Competitive processivity-clamp usage by DNA polymerases during DNA replication and repair

Francisco J.López de Saro, Roxana E.Georgescu, Myron F.Goodman[1] and Mike O'Donnell[2]

Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10021 and [1]Departments of Biological Science and Chemistry, University of Southern California, Los Angeles, CA 90089, USA

[2]Corresponding author
e-mail: odonnel@rockefeller.edu

Protein clamps are ubiquitous and essential components of DNA metabolic machineries, where they serve as mobile platforms that interact with a large variety of proteins. In this report we identify residues that are required for binding of the β-clamp to DNA polymerase III of *Escherichia coli*, a polymerase of the Pol C family. We show that the α polymerase subunit of DNA polymerase III interacts with the β-clamp via its extreme seven C-terminal residues, some of which are conserved. Moreover, interaction of Pol III with the clamp takes place at the same site as that of the δ-subunit of the clamp loader, providing the basis for a switch between the clamp loading machinery and the polymerase itself. *Escherichia coli* DNA polymerases I, II, IV and V (UmuC) interact with β at the same site. Given the limited amounts of clamps in the cell, these results suggest that clamp binding may be competitive and regulated, and that the different polymerases may use the same clamp sequentially during replication and repair.
Keywords: β-clamp/clamp loader/DNA polymerase/ DNA repair/PCNA/RFC

Introduction

Protein clamps have been implicated in all processes of DNA metabolism, including replication, transcription, and various pathways of DNA repair (Warbrick, 2000; Stucki *et al*., 2001). Their biological significance stems, first, from the fact that they are tightly bound to DNA in a topological rather than sequence-specific manner that allows them to slide freely on the nucleic acid (Kong *et al.*, 1992; Stukenberg *et al.*, 1991) and, secondly, from the fact that they can interact with a variety of enzymes to increase their association to DNA. Many proteins that bind to the eukaryotic clamp [proliferating cell antigen (PCNA)] contain a consensus motif (Qxx[I/L]xxFF) that often localizes to the extreme N- or C-terminal regions of the protein (Warbrick, 2000). Polymerases from bacteriophages T4 and RB69 have also been found to interact with their clamps via conserved C-terminal sequences (Berdis *et al*., 1996; Shamoo and Steitz, 1999). Recently, the Y-family polymerase Pol IV of *Escherichia coli* was

also found to interact with the β-clamp via C-terminal residues (Lenne-Samuel *et al*., 2002).

In the case of bacterial chromosomal replicases of the Pol C family, exemplified by DNA polymerase III of *E.coli*, crystal structure information is lacking and no clear map of the clamp–polymerase interaction has been available. The DNA polymerase III core of *E.coli* consists of three subunits: α (DNA polymerase), ε (3′–5′ proof-reading nuclease) and θ. The α DNA polymerase subunit of the Pol III core is responsible for the interaction with β (Stukenberg *et al*., 1991). Deletion studies of the *E.coli* α-subunit have suggested that the β interaction site resides in an internal region of the α-protein, spanning residues 542–991 (α is 129 kDa or 1160 amino acids) (Kim and McHenry, 1996). More recently, and based on limited similarity with other β-binding proteins, Dalrymple *et al*. (2001) proposed a β binding consensus sequence of QLxLF and suggested that β binds at an internal sequence of α at position 918–926. While we cannot exclude a role of internal sequences in the binding to the β-clamp, we have recently performed quantitative binding and functional assays that demonstrate that α binds β mainly through a different site located at the C-terminal 20 residues, and that these residues are required for function of α with β (López de Saro *et al*., 2003). These C-terminal residues of α are also required for the τ switch, which acts on α to regulate polymerase processivity.

In *E.coli* it has been shown that the β-clamp can associate with all five known DNA polymerases in the cell (Pol I–V) to increase their processivity of synthesis (Hughes *et al*., 1991; Bonner *et al*., 1992; Tang *et al*., 2000; Wagner *et al*., 2000; López de Saro and O'Donnell, 2001; Kobayashi *et al*., 2002). These polymerases belong to four different structural families, namely family A (Pol I), family B (Pol II), family C (Pol III) and the newly discovered Y family (Pol IV and V) (Braithwaite and Ito, 1993; Ohmori *et al*., 2001; Filée *et al*., 2002), which have very limited or no apparent sequence similarity between them.

Stimulation of DNA synthesis by the β-clamp is dependent on the topological linkage of the clamp to DNA (Stukenberg *et al.*, 1991). This process involves the transient opening of the protein ring catalyzed by the γ-complex clamp-loader (γ₃δδ′χψ) in a reaction driven by ATP hydrolysis (reviewed in O'Donnell *et al*., 2001). Within the γ-complex, the δ-subunit binds β the tightest and is responsible for opening of the clamp (Turner *et al*, 1999). In combination with the other γ complex subunits, the primed DNA is placed into the open β-ring and then ATP is hydrolyzed, allowing the ring to close around the DNA. Study of the β-clamp interaction with the δ-subunit of the γ-complex and the α-subunit of the Pol III core shows that they compete for β, and thus likely bind β in the same, or nearly the same, location (Naktinis *et al*., 1996).

During lagging strand synthesis, Pol III core rapidly dissociates from the clamp at the end of each completed Okazaki fragment and hops to a new β-clamp on the next RNA-primed site to be extended (O'Donnell, 1987). The β-clamp is left behind on the completed DNA fragment and is free to function with other β-interactive proteins (Stukenberg *et al.*, 1994). These include proteins involved in Okazaki-fragment maturation (Pol I and ligase), replication-associated DNA repair (Pol I and II), mutation-prone lesion bypass (Pol IV and V), and mismatch repair (MutS).

The elucidation of the molecular structure of the γ-complex (Jeruzalmi *et al.*, 2001), and of the δ-subunit of the γ-complex bound to the β-clamp (Jeruzalmi *et al.*, 2001), was the first step in understanding the molecular details of how a protein interacts with β. The main attachment site of δ to β is mediated by δ-residues Leu73 and Phe74, which are highly conserved among prokaryotic δ-subunits (Jeruzalmi *et al.*, 2001). The site on β to which δ binds is a hydrophobic pocket and residues that define the hydrophobic pocket of β are conserved (Jeruzalmi *et al.*, 2001).

The most defined view of how a DNA polymerase interacts with its clamp is provided by the crystal structure of an 11-residue C-terminal peptide of phage RB69 DNA polymerase (a B-family polymerase) bound to its corresponding clamp, the gp45 protein (Shamoo and Steitz, 1999). The interface mainly consists of a hydrophobic pocket in the gp45 clamp, similar to that displayed by δ binding to β. The cocrystal structure of a C-terminal peptide of the cell regulator, p21^{WAF1}, to the human PCNA clamp is also mediated by a hydrophobic pocket in PCNA (Gulbis *et al.*, 1996). In all three systems the clamps have remarkably similar architecture, consisting of six domains organized on either a dimer (β) or trimer (PCNA, gp45), and interaction with the clamp takes place at equivalent locations between domains of the clamps. These similarities suggest that the basic mechanism by which proteins bind to sliding clamps is conserved across all domains of life.

As a first step towards understanding how clamps are used by multiple proteins during DNA synthesis and repair, one aim of the present study is to map in detail the site of interaction of the *E.coli* replicative polymerase, the α-subunit of Pol III, with the β-clamp and to examine whether it binds the same site in β as the δ-subunit of the clamp loader. We find that the C-terminal peptide of the Pol III α subunit inhibits clamp loading by the γ-complex and binding to β by the δ-subunit, suggesting that the α·β and δ·β interactions do indeed occur at the same locus on the clamp. This observation provides the structural basis for the internal competition for clamps in the Pol III holoenzyme replicative machinery (Naktinis *et al.*, 1996).

Another goal of the present study is to determine whether the other *E.coli* polymerases also interact with β in the same locus as α and δ, and if the interaction is mediated by C-terminal residues. We find that DNA polymerase II (Pol II), despite having no sequence similarity with the Pol III α-subunit, also interacts with the β-clamp in the same location, via the C-terminal end of the protein. Pol IV also interacts with β via C-terminal residues, consistent with a previous study (Lenne-Samuel *et al.*, 2002), and we find that it binds β in the same locale as α and Pol II. Furthermore, we find that the sites of interaction between the clamp and DNA polymerases I and V are the same as those of Pol II and Pol III, although these polymerases do not appear to utilize C-terminal residues for this interaction. Overall, we find that all *E.coli* polymerases interact at the same point on the clamp, namely the hydrophobic pocket to which the δ-subunit binds. The implications of these findings with respect to how these enzymes traffic on the β-clamp are discussed.

Results

Characterization of an α-subunit C-terminal peptide interaction with the β-clamp

We have recently demonstrated that the C-terminal 20 residues of the α-subunit are required for functional interaction with β (López de Saro *et al.*, 2003). To define the critical residues that contribute to the α–β interaction, we designed overlapping N-biotinylated peptides spanning the C-terminal region of α and assayed them for interaction with the β-clamp by attaching them to streptavidin-coated microplates and then probing them for ability to bind ^{32}P-β. Radiolabeled β used in these studies contained a six-residue C-terminal extension that can be phosphorylated with protein kinase A and [^{32}P]γ-ATP (Stukenberg *et al.*, 1994; Kelman *et al.*, 1995). We found that peptides corresponding to the C-terminal 20 amino acids interact with the β-clamp, as did a peptide lacking the last residue, but deletion of two residues from the C-terminus abolished the interaction (Figure 1A). Hence, the penultimate F-residue of the α-peptide is required for interaction with β.

Next, we used a protein shift assay (López de Saro and O'Donnell, 2001) to determine whether the C-terminal α-peptide (α1141–1160) complexed to streptavidin in solution is sufficient to bind to ^{32}P-β and to produce a mobility shift in a native polyacrylamide gel. The results showed that streptavidin does indeed produce a slower-mobility complex, presumably consisting of streptavidin·biotin-peptide bound to ^{32}P-β (Figure 1B, lane 4). We also observed that Pol III core retards the mobility of ^{32}P-β in a native gel (Figure 1C, compare lanes 1 and 2). If the polymerase·β interaction requires the α C-terminal sequences, the α1141–1160 peptide may be expected to disrupt this interaction, thus displacing the ^{32}P-β from core polymerase, and the results showed that this is in fact the case (Figure 2C, lane 6). To demonstrate specificity of this interaction further, heterologous clamps were tested for displacement of the Pol III core·^{32}P-β interaction. The addition of excess yeast PCNA clamp or phage T4 gp45 clamp did not displace core from ^{32}P-β (Figure 1C, lanes 4 and 5, respectively). Nor did the α-peptide bind to human ^{32}P-PCNA or inhibit the binding of ^{32}P-PCNA to the cell-cycle regulator p21^{WAFI} (data not shown). As expected from their ability to compete with DNA polymerase for β, the β-binding peptides α1140–1159 and α1141–1160 were inhibitory to Pol III core when added to a replication reaction using primed M13mp18 ssDNA as template; core requires β for synthesis of this substrate (Figure 1D).

Similar binding assays as described above were performed on peptides spanning the N-terminal region of α and the region of the protein from residue 812 to 991 (gray boxes in the key of Figure 1A), with negative results (data not shown). Since the interaction could require a specific
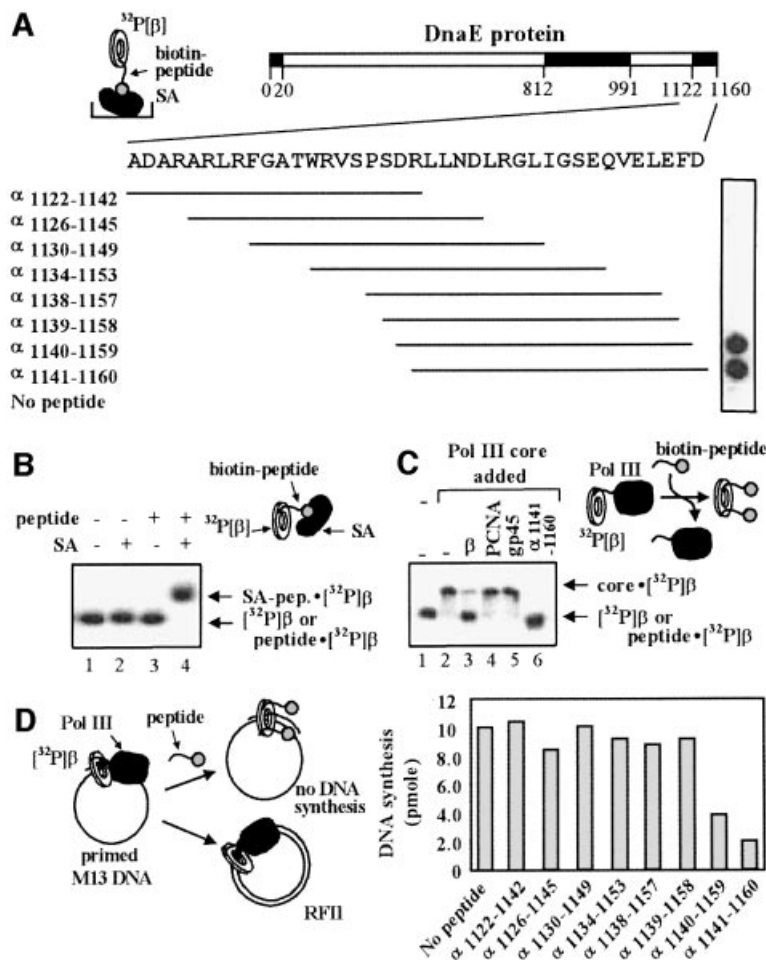
Fig. 1. The extreme C-terminus of the α-subunit of DNA polymerase III interacts with the β-clamp. (**A**) Sequences probed by peptide analysis are shown as stippled boxes in the DnaE protein scheme, but only the analysis of the C-terminal peptides is shown. N-terminal biotinylated peptides were immobilized on streptavidin-coated 96-well plates and probed with ^{32}P-β. It is estimated that 30 nmol of peptide is retained in each well. Peptide sequences used in the microplate assay on the right are shown as lines under the sequence of the C-terminus. Results of the assays are shown to the right. (**B**) Native polyacrylamide electrophoresis was used to separate a complex of ^{32}P-β with biotinylated α1141–1160 bound to streptavidin. Lane 1 contained only ^{32}P-β; the α1141–1160 peptide (220 nM) was added in lanes 3 and 4, and streptavidin (2.2 μM) in lanes 2 and 4, as indicated. (**C**) Gel shift assay of the radiolabeled β-clamp. Native polyacrylamide electrophoresis was performed to separate free ^{32}P-β from ^{32}P-β·core complexes as described in Materials and methods. Pol III core was added in lanes 2–6 and the complex was challenged with either BSA (lane 2), non-labeled *E.coli* β-clamp (lane 3), human PCNA (lane 4), phage T4 gp45 (lane 5) or α1141–1160 peptide (lane 6), as indicated above the gel. (**D**) DNA synthesis is inhibited by α C-terminal peptides. Reactions were performed using primed M13mp18 ssDNA, β, core and γ complex in the presence of 100 μM of each peptide as described in Materials and methods.

conformation not provided by 20-mer peptides, our results do not exclude the possibility that additional sites of contact between α and the β-clamp could be present in this area, as suggested by the studies of Kim and McHenry (1996). On the other hand, our results do not detect the interaction observed by Dalrymple *et al.* (2001), in which a sequence corresponding to region 918–926 of α (sequence IGQADMFGV), fused onto a reporter protein, resulted in an interaction with the β-clamp using a yeast two-hybrid assay. Peptides spanning this region do not show binding to the β-clamp using the assays and conditions described here (data not shown).

Alanine-scan analysis of the α C-terminal peptide

To identify residues at the C-terminus of the α-subunit of Pol III that are critical for interaction with β, we performed an alanine scan analysis of the α1141–1160 peptide. These peptides were used in three different assays: (i) direct

binding of ^{32}P-β to biotinylated peptide immobilized in wells of a microtiter plate (Figure 2A); (ii) ability to displace Pol III core from a ^{32}P-β·core complex observed in a native polyacrylamide gel (Figure 2B); and (iii) ability to displace ^{32}P-β from δ-subunit immobilized in wells of a microtiter plate (Figure 2C). The δ–β displacement assay is based on the previous finding that binding of α and δ to β is sterically exclusive, and that the same single amino-acid changes on β that abolish interaction with α also abolish interaction with δ, suggesting that α and δ share a common site on β (Naktinis *et al.*, 1996). This conclusion is supported here by the finding that peptide α1141–1160 competes ^{32}P-β off the δ subunit (Figure 2C).

While most alanine mutants of the α1141–1160 peptide retain full activity for binding ^{32}P-β, mutation of residues Q_{1154}, L_{1157} and F_{1159} to alanine clearly diminished the activity of the peptides in all three assays. Interestingly, peptide D1160A showed no retention of ^{32}P-β on the
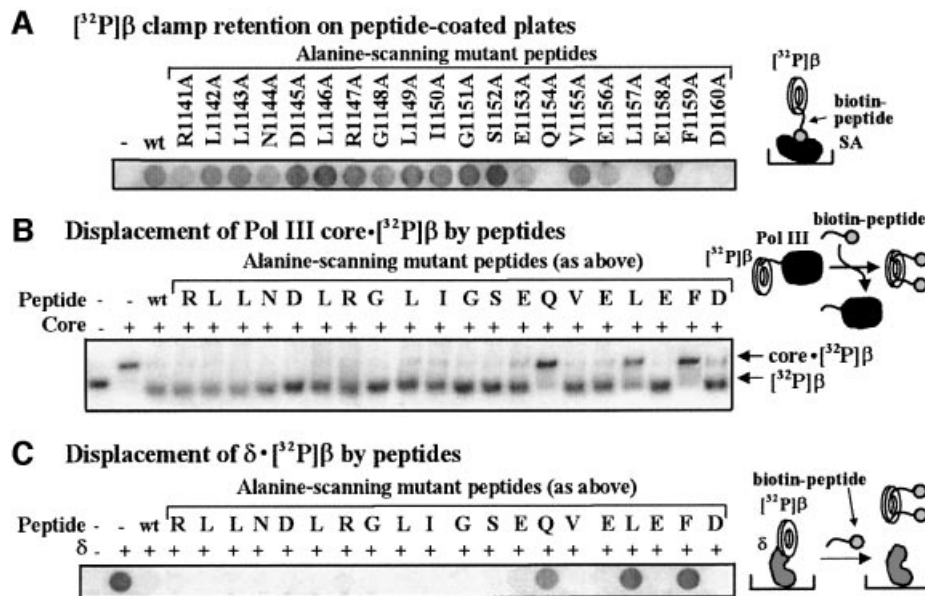
A [^{32}P]β clamp retention on peptide-coated plates

Alanine-scanning mutant peptides

- wt R141A L142A L143A N144A D145A L146A R147A G148A L149A I150A G151A S152A E153A Q154A V155A E156A L157A E158A F159A D160A

[^{32}P]β ↔ biotin-peptide / SA

B Displacement of Pol III core·[^{32}P]β by peptides

Alanine-scanning mutant peptides (as above)

| Peptide | - | - | wt | R | L | L | N | D | L | R | G | L | I | G | S | E | Q | V | E | L | E | F | D |
| Core | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

← core·[^{32}P]β
← [^{32}P]β

Pol III / biotin-peptide / [^{32}P]β

C Displacement of δ·[^{32}P]β by peptides

Alanine-scanning mutant peptides (as above)

| Peptide | - | - | wt | R | L | L | N | D | L | R | G | L | I | G | S | E | Q | V | E | L | E | F | D |
| δ | - | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

biotin-peptide / [^{32}P]β / δ

Fig. 2. Alanine scan analysis of α C-terminal peptide binding to β. (**A**) Peptides were pre-bound to streptavidin-coated microtiter plates and probed with ^{32}P-β as in Figure 1A. A clear spot indicates a critical residue for ^{32}P-β binding. (**B**) Peptides were used to compete Pol III core off the ^{32}P-β·core complex in the native PAGE mobility shift assay, as in Figure 1C. (**C**) Purified δ protein (20 pmol) was fixed to microtiter plates and probed with ^{32}P-β in the presence of the indicated peptide. Retention of ^{32}P-β in the well indicates that a critical residue has been changed to Ala.

microtiter plate (Figure 3A), but was almost as effective as wild-type peptide in competition assays (Figure 3B and C). Since deletion of residue D_{1160} (peptide α 1140–1159) in the experiment shown in Figure 1A did not impair binding to ^{32}P-β, we suggest that the presence of alanine at the position of D_{1160} lowers the affinity of peptide for the β-clamp. The large concentration of peptide used in the competition assays of Figure 1B and C may overcome the deficiency.

Deletion of the C-terminal seven residues of α greatly reduces interaction with the β-clamp
In light of the experiments described above, we deleted the seven C-terminal residues of α that contain the three critical β-interactive residues identified in the alanine scanning experiments. This C-terminal deletion will be referred to here as αΔ7. We then labeled the β-clamp with the fluorescent 488 Oregon Green (OG) probe at the single exposed Cys333, and used fluorescence to determine the affinity of the labeled β-clamp for wild-type α and for αΔC7. Cys333 is on the opposite face of β from where α binds, and OG-labeled $β^{OG}$ was as active as wild-type β in DNA synthesis activity assays with γ-complex and core (data not shown). Titration of α and αΔ7 into $β^{OG}$ reveals that αΔC7 has a 10-fold reduced affinity for the β-clamp (Figure 3A; K_D of 1.16 μM versus 0.11 μM at 100 mM NaCl). The residual binding of αΔC7 for the β-clamp could be due to additional interaction points between the two proteins, perhaps in regions distinct from the C-terminal region. Alternatively, C-terminal residues immediately upstream could participate in binding β via protein backbone contacts with the clamp, as in the p21·PCNA complex (Gulbis *et al.*, 1996). This possibility is supported by our earlier study of αΔ20 (deletion of the C-terminal 20 residues), which binds β 100-fold weaker than wild-type α (López de Saro *et al.*, 2003). In this case,

the upstream residues that contribute peptide backbone contacts to β would not be detected by the alanine scanning experiments described above. This residual affinity of αΔC7 for the β-clamp was clearly salt dependent (Figure 3B), suggesting that the contribution of the last seven residues has a strong hydrophobic component, consistent with the requirement of L_{1157} and F_{1159} in the interaction of α with β.

DNA synthesis assays performed with αΔC7 reconstituted with the ε and θ subunits to form Pol III (αΔ7) core showed that its activity in a β-clamp-dependent replication assay is considerably lower than that of wild-type core (Figure 3C). This result supports the conclusion that the extreme C-terminal seven residues of α are important to functional interaction with β. Both core complexes displayed similar activities in a β-independent extension assay using gapped DNA as a substrate (not shown), indicating that αΔC7 is not impaired in its catalytic activity.

Pol III core interacts with β at the same locus as the δ subunit of the clamp loader
During lagging strand synthesis, the clamp loader (γ-complex) loads β-clamps onto multiple RNA primers. The K_D of γ-complex for β in solution is 8 nM, ~30-fold lower than that of the Pol III core·β complex (250 nM). However, when β is on DNA this affinity is reversed, resulting in a K_D value for the Pol III core·β of <5 nM (Naktinis *et al.*, 1996) and a reduced affinity of the γ-complex for β (Turner *et al.*, 1999; Ason *et al.*, 2000). This DNA-modulated competition between clamp loader and polymerase for β generates a molecular switch that assures an ordered sequence of events during the assembly of the replicase at a primed site (Ason *et al.*, 2003). A previous study on the site of interaction of δ subunit (of γ-complex) with β, and α subunit (of Pol III core) with β, demonstrated that both
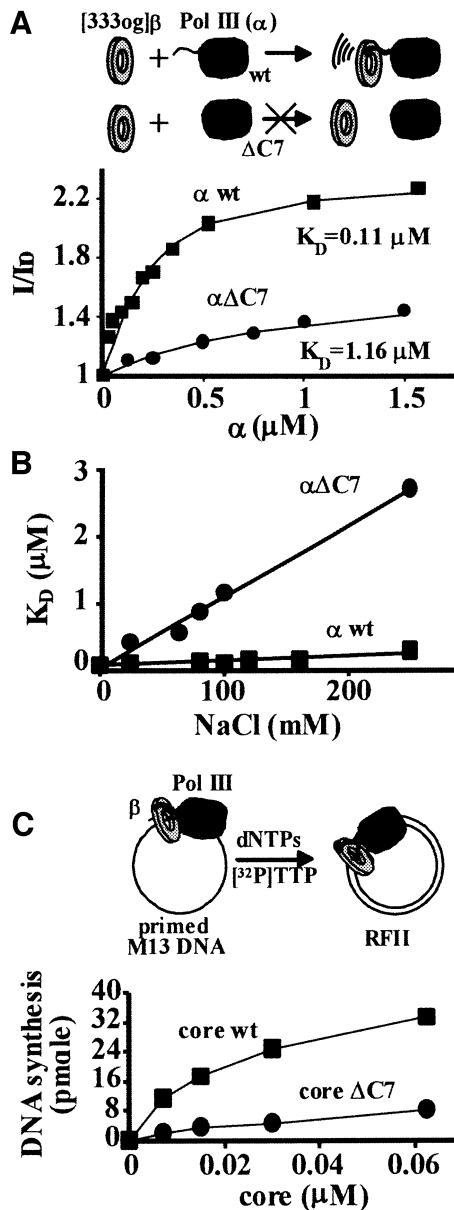
A

B

C

Fig. 3. An α-subunit truncated in the C-terminal seven residues loses affinity and function with the β-clamp. (**A**) Fluorescently labeled β-clamp (β^{OG}) at residue 333 was used in K_D measurements with wild-type α (squares) and αΔC7 (circles). (**B**) K_D measurements were obtained over a 0–230 mM range of NaCl. (**C**) Wild-type core (squares) and core reconstituted using αΔC7 (circles) were assayed for processive DNA synthesis on singly primed M13mp18 ssDNA in the presence of the β-clamp and γ-complex.

proteins interact with the clamp near the C-terminus, which is near the hydrophobic pocket between β domains II and III (Naktinis *et al.*, 1996). Here, as shown in Figure 2C, we find that the 20-mer peptide derived from the C-terminus of α displaces δ from ^{32}P-β. This result indicated that the α and δ proteins bind the same site on β, rather than competing by steric occlusion, in which two large proteins bind different sites on β, but are too close for both α and δ to bind β at the same time.

The ability of the α C-terminal peptide to compete δ from β suggests that this peptide may inhibit the clamp loading reaction. Indeed, the peptide inhibits loading of the
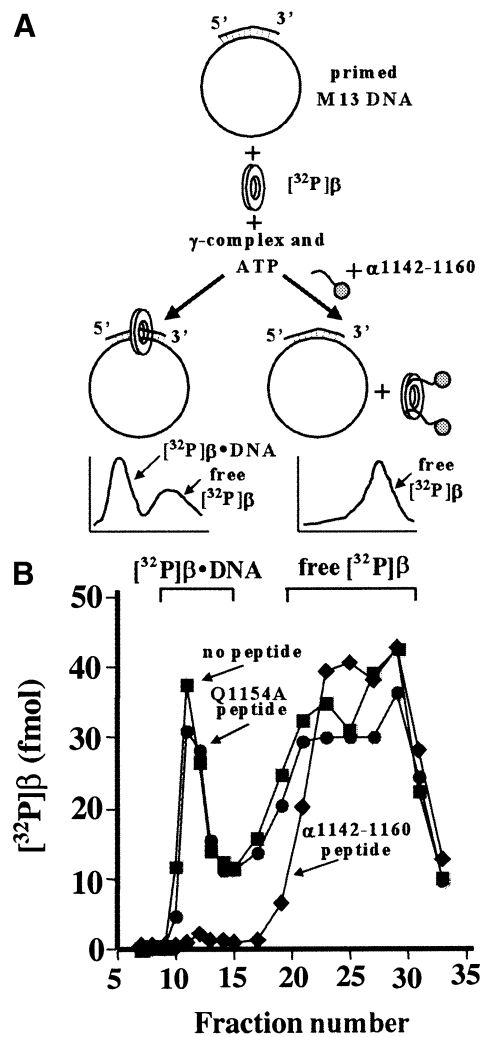
A

B

Fig. 4. The α C-terminal peptide inhibits β-clamp loading by the γ-complex. (**A**) In the presence of ATP, the γ-complex (γ₃δδ'χψ) loads ^{32}P-β onto primed M13mp18 ssDNA, resulting in a complex with a molecular mass that elutes earlier than free ^{32}P-β in gel filtration. (**B**) The plot shows the gel filtration profiles of reactions without peptide added (squares), or with a peptide that does not interact with ^{32}P-β (Q1154A) (circles), or with wild-type α1141–1160 peptide that binds and inhibits the clamp loading reaction (diamonds).

β-clamp on DNA by γ-complex, but a related peptide mutated in a single residue (Q1154A) does not (Figure 4). These results further support the conclusion that the site of interaction of δ and α with β is the same, namely the hydrophobic pocket between domains II and III of the β ring, which is near the β C-terminus.

Conserved residues at the C-terminus of Pol II, Pol III and Pol IV interact at the same site of β

The α C-terminal residues identified by alanine scanning as being essential for interaction with β appear to be present in the C-terminal sequences of Pol II and Pol IV, although the spacing is somewhat different (Figure 5A). A peptide corresponding to the C-terminus of Pol IV has been shown recently to interact with β directly, and a C-terminal deletion of this Y-family polymerase no longer functions with β (Lenne-Samuel *et al.*, 2002). The experiment shown in Figure 5B (upper panel)

demonstrates that immobilized peptides derived from the C-terminus of either Pol II or Pol IV interact with ^{32}P-β and retain ^{32}P-β in ELISA plates. If these Pol II and Pol IV peptides bind β at the same site, they should be capable of competing ^{32}P-β off δ protein. The experiment shown in Figure 5B (lower panel) illustrates that this prediction is valid. Immobilized δ retains ^{32}P-β in the well of a microtiter plate, and the addition of C-terminal peptide derived from α, Pol II or Pol IV displaces ^{32}P-β from the immobilized δ. Further, the Pol II and Pol IV proteins also displace Pol III core from the Pol III core·β complex in the native PAGE assay, as shown in Figure 5C. The 20-mer peptides derived from the C-terminus of Pol II and Pol IV also inhibit β-dependent replication by Pol III core (data not shown).

The alignment of the C-terminal tails of various polymerases reveals slight differences in the presence and/or spacing of the residues that are important for α to bind β (see Figure 5A). To assess the importance of these variations to the relative affinity to β, we compared the Pol II and Pol III C-terminal peptides for their effectiveness in disrupting polymerase·clamp complexes. In Figure 5D (upper panel) we titrated the 20-mer peptide of Pol II into the ^{32}P-β·Pol III core complex, and in the lower panel, the C-terminal Pol III α 20-mer peptide was titrated into ^{32}P-β·Pol II complex. The results demonstrate that the peptides have similar efficiencies in disrupting the polymerase–clamp complex.

We next determined the apparent K_D of Pol III core·β (51 ± 9 nM) and Pol II·β (120 ± 23 nM) using β^{OG} (Figure 5E, upper panels). To compare the relative affinity of the C-terminal 20 residues of both polymerases for β, we used synthetic peptides labeled at their N-terminus with rhodamine (TAMRA); the apparent K_D values calculated from titration measurements were 3.2 μM for the α peptide and 3.5 μM for the Pol II peptide, revealing that despite the differences in sequence, both peptides bind to the clamp with very similar affinities (Figure 5E, lower panels). Calculation of the free energy of the polymerase·β interaction indicates that ~75% of the interaction energy between Pol III and β resides in the C-terminal 20 residues (ΔG = 9.94 kcal/mol for Pol III·β, ΔG = 7.50 kcal/mol for Pol III peptide·β) as does 79% of the interaction energy

between Pol II and β (ΔG = 9.45 kcal/mol for Pol II·β, ΔG = 7.44 kcal/mol for Pol II peptide·β).

DNA polymerases I and V (UmuC) also interact with β in the same locus as Pol III

Our previous studies have shown that the β-clamp can stimulate the Klenow fragment of DNA polymerase I in synthesis on SSB-coated, singly primed M13mp18 ssDNA (López de Saro and O'Donnell, 2001). However, the C-terminus of the Klenow fragment lacks a sequence containing the β-binding residues of the Pol III α-subunit. Consistent with this, we detected no binding to the β-clamp of a C-terminal 20-residue peptide derived from
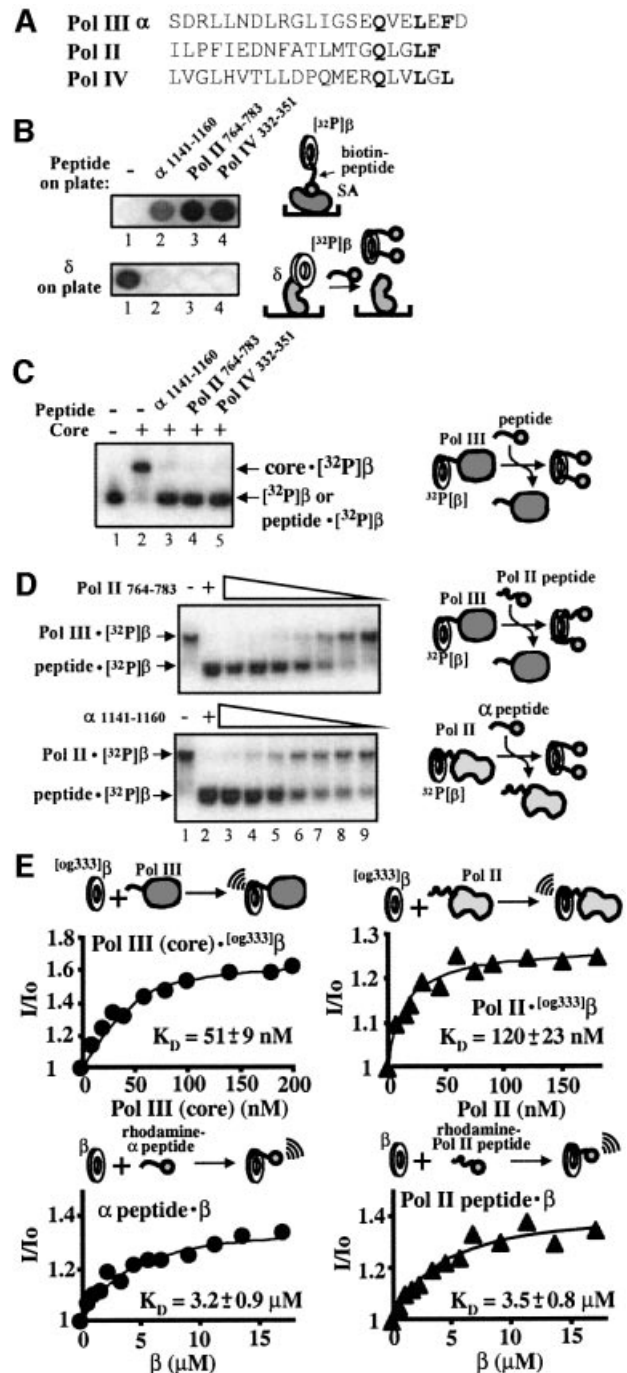

Fig. 5. Pol II and Pol IV interact with the β-clamp via extreme C-terminal sequences. (**A**) C-terminal sequences from Pol III, Pol II and Pol IV. Conserved residues that correspond to residues in *E.coli* α needed to bind β highlighted. (**B**) Synthetic biotinylated peptides derived from the C-terminus *E.coli* Pol II and Pol IV bind ^{32}P-β as determined using streptavidin-coated microtiter plates (upper panel), and by ability to disrupt the δ·^{32}P-β complex (lower panel). (**C**) C-terminal peptides from Pol II and Pol IV displace Pol III core from ^{32}P-β·core complex in a native PAGE assay (as in Figure 1C). (**D**) The native PAGE assay was used to challenge the ^{32}P-β·core complex with C-terminal Pol II peptide (Pol II764–783) (left panel) or the ^{32}P-β·Pol II complex with a C-terminal α peptide (α1141–1160) (right panel). Peptide was added in reactions 3–10 as follows: 500, 500, 250, 125, 62.5, 31.2, 15.6 and 7.8 μM, respectively. Concentrations of Pol III core (left panel) and Pol II (right panel) were 500 nM and 925 nM, respectively. (**E**) Fluorescence K_D measurements of the interaction between Pol III core (upper left panel) or Pol II to β^{OG} as described in the Materials and methods. The lower panels show the interaction of wild-type β with rhodamine-labeled peptides corresponding to the C-terminal 20 residues of α (left panel) and Pol II (right).
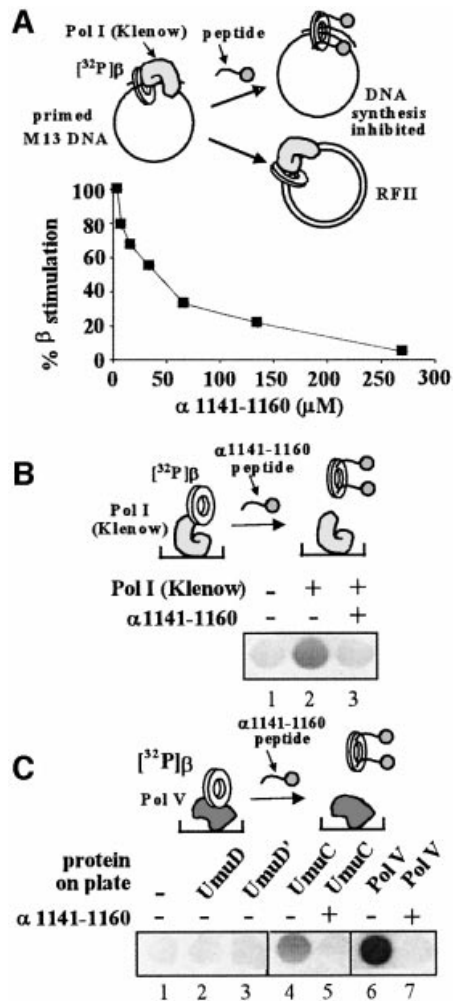
Fig. 6. Pol I (Klenow fragment) and Pol V also bind the β-clamp in the same locus as δ, Pol III, Pol II and Pol IV. (**A**) Stimulation of DNA synthesis by the β-clamp is inhibited by peptide α1141–1160. The plot shows the result of a titration of the α1141–1160 peptide into a β-stimulated Pol I Klenow fragment DNA replication assay on primed M13mp18 ssDNA. β was loaded on the primed template prior to the addition of Pol I. (**B**) Pol I Klenow fragment (50 pmol) was fixed in microtiter plates and assayed for binding to ^{32}P-β in the presence or absence of α1141–1160 peptide (100 μM). (**C**) UmuD, UmuD′, UmuC or Pol V (UmuD′$_2$UmuC) (50 pmol each) were fixed in a microtiter plate and probed with ^{32}P-β. In reactions 5 and 7, 100 μM α1141–1160 peptide was added.

the Klenow sequence (data not shown). However, the Pol III α subunit peptide (α 1141–1160) did inhibit β-stimulated DNA synthesis by Pol I Klenow fragment (Figure 6A). In this experiment the β-clamp was loaded onto the primed DNA before the peptide and Pol I (Klenow fragment) were added. Hence, the observed inhibition is likely the result of disruption of the Pol I·β contact by the peptide. Further, ^{32}P-β is retained in wells by immobilized Klenow fragment (Figure 6B), suggesting that the site on β that is bound by the α C-terminal peptide and the Pol I Klenow fragment is the same, and that both polymerases interact with the clamp in a competitive manner.

Finally, we turned our attention to Pol V of *E.coli*, a heterotrimeric polymerase of the Y-family with the subunit structure UmuD′$_2$UmuC (Woodgate *et al.*, 1989;

Bruck *et al.*, 1996) that is involved in translesion synthesis (Tang *et al.*, 2000). UmuD′ (115 amino acids) is produced by RecA-induced proteolytic removal of an N-terminal peptide from UmuD (139 amino acids). We examined 20-mer peptides derived from the N- and C-terminal sequences of UmuD and UmuC for binding to the β-clamp, with negative results. We then fixed the full UmuD, UmuD′, UmuC and Pol V complex (UmuD′$_2$UmuC) to ELISA plates and probed the immobilized protein with ^{32}P-β. As shown in Figure 6C, only UmuC and Pol V complex showed an interaction with ^{32}P-β, and these interactions were disrupted by the addition of the Pol III α C-terminal peptide (α 1141–1160). These results indicate that the main connection between Pol V and β is mediated by the single UmuC subunit, and that it binds to β in the same location as Pol III and the other DNA polymerases. Consistent with this, Dalrymple *et al.* (2001) have reported, on the basis of sequence similarity, a putative binding site on UmuC for the β-clamp that is situated ~62 residues from the C-terminus, and more recently it has been shown that removal of these residues abolishes the lesion bypass ability of Pol V (Becherel *et al.*, 2002). This sequence (QLNLF) resembles the motifs present in the other *E.coli* DNA polymerases. An interaction between UmuD and β has been reported using crosslinking assays (Sutton *et al.*, 1999). Despite identification of a UmuC-β interaction, we cannot exclude the possibility that UmuD or UmuD′ may interact with β in a fashion undetected under the conditions of this report.

Discussion

Protein clamps play a central role in DNA metabolism, either as processivity factors for DNA polymerases or to orchestrate complex multienzymatic reactions. In this report we performed a detailed analysis of a β-clamp interaction motif in the main replicase, a C-family DNA polymerase. Peptide mapping revealed critical residues at the extreme C-terminus of the α subunit needed to function with β, consistent with our previous finding that this region is critical for the sequential binding and release of the clamp during Okazaki-fragment synthesis. We find that the α subunit of Pol III interacts with the β-clamp at the same locus as the δ-subunit of the γ-complex, providing the basis for the competitive switch between the loading machinery and the polymerase. We have shown that this competitive switch can occur for all five *E.coli* polymerases interacting with the β-clamp at a common locus.

A peptide tail in Pol II, III and IV interact with the β-clamp

This report demonstrates that *E.coli* Pol II, III and IV interact with the β-clamp via the C-terminal tail of these polymerases. It has been hypothesized that attachment via a flexible C-terminal extension could allow for the clamp and polymerase to rotate with respect to one another, perhaps aiding the 3′ terminus of DNA to switch from the polymerization to the exonuclease site (Shamoo and Steitz, 1999). Some degree of flexibility would also be advantageous if two different polymerases were to bind the same clamp concurrently, as some models have proposed. Since the β-clamp is a homodimer, two
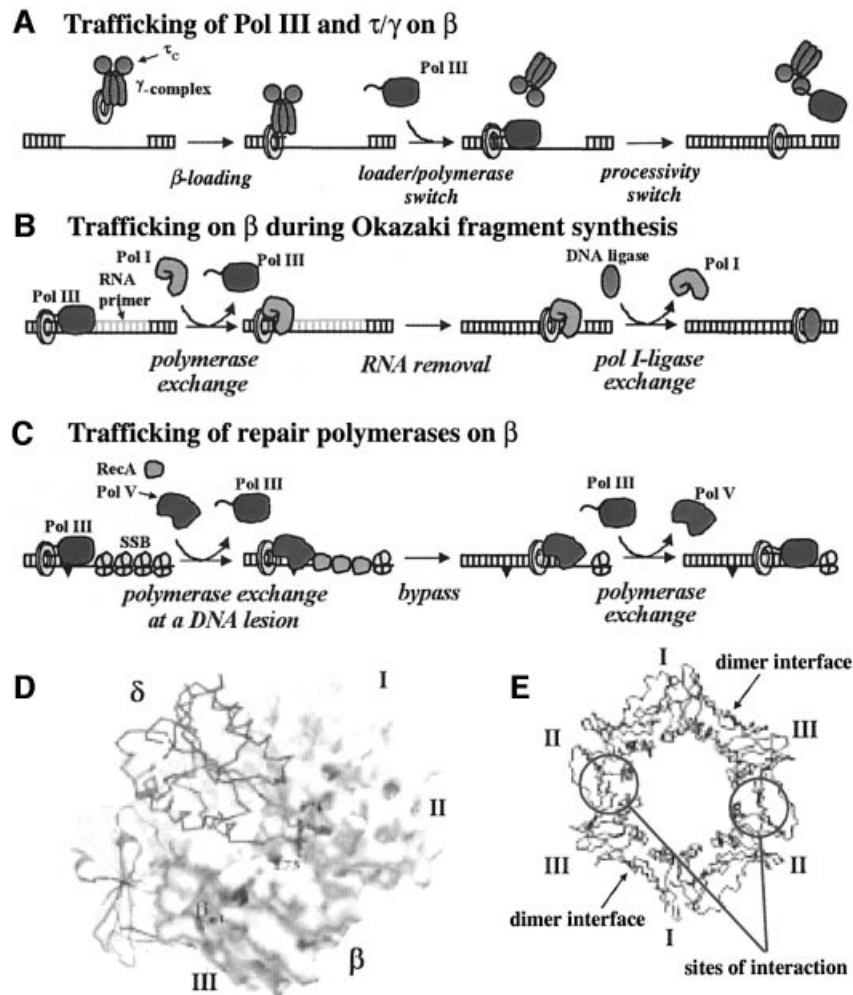
Fig. 7. Trafficking of various proteins on β-clamps at different stages of replication and repair. (**A**) The γ-complex loads β-clamps on DNA, which are then used for processive elongation by Pol III core. These proteins trade places on β. The affinity of these proteins for β is modulated by whether β is on or off DNA, thus regulating this traffic flow as described in the text. (**B**) Pol I and ligase are thought to interact sequentially during removal of the RNA primer and sealing of the nick during Okazaki fragment synthesis. β-clamps interact with repair proteins which may utilize β for genome surveillance directly after Okazaki fragment maturation. (**C**) Lesion bypass polymerases IV and V are thought to replace Pol III at sites of replication blocks or damage. Pol III may take over with β again after bypass is complete. (**D**) Model of the crystal structure of δ-subunit bound to the β-clamp. Domains of the β-dimer are labeled I, II and III. δ interacts with β via two residues of an exposed loop (Leu73 and Phe74) that bind a hydrophobic pocket between domains II and III of β. (**E**) Location of the sites of interaction on β of the DNA polymerases and the δ-subunit.

hydrophobic pockets could potentially be available for binding two proteins. The arrangement of the subunits places both sites on the same face of the ring, which may create steric constraints that preclude binding of more than one protein at a time. No conclusive evidence has been presented in favor of or against this possibility, but the alternative model, one involving sequential binding by different proteins, is also possible. In this case, different proteins would compete for attachment to the clamp, and traffic flow on β could be regulated by other factors, such as DNA structure or post-translational modifications of the clamp.

Competition of proteins for the same clamp has been documented in the case of the γ-complex and the Pol III core, which switch their affinity for β-clamps sequentially during the first steps in processive DNA synthesis (illustrated in Figure 7A). The switch is regulated by whether DNA is inside the β-ring or not (Stukenberg *et al.*, 1994). When β is in solution (i.e. not on DNA), γ-complex

binds β tighter than core, but when DNA goes through β, core develops a much greater affinity for β and takes the ring over from the clamp loader (Ason *et al.*, 2003). A second switch, also dependent on DNA structure, occurs at the end of processive Okazaki-fragment synthesis. In this case Pol III core is released from the β-clamp only when synthesis is complete, allowing core to rapidly recycle to a β-clamp at a new primed site for the synthesis of the next Okazaki fragment (O'Donnell, 1987; Stukenberg *et al.*, 1991). In a recent study we have demonstrated that the C-terminus of the α-subunit is also a target for binding by the τ-subunit of the clamp loader (López de Saro *et al.*, 2003). The τ-subunit senses DNA structure, and when it lacks ssDNA template, τ binds the C-terminus of Pol III core and displaces it from β and DNA as illustrated in Figure 7A (Leu *et al.*, 2003). This 'processivity switch' is mediated by direct competition between a DNA-sensing protein (τ) and the β-clamp for a common site on the Pol III α-subunit and reveals an elegant mechanism by which

polymerase trafficking on clamps can be connected to the different structures of the DNA substrate and product.

Analogous competition mechanisms for clamps regulated by DNA structure may be discovered for other clamp-related reactions. For example, the clamp left by Pol III core at the completed Okazaki fragment may be sequentially used by Pol I and DNA ligase for RNA primer removal and sealing of the nick, respectively (see key to Figure 7B). Perhaps Pol I binds β tightest when RNA is present, but loses affinity for β when the processing job is complete. Ligase may interact most favorably with β at the nicked site created by Pol I action. Besides polymerases and ligase, MutS has also been shown to bind β (López de Saro and O'Donnell, 2001). Hence, either before or following Okazaki-fragment processing, β-clamps left on DNA may serve as markers of newly synthesized DNA for genome surveillance by repair proteins or other DNA modifying proteins before being recycled from DNA for use at other locations.

Traffic flow on β during bypass of a DNA lesion

The SOS response in *E.coli* includes the induction of three polymerases; Pol II, Pol IV and Pol V (Goodman, 2002). It has been suggested that these polymerases are called into action to bypass DNA damage during chromosomal replication, and that their diversity reflects the variety of damaged DNA that can be encountered (Goodman and Tippin, 2000; Sutton and Walker, 2001; Wagner *et al*, 2002). The finding that they interact with the β-clamp on the same locus as Pol III core suggests that they compete with core for β at sites of DNA damage (see Figure 7C). The levels of Pol V (UmuC subunit) in the cell rise to ~200 copies per cell upon induction (Woodgate and Ennis, 1991), ~10-fold higher than the intracellular level of Pol III core, which is present at a concentration of ~20 copies per cell (Baker and Kornberg, 1992).

A combination of DNA structural elements or signals from the stalled replication fork could be responsible for the selection of the polymerase that resides with β at a 3′ terminus at any given time. However, the fact that the repair polymerases and Pol III core compete for the same site on the clamp suggests that control of polymerase selection may be exercised at the level of their affinity for the clamp. In the case of Pol V, action with β requires RecA, which is also induced in the SOS response (Pham *et al*., 2001, 2002). Pol III core, on the other hand, is inhibited by RecA. Hence, one may expect that Pol III core, stalled at a DNA lesion, may be displaced by RecA, and at the same time the presence of RecA and β would recruit Pol V (Figure 7C) (Pham *et al*., 2001). After lesion bypass and disassembly of the RecA filament (due to inherent instability of RecA), Pol V would lose affinity in the absence of RecA and dissociate from β (Pham *et al*., 2001). Pol III core could then reassociate with β to resume chain extension.

Similarity within the clamp-binding motifs

Despite the lack of sequence similarity between Pol II, III and IV of *E.coli*, they have evolved similar C-terminal sequence motifs that bind to β. The sequence motif consists of the pattern QxxL(x)F. This sequence is somewhat reminiscent of the PCNA consensus binding motif (Qxx[I/L]xxFF) (Warbrick, 2000) and the β binding

motif (QLxLF) suggested by Dalrymple *et al*. (2001). This is even more remarkable in view of the fact that β and PCNA share no sequence similarity, despite having a common fold. Clamp-binding motifs could either be ancient and reflect a common ancestor, or may have occurred as a result of convergent evolution.

The finding that the clamp loader and all the polymerases present in the *E.coli* cell interact with the β-clamp at the same location suggests that this site may be an attractive target for the development of DNA replication inhibitors. This is especially true considering that many proteins interact with the same hydrophobic pocket on β. Overcoming drug binding to this pocket would likely impair its binding to several proteins at once, thus making the appearance of antibiotic-resistant variants less likely.

Materials and methods

Materials

HPLC-purified, N-terminal biotinylated 20-mer peptides corresponding to N- and C-terminal sequences of Pol III α, Pol II and Pol IV were purchased from Bio-synthesis, Inc.; N-terminal biotinylated 20-mer peptides corresponding to internal regions of the α-protein and the Ala-scanning peptide series shown in Figure 2 were purchased from Chiron Technologies; they were synthesized using Multipin peptide technology, which leaves a diketopiperidine linker at the C-terminus of the peptide. Streptavidin-coated, 96-well microtiter plates were from Costar, and non-streptavidin plates (MaxiSorp) were from Nalge Nunc, Inc. Streptavidin was from Sigma Co. Labeled nucleotides were from Dupont, New England. Nuclear and unlabeled nucleotides were from Pharmacia-LKB.

Proteins were purified as described previously: α, ε, θ (Studwell and O'Donnell, 1990) and β (Kong *et al*., 1992). ^{32}P-β was labeled using γ-^{32}P ATP as described previously (Kelman *et al*., 1995). Pol III core was reconstituted from isolated subunits, then purified from unbound proteins as described (Studwell-Vaughn and O'Donnell, 1991).

Microplate assays

Microplate assays were performed as described previously by Yuzhakov *et al*. (1999). N-terminally biotinylated peptides (100 pmol) were added to streptavidin-coated 96-well plates in 30 μl PBST buffer [0.01 M phosphate-buffered saline pH 7.2 (PHS) supplemented with 0.1% v/v Tween 20] and incubated for 1 h at 23°C. They were then washed three times by the addition and removal of 100 μl PBST buffer. ^{32}P-β was added (90 nM) directly to wells in a volume of 30 μl and incubated for 1 h, after which each well was washed three times with 50 μl PBST buffer and the plates were analyzed using a PhosphorImager (Molecular Dynamics).

MaxiSorp plates were used in 96-well plate assays in which other proteins were immobilized. Proteins (100 pmol of either δ, Pol I, Pol IV, UmuC, UmuD or UmuD′) were added directly to the plate in 30 μl of water and the plates were dried at 23°C. Plates were then blocked with 5% non-fat dry milk in PBST for 1 h and washed. ^{32}P-β (90 nM in 30 μl PBST buffer) was then added and the plates analyzed using a PhosphorImager.

Native gel mobility shift assays

Native gel electrophoresis assays using ^{32}P-β were performed as described by López de Saro and O'Donnell (2001). Reactions (15 μl) contained 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 4% glycerol, 50 μg/ml bovine serum albumin (BSA), 100 mM NaCl, 5 mM DTT, 200 nM Pol III core (as indicated) and 90 nM ^{32}P-β (150 d.p.m./fmol). When present, either of the following components were added: 4 μg *E.coli* β, 4 μg human PCNA, 4 μg T4gp45 or 25 pmol α1141–1161 peptide. Reactions were incubated at 37°C for 5 min before loading 4 μl onto a 4% native polyacrylamide gel. Electrophoresis was performed using 40 mM Tris-acetate (pH 8.0), 1 mM EDTA, at 4°C and developed at 17 mA for 90 min. Gels were dried and detection of ^{32}P-β was performed using a PhosphorImager.

Replication assays

DNA synthesis assays were performed using M13mp18 phage circular ssDNA (1.7 nM) primed with a DNA 30-mer. Reactions included 0.42 μM ssDNA-binding protein (as tetramer), 2 nM γ-complex (γ$_3$δδ′χψ) 30 nM β (as dimer), and either 25 nM α, Pol III core or Pol I Klenow fragment, as

indicated, in 25 µl 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 4% glycerol, 40 µg/ml BSA, 5 mM DTT, 8 mM MgCl$_2$, 0.5 mM ATP, 60 µM each of dGTP, dATP and dCTP, and 20 µM [α-^{32}P]dTTP. When present, peptide was added at the concentrations indicated prior to shifting to 37°C. Reactions were incubated for 30 s (Pol III) or 30 min (Pol I Klenow fragment) at 37°C and stopped with 25 µl of 1% SDS, 50 mM EDTA, then analyzed for nucleotide incorporation by spotting onto DE81 filter papers as described previously (Studwell and O'Donnell, 1991).

Clamp loading assay

Clamp loading assays of ^{32}P-β onto singly primed M13mp18 ssDNA by γ-complex were performed as described for replication reactions, but in the absence of DNA polymerase or dNTPs. Reaction volumes were 100 µl of 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 4% glycerol 40 µg/ml BSA, 5 mM DTT, 8 mM MgCl$_2$, 0.5 mM ATP and incubation was for 5 min at 37°C. Reactions were then applied to a 5 ml BioGel A15M column (BioRad) pre-equilibrated with 20 mM Tris–HCl (pH 7.5), 0.1 mM EDTA, 4% glycerol, 40 µg/ml BSA, 5 mM DTT, 8 mM MgCl$_2$ and 100 mM NaCl. Chromatography was performed at 22°C and fractions of 180 µl were collected and analyzed by liquid scintillation counting.

K_D values using fluorescent $β^{OG}$

The β-subunit can be uniquely labeled at Cys333 using maleimide derivatives (Griep and McHenry, 1988). β (3 mg) was labeled using OG 488 maleimide (Molecular Probes) in 1 ml of 50 mM potassium phosphate (pH 7.5) and 100 mM NaCl. The OG maleimide (90 nmol) was dissolved in 100 µl DMSO, then 80 µl was added to β with gentle stirring at 4°C, followed by overnight incubation at 4°C in the dark. $β^{OG}$ was separated from unreacted reagent on a 50 ml column of BioGel 6 and contained ~0.91 molecules of OG per β monomer as determined from protein absorbance at 280 nM (ε280 = 14 890/M/cm) and OG at 490 nm (ε491–76 000/M/cm).

Titration of wild-type α or core αΔC7 into $β^{OG}$ was performed as follows. Reactions contained 50 nM $β_2^{OG}$ and 1.5 µM α in 60 µl 20 mM Tris–HCl (pH 7.5), 0.5 mM EDTA, 1 mM DTT and 0–250 mM NaCl (as indicated) on ice. Titrations of Pol III core or Pol II were performed in a similar buffer supplemented with 100 mM NaCl. Reactions were shifted to 22°C for 15 min then 51 µl was placed in a 3 X 3 mm cuvette. Excitation was at 490 nm and emission was monitored from 500–600 nm in a PTI Quantamaster spectrofluorimeter. Fluorescence emission at 517 nm was used for analysis. Additional titrations of wild-type α into high concentrations of $β_2^{OG}$ (250 nM and 500 nM) showed a α:$β_2$ =1:1 stoichiometry. As a consequence, data points were fit according to the model, A + B → AB using the Origin software (Microcal, Inc.). Reactions using rhodamine (TAMRA)-labeled peptides contained 1 µM peptide and β-subunit as indicated. Excitation was at 490 nm, and the emission was monitored from 500–600 nm. Fluorescence emission at 517 nm was used for analysis as above.

Acknowledgements

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