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Protein trafficking on sliding clamps

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The sliding clamps of chromosomal replicases are acted upon by both the clamp loader and DNA polymerase. Several other proteins and polymerases also interact with the clamp. These proteins bind the clamp at the same spot and use it in sequential fashion. First the clamp loader must bind the clamp in order to load it onto DNA, but directly thereafter the clamp loader must clear away from the clamp so it can be used by the replicative DNA polymerase. At the end of replication, the replicase is ejected from the clamp, which presumably allows the clamp to interact with yet other proteins after its use by the replicase. This paper describes how different proteins in the *Escherichia coli* replicase, DNA polymerase III holoenzyme, coordinate their traffic flow on the clamp. The mechanism by which traffic flow on the β clamp is directed is based on competition of the proteins for the clamp, where DNA structure modulates the competition. It seems likely that the principles will generalize to a traffic flow of other factors on these circular clamp proteins.

Keywords: sliding clamps; protein trafficking; protein switch; DNA polymerase; clamp loader; replisome

1. INTRODUCTION

The replication machinery of cellular chromosomes use a three-component strategy to attain a tight grip on DNA (Kornberg & Baker 1992; O'Donnell *et al.* 2001). The sliding clamp component is a ring shaped protein that encircles the duplex and binds the polymerase, tethering it to DNA for high processivity (Kong *et al.* 1992). The third component is a multi-subunit clamp loader that uses ATP to open the clamp protein and load it onto primed DNA (Jeruzalmi *et al.* 2002). All cellular organisms use this three-component strategy, including prokaryotes, eukaryotes and archaeal cells (see figure 1; table 1). Most phage and viral replicases achieve processivity in a different way, using only two components, polymerase coupled to a single subunit processivity factor (i.e. T7 DNA polymerase + thioredoxin). However, the T4 bacteriophage uses the same three-component strategy as cellular organisms (table 1).

At a growing replication fork it is generally presumed, and in some cases shown, that two DNA polymerases function together for simultaneous duplication of both parental DNA strands (Baker & Bell 1998). The polymerases are also coupled to a hexameric helicase for duplex unwinding, and a primase to initiate lagging strand fragments (Kim *et al.* 1996). Replication of duplex DNA is a complicated process and requires many different events to be coordinated with one another in a precise fashion. To illustrate an underlying principle of how event flow is controlled in a dynamic machine, this report will focus on the replication proteins at an *Escherichia coli* replication fork, shown in figure 2*a*.

The DnaB hexameric helicase encircles the lagging strand, and tracks along it to peel apart the parental strands. The replicase, DNA polymerase III holoenzyme, directly couples to the helicase via the β subunits, and this connection accelerates helicase unwinding 20–40-fold, to *ca.* 1 kilobase (kb) s^{-1} (Kim *et al.* 1996). The core polymerase is a heterotrimer of α (the DNA polymerase), ε (the proofreading 3′–5′ exonuclease) and θ. The holoenzyme contains two core polymerases which are attached to two τ subunits within the clamp loader. There is only one clamp loader in the holoenzyme particle; it consists of seven subunits (γ_1, τ_2, δ_1, δ'_1, χ_1 and ψ_1). Only five of these subunits are essential for clamp loader action, γ, τ_2, δ and δ′, and they form a circular heteropentamer (Jeruzalmi *et al.* 2002). The χ and ψ subunits (not shown in figure 2) stabilize the clamp loader to salt and are involved in the switch of the RNA primer from primase to the clamp loader. The mechanism of the clamp loader has been reviewed (O'Donnell *et al.* 2001; Jeruzalmi *et al.* 2002) and will not be described in detail here, but some information on the γ and τ motor subunits is central to this report. The γ and τ subunits are the only subunits that interact with ATP and thus are the motors of the clamp loading machine. Both γ and τ are encoded by the *dnaX* gene; τ (71 kDa) is the full-length product while γ (47 kDa) is the N-terminal 2/3, as it is truncated by a translational frameshift (Flower & McHenry 1990; Tsuchihashi & Kornberg 1990). The C-terminal 24 kDa region unique to τ, referred to here as τ_c, binds to both the core polymerase and to DnaB (McHenry 1982; Studwell-Vaughan & O'Donnell 1991; Kim *et al.* 1996; Yuzhakov *et al.* 1996).

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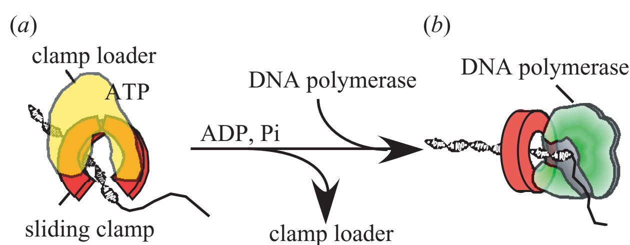

Figure 1. Three components of chromosomal replicases. High processivity is achieved through use of a circular sliding clamp protein. The sliding clamp is placed onto a primed site by a multiprotein clamp loader (*a*) which couples ATP hydrolysis to the clamp loading process. After the clamp loader departs from the clamp, the DNA polymerase associates with the clamp (*b*) for highly processive chain elongation.

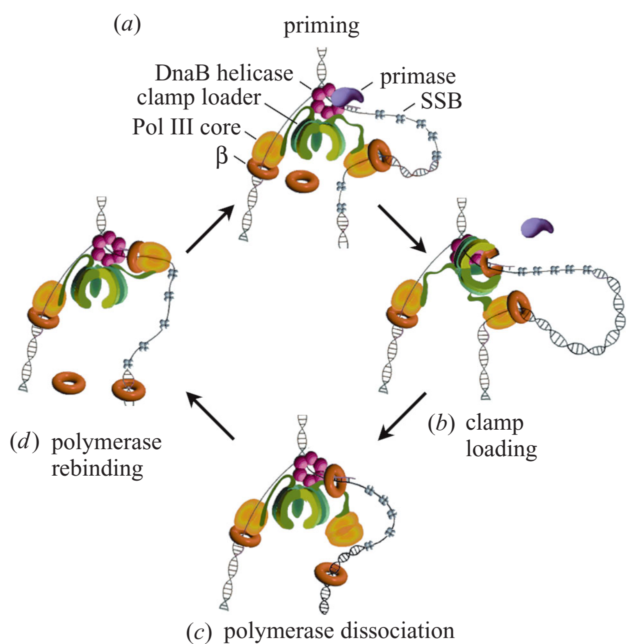

Figure 2. Overview of traffic flow on β during the lagging strand. (*a*) This illustrates the architecture of the *Escherichia coli* replisome. At the centre, the γ complex clamp loader organizes the other proteins through the two τ subunits which bind two Pol III cores and connect to the DnaB hexamer helicase. Primase synthesizes an RNA primer to initiate an Okazaki fragment. (*b*) The clamp loader assembles a clamp onto the RNA primer. (*c*) The τ processivity switch is activated upon completion of an Okazaki fragment. This switch results in ejecting core from β, leaving the β clamp on the completed fragment. (*d*) The core polymerase associates with the new β clamp on the next RNA primer to start extension of the primer into a new Okazaki fragment.

2. LAGGING STRAND CYCLE

Replication fork operations require a flow of numerous events which must occur in a particular order (figure 2*a*–*d*). This is most evident on the lagging strand which requires significantly more action than the leading strand because it must be made in a direction opposite that of fork movement. Each of the thousands of Okazaki fragments synthesized during *E. coli* chromosome duplication follows a repeated series of events with a cycle time of

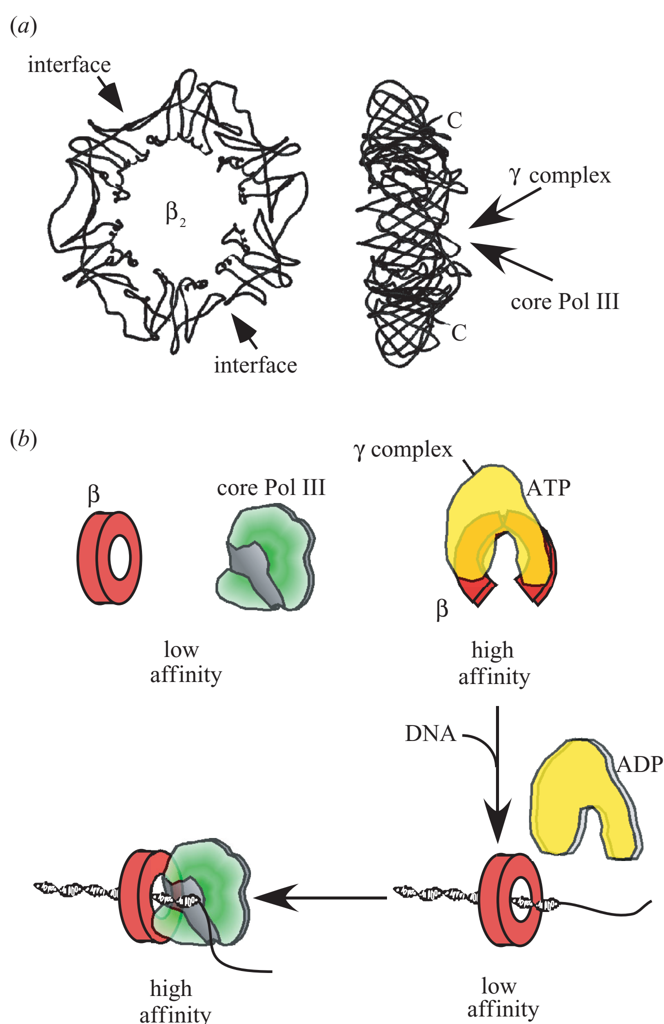

Figure 3. Traffic flow on the β clamp is dictated by DNA structure. (*a*) This shows the front and side views of the *Escherichia coli* β clamp. The DNA polymerase and γ complex clamp loader both bind the same side of the β clamp, and in fact, compete for it. (*b*) This outlines how event flow is controlled on β. When β is not on DNA, the γ complex has a much higher affinity for β than the core. However, once β is loaded onto primed DNA, the γ complex loses affinity for the β–DNA complex. At the same time, the core develops a much higher affinity for the β–DNA complex.

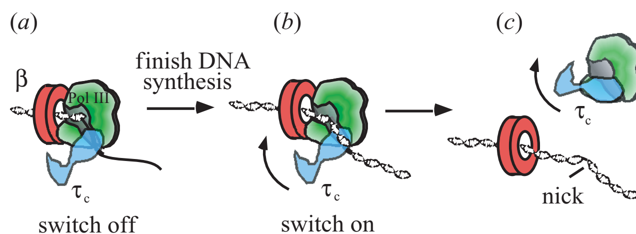

Figure 4. Traffic flow of polymerase off β at the end of an Okazaki fragment. The completion of an Okazaki fragment triggers the τ subunit processivity switch that disengages core polymerase from β. (*a*) This illustrates the core and β extending DNA. The τ subunit binds the core via the C-terminal 24 kDa section of τ, and only this 'τ_c' section of τ is shown in the diagram. (*b*) Upon completing chain extension to fill in the fragment, the τ_c senses completion of DNA. (*c*) The τ_c switch disengages the core from β, causing the core to release from the β–DNA complex.

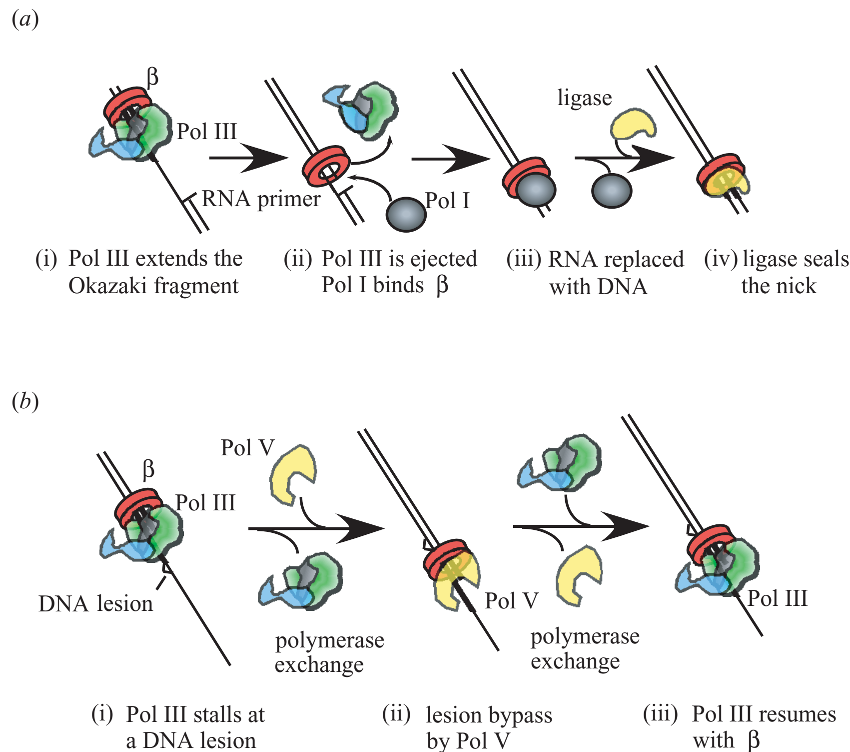

Figure 5. Proposed protein trafficking on β after use by Pol III. Several proteins function with DNA sliding clamps. (*a*)(i) This suggests how Pol III, Pol I and ligase may coordinate their traffic flow on β. After the processivity switch ejects Pol III from β at the end of an Okazaki fragment, Pol I may target the abandoned β (ii) for function in removing the RNA primer with its 5′–3′ exonuclease activity (iii). After Pol I action, ligase is required to seal the nick, and the β clamp may help target ligase to its site of action (iv). (*b*) This proposes how Pol III and Pol IV may coordinate their action on β during lesion bypass. Upon Pol III–β encountering a lesion (i), Pol III may dissociate from β, allowing Pol IV to associate with β at the lesion site (ii). After lesion bypass (iii), Pol V may dissociate from β, allowing Pol III to reassociate with β past the lesion (iv).

1–3 s. First, primase must receive the signal to synthesize an RNA primer, after which it remains attached to the primed site to prevent primer dissociation and protect it against destruction by nucleases. Then the clamp loader dislodges primase in order to load β onto the primed site. After this, the core polymerase must recruit the clamp from the clamp loader. During extension of the primer, the core–β complex remains tightly bound to DNA and to the rest of the replisome machinery, producing a DNA loop (see figure 2*b*).

Upon completing the Okazaki fragment, the core–β collides with the RNA primer of the previous Okazaki fragment (figure 2*c*). At this juncture, the core polymerase must dissociate from the DNA, freeing it for synthesis of the next Okazaki fragment. It seems unlikely that the core will leave the DNA easily and rapidly, as it is held tightly to the template by the β ring. However, studies have shown that the core polymerase indeed rapidly cycles off DNA, but only when synthesis is completely finished (Stukenberg *et al.* 1994). In doing so, the core leaves behind the β clamp on DNA, and therefore must reassociate with a new clamp that has been loaded on the next RNA primer by the clamp loader (figure 2*d*). The leftover β clamps must be recycled off DNA as well, and in fact the clamp loader also unloads clamps from DNA in an ATP-dependent reaction (Stukenberg *et al.* 1994). The RNA primer is then removed by the action of DNA polymerase I, and finally the resulting nick can be sealed by ligase (not shown).

This report will deal with two of the events that occur during the lagging strand cycle. First, how does the clamp transit from the clamp loader to the polymerase? And second, how does the tightly bound polymerase dissociate from β and DNA upon completing an Okazaki fragment? The first of these events was explored a few years ago (Naktinis *et al.* 1996), but will be summarized here, as it relates in significant ways to the mechanism by which the core is released from β upon completing DNA.

3. EVENT FLOW 1: CLAMP TRANSFER FROM CLAMP LOADER TO POLYMERASE

Transit of the clamp from the clamp loader to the polymerase is rooted in the architecture of the β clamp itself. The β clamp is a head-to-tail dimer which results in structurally distinct faces (figure 3*a*; Kong *et al.* 1992). One face has several loops and protruding C-terminal residues, and the other face is relatively flat. Initially, we thought that the clamp loader would function with one side of β and that the polymerase would function with the other, but this raised the issue of why the unloading action of the clamp loader did not occur during use of the ring by the DNA polymerase. Obviously, clamp unloading during polymerase action would be disasterous for processive replication. However, protein–protein interaction studies demonstrated that the polymerase and clamp loader actually bind to the same face of β, the one from which the C-termini protrude, and in fact they compete with one

another for β (Naktinis *et al.* 1996). Moreover, DNA modulates this interaction and determines which protein wins the competition for β (figure 3*b*). In the absence of DNA, the clamp loader binds to β much tighter than the polymerase. But once on primed DNA, the β complex loses affinity for β and core polymerase develops a much tighter interaction with β. Thus, primed DNA determines the event flow from the β–clamp loader complex to the β–core complex. It also answers the question of how clamp unloading is coordinated with polymerization. Polymerase binds β tightly while it is on DNA, and thus the clamp loader does not have access to the clamp for unloading it from DNA. However, when the polymerase completes DNA synthesis, and pops off β and DNA, the β clamp is left alone on DNA and is now susceptible to the γ complex for clamp unloading.

4. EVENT FLOW 2: POLYMERASE TRANSFER FROM CLAMP TO SOLUTION

How does the polymerase know when DNA is completed, and use this information to disengage from β at the end of an Okazaki fragment synthesis cycle? We refer to this process as the 'processivity switch' to reflect the abrupt transition in polymerase behaviour from being highly processive to being fully distributive. The intelligence behind this processivity switch is based on an internal competition reaction between holoenzyme subunits (Leu *et al.* 2003). The competition is modulated by DNA structure, similar to the case described above in which β trades places from the clamp loader to the polymerase. The central actor in this reaction is τ, which senses the difference between ssDNA and dsDNA and modulates the affinity of the core for β. On primed DNA, the τ switch is turned off and the β–core interaction is unperturbed. But upon completing the template, τ is switched on and it prevents the core from binding to β, thereby releasing the core from β and DNA as illustrated in figure 4. This effect can be measured experimentally by using fluorescent tagged β, as described in our earlier study (Leu *et al.* 2003). Using this method, the magnitude of the effect of τ on the K_d of the core–β interaction is presented in table 2. In the absence of τ, the core–β complex forms on primed DNA with a K_d of 18 nM. This value is not significantly altered by the presence of τ. But using dsDNA with a single nick, to mimic the end of an Okazaki fragment, τ reduces the affinity of the core–β interaction over 30-fold. Further examination of this reaction showed that the core retains tight affinity for β even on DNA having a ssDNA gap of only one nucleotide (Leu *et al.* 2003). Filling the gap results in flipping the τ processivity switch causing the core to dissociate from β and DNA.

Exactly how does the τ processivity switch work? The T4/RB69 phage systems demonstrate that the C-terminal residues of the polymerase are essential to maintain productive contact with the gp45 sliding clamp protein (Berdis *et al.* 1996; Shamoo & Steitz 1999). Hence, if the same were true for the *E. coli* core/β system, one may propose that τ binds the C-terminal residues of the α DNA polymerase subunit, only in response to completing a section of DNA. The consequence of this would be to

Table 1. Clamps and clamp loaders in various branches of life.

organism	clamp	clamp loader
Escherichia coli	β	γ complex ($\gamma_1\tau_2\delta_1\delta'_1\chi_1\psi_1$)
Archae	PCNA	RFC (RFC-L: RFC-S, either 1 : 4 or 2 : 3)
yeast	PCNA	RFC (one copy each of RFC1, 2, 3, 4, 5)
human	PCNA	RFC (one copy each RFC1, 2, 3, 4, 5)
T4 phage	gp45	gp44/62 complex (4 : 1 stoichiometry)

Table 2. τ modulates core–β interaction in response to DNA. The β clamp was modified at Cys333 with the Oregon Green fluorophore. Core was titrated into reactions containing a mixture of fluorescent tagged β, τ (where present) and the indicated DNA substrate. Data are from Leu *et al.* (2003).

DNA type	addition	K_d (nM) core–β
primed	τ	16 ± 5
duplex	none	18 ± 5
duplex	τ	> 600

disengage the core polymerase from β, specifically upon finishing DNA.

The two cases of event flow during replication fork progression that have been described above have in common an internal competition reaction between components of the holoenzyme for another subunit. In the first case, event flow during the passage of the clamp from the clamp loader to the polymerase is based in competition for β between the α subunit of core, and the δ subunit of the γ complex. The second event, the processivity switch, is based in competition between β and τ for α.

Although not described so far in this report, the event flow dictating hand off of the primed site from primase to the clamp loader has also been shown to be based on a competition reaction (Yuzhakov *et al.* 1999). In this case, primase must bind SSB to remain associated with the RNA primed site. But primase must be removed for a clamp to be loaded onto it by the γ complex clamp loader. To remove primase, the primase-to-SSB contact is broken by the χ subunit of the γ complex, which binds SSB in a competitive fashion with the primase and displaces primase from the primed site (Yuzhakov *et al.* 1999).

5. PROTEIN TRAFFICKING ON SLIDING CLAMPS

Clamps from a variety of systems have very similar structures (Kong *et al.* 1992; Krishna *et al.* 1994; Gulbis *et al.* 1996; Shamoo & Steitz 1999; Moarefi *et al.* 2000). Crystal structure analysis of clamps in complex with their substrates suggests that proteins have a common method of attachment to clamps. The three existing co-crystal structures, human PCNA–p21 peptide (a cell cycle regulator (Gulbis *et al.* 1996)), phage RB69 polymerase peptide–clamp (Shamoo & Steitz 1999), and *E. coli* δ–β

(Jeruzalmi *et al.* 2001), all reveal a major interaction with a small hydrophobic pocket located in an analogous spot on each of the clamps. Interaction of various proteins with β are possibly all localized to the same small hydrophobic pocket.

Sliding clamps of replicases are used by numerous proteins, not just the replicative DNA polymerase. This has especially been shown to be the case with PCNA, which interacts with numerous proteins in repair, DNA modification and cell cycle control, as well as with several DNA polymerases (Warbrick 2000). In many cases the PCNA attachment site on the interacting protein can be inferred from a sequence search, as the binding motif to PCNA is conserved in some proteins. A common motif is in keeping with the idea that all these proteins bind the clamp at the same location and predicts that they all compete with one another. What determines their event flow?

An outline of event flow for some of these 'extra-replisomal' uses of the clamp may be hypothesized from studies in *E. coli*. The β clamp has been shown to function with ligase and DNA polymerase I, as well as yet other proteins such as MutS and other DNA polymerases (Tang *et al.* 1999, 2000; Lopez de Saro & O'Donnell 2001; Lenne-Samuel *et al.* 2002). An event flow for multiple use of β in Okazaki fragment maturation can be hypothesized as illustrated in figure 5*a*. After completion of an Okazaki fragment, the τ processivity switch ejects the core from DNA, leaving β behind on the finished Okazaki fragment. The β clamp is abandoned precisely where DNA polymerase I (Pol I) action is needed to remove the initiating RNA primer, and β may help target Pol I to this area. Following this job, ligase is needed to join the fragments and after Pol I leaves β, the clamp should be in position to target ligase to its site of action. How does ligase trade places with Pol I? Perhaps the affinity of Pol I for β is diminished when all RNA residues have been excised. Another possibility is that ligase develops a tighter affinity for β after Pol I produces a ligatable nick (i.e. when 3′ and 5′ termini are both DNA) and outcompetes Pol I for β.

Trafficking of different polymerases on sliding clamps is hypothesized for lesion bypass in figure 5*b*. In the case shown in the figure, *E. coli* Pol III–β runs into a lesion on the template strand and is blocked from forward progression. Perhaps at this point the affinity of Pol III for β may decrease, or other proteins involved in lesion bypass, such as RecA, may push Pol III off the β clamp. This would then leave β available for use by an alternative DNA polymerase, such as DNA polymerase V which requires β for bypass of lesions on DNA (Tang *et al.* 2000). The exact mechanisms by which protein trafficking on sliding clamps occurs is an exciting avenue for future studies. In light of the event flows that have been described here in the *E. coli* system, it seems likely that some of these additional protein trafficking events will be characterized by competition reactions that are modulated by the changing nature of the DNA as the reactions transpire.

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GLOSSARY

DnaB: *E. coli* DnaB hexameric helicase
dsDNA: double-stranded DNA
PCNA: proliferating cell nuclear antigen
SSB: single-stranded DNA-binding protein
ssDNA: single-stranded DNA