). First, DnaA bound to DnaA boxes, I- and τ-sites within *oriC* unwinds a region near the left border, and then directs the loading of a $DnaB_6$-$DnaC_3$ complex on each of the separated strands of DNA. In the DnaB-DnaC complex, DnaB helicase is inactive and awaits the release of DnaC. Next, primase interacts with the C-terminal region of DnaB to induce the dissociation DnaC. We suggest that this step is coupled with the translocation of DnaB on the parental DNA strand and primer synthesis. Primase then synthesizes the first primer (in red; arrows denote the 3′-end) that is used for leading strand synthesis by DNA polymerase III holoenzyme whereas subsequent primers (as represented by the primer in blue) are used for Okazaki fragment synthesis. Thus, the transition from the stage of initiation to the elongation phase of chromosomal DNA replication is marked by a discrete event: the interaction of primase with DnaB.

The molecular events in the initiation of DNA replication in *E. coli* are strikingly similar to those that occur at a eukaryotic replication origin. Indeed, DnaA and DnaC are comparable in structure to Cdc6 and Orc1, a component of the origin recognition complex (Erzberger et al., 2002). Like DnaC, Cdc6 in a process dependent on its ability to hydrolyze ATP with Ctd1 loads the eukaryotic Mcm2-7 complex, which likely functions as the replicative DNA helicase, at chromosomal origins (Randell et al., 2006). Like DnaB, the Mcm2-7 complex is recruited in an inactive form and must be activated after loading (Borlado and Mendez, 2008; Francis et al., 2009; Labib and Gambus, 2007; Sheu and Stillman, 2006). Thus, the work described herein has broader implications, and may provide insight into the process of initiation in eukaryotic cells.

Experimental Procedures

DNAs, strains, proteins, and reagents

Plasmids and *E. coli* K12 strains are described in Table S1 (supplemental data). The 5′-biotinylated oligonucleotide for biosensor experiments was synthesized by an in-house facility. Replication proteins including a mutant DnaC carrying an L11Q substitution have been described (Ludlam et al., 2001; Marszalek and Kaguni, 1994). Mutant DnaC proteins were purified as described in Figure S1 (supplemental data). Commercial reagents were BSA,

horseradish peroxidase conjugated to goat anti-rabbit IgG (Jackson Laboratories), ribo- and deoxyribonucleotides (Pharmacia P-L Biochemicals), [α-^{32}P] ATP (Amersham Biosciences), and [methyl-^{3}H] dTTP (MP Biochemicals). Affinity-purified rabbit polyclonal antibodies for DnaB and DnaC, and rabbit antiserum that specifically recognizes the C-terminal region of DnaA (amino acids 370-467) were prepared in our laboratory.

DNA replication assays

Reactions (25 μl) with M13 A-site ssDNA or with M13*oriC*2LB5 supercoiled DNA were incubated at 30 °C for 15 or 30 min, respectively, to measure DNA synthesis, and were assembled with DnaA (55 ng) and the indicated amounts of wild type DnaC, R216A, or R220A as described (Chodavarapu et al., 2008; Marszalek and Kaguni, 1994). DNA synthesis was measured by liquid scintillation spectrometry of acid-insoluble radioactivity.

Isolation of the prepriming complex

Except where noted, reactions (85 μl) contained 10-fold greater amounts of proteins and DNA in a standard reaction for *oriC* plasmid replication, and included M13*oriC*2LB5 or M13ΔE101 (2 μg), SSB (200 ng), HU (α dimer; 50 ng), DnaA (920 ng), DnaB (1 μg), and wild type or mutant DnaC (500 ng) as indicated in buffer containing 40 mM Hepes-KOH pH 8.0, 40 mM potassium glutamate, 4% sucrose, 0.5 mM magnesium acetate, 5 mM ATP or ATPγS as indicated, 4 mM DTT, and 1 mg/ml BSA. The components for priming and DNA replication (primase, β clamp and DNA polymerase III* to reconstitute DNA polymerase III holoenzyme, and CTP, GTP, and UTP each at 0.5 mM) were omitted unless indicated. After incubation for 10 min at 37°C, the prepriming complex was isolated at 22°C by gel filtration chromatography (Sepharose 4B, 0.7 × 8.6 cm, Amersham Biosciences) in the buffer described above. Aliquots (20 μl) of the void volume fractions (120 μl) were placed on ice, magnesium acetate was adjusted to 10 mM, and components for priming and DNA replication were added at the standard amounts for *oriC* plasmid replication to a final volume of 25 μl. After incubation at 30°C for 30 min. DNA synthesis was measured as described above.

Quantitative analysis of the prepriming complex

The amount of M13*oriC*2LB5 DNA in the isolated prepriming complex was quantified by agarose gel electrophoresis relative to known amounts of this DNA as described (Carr and Kaguni, 2001). Concurrently, the amounts of DnaA, DnaB, and wild type or mutant DnaC protein were quantified by immunoblotting relative to known amounts of the respective proteins, which were analyzed in parallel to prepare standard curves. The filters (BA-85, Whatman) were probed with either antiserum that specifically recognizes DnaA protein (Chodavarapu, HU 2008), or with affinity-purified polyclonal antibody to detect DnaB or DnaC. The chemiluminescent signal (SuperSignal, Pierce) of immune complexes detected with horseradish peroxidase conjugated to the secondary antibody was quantified with a Kodak 4000R Image Station.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by grant GM33992 from the National Institutes of Health and by the Michigan Agricultural Experiment Station. We thank Dr. Magdalena M. Felczak for her expert assistance in this work. The authors do not have a financial conflict of interest that might be construed to influence the results presented in the manuscript, or the interpretation of these results.

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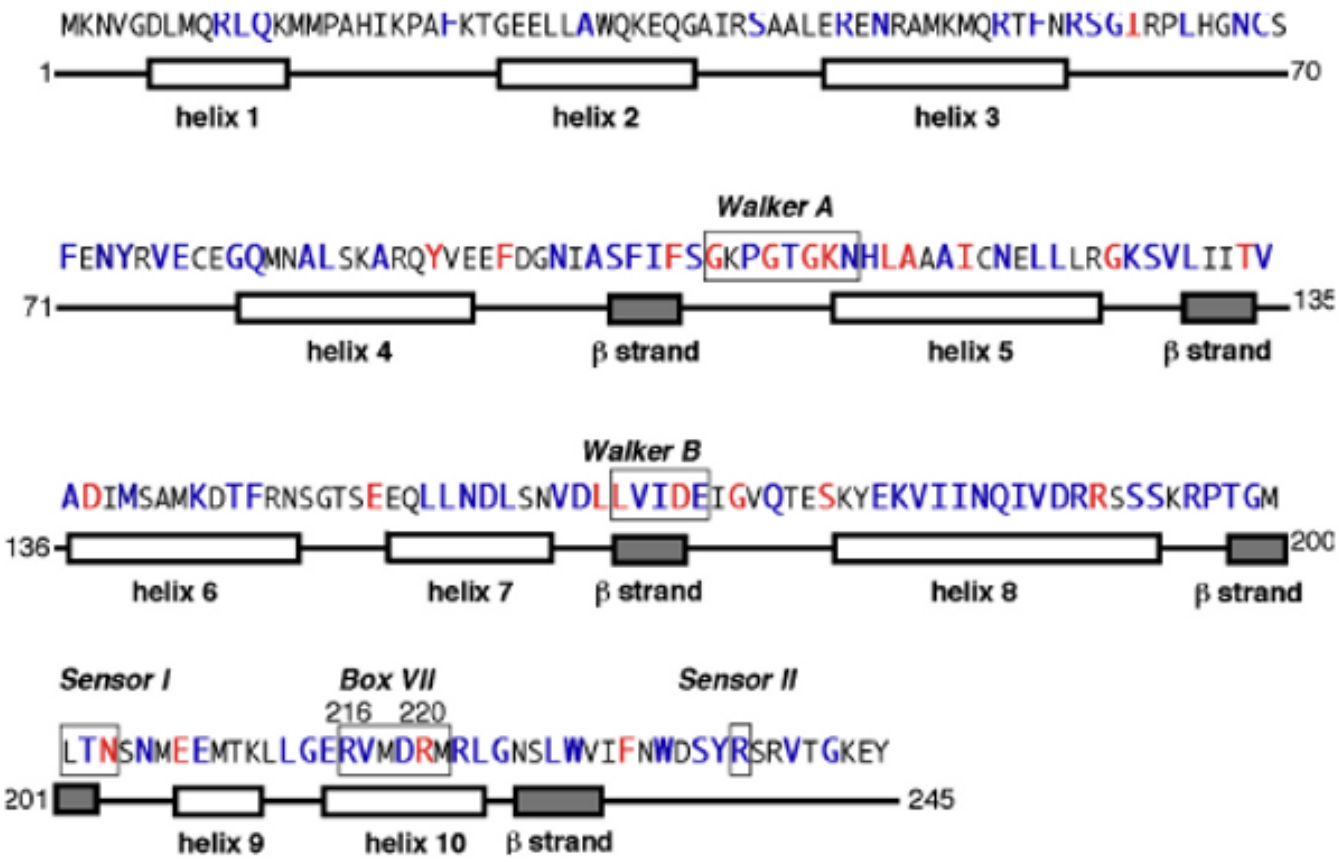


Figure 1. Primary and secondary structure of DnaC, and its amino acid sequence motifs

Comparison of the amino acid sequence of *E. coli* DnaC with its homologs reveals amino acid residues that are conserved (red) and moderately conserved (purple) (Ludlam et al., 2001). Its secondary structure is based on secondary structure prediction (residues 1-70) and a homology model derived from the ATP binding region of *A aeolicus* DnaC (residues 43-235) (Ludlam et al., 2001; Mott et al., 2008). The numbers at the right correspond to residues of *E. coli* DnaC. The boxed residues denote Walker A and B, Sensor I and II, and Box VII motifs conserved among AAA+ proteins.

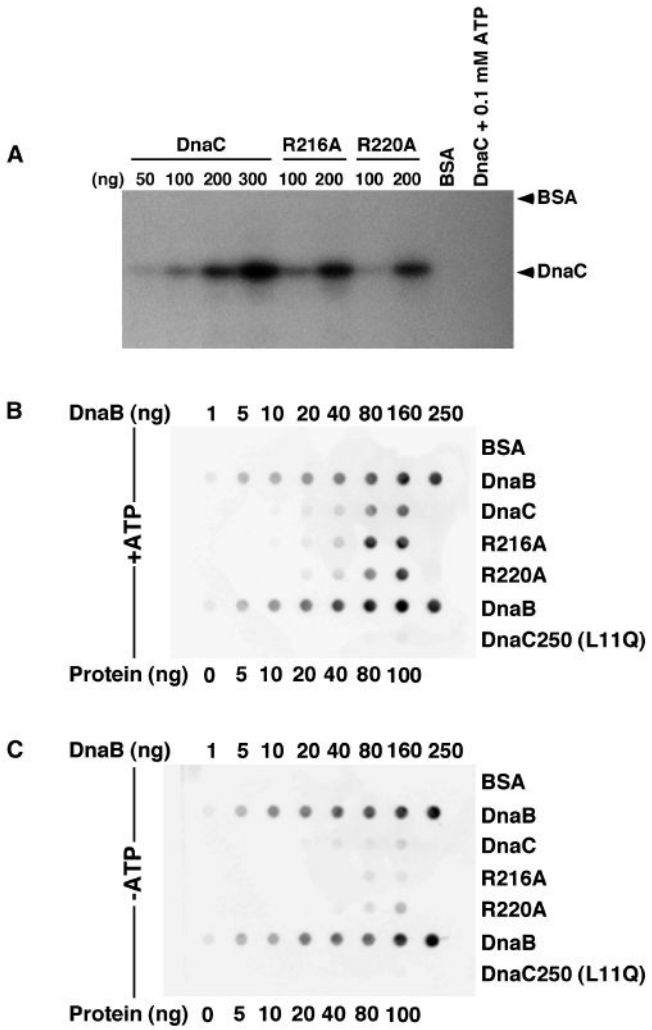


Figure 2. R216A and R220A are active in ATP binding, which stimulates their ability to interact with DnaB

(**A**) UV crosslinking of [α-^{32}P] ATP (10 μCi) was performed with duplicate samples essentially as described (Ludlam et al., 2001), but only one of each pair is shown because the results are essentially identical. As negative controls, BSA (200 ng) was used in place of wild type DnaC, or wild type DnaC (200 ng) and [α-^{32}P] ATP were incubated with 0.1 mM ATP. The electrophoretic mobilities of BSA and DnaC, which were visualized by staining with Coomassie Brilliant Blue before autoradiography, are indicated at the right. (**B**, **C**) Protein overlay analysis was performed to measure the interaction of DnaB with DnaC as described (Ludlam et al., 2001). Values at the top of each panel indicate the amounts of immobilized DnaB in the second and sixth rows that serve as a positive control for the detection method, and a reference to compare the relative amount of binding of DnaB to immobilized DnaC. The bottom of each panel indicates the amounts of BSA, or wild type or mutant DnaC present in the respective column. DnaC250 carries an L11Q substitution, which distrupts the ability of DnaC to interact with DnaB (Ludlam et al., 2001). After protein immobilization using a Bio-Rad dot blot apparatus, the filters (BA-85, Whatman) were incubated with DnaB (1 μg/ml) in buffer (40 mM Hepes-KOH pH 8.0, 20 mM Tris-HCl, pH 7.6, 10 mM magnesium chloride, 4% sucrose, 4 mM DTT, and 0.1 mg/ml BSA) lacking or supplemented with 1 mM ATP. The

washed filters were then incubated with affinity-purified antibody that specifically recognizes DnaB. Immune complexes were detected as described in *Experimental Procedures*.

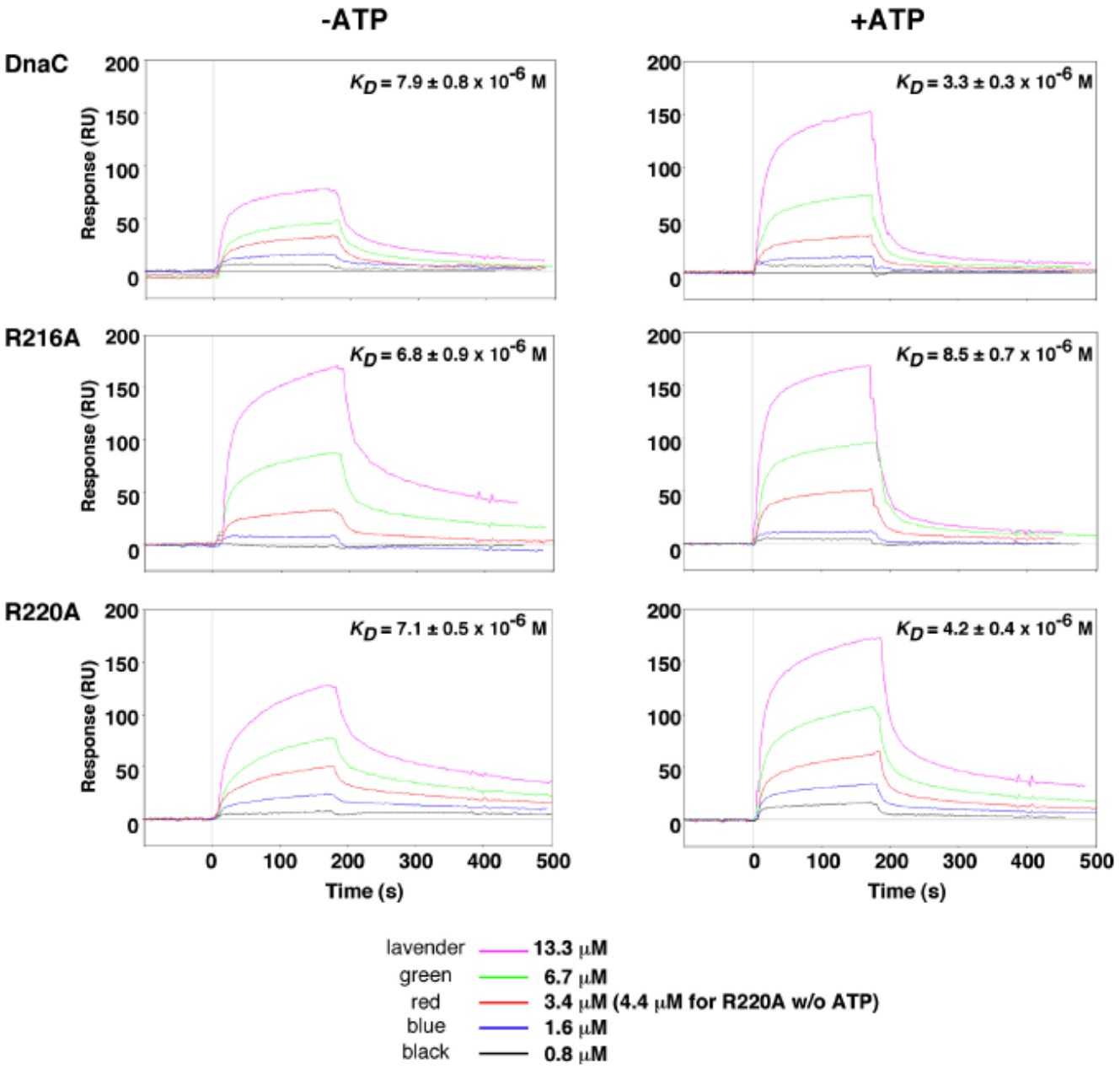


Figure 3. ATP stimulates the binding of wild type DnaC and R220A but not R216A to ssDNA

The interaction of wild type or mutant DnaC protein at the concentrations indicated with a 5′-biotinylated oligonucleotide (CATCCAATAAATCATACACAAGGC; 200 resonance units immobilized onto a streptavidin-coated sensor chip) was measured by surface plasmon resonance (Biacore 2000 biosensor, GE Healthcare). Binding was measured in duplicate at 22 °C at a flow rate of 5 μl min-^{1} in buffer containing 40 mM Hepes-KOH pH 8.0, 40 mM potassium glutamate, 4% sucrose, 0.5 mM magnesium acetate, and 4 mM DTT, and supplemented with 150 μM ATP where indicated. Because both sensorgrams at each concentration were so similar, only one binding isotherm is shown. After each measurement, the residual protein was removed from the chip with 2 M NaCl for 2 min. The background signal from a reference flow cell corresponding to a streptavidin-coated surface was subtracted from each data set. As the data did not conform with a 1:1 Langmuir interaction model, the

sensorgrams were fit globally to estimate the on-rate (k_a), the off-rate (k_d) and the dissociation constants ($K_D = k_a/k_d$) using BIAevaluation 3.1 software.

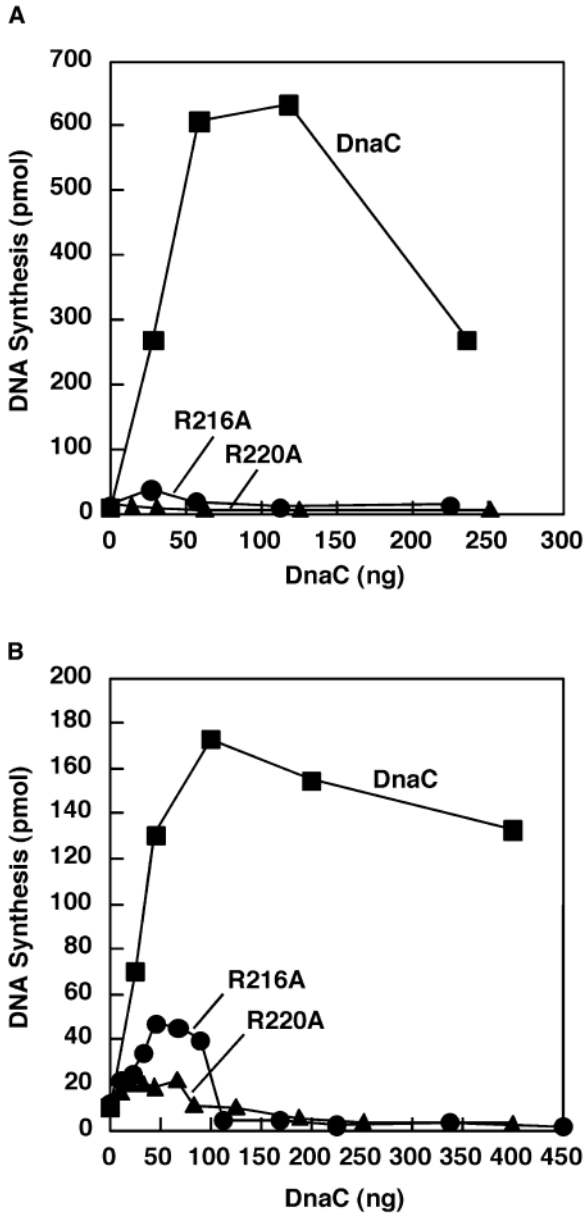
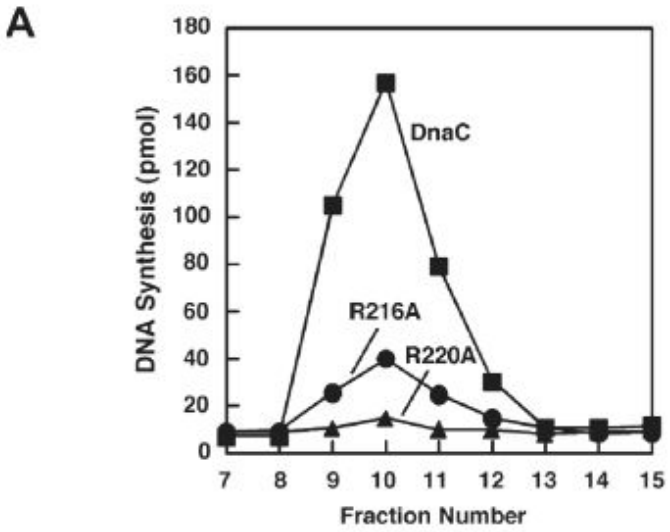
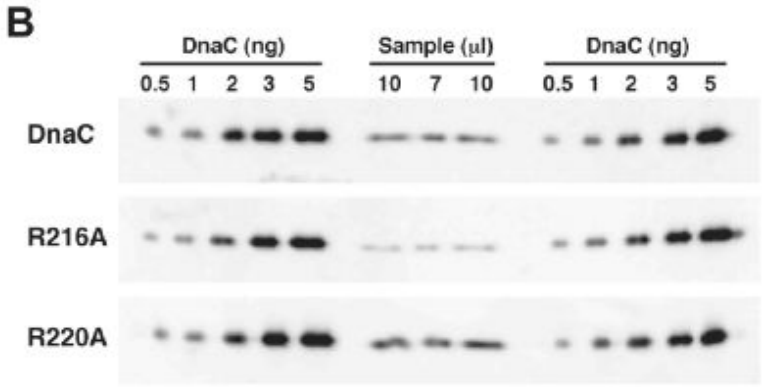


Figure 4. R216A and R220A are defective in DNA replication in vitro

DNA replication with the indicated amounts of DnaC, R216A, or R220A was measured in a reconstituted system of purified proteins with either M13*oriC*2LB5 supercoiled DNA (**A**) or M13 A-site ssDNA (**B**) as described in *Experimental Procedures*.



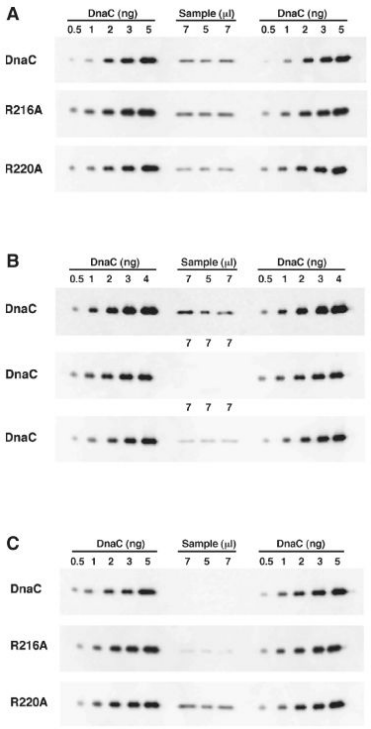


C

Protein	DnaA			DnaB			DnaC		
	Monomers/*oriC*	Range	n	Monomers/*oriC*	Range	n	Monomers/*oriC*	Range	n
DnaC	9.2 ± 1.2	8.3-10.8	3	11.3 ± 0.9	10.7-13.0	3	5.9 ± 0.9	4.3-6.8	4
R216A	8.0 ± 0.2	7.8-8.2	2	12.5 ± 0.5	12.2-12.9	2	2.6 ± 0.5	1.9-3.1	3
R220A	8.1 ± 1.7	6.3-9.9	2	11.7 ± 0.5	11.3-12.2	2	5.3 ± 1.5	3.2-6.8	3

Figure 5. R216A and R220A are able to deliver DnaB to *oriC*, but the prepriming complex is inactive

The prepriming complex was assembled with DnaC, R216A or R220A and isolated as described in *Experimental Procedures*. (**A**) Portions (20 µl) of the eluted fractions were assayed for DNA replication activity after incubation at 30°C for 30 min with CTP, GTP, UTP, dNTPs, SSB, primase, DNA polymerase III holoenzyme and DNA gyrase at levels used in standard replication reactions. (**B**) In parallel, the amount of $DnaC^{+}$, R216A, or R220A in the respective isolated prepriming complex at the volumes indicated was measured by quantitative immunoblotting relative to a standard curve obtained with known amounts of DnaC, which were analyzed in duplicate. (**C**) The amounts of DnaA, DnaB, and DnaC (wild type or mutants) were quantified and are reported relative to the amount of the *oriC*-containing plasmid (see *Experimental Procedures)*. The column labeled “n” indicates the number of experiments.



Reaction Conditions	DnaA		DnaB		DnaC	
	Monomer per *oriC*	n	Monomer per *oriC*	n	Monomer per *oriC*	n
DnaA, DnaB, DnaC, and 5 mM ATPγS	6.5 ± 0.22	2	10.5	1	5.9	1
DnaA, DnaB, R216A, and 5 mM ATPγS	6.9 ± 0.45	2	10.8	1	6.0	1
DnaA, DnaB, R220A, and 5 mM ATPγS	7.1 ± 0.2	2	8.8	1	5.5	1

Reaction Conditions	DnaA		DnaB		DnaC	
	Monomer per *oriC*	n	Monomer per *oriC*	n	Monomer per *oriC*	n
DnaA, DnaB, DnaC, primase, and ATP	10.1	1	11.8	1	5.2	1
DnaA, DnaB, DnaC, primase, and rNTPs	7.9 ± 0.8	2	12.3 ± 0.9	2	None detected	2
DnaA, DnaB, DnaC, DNA pol III, and rNTPs	8.1 ± 1.1	2	11.4 ± 0.8	2	1.9 ± 0.3	2

Reaction Conditions	DnaA		DnaB		DnaC	
	Monomer per *oriC*	n	Monomer per *oriC*	n	Monomer per *oriC*	n
DnaA, DnaB, DnaC, primase, DNA pol III, and rNTPs	10.3	1	11.6	1	None Detected	2
DnaA, DnaB, R216A, primase, DNA pol III, and rNTPs	10.3	1	11.1	1	1.6 ± 0.2	2
DnaA, DnaB, R220A, primase, DNA pol III, and rNTPs	9.9	1	9.6	1	4.8 ± 0.2	2

Figure 6. Primase induces the dissociation of DnaC but not R216A or R220A from DnaB

In each panel at the left, the indicated volumes of the isolated prepriming complex were analyzed concurrently with known amounts of DnaC that were used for a standard curve. (**A**) The prepriming complex was assembled with DnaC, R216A or R220A as described in *Experimental Procedures*, but in buffer containing 5 mM ATPγS instead of ATP. After isolation in this buffer, the stoichiometry of DnaA, DnaB, and either DnaC, R216A or R220A per *oriC* plasmid was calculated. (**B**) Reactions to assemble the prepriming complex also contained ATP (5 mM) or all four ribonucleotides (0.5 mM each except ATP at 5 mM), and primase (1.1 μg) or DNA polymerase III holoenzyme (DNA pol III) reconstituted with the β subunit (0.5 μg) and DNA polymerase III* (3 μg) as indicated at ten-fold greater levels than those used in standard replication reactions. The immunoblots at the left show the amount of DnaC in the isolated complex assembled with primase (top and middle) or DNA polymerase III holoenzyme (bottom). (**C**) Reactions for prepriming complex formation contained DnaC, R216A or R220A, all four ribonucleotides, and both primase and DNA polymerase III holoenzyme at the amounts described above. The immunoblots at the left show DnaC, R216A, and R220A in the isolated complex.

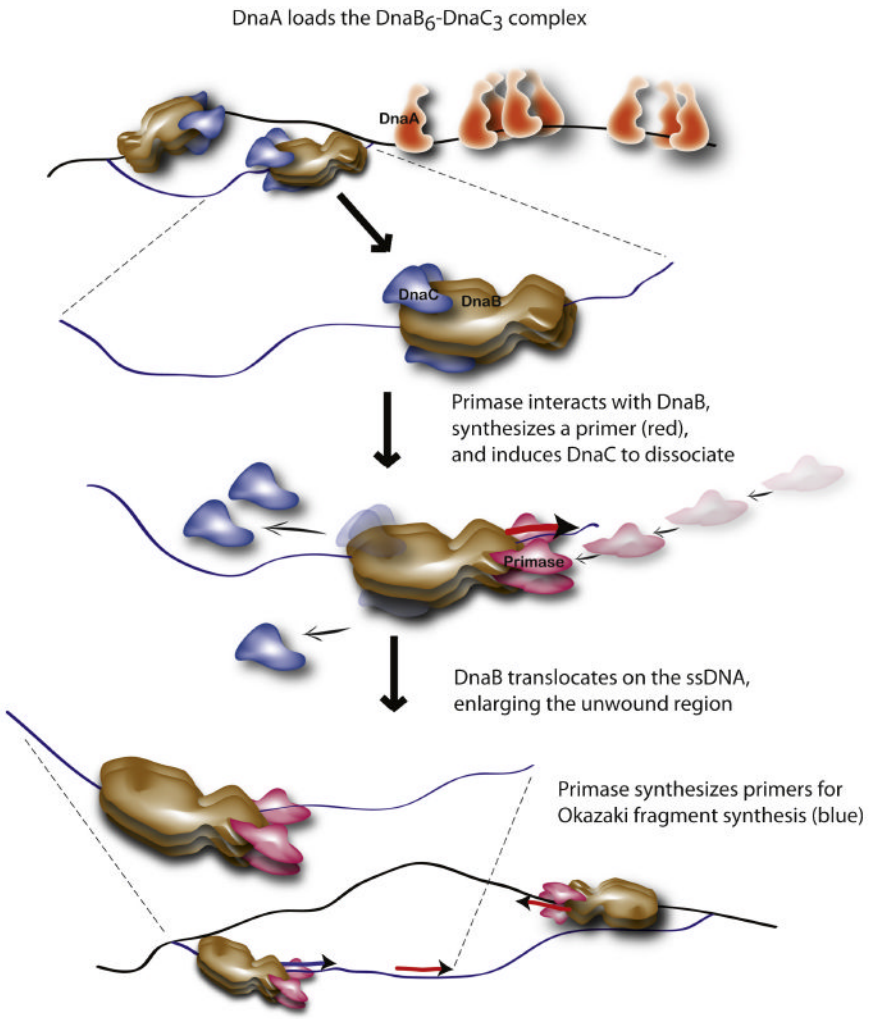


Figure 7. The interaction of primase with DnaB defines the switch from initiation to the elongation phase of chromosomal DNA replication

Please see the *Discussion* for details.