Chapter 15
PCNA Structure and Function: Insights from Structures of PCNA Complexes and Post-translationally Modified PCNA

Lynne M. Dieckman, Bret D. Freudenthal, and M. Todd Washington

Abstract Proliferating cell nuclear antigen (PCNA), the eukaryotic DNA sliding clamp, forms a ring-shaped homo-trimer that encircles double-stranded DNA. This protein is best known for its ability to confer high processivity to replicative DNA polymerases. However, it does far more than this, because it forms a mobile platform on the DNA that recruits many of the proteins involved in DNA replication, repair, and recombination to replication forks. X-ray crystal structures of PCNA bound to PCNA-binding proteins have provided insights into how PCNA recognizes its binding partners and recruits them to replication forks. More recently, X-ray crystal structures of ubiquitin-modified and SUMO-modified PCNA have provided insights into how these post-translational modifications alter the specificity of PCNA for some of its binding partners. This article focuses on the insights gained from structural studies of PCNA complexes and post-translationally modified PCNA.

Keywords DNA replication • DNA repair • DNA polymerase • Processivity factor • Protein-protein interactions • Sliding clamp

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15.1 Introduction

DNA sliding clamp proteins are found in all three domains of life. Despite little sequence homology among the sliding clamps from bacteria, archaea, and eukaryotes, these proteins have similar overall structures. They all form ring-shaped proteins that encircle double-stranded DNA. These sliding clamps are most widely known for their ability to confer high processivity to classical DNA polymerases – those involved in normal DNA replication and repair. Sliding clamps, however, do far more than this; they form mobile platforms on the DNA that recruit many of the enzymes involved in DNA replication, repair, and recombination.

In eukaryotes, the sliding clamp protein is proliferating cell nuclear antigen (PCNA). In a recent review article on PCNA, this protein was called the "maestro of the replication fork" (Moldovan et al. 2007). This is indeed an apt metaphor because PCNA coordinates the recruitment of many proteins to sites of DNA replication and in many cases regulates their activities. In this capacity, PCNA plays a critical role in a wide range of nuclear processes including DNA replication, translesion DNA synthesis, base excision repair, nucleotide excision repair, mismatch repair, recombination, chromatin assembly and remodeling, sister chromatid cohesion, and cell cycle control (Maga and Hubscher 2003; Moldovan et al. 2007; Naryzhny 2008; Tsurimoto 1999; Zhuang and Ai 2010).

The X-ray crystal structure of eukaryotic PCNA was first determined in 1994 (Krishna et al. 1994). Over the last 18 years, various X-ray crystal structures of PCNA bound to peptides derived from PCNA-binding proteins have been determined. More recently, X-ray structures and lower-resolution structures of PCNA bound to full-length protein partners have been determined. These structures have provided valuable insights into how PCNA recognizes PCNA binding proteins and recruits them to replication forks.

A paradigm that has emerged over the last decade is that the specificity of PCNA for some of its binding partners is regulated by post-translational modifications of PCNA (Bergink and Jentsch 2009; Shaheen et al. 2010; Ulrich 2009; Ulrich and Walden 2010; Watts 2006). For example, mono-ubiquitylation of PCNA facilitates translesion synthesis by recruiting non-classical DNA polymerases to stalled replication forks. SUMOylation of PCNA inhibits unwanted recombination by recruiting anti-recombinogenic helicases to replication forks. Recently, X-ray crystal structures of ubiquitin-modified and SUMO-modified PCNA have been determined, and these have provided insights into how these modifications alter the specificity of PCNA for some of its binding partners (Freudenthal et al. 2010, 2011).

This review article focuses on the insights gained in recent years from structural studies of PCNA, PCNA complexes and post-translationally modified PCNA. In particular, we will discuss how PCNA recognizes many PCNA-interacting proteins. We will discuss how these structures begin to help us understand how PCNA regulates the activity of some of these proteins and how PCNA facilitates multi-step enzymatic processes on DNA. Finally, we will discuss how ubiquitin and SUMO modifications impact the function of PCNA.

15.2 Structure of PCNA

Sliding clamps from bacteria, archaea and eukaryotes all form ring-shaped proteins with pseudo-sixfold symmetry. However, there are differences in the number of domains that comprise each subunit and the way that the subunits assemble to form the ring. For example, the bacterial sliding clamp, called the β clamp, is a component of the DNA polymerase III holoenzyme. It is a ring-shaped homo-dimer with each subunit containing three domains (Kong et al. 1992). By contrast, PCNA, the eukaryotic sliding clamp, is a ring-shaped homo-trimer with each subunit containing two domains (Krishna et al. 1994). The archaeal sliding clamp is also called PCNA. Like eukaryotic PCNA, it is a ring-shaped trimer with each subunit containing two domains. However, in some archaeal species, PCNA is a homo-trimer and in others it is a hetero-trimer.

The X-ray crystal structure of eukaryotic PCNA shows that each subunit consists of two independent and similarly folded domains (Fig. 15.1) (Krishna et al. 1994). The N-terminal domain (residues 1–117) is referred to as domain A, and the C-terminal domain (residues 135–258) is referred to as domain B. These independent domains are held together by an extended β sheet across the interdomain boundary on each subunit. Furthermore, the two domains are connected through a long, flexible linker (residues 118–134) called the interdomain connector loop (IDCL). The three subunits assemble in a head-to-tail manner with domain A of one subunit interacting with domain B on an adjacent subunit. This interaction is stabilized through an extended β sheet comprised of β strands from domain A of one subunit and β strands from domain B of an adjacent subunit at each subunit interface.

The PCNA ring has a diameter of approximately 80 Å. The central hole in the ring has a diameter of approximately 35 Å. The outer surface of the PCNA ring is a circular collar of the aforementioned six β sheets (three interdomain β sheets and three intersubunit β sheets). The inner surface of the PCNA ring is a set of 12 α helices, two from each domain. While the overall electrostatic potential of PCNA is negative, the inner surface is positively charged due to the presence of lysine and arginine residues on these α helices. These localized positive charges facilitate the passage of the negatively charged DNA through the central hole.

The PCNA ring is approximately 30 Å wide and contains distinct front and back faces. The front face of PCNA contains the IDCL and is involved in many protein-protein interactions (see Sect. 15.3 below). This is notable as many replication proteins, such as DNA polymerases and DNA ligases, carry out their operations on the DNA at the front face of the PCNA ring. The role of the back face of PCNA is currently less clear. The back face is emerging as a site of PCNA post-translational modification and is likely involved in recruiting protein factors to replication forks and holding them in reserve until they are needed on the front face of PCNA (see Sect. 15.5 below).

X-ray crystal structures of the bacterial β clamp and of eukaryotic PCNA bound to DNA show that as the DNA passes through the central hole of the ring, it is tilted significantly away from the axis of symmetry (Georgescu et al. 2008). The angle of

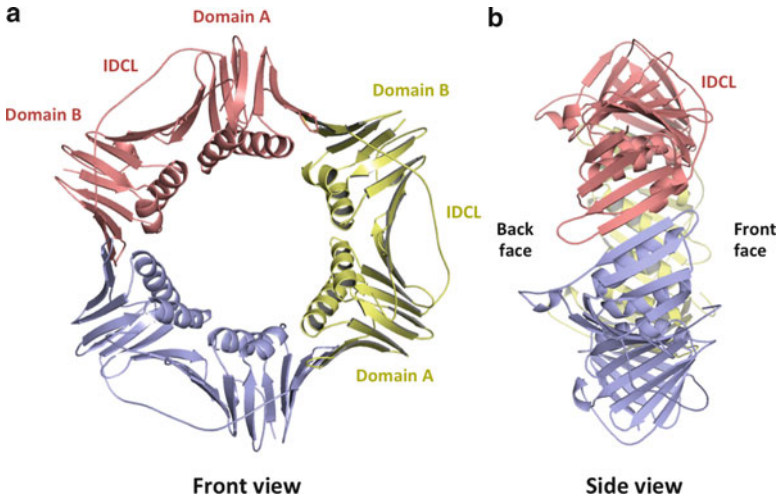

Fig. 15.1 The structure of PCNA. Ribbon diagram of the PCNA trimer (PDB ID: 1PLQ) shown from the *front* (**a**) and the *side* (**b**) with the individual PCNA subunits colored *red*, *yellow* and *blue*. The inter-domain connector loop (IDCL) is indicated

the DNA is 22° in the case of the β clamp and 40° in the case of the PCNA. In addition, single-particle electron microscopy analysis of other PCNA-containing complexes also shows that the DNA is tilted as it passes through the central hole of the PCNA ring (see 15.3.3 below) (Mayanagi et al. 2009, 2011). Moreover, single-molecule studies have shown that PCNA can diffuse along the DNA in two distinct modes (Kochaniak et al. 2009). The first mode involves rotation and translation as it tracks the helical pitch of the DNA duplex. The second mode, which is less common, involves faster translation that does not involve tracking the helical pitch. This angular, rotational, and translational flexibility of PCNA on DNA may allow it to accommodate the many diverse proteins with which it must interact.

15.3 Structures of PCNA Complexes

PCNA provides a structural platform for many cellular processes including DNA replication and repair. To do this, PCNA must interact with many of the enzymes involved in these processes. Structural studies of PCNA bound to several of its binding partners have been carried out and these have provided valuable insights into how PCNA interacts with these proteins. We will first discuss X-ray crystal structures of PCNA bound to peptides derived from a variety of PCNA-interacting proteins. We will then discuss X-ray crystal structures of PCNA bound to full-length PCNA-interacting proteins. Finally, we will discuss the architecture of other PCNA-containing complexes determined by single-particle electron microscopy and small angle X-ray scattering (SAXS).

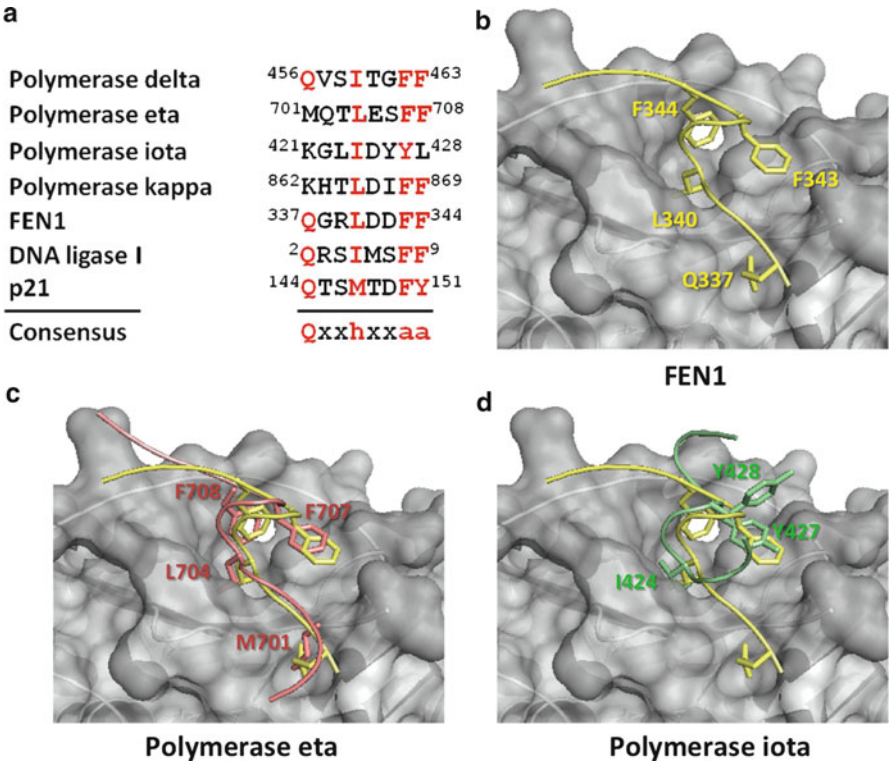

Fig. 15.2 Structures of PCNA bound to PIP peptides. (**a**) Sequence alignment of PIP peptides from several human PCNA-binding proteins. In the PIP consensus sequence, the 'h' can be isoleucine, leucine or methionine, and the 'a' can be phenylalanine or tyrosine. (**b**) The structure of the canonical PIP motif from FEN1 binding to PCNA (PDB ID: 1U7B) is shown in *yellow*. (**c**) The structure of the PIP motif from DNA polymerase η bound to PCNA (PDB ID: 2ZVK) shown in *red* overlaid with the structure of the PIP motif from FEN1 shown in *yellow*. (**d**) The structure of the PIP motif from DNA polymerase ι bound to PCNA (PDB ID: 2ZVM) shown in *green* overlaid with the structure of the PIP motif from FEN1 shown in *yellow*

15.3.1 Structures of PCNA Bound to PIP Peptides

Many proteins that bind PCNA do so through a conserved PCNA-interacting protein (PIP) motif (Hingorani and O'Donnell 2000; Maga and Hubscher 2003; Tsurimoto 1999). The PIP motifs of several proteins are shown in Fig. 15.2a. These motifs usually interact with PCNA on a single subunit in a region between the two domains near the IDCL. The canonical PIP motif contains eight amino acid residues. The conserved glutamine of the PIP motif normally inserts into a small pocket in PCNA (Fig. 15.2b). The last five residues of the PIP motif, which include the conserved hydrophobic residue (methionine, leucine, or isoleucine) and the two conserved phenylalanine or tyrosine residues, form a 3_{10} helix that binds in a large hydrophobic pocket between the two domains and also contacts the IDCL. Generally,

the structure of PCNA is not changed upon the binding of PIP peptides; only small alterations in the structure of the IDCL are observed.

PIP motifs are often thought to be a flexible tether that anchors the PCNA-binding protein to PCNA. PIP motifs are often found at the C-termini of PCNA binding proteins, such as classical DNA polymerase δ (the p66 subunit), non-classical DNA polymerase η, and the cyclin-dependent kinase inhibitor p21. PIP motifs, however, can occur elsewhere in the primary structure of the PCNA-binding proteins, including the N-termini (such as DNA ligase I) and the interiors of the proteins (such as non-classical DNA polymerase ι). Deletion of the PIP motif or mutations in its conserved residues can significantly weaken or abolish PCNA interactions *in vivo* and *in vitro*. Thus, even though the PCNA-PIP interactions involve rather small regions of these proteins, these interactions are often necessary to recruit many enzymes to replication forks.

Classical DNA polymerases are responsible for synthesizing DNA during DNA replication and DNA repair. They achieve high processivity by interacting with PCNA, and this interaction is dependent on their PIP motifs. DNA polymerase δ is the classical polymerase that is responsible for lagging strand synthesis in eukaryotes (see Chap. 13, this volume). In humans, DNA polymerase δ is composed of four subunits (p125, p66, p50, and p12). The catalytic activity resides in the p125 subunit. DNA polymerase δ interacts with PCNA via the PIP motif on the p66 subunit. The X-ray crystal structure of PCNA bound to the PIP peptide of p66 shows that the PIP motif forms the normal 3_{10} helix that fits into the large hydrophobic pocket of PCNA (Bruning and Shamoo 2004).

Upon encountering DNA damage in the template strand, the replication fork stalls. This is because classical DNA polymerases are unable to incorporate nucleotides across from damaged DNA templates. Non-classical DNA polymerases, such as DNA polymerases η, κ, and ι, are recruited to stalled replication forks to carry out translesion synthesis (Prakash et al. 2005; Prakash and Prakash 2002; Washington et al. 2009). The recruitment of these non-classical DNA polymerases is governed in part by the mono-ubiquitylation of PCNA; this aspect of non-classical polymerase recruitment will be described later (see Sect. 15.5 below). Nevertheless, the PIP motifs of these non-classical polymerases are necessary for their recruitment to stalled replication forks.

The X-ray crystal structures of PCNA bound to the PIP motifs of DNA polymerases η, κ, and ι have been determined (Hishiki et al. 2009). The structures of the PIP motifs of DNA polymerases η and κ are similar to that of the classical DNA polymerase δ in that they form the normal 3_{10} helix (Fig. 15.2c). There are, however, some minor differences in the specific contacts made by these PIP motifs, because the sequences of the PIP motifs of these non-classical polymerases differ slightly from the PIP consensus sequence. For example, neither of these PIP motifs have the conserved glutamine residue. DNA polymerase η, for instance, has a methionine residue that inserts into the small pocket where the glutamine normally fits. The structure of the PIP motif of DNA polymerase ι, however, differs significantly from that of any other PIP motif structure. It does not form the normal 3_{10} helix, but instead forms a β-bend-like structure (Fig. 15.2d). Taken together, it is likely that

the divergence of the non-classical polymerase PIP motifs from the consensus PIP sequence reduces their affinities for PCNA relative to other PIP motifs (Hishiki et al. 2009). This could be important for preventing the recruitment of non-classical polymerases to replication forks until the PCNA is mono-ubiquitylated and their activities are needed.

In the X-ray crystal structures of PCNA bound to some PIP peptides, secondary contacts (i.e., those that occur outside of the PIP motif) are observed between PCNA and the portions of the peptide flanking the PIP motif. For example, DNA ligases catalyze the linkage of 5′phosphates and a 3′OH groups during DNA repair and Okazaki fragment processing (see Chap. 17, this volume). The yeast Cdc9 DNA ligase has a PIP motif that forms the conventional 3_{10} helix. However, the residues flanking the N-terminal sides of the PIP motif form an anti-parallel β-sheet with the C-terminus of PCNA (Vijayakumar et al. 2007).

The presence of DNA damage triggers an increase in expression of the tumor suppressor protein p21 leading to DNA replication arrest. The inhibition of DNA replication by p21 requires that it bind directly to PCNA (Flores-Rozas et al. 1994; Gibbs et al. 1997; Waga et al. 1994). The X-ray crystal structure of the p21 PIP motif bound to PCNA reveals that this PIP motif binds in the normal manner. However, secondary contacts between PCNA and the peptide in the regions immediately flanking the PIP motif are observed. The N-terminal and the C-terminal flanking regions form anti-parallel β-sheets with the C-terminus and the IDCL of PCNA, respectively (Gulbis et al. 1996). It has been suggested that these extensive interactions are responsible for the higher affinity PIP motif-PCNA interaction observed with the p21 PIP motif relative to other PIP motifs. This tighter binding may allow the p21 PIP to inhibit DNA replication by effectively competing with DNA polymerases for binding PCNA.

15.3.2 Structures of PCNA Bound to Full-Length Proteins

While most structures of PCNA have been of complexes of PCNA with PIP motif peptides, a few structures have been determined of complexes of PCNA with full-length proteins. These have provided insights into the secondary contacts between PCNA and PCNA-binding proteins that occur in addition to and alongside the contacts mediated by PIP motifs. For example, the X-ray crystal structure of PCNA bound to full-length flap endonuclease 1 (FEN1), which catalyzes the removal of 5′ single-stranded DNA overhangs that occur during DNA repair and during the processing of the ends of Okazaki fragments (see Chap. 16, this volume), has been determined (Fig. 15.3a) (Sakurai et al. 2005). FEN1 consists of a nuclease core domain (residues 1–332) and a C-terminal tail region (333–380). The main PCNA-interacting interface of FEN1 is the N-terminal half of the C-terminal tail region, which contains a PIP motif.

Although the primary contact made between FEN1 and PCNA is mediated by the PIP motif, there are secondary contacts between PCNA and the regions flanking

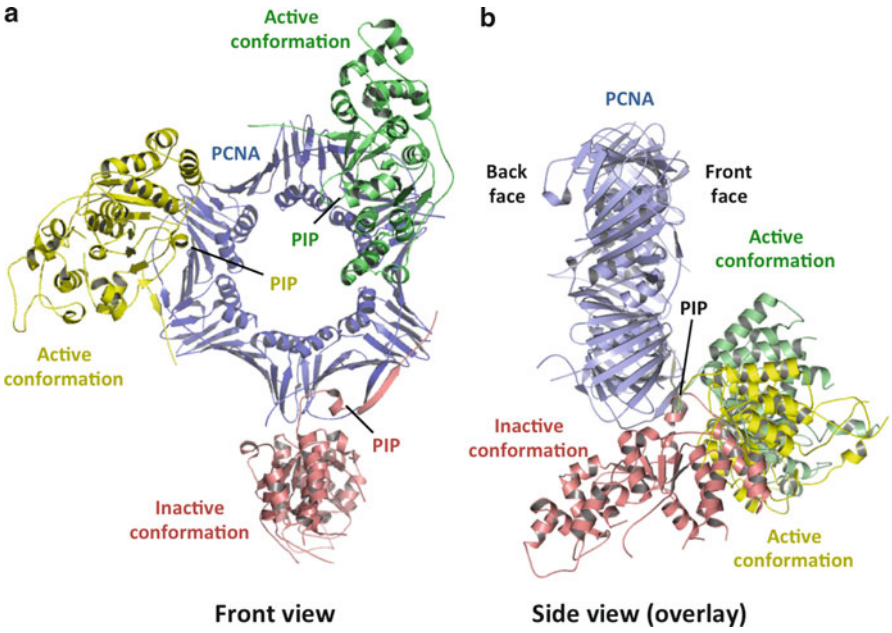

Fig. 15.3 Structure of PCNA bound to FEN1. (**a**) Ribbon diagram of the PCNA trimer shown in *blue* bound to three molecules of FEN1 shown in *red*, *yellow* and *green* (PDB ID: 1UL1). (**b**) Overlay showing the three positions of FEN1 relative to the PCNA subunit to which they are bound. The PCNA is shown in *blue*, the inactive conformation is shown in *red*, and the active conformations are shown in *yellow* and *green*

the PIP motif and between PCNA and the core domain of FEN1. Residues of the core domain make several intramolecular contacts with the PIP motif as well as several intermolecular interactions with both the IDCL and C-terminus of PCNA. Moreover, the core domain of FEN1 is connected to its C-terminal tail through a 4-residue linker. It has been suggested that this linker acts as a hinge to allow the core domain of FEN1 to be positioned near its DNA substrate.

The structure of the FEN1-PCNA complex had three FEN1 molecules bound to PCNA, and each FEN1 molecule was in a different position relative to the PCNA subunit to which it was bound (Fig. 15.3b). One of the observed FEN1 positions had the active site of the core domain swung away from the front face of PCNA, and this may represent an inactive conformation of FEN1. In the other two positions, the core domain is located closer to the PCNA central cavity near the expected position of the DNA. These latter positions mat reflect active conformations in which FEN1 can bind the DNA flap and bring itself into a position to cleave it.

Replication factor C (RFC) is the ATP-dependent clamp loading protein that binds to PCNA, opens the ring, and deposits it on the DNA. The structure and mechanism of RFC is described in more detail in a companion chapter (Chap. 14, this volume). Here, however, we will briefly mention the key features observed in the X-ray crystal structure of the RFC-PCNA complex (Bowman et al. 2004). RFC

sits on the front face of the closed PCNA ring. The five subunits of RFC form a right-handed spiral that is tilted by approximately 9° relative to the threefold axis of PCNA. Only three of the five subunits of RFC (RFC-A, RFC-B, and RFC-C) make contacts with the PCNA. In the case of RFC-A and RFC-C, these are contacts mediated by PIP motifs. RFC-B, by contrast, makes several secondary contacts with PCNA at the intersubunit regions.

15.3.3 Low Resolution Structures of PCNA Complexes

Lower resolution approaches, such as small angle X-ray scattering (SAXS) and single particle electron microscopy (EM), have been used to examine the architecture of other PCNA complexes. Although much of this work has been done using archaeal PCNA from either *Sulfolobus solfataricus* or *Pyrococcus furiosus*, these studies have uncovered principles about PCNA complexes that are likely applicable to eukaryotic systems. Like eukaryotic PCNA, *P. furiosus* PCNA is a homotrimer. Each subunit has a similar overall fold to eukaryotic PCNA including a protein binding pocket near the IDCL (Matsumiya et al. 2001). Thus *P. furiosus* PCNA trimers have three identical protein binding sites. Unlike eukaryotic PCNA, *S. solfataricus* PCNA is a heterotrimer comprised of three subunits: PCNA1, PCNA2, and PCNA3. These three subunits share the same overall fold with one another and with eukaryotic PCNA (Williams et al. 2006). Consequently, *S. solfataricus* PCNA trimers have three distinct protein binding sites.

The architecture of the *S. solfataricus* PCNA bound to DNA ligase in the absence of DNA was examined using SAXS (Pascal et al. 2006). The ligase and PCNA trimer form a 1:1 complex with the ligase binding to the PCNA3 subunit. *Ab initio* shape predictions suggested that DNA ligase has a preferred orientation with respect to the PCNA ring and is extended out from the side of the ring. Structures of PCNA and DNA ligase obtained from X-ray crystallography were docked into the SAXS molecular envelope showing that the DNA ligase was in the open conformation. It is suggested that the interface between the DNA ligase and the PCNA is malleable enough to accommodate the conformational change in the DNA ligase from the open state to the closed state that is needed for catalysis when DNA is present.

Insight into the architecture of PCNA-DNA ligase complex in the presence of DNA came from single particle EM studies of *P. furiosus* PCNA and DNA ligase (Mayanagia et al. 2009). The 3D map, with a resolution estimated to be 15 Å, revealed a two-tier structure. The lower tier was a hexagonal ring into which the structure of PCNA nicely fits. The upper tier was crescent-shaped and corresponded well to the structures of the domains of the DNA ligase. The DNA was visible in the 3D map as a rod-shaped component that went through the center of the PCNA ring. The DNA ligase wrapped half way around the DNA. In this complex, the DNA was tilted about 16° from the threefold axis of the PCNA ring.

Single particle EM studies were also used to examine the structure of the complex of *P. furiosus* PCNA and DNA polymerase B bound to DNA (Mayanagi et al. 2011).

Again, the 3D map, with a resolution estimated to be 19 Å, was a two-tier structure. The lower layer corresponded to PCNA, and the upper layer corresponded to DNA polymerase B. The DNA was visible through the central channel of PCNA and was tilted about 13° from the threefold axis of PCNA. Interestingly, the DNA polymerase directly contacted the PCNA trimer at two sites. One contact site was the normal interaction mediated through the PIP motif of the polymerase. The other contact site was with a different PCNA subunit than the one contacted by the PIP motif. It has been suggested that this secondary contact helps to properly orient the polymerase, which is difficult to do with only a PIP-mediated contact as this latter contact is rather flexible. This secondary contact may also preclude other proteins from binding at this other subunit.

The Msh2-Msh6 protein recognizes DNA mismatches and initiates mismatch repair. The architecture of the complex of eukaryotic PCNA and the Msh2-Msh6 mismatch repair protein was analyzed by SAXS (Shell et al. 2007). First, the N-terminal region of Msh6, which contains a PIP motif and binds tightly to PCNA, was shown by SAXS to be intrinsically disordered. Upon binding to PCNA, the N-terminal region does not acquire structure suggesting that this region functions as a disordered tether. SAXS analysis was also performed on the full Msh2-Msh6 protein bound to PCNA, and these results did not favor a model in which the folded regions of the Msh2-Msh6 protein directly contacted the PCNA ring. Instead they suggested that the interaction is solely mediated through the long, unstructured tether. This tether likely allows the structured regions of Msh2-Msh6 to remain associated with PCNA, but also reach around other protein factors at the replication fork in search of mismatches. It is likely that this type of PCNA interaction is common, because other proteins containing PIP motifs also have adjacent regions predicted to be intrinsically unstructured (Shell et al. 2007).

15.3.4 Unresolved Issues

PCNA interacts with a variety of proteins. How does PCNA discriminate between these different partners? How does PCNA regulate when a protein should be recruited to a replication fork or released from a replication fork? Because most PIP motifs make very similar contacts with PCNA, they are unlikely to contribute much toward this specificity. Notable exceptions include the non-classical DNA polymerases and p21. The PIP motifs of non-classical polymerases are thought to bind PCNA with lower affinity than those of classical DNA polymerases (Hishiki et al. 2009). This could be important for preventing non-classical polymerases from binding PCNA until PCNA is mono-ubiquitylated. By contrast, the PIP motif of p21 binds PIP with higher affinity than those of classical polymerases (Bruning and Shamoo 2004). This could be important for arresting DNA replication when there is DNA damage.

In most cases, the specificity of PCNA for its binding partners probably comes from contacts outside the PIP motif. Secondary contacts between PCNA and PCNA-binding proteins involving regions that immediately flank the PIP motif or elsewhere on the PCNA-binding protein likely play a major role in specificity. This emphasizes the

need for additional X-ray crystal structures of PCNA bound to full-length proteins. Moreover, contact with the DNA may also play an important role in specificity in cells. For example, specificity for FEN1 likely arises in part due to the secondary contacts observed in the X-ray crystal structure and in part due to the presence of a DNA substrate containing a 5′ flap. Moreover, post-translational modifications of PCNA such as mono-ubiquitylation and SUMOylation clearly control the specificity of PCNA interactions (see Sect. 15.5 below). Similarly, post-translational modifications of PCNA-binding proteins such as phosphorylation have been observed with p21 and FEN1, and these modifications inhibit PCNA binding (Henneke et al. 2003; Scott et al. 2000). Further studies will be needed to flesh out some of these mechanisms and uncover yet others.

Many of the processes in which PCNA participates are multi-step processes that involve the handing off of the DNA from one enzyme to another. For example, in Okazaki fragment processing, FEN1 must cleave off the 5′ flap on the DNA before handing it off to DNA ligase that seals the nick (see Chap. 16, this volume). How does such a DNA handoff occur? One possibility is that PCNA forms toolbelts by simultaneously binding several different enzymes and that these toolbelts facilitate the handoff. For example, the simultaneous binding of DNA polymerase, FEN1, and DNA ligase to a single PCNA trimer has been observed in *S. solfataricus* (Dionne et al. 2003). Currently, there is no clear evidence for eukaryotic PCNA functioning as a toolbelt but this seems to be a very likely scenario.

15.4 Structures of Mutant PCNA Proteins

A variety of PCNA mutant proteins have been identified that increase the sensitivity of cells to DNA damaging agents (Ayyagari et al. 1995). Here we will focus on two mutant proteins that block translesion synthesis. The first of these mutant proteins, which was identified in a yeast genetic screen, has a glycine to serine substitution at residue 178 (Zhang et al. 2006). Yeast cells producing this G178S mutant form of PCNA have an increased sensitivity to DNA-damaging agents and are completely defective in translesion synthesis. Interestingly, this mutant form of PCNA functions normally in all other respects, such as DNA replication and repair. The second of these mutant proteins, which was identified in another yeast genetic screen, has a glutamate to glycine substitution at residue 113 (Amin and Holm 1996). Yeast cells producing this E113G mutant protein have a very similar phenotype to those with the aforementioned G178S substitution.

Gly178 is located in domain B at the subunit interface of the PCNA trimer. Glu-113 is located in domain A at the subunit interface directly across from Gly-178 on the adjacent subunit (Fig. 15.4a). Steady state kinetic studies show that while wild-type PCNA stimulates incorporation by the non-classical DNA polymerase η opposite an abasic site, the G178S PCNA protein actually inhibits incorporation opposite this DNA lesion (Freudenthal et al. 2008). Similarly, the E113G PCNA mutant protein is unable to stimulate incorporation by DNA polymerase η opposite

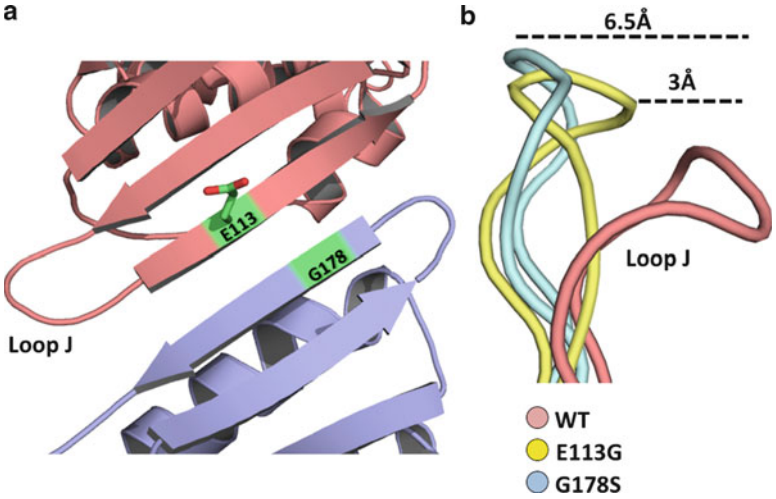

Fig. 15.4 Structures of the G178S and E113G PCNA mutant proteins. (**a**) A ribbon diagram of the subunit interface of PCNA with domain A of one subunit shown in *red* and domain B of the adjacent subunit shown in *blue*. The position of Glu113, the position of Gly178 and loop J are indicated. (**b**) The structure of backbone of loop J in wild-type PCNA protein shown in *red* (PDB ID: 1PLQ) is superimposed on the structures of the backbones of loop J in the E113G PCNA mutant protein shown in *yellow* (PDB ID: 3GPM) and the G178S PCNA mutant protein shown in *blue* (PDB ID: 3F1W)

this lesion (Freudenthal et al. 2008). Furthermore, the E113G PCNA mutant protein is unable to stimulate the activity of non-classical DNA polymerase ζ involved in translesion synthesis (Northam et al. 2006). The E113G PCNA mutant protein, however, is capable of being mono-ubiquitylated on Lys164 (Northam et al. 2006), which is required for translesion synthesis *in vivo* (see Sect. 15.5 below). This suggests that the inability of these PCNA mutant proteins to support translesion synthesis is independent of their mono-ubiquitylation.

X-ray crystal structures of the G178S and E113G PCNA mutant proteins have provided insight into how these substitutions disrupt translesion synthesis by non-classical DNA polymerases (Freudenthal et al. 2008, 2009). The G178S PCNA mutant protein has little effect on the structure of domain B, which is the domain in which the amino acid substitution occurs. Instead, a significant, local structural change occurs in domain A of the adjacent subunit. This difference between the G178S PCNA mutant protein and the wild type PCNA structures is limited to a single, extended loop (residues 105–110), which is called loop J. In the mutant protein structure, loop J adopts a very different conformation in which the protein backbone has moved by as much as 6.5 Å from its position in the wild type structure (Fig. 15.4b) (Freudenthal et al. 2008). The E113G mutant protein structure has a similar, but somewhat smaller (only about 3 Å) shift in loop J (Freudenthal et al. 2008). These structures suggest a key role for loop J in facilitating translesion synthesis by non-classical polymerases, perhaps as a novel site of a secondary contact between the polymerases and PCNA.

15.5 Structures of Post-translationally Modified PCNA

The recruitment of proteins to sites of replication via interactions with PCNA is regulated in some cases by post-translational modifications of PCNA. For example, PCNA is mono-ubiquitylated on Lys164 by Rad6 (an ubiquitin-conjugating enzyme) and Rad18 (an ubiquitin ligase) in a DNA damage-dependent manner (Hoege et al. 2002; Stelter and Ulrich 2003). The mono-ubiquitylation of PCNA is required for translesion synthesis by non-classical DNA polymerases. Several of these non-classical polymerases contain ubiquitin-binding motifs (Bienko et al. 2005) and the switch between the classical and non-classical DNA polymerases only occurs when PCNA is mono-ubiquitylated (Zhuang et al. 2008).

The mono-ubiquitin on Lys164 can be converted to Lys63-linked poly-ubiquitin chains by the Mms2-Ubc13 complex (an ubiquitin conjugating enzyme) and Rad5 (an ubiquitin ligase) (Hoege et al. 2002). The poly-ubiquitylation of PCNA is required for an error-free damage bypass pathway that is currently poorly understood. It has been suggested that this pathway involves switching of the stalled replicative polymerase from the damaged template to the newly synthesized sister strand.

In addition to mono-ubiquitylation and poly-ubiquitylation, PCNA is also subject to SUMOylation on Lys164 by Ubc9 (a SUMO conjugating enzyme) and Siz1 (a SUMO ligase) (Hoege et al. 2002). PCNA SUMOylation inhibits unwanted recombination by recruiting the anti-recombinogenic Srs2 helicase (Papouli et al. 2005; Pfander et al. 2005) which contains a SUMO-binding motif. The Srs2 helicase then disrupts the Rad51 nucleoprotein filaments needed to carry out the strand exchange reaction (Krejci et al. 2004; Veaute et al. 2003). SUMOylation has also been observed to a lesser extent on Lys127 on the IDCL but the biological implications of this SUMOylation are unclear.

Our understanding of the structural and mechanistic basis of the recruitment of these factors to post-translationally modified PCNA has come in part from recently determined X-ray crystal structures of ubiquitin-modified and SUMO-modified PCNA (Freudenthal et al. 2010, 2011). In the sections that follow we discuss these structures and their implications.

15.5.1 Structure of Ubiquitin-Modified PCNA

Obtaining sufficient quantities of ubiquitin-modified PCNA for X-ray crystallography had been an obstacle for years. A breakthrough came when it was shown that large amounts of ubiquitin-modified PCNA could be created by splitting the PCNA protein into two polypeptides at residue 164 (the site of ubiquitylation) (Freudenthal et al. 2010). These two polypeptides self-assemble *in vivo*. This allowed the ubiquitin to be fused in-frame to the C-terminal portion of the split PCNA generating a split ubiquitylated PCNA analog that supported UV resistance *in vivo* and translesion synthesis *in vitro* (Freudenthal et al. 2010). This analog allowed for the determination of the X-ray crystal structure of ubiquitin-modified PCNA.

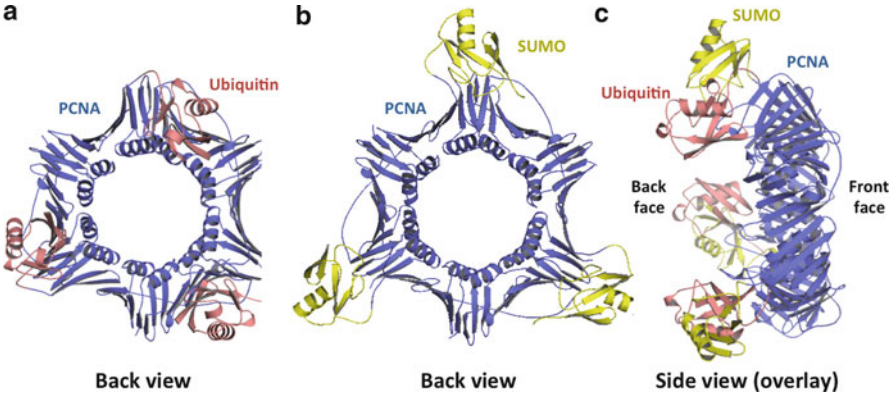

Fig. 15.5 Structures of ubiquitin-modified and SUMO-modified PCNA. (**a**) Ribbon diagram of the ubiquitin-modified PCNA trimer (PDB ID: 3L10) shown from the back with the PCNA ring shown in *blue* and the ubiquitin moieties shown in *red*. (**b**) Ribbon diagram of the SUMO-modified PCNA trimer (PDB ID: 3PGE) shown from the back with the PCNA ring shown in *blue* and the SUMO moieties shown in *yellow*. (**c**) Overlay of the structures of ubiquitin-modified PCNA and SUMO-modified PCNA shown from the side

The structure of ubiquitin-modified PCNA shows that the ubiquitin moiety occupies a position on the back face of the PCNA ring (Fig. 15.5a) (Freudenthal et al. 2010). It interacts primarily with a long loop on the back of PCNA called loop P (residues 184–195). Moreover, the attachment of ubiquitin to PCNA does not alter the conformation of the PCNA in any significant way. This suggests that the ubiquitin moiety does not act as an allosteric modifier to increase the affinity of PCNA for the non-classical polymerase. Instead, it argues for a simpler model in which the ubiquitin moiety provides an additional binding surface to which the non-classical polymerases can attach.

The position of the ubiquitin moiety on the back face of PCNA is consistent with a variation of the typical toolbelt model. In the typical toolbelt model, different PCNA-binding partners interact with different subunits on the front face of the PCNA ring. In the case of PCNA ubiquitylation, PCNA should be able to interact with other protein factors such as the classical DNA polymerase on its front face, while at the same time binding non-classical polymerases on its back face. Here the non-classical polymerase can be held in reserve until needed without interfering with on-going activity on the front face of the PCNA ring. While there is not yet experimental evidence for such a toolbelt in eukaryotes, there is convincing experimental evidence for the analogous toolbelt in prokaryotes. The classical DNA polymerase III and the non-classical DNA polymerase IV have been shown to simultaneously bind the β sliding clamp (Indiani et al. 2005).

The residues of ubiquitin that interact with the non-classical polymerases have been mapped by NMR spectroscopy (Bomar et al. 2007). In the case of DNA polymerase η, this interaction is mediated by the same hydrophobic residues (Leu7, IIe44, and Val70) that ubiquitin uses to interact with a wide range of other proteins. It turns out that these residues of ubiquitin are buried at the ubiquitin-PCNA interface (Freudenthal et al. 2010). This means that the conformation of

ubiquitin-modified PCNA observed in this structure is not the conformation to which the non-classical polymerases bind. This implies that the ubiquitin moiety must either be capable of re-orienting itself on the back face of the PCNA ring or be capable of moving around to occupy other positions on the PCNA ring including possibly the side of the ring. Such alternative conformations would be necessary to recruit the non-classical polymerase.

15.5.2 Structure of SUMO-Modified PCNA

Once it was shown that one could obtain sufficient quantities of ubiquitin-modified PCNA for structural studies using the split/fusion strategy, large quantities of SUMO-modified PCNA were produced using the same approach (Freudenthal et al. 2011). The X-ray crystal structure of SUMO-modified PCNA was then determined. In this structure, the SUMO was also found to be on the back face of the PCNA ring interacting predominantly with loop P of PCNA (Fig. 15.5b) (Freudenthal et al. 2011). Interestingly, the SUMO occupied a different, more radial, position on the PCNA relative to the position occupied by ubiquitin in the ubiquitin-modified PCNA structure (Fig. 15.5c).

The attachment of SUMO does not change the structure of PCNA suggesting that allosteric models for the recruitment of the anti-recombinogenic Srs2 helicase are unlikely. Instead, the SUMO moiety likely provides an additional binding surface to which Srs2 can bind. Moreover, the finding of the SUMO moiety on the back face of the PCNA ring also argues for a toolbelt model. In such a model, Srs2 could be recruited to the back face of PCNA where it can be held in reserve until needed.

15.5.3 Unresolved Issues

Understanding precisely how ubiquitin and SUMO modifications regulate the recruitment of non-classical polymerases and anti-recombinogenic helicases to replication forks requires that we learn more about the dynamics of these modified PCNA proteins. What other conformations do ubiquitin-modified PCNA or SUMO-modified PCNA adopt? Which of these conformations recruit the polymerase or helicase to the replication fork? Which of these conformations supports the enzymatic activity of the polymerase or helicase? Answering these questions will require further structural studies of the modified PCNA proteins as well as structures of the modified PCNA proteins bound to target proteins. It is likely that a combination of high resolution approaches such as X-ray crystallography and low resolution approaches such as single-particle EM and SAXS will be required.

Despite an intense effort, it remains unclear whether one subunit or all three subunits of PCNA are modified in cells. This raises the possibility that PCNA trimers may have some subunits mono-ubiquitylated and other subunits SUMOylated at the same time. Do the ubiquitin and SUMO modifications work together? Does the

SUMO modification inhibit recombination and therefore allow the ubiquitin modification time to promote translesion synthesis? The ability to produce constitutively ubiquitylated or constitutively SUMOylated PCNA in cells using the split/fusion strategy may help answer these questions.

Finally, the details of the error-free damage bypass pathway promoted by Lys63-linked poly-ubiquitylation of PCNA are still unclear. What protein factors are recruited to replication forks when PCNA is poly-ubiquitylated? How do these proteins allow replication to proceed past the DNA damage? These questions are important because, unlike translesion synthesis, this damage bypass pathway does not contribute to genome instability.

15.6 Concluding Remarks

All of the structural studies discussed here have contributed to our understanding of the complex and dynamic processes in which PCNA participates. They have revealed how PCNA binds PIP motifs from various PCNA-binding proteins, and they have suggested mechanisms by which these interactions may be regulated. Nevertheless, many unanswered questions remain regarding the regulation of these processes. One important issue deals with how PCNA recognizes specific binding partners in certain circumstances. Does this involve secondary contacts between PCNA and its binding partner outside the PIP motif? Does this involve post-translational modifications of either PCNA or its binding partner? Some of the structural studies discussed here represent the first steps toward addressing these issues.

Another important issue deals with how the DNA substrate is handed off from one PCNA binding partner to another. Does this involve the sequential binding of these enzymes or does PCNA form specific toolbelts among the three protein-binding sites on the front face of the PCNA ring to facilitate this handoff? Do ubiquitin and SUMO modifications also allow PCNA to function as a toolbelt by opening up new binding sites on the back face of the PCNA ring? The answers to these questions await further biochemical and structural studies of complexes of unmodified and modified PCNA.

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